V-ATPase upregulation during early pregnancy: a possible link to establishment of an inflammatory response during preimplantation period of pregnancy

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Abstract

Various mechanisms exist to prevent a potentially deleterious maternal immune response that results in compromising survival of semiallogeneic fetus. In pregnancy, there is a necessary early preimplantation inflammatory stage followed by a postimplantation anti-inflammatory stage. Thus, there is a biphasic 'immune response' observed during the course of pregnancy. We provide the evidence that capacitation of sperm induced the expression of $\alpha 2$ isoform of V-ATPase (ATP6V0A2 referred to as $\alpha 2V$), leukemia inhibitory factor (*Lif*), *Il1b*, and *Tnf* in the sperm. Capacitated sperm also released cleaved N-terminal domain of $\alpha 2V$ -ATPase ($\alpha 2V$ NTD), which upregulates the gene expression of *Lif*, *Il1b*, *Tnf*, and monocyte chemotactic protein-1 (*Ccl2* (*Mcp1*)) in the uterus. Unfertilized eggs had low $\alpha 2V$ expression, but after fertilization, the expression of $\alpha 2V$ increased in zygotes. This increased level of $\alpha 2V$ expression was maintained in preimplantation embryos. Seminal plasma was necessary for upregulation of $\alpha 2V$ expression in preimplantation embryos, as mating with seminal vesicle-deficient males failed to elicit an increase in $\alpha 2V$ expression in preimplantation embryos. The infiltration of macrophages into the uterus was significantly increased after insemination of both sperm and seminal plasma during the preimplantation period of pregnancy. This dynamic infiltration into the uterus corresponded with the uterine $\alpha 2V$ expression through the induction of *Ccl2* expression. Furthermore, the polarization ratio of M1:M2 (pro-inflammatory/anti-inflammatory) macrophages in the uterus fluctuated from a ratio of 1.60 (day 1) to 1.45 (day 4) when female mice were inseminated with both sperm and seminal plasma. These data provide evidence that exposure to semen may initiate an inflammatory milieu by inducing $\alpha 2V$ and cytokine/chemokine expression, which triggers the influx of macrophages into the preimplantation uterus during the onset of pregnancy and ultimately leads to successful pregnancy outcome.

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Introduction

Sperm capacitation is the final step of sperm maturation. This phenomenon occurs in the reproductive tract of the female for a finite period before the sperm can fertilize the egg (Austin 1951, Chang 1951). During capacitation, sperm undergo several physiological changes that start with ejaculation. Several other changes arise over a longer period in the female tract. However, a very early event in sperm capacitation is the activation of sperm motility. *In vivo* imotile sperm are stored in the cauda epididymis and the vigorous movement of flagellum starts when sperm are released from the epididymis and come into contact with the high concentration of HCO_3^- and Ca^{++} present in the seminal fluid. Capacitation may be induced *in vitro* in appropriate media (Visconti 2009). *In vitro* capacitation media contains a protein source,

usually BSA, and ions including HCO_3^- and Ca^{++} . Previously, it was reported that during sperm capacitation, BSA acts as a sink for the depletion of cholesterol from sperm plasma membrane and that HCO_3^- and Ca^{++} ions stimulate protein tyrosine phosphorylation through cAMP. Finally, through this increase in protein tyrosine phosphorylation, sperm acquire fertilizing capacity (Salicioni *et al.* 2007). Several studies on rodent, livestock species, and humans suggest that the introduction of ejaculated sperm into the maternal reproductive tract stimulates several molecular and cellular changes, with physiological consequences bearing on conception and pregnancy (Robertson 2007). Factors associated with sperm and seminal fluid have potential biological effects in the mother, which includes cytokines, sex hormones, and prostaglandins (Aumuller & Riva 1992, Maegawa *et al.* 2002).

These molecules target the cells of the cervix, uterus, and oviduct and induce early inflammatory responses important for the establishment of successful implantation (Manjunath *et al.* 1994, Robertson *et al.* 2006).

In addition to DNA, sperm contribute RNA and proteins to the ova, which are crucial for early embryonic development (Ainsworth 2005). We tested mouse sperm for the presence of an important immune regulatory molecule a2V and studied its possible immune-regulatory role during capacitation and early embryonic development. Earlier studies demonstrated that a2V is one of the first molecules expressed in pregnancy that has regulatory effects on the maternal immune response (Lee *et al.* 2001). Sun-Wada *et al.* (2002) have previously reported that a2V was expressed in mature sperm. Skinner *et al.* (1999) showed that expression of V-ATPase subunits in the bovine endometrium was crucial for trophoblast invasion and cellular communication during the window of implantation. Additionally, the acidic environment regulated by V-ATPase is important for the development of the preimplantation embryo following implantation (Sun-Wada *et al.* 2000). Our recent studies have shown that a2V affects cytokine and chemokine expression and may be a key factor in the early control of the inflammatory process necessary for implantation and placentation (Ntrivalas *et al.* 2010, Jaiswal *et al.* 2011). Several inflammatory cytokines are known to be important in implantation (Chaouat *et al.* 2007). Leukemia inhibitory factor (LIF) is known to be mandatory for implantation in mice (Stewart *et al.* 1992, Aghajanova 2010). It is expressed in the endometrium maximally during the implantation period; the LIF receptor is localized in villous and extravillous trophoblasts. LIF is involved in mediating interactions between maternal decidual leukocytes and trophoblasts. LIF enhances HLA-G expression in trophoblasts and supports the establishment of maternal immune tolerance at the feto-maternal interface (Jones *et al.* 1997). *Hoxa10* gene is a leading candidate for regulating differentiation of the uterine endometrium in the preparation for blastocyst implantation (McGinnis & Krumlauf 1992, Krumlauf 1994). The uteri of *Hoxa10*-deficient mice are not supportive of implantation (Satokata *et al.* 1995, Taylor *et al.* 1997).

Previously, we have shown that a2V can regulate IL1B as well as IL1A with little or no subsequent increase in TNF secretion (Ntrivalas *et al.* 2007, Kwong *et al.* 2011). This process 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 (Ntrivalas *et al.* 2007). ATP binding to P2X7 causes the influx of potassium ions and the activation of the inflammasome (Kwong *et al.* 2011). The second mechanism is the cleavage and release of soluble

N-terminus portion (a2NTD) from a2V. This a2NTD peptide is responsible for the selective increase in transcription and secretion of several different cytokines and chemokines important in maintaining specific immune responses (Ntrivalas *et al.* 2010, Kwong *et al.* 2011).

One of the key events mediated by chemokines and cytokines is the regulation of migration of specific mononuclear cells to the uterus during pregnancy (Hunt & Pollard 1992, Hunt *et al.* 2000, Mor & Koga 2008). Macrophages and NK cells infiltrate as the most prominent leukocyte populations in the uterine tissues during the first trimester of pregnancy (Gomez-Lopez *et al.* 2010). These cells are important modulators of vascular remodeling and immune activity in the uterus and developing placenta (Hunt *et al.* 1985, Moffett-King 2002, Tabiasco *et al.* 2006). Recently, two distinct subsets of macrophages have been defined. Type 1 or M1 macrophages have the traditional characteristics of cytotoxicity and the release of inflammatory cytokines and chemokines. Type 2 or M2 macrophages are found associated with tumors and tumor angiogenesis (Mantovani *et al.* 2002, Gordon 2003, Mosser 2003). M2 macrophages are associated with the resolution of inflammation and also with angiogenesis (Mantovani *et al.* 2002).

Previously, we have shown that a2V regulates a delicate network of cytokines and chemokines that coordinates the recruitment of M1 and M2 macrophages to the feto-maternal interface for successful placental development and growth (Jaiswal *et al.* 2011). In this study, we correlated the pattern of cytokine and chemokine expression with macrophage movement into the uterus during implantation and show that macrophages migrate in an order of the reflective events induced by the upregulation of uterine a2V expression and monocyte chemoattractant protein-1 (*Mcp1*) upon female exposure to sperm accompanied with seminal plasma. We conclude that capacitation induces a2V, *Lif*, *IL1b*, and *Tnf* expression in the sperm. Capacitation also brings the release of a2NTD that, in turn, induces maternal inflammatory cytokines such as *Lif*, *IL1b*, *Tnf*, and *Ccl2* (*Mcp1*) expression. The exposure of the uterus with sperm accompanied by seminal plasma induces the accumulation of macrophages before blastocyst implantation on day 4 of pregnancy through upregulation of a2V and *Ccl2*.

Results

Sperm capacitation induces a2V and cytokine expression

We first looked for a2V expression in sperm collected from the cauda epididymis. a2V was not detected in sperm before capacitation (Fig. 1). However, the

Figure 1 Capacitation of sperm induces a2V expression in sperm. A, The photomicrograph shows immunolocalization of a2V in sperm recovered from epididymis. Green (FITC) shows localization of a2V and blue (DAPI) shows nuclear stain. Panel I shows that no expression occurs before capacitation. Panel II shows lower expression of a2V in noncapacitated sperm recovered from KR without calcium and BSA. Panel III shows higher expression of a2V in capacitated sperm recovered from KR with calcium and BSA. Data are representative of five experiments.

expression of a2V was increased after incubation with capacitation buffer (KR buffer with calcium and BSA). After 90 min incubation with capacitation buffer, a2V was abundantly detected in the sperm (Fig. 1). Real-time PCR studies confirmed that the gene expression of a2v, *Lif*, *Il1b*, and *Tnf* was significantly higher in the sperm recovered after 90 min incubation with capacitation buffer when compared with their respective controls (Fig. 2A, B, C and D). The *Il10* gene expression was not detected in the capacitated sperm. This finding suggests that capacitation induces a2V and *Lif*, *Il1b*, and *Tnf* cytokine expression in sperm.

a2NTD induces inflammatory cytokine expression in the prepared uterus

The a2NTD peptide was detected in the supernatants recovered after sperm capacitation (Supplementary Figure S2, see section on supplementary data given at the end of this article). In order to prove that a2V expression directly led to cytokine expression *in vivo*, recombinant a2NTD was injected into the uterus during proestrous stages of females and different cytokine expression was measured. As shown in Fig. 3, only the uterine horn that was injected with a2NTD showed significantly higher expression of *Lif*, *Il1b*, *Tnf*, and *Ccl2*. However, this was not seen in the a2NTD-injected uterine horn of randomly selected female mice that were not in the proestrous stage, where the cytokine expression was almost undetectable (Supplementary Table S1, see section on supplementary data given at the end of this article). These data indicate that a2NTD interacts with uterine epithelium of the prepared uterus and induces the expression of *Lif*, *Il1b*, *Tnf*, and *Ccl2* *in vivo* (Fig. 3A, B, C and D).

Expression of a2V in the preimplantation embryos recovered from intact or seminal plasma-deficient matings

Immunocytochemistry studies showed that a very low level of a2V expression was observed in the mature ova recovered from female mice (Fig. 4). However, a2V expression was significantly increased after fertilization in the preimplantation embryos recovered from intact (INT) mating as shown in Fig. 4. The zygote and two-cell stage embryos were highly positive for a2V and had a distinct peripheral expression pattern. The staining of a2V was brightest in the morula and blastocyst recovered from INT mating. However, in seminal plasma-deficient (SVX) mating, very low expression of a2V was seen at different stages of the preimplantation embryo. However, expression of a2V was decreased in the implanted blastocyst recovered from both the groups (Fig. 4). This differential expression of a2V in preimplantation embryos recovered from the INT or SVX mating clearly suggests that a2V expression is the result of the presence of seminal plasma. These data showed that the exposure to both sperm and seminal plasma in INT mating contributes to increased expression of a2V in preimplantation embryos.

The expression of a2v and *Lif* mRNA was measured by real-time PCR in preimplantation embryos recovered from mating. As shown in Supplementary Figure S1, see section on supplementary data given at the end of this article, *Lif* was not detectable in the zygote (day 1) and two-cell stage embryos (day 2), whereas a2v gene expression was found in zygotes and increased in two-cell embryos. However, on day 4, a2v expression decreased and *Lif* expression increased dramatically in blastocyst. After implantation on day 6, the expression of both a2v and *Lif* was not detected in implanted embryos.

Figure 2 Capacitation of sperm induces the gene expression of a2v and different cytokines. Gene expression of (A) a2v, (B) *Lif*, (C), *Il1b*, and (D) *Tnf* in sperm recovered from after 90 min incubation at 37 °C in 5% CO₂ in KR buffer with or without calcium and BSA. a2v, *Lif*, *Il1b*, and *Tnf* gene expression was significant higher in the capacitated sperm recovered from KR with calcium and BSA when compared with sperm recovered before capacitation or from KR buffer without calcium and BSA. Data are from five independent experiments (mean $\pm$ S.E.M.). **Significant difference vs respective control, $P < 0.01$.

Lif expression remained undetectable, but a low level of a2v expression was observed in the embryos recovered on day 8 of pregnancy (Supplementary Figure S1).

Expression of a2v, *Ccl2*, *Lif*, and *Hoxa10* in the uterus recovered from intact, seminal plasma-deficient, and vasectomized matings

The real-time PCR studies showed that the levels of gene expression of a2v, *Ccl2*, and *Lif* were significantly increased from days 1 (time period after few hours of insemination) to 4 in the uterus recovered from the INT mating when compared with seminal plasma-deficient (SVX) or vasectomized (VAS) matings (Fig. 5A, B and C). The increased gene expression of a2v on day 4 in the uterus of the intact mating corresponds with the increased gene expression of *Ccl2* and *Lif* (Fig. 5A, B and C). *Hoxa10* gene expression was similar in all studied groups on day 1, but its expression was significantly decreased in the SVX or VAS matings on day 4 when compared with the INT mating (Fig. 5D). By immunohistochemistry, we examined the localization of a2v in the uterus during preimplantation period of pregnancy. Immunohistological staining in the uterus showed that the density of a2v expression followed the same pattern as its gene expression (Fig. 6). On day 1, a2v protein primarily localized to the stromal cells of the uterus (Fig. 6A). Its density of expression was significantly increased near the window of implantation on day 4 uterus recovered from intact mating (ISIS = 5.67 ± 0.86 , day 1; ISIS = 13.45 ± 1.25 , day 4) (Fig. 6D and G). On day 4, a2v protein was localized to the glandular and luminal epithelium as well as stromal cells in the uterus. However, in SVX or VAS matings, the a2v expression was not increased near the window of implantation on day 4 (ISIS = 04.98 ± 1.89 , SVX; 05.22 ± 1.14 , VAS) (Fig. 6E, F, H and I). These data demonstrate that the expression of a2v, *Ccl2*, and *Lif* was significantly upregulated in the uterine horn, which was exposed to sperm accompanied with seminal plasma (INT mating), as their expression was not increased when females were mated with SVX or VAS males. Moreover, for the consistent gene expression of *Hoxa10*, the exposure of both sperm and seminal plasma to the uterus is crucial, as its expression was decreased in uterus recovered from SVX or VAS matings.

Infiltration of M1 and M2 macrophages in the uterus recovered from intact, seminal plasma-deficient, and VAS matings

Ccl2 is well known to amplify the recruitment of macrophages and other immune effector cells into reproductive tissues (Critchley *et al.* 1996, Jones *et al.* 1997). By immunohistochemistry, we examined the infiltration and polarization of M1 (ITGAX) (Fig. 7) and M2 (MRC1) (Fig. 8) macrophages in the uterus recovered

Figure 3 a2NTD induces cytokine expression in uterus. The bar diagrams show the gene expression of (A) *Lif*, (B) *Il1b*, (C) *Tnf*, and (D) *Ccl2* in uterine horn recovered from control and 10 ng intrauterine a2NTD-injected BALB/c females. *Lif*, *Il1b*, *Tnf*, and *Ccl2* gene expression was higher in the a2NTD-injected horn than their respective controls. Data are from four independent experiments (mean $\pm$ S.E.M.). **Significant difference vs respective control, $P < 0.01$.

during the preimplantation period of pregnancy. The number of M1 and M2 macrophages was significantly ($P \leq 0.05$) higher in the uterus recovered from INT mating when compared with the SVX or VAS matings on both day 1 (time period after few hours of insemination) and day 4 (near the window of implantation) (Figs 7 and 8). There was significantly higher expression of a2V in the day 4 uterus of the INT mating, which corresponds to a significantly higher level of *Ccl2* expression as well as increased infiltration of M1 and M2 macrophages when compared with the SVX or VAS matings. The levels of infiltration of M1 and M2 macrophages directly correlated with the expression of *Ccl2*, which corresponded to the expression of a2V in the uterus during different days of pregnancy. The polarization pattern of macrophages on days 1 and 4 was skewed to the M1 phenotypes (M1/M2 = 1.6, day 1; 1.45, day 4) (Fig. 8H) in the uterus recovered from INT mating. However, this change was not seen in the SVX or VAS matings (Fig. 8H). These data indicate that the higher expression of uterine a2V corresponds with the dynamic infiltration of M1 and M2 macrophages through the induction of *Ccl2* expression. This strengthens the hypothesis that a2V regulates the delicate cytokine and chemokine networks and coordinates the recruitment of macrophages important in successful implantation.

Discussion

We report for the first time that sperm capacitation induces the expression of the immune regulatory molecule a2V and the cytokines *Lif*, *Il1b*, and *Tnf* in sperm. It is a well-known fact that capacitation is the final stage of sperm maturation (Austin 1951, Chang 1951). Our data now provide clear evidence that capacitation of sperm is not only a step of maturation but also induces the cytokines necessary for the onset of pregnancy. The role of maternal immune responses to the developing embryo has been intensely studied. It is known that various mechanisms exist to prevent a potentially deleterious maternal immune response, which, would result in compromising the survival of the semiallogeneic fetus (Chaouat *et al.* 2010). However, it is also known that several inflammatory cytokines, which are potentially harmful to the fetus, are necessary for successful implantation during preimplantation period (Chaouat *et al.* 2007, 2010). Thus, there is a biphasic 'immune response' required during the course of a successful pregnancy. There is an early inflammatory stage, followed by an anti-inflammatory stage required to prevent the detrimental effect on pregnancy (Mor *et al.* 2011). We provide evidence that sperm can initiate this unique immune process upon capacitation by inducing both a2V and different cytokines such as *Lif*, *Il1b*, and *Tnf* expression in the sperm. Thus, it is a paternal factor that initiates an essential inflammatory milieu required for successful implantation.

Figure 4 Expression of a2V in preimplantation embryos. Photomicrograph shows immunolocalization of a2V in different stages of preimplantation embryos recovered from females mated with intact or SVX mating. FITC-stained with anti-a2V antibodies (green) and DAPI nuclear stain (blue). Data are representative of four experiments.

Our previous studies showed that the key portion of the a2V protein is a2NTD, the N-terminal portion that is released from the activated monocyte and induces the cytokines secretion (Kwong *et al.* 2011). In this study, we showed that capacitated sperm secrete a2NTD in the surrounding milieu. To confirm whether a2NTD stimulates cytokine secretion in the female uterus, we injected a2NTD intrauterine into the prepared uterine horn (proestrous stages uterus). a2NTD induced the expression of different cytokine expression such as *Lif*, *Il1b*, *Tnf*, and *Ccl2* in the injected uterine horn. Thus, we suggest that following capacitation of the sperm in the proestrous stage uterus, sperm initiates inflammation in the uterine horn by the release of the immune regulatory molecule a2NTD, which in turn induces the expression of inflammatory cytokines.

Capacitation has been well studied and involves a cascade of biochemical and physiological changes including changes in metabolism and sperm motility, actin polarization, and complex signal transduction (Breitbart 2003). However, little is known about the protein translation that occurs during capacitation. The mature sperm has a reservoir of a complex population mRNA, which aid in the replacement of degraded proteins and which are critical for postfertilization events (Ostermeier *et al.* 2002, 2004, 2005). Ejaculated sperm are capable of using mRNA transcripts for protein translation during capacitation in the female reproductive tract (Miller & Ostermeier 2006). In the capacitation-dependent process, nuclear-encoded mRNA can be translated by the mitochondrial type ribosomes whereas

cytoplasmic machinery is not involved. In a recent viewpoint, mature sperm has a capacity of *de novo* protein translation (Gur & Breitbart 2008).

Recent reports suggested that sperm deliver not only the male genome to the ova but also several crucial molecules such as RNA and proteins required for the fertilization and early embryonic development (Ostermeier *et al.* 2002, 2004, 2005, Ainsworth 2005). We detected the presence of both crucial immune regulatory molecules a2V and *Lif* in the capacitated sperm. After fertilization in INT mating, the expression of a2V is remarkably greater in the zygote than in the unfertilized egg. These data show that a high level of a2V expression is maintained in the developing preimplantation embryos recovered from intact mating until the time of implantation. Moreover, we showed that developing blastocysts express both a2V and *Lif*. These molecules are known to play a vital role in the establishment of successful implantation. Gene knockouts of *Lif* and LIF antagonist studies on mice have shown that deleting the *Lif* receptor gene or impeding the interaction of *Lif* with the receptor results in implantation failure (Stewart *et al.* 1992). However after implantation, neither a2v nor *Lif* expression was detected in the implanting blastocyst. In contrast to SVX mating, the preimplantation stage embryo had lower expression of a2V. Together, these results indicate that in INT mating, the active immune response is established as shown by the presence of a2V and LIF before embryo implantation and then ceased after implantation. In contrast to SVX mating, lower a2V expression indicates the importance of seminal plasma

Figure 5 Gene expression of *a2v*, *Ccl2*, *Lif*, and *Hoxa10* in the uterus. The bar diagrams shows the gene expression of (A) *a2v*, (B) *Ccl2* (C) *Lif*, and (D) *Hoxa10* in the uterine horn recovered from females mated with INT, SVX, or VAS males. Data are from four independent experiments (mean $\pm$ s.e.m.). **Significant difference between groups, $P < 0.01$. *Significant difference between groups, $P < 0.05$.

as a key regulator that influences the local immune environment during pregnancy.

The remarkable finding of this study was the infiltration of macrophage subsets to the uterus during the preimplantation period. Our data suggests that during the preimplantation period, the microenvironment of female uterus becomes inflammatory in INT mating. It has been previously reported that within the hour of natural insemination, the surge of proinflammatory cytokines induces the infiltration of inflammatory leukocytes including macrophages, dendritic cells, and granulocytes into the female uterus. This response is short-lived and is resolved near the window of implantation with the decline of proinflammatory cytokines and chemokines (Robertson *et al.* 1992, 1996). Our data quantifying uterine M1 and M2 macrophages and their polarization ratios clearly show that within a few hours of insemination, on day 1 of pregnancy, the number of macrophages migrating into the uterus increased until the window of implantation on day 4. These macrophages were mainly skewed to M1 macrophages as the ratios ranged from 1.6 to 1.45 in the INT mating. However, in SVX or VAS matings, the infiltration of M1 and M2 macrophages was lower during the preimplantation period of pregnancy. Moreover, the polarization pattern was mainly skewed to M2 phenotypes on day 4 of pregnancy in the SVX mating. These data also indicate that the dynamic infiltration of M1 and M2 macrophages was corresponded with higher expression of uterine *a2v* through the induction of *Ccl2* expression. Cumulatively, these results provide evidence that the presence of the early inflammatory phase of pregnancy may be a reflective event of upregulation of *a2v* and *Ccl2* in the uterus recovered from the INT mating where the uterus gets exposed to seminal plasma as well as sperm for successful pregnancy outcome.

It is intriguing that control of the immune response thought to be vital in the mother to maintain pregnancy may develop actively by the male. It has been previously reported that male semen contributes to the events through which maternal tolerance is established at the outset of pregnancy (Manjunath *et al.* 1994, Robertson *et al.* 2009). We have previously shown that the *a2v* expression was significantly higher in the preimplantation embryos and placentas of nonabortion-prone ($\text{♀BALB/c} \times \text{♂BALB/c}$ and $\text{♀CBA} \times \text{♂BALB/c}$) matings compared with the abortion-prone ($\text{♀CBA} \times \text{♂DBA}$) matings. The *a2v* expression in preimplantation embryos and placenta was significantly higher when BALB/c males were used than when DBA male were used. This highlights the importance of the male contribution to the amount of *a2v*. It can be postulated that a low paternal contribution of *a2v* may be responsible for the severe pregnancy complications observed in mothers from recurrent miscarriage (Jaiswal *et al.* 2011). In this study, we showed that lower *a2v* expression in both

Figure 6 Distribution of a2V protein in the mouse uterus on different days of pregnancy. The brown staining indicates that a2V protein localization in the uterine horn recovered from females mated with INT, SVX, or VAS males. (A) Day 1, INT mating; (B) day 1, SVX mating; (C) day 1, VAS mating; (D and G) day 4, INT mating; (E and H) day 4, SVX mating; (F and I) day 4, VAS mating; (J) negative control ($n=4$ for each group). GE, glandular epithelium; LE, luminal epithelium; S, stroma; (40 $\times$). Data are representative of four experiments. Data shown in the table is expressed in mean $\pm$ S.E.M. ¹Immunostaining index score (ISIS)=stained area score (SAS) $\times$ immunostaining intensity score (IIS). *Significant difference between groups, $P<0.05$.

preimplantation embryos and uterus was directly associated with the reduced fertility rate in the SVX mating. No live fetuses were observed in the uteri recovered from females mated with SVX-deficient males (data not shown). These data are in agreement with the previous reports that removal of the seminal vesicle reduces the fertility rate in the mouse (Peitz & Olds-Clarke 1986).

In summary, this study provides new insights into the role of capacitation in facilitating pregnancy through the regulation of immune process, which is characterized by the expression of a2V and inflammatory cytokines in capacitated sperm. Given the critical role that the release of a2V subcomponent a2NTD from the capacitated sperm and its involvement in the expression of inflammatory cytokines, there is strong evidence that it is an essential molecule for onset of pregnancy. Following fertilization of the ova, the embryo at the maternal interface takes over the regulation of the local immune response by expressing a2V. In the postmating period, the inflammatory phase was confirmed by the recruitment of M1 macrophages in the early pregnant uterus, which is the reflective event of the upregulation a2V and *Ccl2* expression. This study also provides evidence that the exposure of both sperm and seminal plasma is crucial for facilitating pregnancy through

upregulation of a2V and panel of genes that confers uterine receptivity near the window of implantation.

Materials and Methods

Mice and surgical treatments

Inbred strains of BALB/c (H-2^d) mice (6–7 week, 18–20 g) were used for this study (Jackson Laboratories, Bar Harbor, ME, USA). The housing and handling of experimental animals were in accordance with the guidelines of Institutional Animal Care and Use Committee.

Seminal plasma-deficient (SVX) and VAS BALB/c males were prepared as described by Robertson *et al.* (1996) with slight modifications. Male mice were anesthetized by the i.p. dose of ketamine (100 mg/kg body weight) and xylazine (16 mg/body weight). Male mice were VAS by bilateral ligation of the vas deferens, about 4–5 mm apart and cut out a section of vas deferens from in between the knots through a transverse incision at the abdominal wall. For SVX males, seminal vesicles were removed through a transverse incision in the abdominal wall after ligation and serving of the proximal tubule at the base of the gland. The body wall was sutured and skin was closed up using two clips to hold the wound closed. The surgical males were allowed to recover for at least 10 days before mating. VAS males were proven incapable of fertilization by examination of females 8 days after mating. For experimental matings, females were individually caged with intact (INT) SVX and VAS stud

Figure 7 Infiltration of M1 macrophages in the uterus. Distribution of M1 macrophages (black arrow) in mouse uterus recovered from females mated with INT, SVX, or VAS males. (A) Day 1, INT mating; (B) day 1, SVX mating; (C) day 1, VAS mating; (D) day 4, INT mating; (E) day 4, SVX mating; (F) day 4, VAS mating; (G) negative control for staining of M1 and M2 macrophages ($n=4$ for each group) (40 $\times$). Panel H shows the quantification of M1 macrophages in the uterus recovered from intact, SVX, or VAS mating on days 1 and 4 of pregnancy. Data are representative of four experiments (A, B, C, D, E, F and G) or are four independent experiments (H; mean $\pm$ S.E.M.). *Significant difference between groups, $P < 0.05$.

males. Females mated with INT males (intact or INT mating) and VAS males (VAS mating) were checked for the presence of vaginal plug for the confirmation of mating. Mating with SVX males (SVX mating) was detected by the presence of sperm in the vaginal smear prepared on every morning. The confirmation of mating was considered as day 1 of pregnancy.

Recovery of embryos and uterine horn

Embryos were recovered from pregnant females mated with INT or SVX. The females were selected for the retrieval of zygotes between 12 and 13 h on the day of the appearance of vaginal plugs, i.e. day 1, ~ 14 hpc from oviduct in a sterile petridish with sterile PBS. The two-cell embryos were recovered between 14 and 15 h the following day of the appearance of vaginal plugs, i.e. day 2, ~ 40 hpc from oviduct. The compact morula and blastocysts were recovered on day 4, ~ 84 – 90 hpc from uterus respectively. These preimplantation embryos were used for further studies. The uterine horns were recovered from pregnant females mated with INT, SVX, or VAS

males on days 1 (~ 14 hpc) and 4 (~ 90 hpc) of pregnancy and used for further studies.

Localization of a2V in sperm and different preimplantation stages embryos

Sperm were collected from epididymis of 8 week BALB/c males and incubated with Krebs–Ringer (KR) buffer (Sigma Chemicals Co.) with or without calcium and BSA for 90 min at 37 $^{\circ}$ C in 5% CO_2 . KR buffer contains HCO_3^- and induces the capacitation of sperm in the presence of calcium and BSA (named as capacitation buffer). After incubation with these buffers, sperm were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and 3% BSA, and placed in a 1:100 dilution (20 $\mu\text{g}/\text{ml}$) of a mouse MAB to a2V (named as 2C1) in 1% BSA in PBS (BSA–PBS) overnight at 4 $^{\circ}$ C. After washing with BSA–PBS, the specimens were incubated with secondary antibody, FITC rabbit polyclonal antimouse IgG (Dako) at a 1:20 dilution in BSA–PBS for 45 min at room temperature. To visualize the nuclei, the sperm were counterstained with

Figure 8 Infiltration of M2 macrophages in the uterus. Distribution of M2 macrophages (black arrow) in mouse uterus recovered from females mated with INT, SVX, or VAS mating mating. (A) Day 1, INT mating; (B) day 1, SVX mating; (C) day 1, VAS mating; (D) day 4, INT mating; (E) day 4, SVX mating; (F) day 4, VAS mating ($n=4$ for each group) (40 $\times$). Panel G shows the quantification of M2 macrophages receptively in the uterus recovered from intact, SVX, or VAS mating on days 1 and 4 of pregnancy. Panel H shows the polarization of M1 and M2 macrophages in the uterus. Data are representative of four experiments (A, B, C, D, E and F) or of four independent experiments (G and H; mean $\pm$ s.e.m.). *Significant difference between groups, $P<0.05$.

4',6-diamidino-phenylindole (DAPI; 0.5 μ g/ml). After mounting the specimens on slides with vectashield, antigen distribution was examined under a Nikon eclipse TE2000-S fluorescence microscope (Nikon Instrument, Inc., Melville, NY, USA). One group of sperm was directly used for a2V immune-staining as described earlier without any incubation with KR buffers. Experiment was repeated five times (number of animals used=10). Embryos at different stages of preimplantation were collected from females mated with INT and SVX males and stained for immunolocalization of a2V as described earlier for sperm. The number of animals was three for each day of pregnancy studied. Experiments were repeated four times.

Gene expression of a2v and different cytokines in sperm

Sperm were collected and incubated as described earlier. Sperm were washed three times and resuspended in 50 μ l

sterile PBS and used for extraction of total cellular RNA using High Pure RNA Isolation Kit (Roche Diagnostics GmbH) according to the manufacturer's instructions. RNA samples were stored at -80°C until further use. The cDNA was generated from total cellular RNA by RT at 50°C for 60 min using random hexamer primer of transcript first-strand cDNA synthesis kit (Roche Diagnostics GmbH). Multiplex real-time PCR was performed by TaqMan gene analysis using the Applied Biosystems StepOne Real-time PCR system for a2v, and different cytokines *Lif*, *Il1b*, *Tnf*, and *Il10* were measured and normalized to the *Gapdh* housekeeping gene. Real-time PCR was performed using universal PCR master mix reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The prevalidated TaqMan gene expression assays for *Atp6v0a2* (Mm00441848_m1), *Il1b* (Mm00434228_m1), *Tnf* (Mm00443258_m1), *Lif* (Mm00434761_m1), *Ccl2* (Mm00441242_m1), *Il10* (Mm00439614_m1),

and *Gapdh* (part number 4352339E) were purchased from Applied Biosystems. Experiments were repeated five times (number of animals used=5).

Gene expression of *a2v* and *Lif* in different preimplantation stage embryos and uterus

Embryo

The gene expression of *a2v* and *Lif* in the different stage preimplantation embryos was analyzed by TaqMan gene expression Cell-to-Ct kit according to the manufacturer's protocol with slight modifications. A total of 25 embryos were collected at each stage, washed, and resuspended in 10 µl sterile PBS and used to prepare cell lysates for RT-PCR. Multiplex real-time PCR was performed for *a2v* and *Lif* and normalized to the *Gapdh* housekeeping gene. The prevalidated TaqMan gene expression assays were used as mentioned earlier. The number of animals was four for each day of pregnancy studied. Experiments were repeated three times.

Uterus

Total cellular RNA was extracted by Melt Total nucleic acid isolation system (Ambion, Austin, TX, USA) according to the manufacturer's protocol. Quantity and integrity of RNA were confirmed by the ratio at 260:280 nm and electrophoresis was performed on 1.5% native agarose gel to visualize 18S and 28S rRNA subunits. Samples were stored at -80 °C until further use. The cDNA was generated from 1 µg total cellular RNA by RT at 50 °C for 60 min using random hexamer primer of transcript first-strand cDNA synthesis kit (Roche Diagnostics GmbH). Multiplex real-time PCR was performed for *a2v*, *Lif*, *Ccl2*, and *Hoxa10* as mentioned earlier. The prevalidated TaqMan gene expression assays were used as mentioned earlier. Experiments were repeated four times.

Immunohistochemistry

The uterine horns were collected from females mated with INT, SVX, or VAS males on days 1 and 4 of pregnancy. These uterine horns were used for the immunostaining of *a2v* and different macrophage phenotypes (M1 and M2 macrophages). The uterine horns 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 till the tissue was sunk. The tissues were snap frozen in the OCT (Tissue-Tek, Torrance, CA, USA) by liquid nitrogen. Frozen tissue was stored at -80 °C until further use. Frozen 5 µm sections from frozen tissues were mounted onto saline-coated glass slides (Dako, Carpinteria, CA, USA) and stored at -80 °C until used. The Dako EnVision+ System-HRP (DAB) (Dako) 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 MAB to *a2v* in 1% BSA-PBS for 1 h. After washing, sections were incubated with secondary antibody-labeled polymer HRP antmouse IgG1. The 3,3'-diaminobenzidine chromogen was used as substrate for

the EnVision+ HRP system according to the manufacturer's instructions. A mouse IgG1 isotype control (Abcam, Cambridge, MA, USA) was used at the same concentration as the primary antibody. The sections were counterstained with Meyer's 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, Weesp, The Netherlands) and high-resolution camera (Canon G10, Japan). 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 percentage of stained tissue and immunostaining intensity was calculated according to the method described in Teixeira Gomes *et al.* (2009). Then, immunostaining index score (ISIS) was generated by the equation stained area score (SAS) multiplied by the immunostaining intensity score (IIS): $ISIS = SAS \times IIS$.

We used antibodies targeted to ITGAX and MCR1 for M1 and M2 subtypes respectively (Aron-Wisniewsky *et al.* 2009, Fujisaka *et al.* 2009, Jaiswal *et al.* 2011). The Dako EnVision+ System HRP (AEC) (Dako) was used to stain the frozen sections according to the manufacturer's instructions with slight modification. 1:100 and 1:25 dilutions were used for ITGAX and MCR1 (Abcam) receptively. The number of macrophages was counted in at least ten randomly chosen areas per uterine section at 40× view. The number of animals was four for each group. Six sections were analyzed per animal.

I.u. injection of a2NTD

BALB/c females were injected i.p. with 5 IU pregnant mare serum gonadotropin (PMSG; Folligon, Sigma) and human chorionic gonadotropin (hCG; Chorulon, Intervet, Boxmeer, Holland). After 5 h of hCG injection, a single dose of 10 ng *a2NTD* in 15 µl sterile PBS was injected in one of the uterine horns. The control received 15 µl sterile PBS by the same route. Uterine horns were recovered after 24 h of injection and stored at -80 °C until use. Total cellular RNA was extracted by Melt Total nucleic acid isolation system (Ambion) according to the manufacturer's protocol. The cDNA was generated from 1 µg total cellular RNA by RT at 50 °C for 60 min using random hexamer primer of transcript first-strand cDNA synthesis kit (Roche Diagnostics GmbH). The multiplex real-time PCR was performed for different cytokines *Lif*, *Il1b*, *Tnf*, *Ccl2*, and *Il10* as mentioned earlier. Experiments were repeated three times. The numbers of animals were four for each group.

Statistical analyses

The results of each experiment were analyzed by one-way ANOVA with Duncan's multiple range test for comparison of the significance level (*P*) between groups. A *P*<0.05 value was considered as a significant difference between the values compared.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-12-0036>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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