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Apoptosis-associated speck-like protein containing a CARD is not essential for lipopolysaccharide-induced miscarriage in a mouse model

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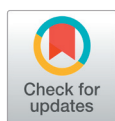
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Abstract

A disrupted immune system during pregnancy is involved in pregnancy complications, such as spontaneous abortion, preeclampsia, and recurrent pregnancy loss. This study examined the role of toll-like receptor (TLR) 4 and ASC (apoptosis-associated speck-like protein containing a CARD [c-terminal caspase recruitment domain]) in pregnancy complications using a lipopolysaccharide (LPS)-induced miscarriage mice model. Incidences of miscarriage and embryonic resorption were examined at 9.5 days of pregnancy in wild-type (WT), ASC knockout (KO), and TLR4 KO mice after injecting them with LPS. The fetuses and placenta were obtained after sacrifice at 15.5 days of pregnancy. A significantly lower frequency of fetus absorption was found in TLR4 KO mice, whereas corresponding absorption outcomes were strongly induced in the WT and ASC KO mice upon an LPS injection. As expected, TLR4 KO mice were resistant to LPS-induced abortion. A histological analysis of the miscarried placenta showed increasing levels of the eosin staining of spongiotrophoblast cells without any obvious difference between WT and ASC KO mice. These results suggest that TLR4 KO mice are resistant to LPS, which affects pregnancy persistence, whereas WT and ASC KO mice show high miscarriage rates due to LPS. Moreover, the ASC adaptor is not directly involved in LPS-induced miscarriages, and the NLRP3 inflammasome can be activated by other proteins in the absence of ASC.

Key words: ASC (apoptosis-associated speck-like protein containing a CARD), inflammation, miscarriage, pregnancy, preterm birth



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Introduction

Mammalian pregnancy is an important period of reproduction when a female carries their offspring. In the case of humans, intrauterine gestation is a unique immunological process caused by the semi-allograft nature of the fetus. On the other hand, the process demands significant changes in physiology

and immunology of females, which result in an increased health risk of both the newborn and mother (Nepomnaschy et al., 2007). Therefore, in mammals, the placenta is a vital organ for managing pregnancy (Robinson and Klein, 2012). The placental fetomaternal interface comprises numerous maternal immune and inflammatory cells. These cells play a crucial role in immune and inflammatory responses (Nepomnaschy et al., 2007). Aluvihare et al. (2004) reported that these cells might affect placental development and function. Moreover, excessive inflammatory responses caused pregnancy complications, including preterm delivery, poor fetal growth, and preeclampsia (Robinson and Klein, 2012). In the gestation period, it is important to protect the mother and fetus from infections caused by pathogens, including bacteria and viruses. Maternal infection is a major cause of spontaneous abortion in humans during the first trimester of pregnancy. Miscarriage is the process, which shows the spontaneous loss of pregnancy before the fetus has reached viability (Denny et al., 2015).

When most clinical examinations show that excessive uterine inflammations caused pregnancy complications, including fetal resorption or miscarriage (Mulla et al., 2013; Shirasuna et al., 2015). Many studies have evidence regarding spontaneous abortions caused by maternal infections related to bacterial infections, such as bacterial vaginosis, mycoplasmosis, toxoplasmosis, and listeriosis (Griebel et al., 2005; Mulla et al., 2013). Furthermore, the anatomical conditions of the female genital tract are affected by spontaneous abortion within the early pregnancy days. Garcia-Engudanos et al. (2002) and Li et al. (2002) reported that uterine congenital malformations (unicornuate uterus, uterus didelphys, bicornuate uterus, and septate uterus), acquired uterine defects (Asherman's syndrome, diethylstilbestrol [DES] exposure, and uterine leiomyomata) are the anatomical factors that affect spontaneous abortions. On the other hand, stress, nutrition factors, age, previous miscarriage, maternal diseases, living habits, and microbial infection are the factors responsible for miscarriage (Garcia-Engudanos et al., 2002; Li et al., 2002; Pandey et al., 2005).

LPS is the main component of the bacterial cell wall. LPS-induced inflammation is a frequently used model and well-documented process to study induced spontaneous abortion (Silver et al., 1997; Robertson et al., 2007; Yi et al., 2021). According to Aisemberg et al. (2013), LPS induces the release of numerous biomedical mediators, including cytokines, arachidonic acid, metabolites, nitric oxide, and toxic oxygen radicals. The innate immunity is a mechanism that activates the signaling system in the cells by recognizing the molecular pattern of pathogens by pattern-recognition receptors (PRRs) present in the membrane of macrophages, thereby promoting the production and secretion of inflammatory cytokines. In particular, toll-like receptors (TLRs) present in the cell membrane and NOD-like receptors (NLRs) present in the cytoplasm play a role in PRRs (Schroder and Tschopp, 2010; Yang et al., 2019). Among the NLRs, NLRP3 (NLR family pyrin domain containing 3) is the most well-known protein that is activated by various pathogen-associated molecular patterns (PAMPs). Activated NLRP3 forms a multi-protein complex called NLRP3 inflammasome that recruits pro-caspase-1 via the adaptor molecule ASC (apoptosis-associated speck-like protein containing a CARD [c-terminal caspase recruitment domain]) and cleaves pro-caspase-1 to caspase-1. At the same time, pro-IL-1 and pro-IL-18, which are generated by the TLR4/NF- κ B mediated pathway, are cleaved to IL-1 and IL-18 by caspase-1 (Bauernfeind et al., 2009). An excessive amount of IL-1 β may cause pregnancy complications, such as spontaneous miscarriage (Mulla et al., 2013). Therefore, the present study compared miscarriage and embryonic resorption in wild type (WT), ASC knockout (KO), and TLR4 KO mice after being injected with LPS during pregnancy.

Materials and Methods

Animals

C57BL/6 (WT; six weeks of age) mice were purchased from Samtako Bio Korea (Osan, Korea). The ASC KO mice were donated by Prof. Jong-Hwan Park (Chonnam National University), and the TLR4 KO mice are described elsewhere (Kawai and Akira, 2007). The animals were housed in a facility at 22 - 25°C with a humidity of 50 ± 10% with a 12 hrs light/dark cycle and were fed normal chow containing no animal proteins or microbes. A female mouse was mated overnight with two male mice. Subsequently, the presence of a vaginal plug in the mice was checked and designated as day 0.5 of gestation. All mouse experiments were performed according to the guidelines from the Animal Care and Use Committee (ACUC) of Chungbuk National University (CBNUA-1547-21-01).

Mice models prepared for LPS- induced miscarriage

The mice were injected with intraperitoneally LPS (Sigma-Aldrich, St. Louis, MO, USA) and 0.9% NaCl saline (control) at day 9.5 of gestation: (i) 30 µg·kg⁻¹ of LPS and (ii) 30 µg·kg⁻¹ of saline were injected into separated groups of mice. The mice were sacrificed on day 15.5 of pregnancy to obtain fetuses and placentas (Fig. 1). The uteri of the animals were dissected, and the litter size and absorbed fetus of each mouse were observed and calculated. The frequency of absorption rate was calculated using the following formula: number of the absorbed fetus/ number of the total litter size.

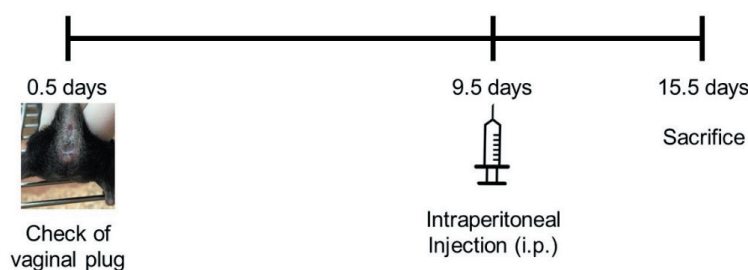


Fig. 1. Experimental schedule of the lipopolysaccharide (LPS) or saline (a control) injection. After mating, the presence of a vaginal plug in the mice was checked and designated as day 0.5 of gestation. LPS was injected at day 9.5 of gestation, and then the mice were sacrificed on day 15.5 of pregnancy.

Histology

The placenta isolated from the mice were fixed in 10% neutral-buffered formalin (Sigma), and the fixed tissues were dehydrated through a graded series of ethanol (50, 70, 80, 90, and 100%) and cleared in xylene. Subsequently, the tissues were embedded in paraffin and sectioned at a 5 µm thickness. Finally, the placental sections were deparaffinized in xylene and dehydrated through a graded series of ethanol (100, 90, 80, and 70%) and stained with hematoxylin for 5 min. Finally, the sections washed in tap water were counterstained with eosin for 15 min, and the sections were dehydrated with ethanol (70, 80, 90, and 100%) and cleared in xylene. The placental sections were then mounted with a mounting solution (Sigma) for further observation.

Statistical analysis

The data were processed using one-way ANOVA in GraphPad PRISM (GraphPad Software, San Diego, CA, USA) with a completely randomized design. Tukey's multiple comparison test was used to compare the results of the individual treatments. The data are expressed as the mean \pm SEM. Results with p values < 0.05 , 0.01 , or 0.001 were considered significant.

Results and Discussion

TLR4 KO mice were resistant to LPS-induced miscarriage, but ASC KO mice were not

The WT, ASC KO, and TLR4 KO mice injected with LPS or saline (a control) were sacrificed on day 15.5 and dissected carefully to obtain the uterine sections, including the placenta and fetus (Fig. 2). The normal morphology of the fetus and placenta was observed in mice injected with saline (Fig. 2A - C). In the WT and ASC KO mice, the LPS injection induced a miscarriage, and most of the fetuses were absorbed and could not be obtained after dissection. The size of the placenta also decreased significantly (Fig. 2A' and B'). On the other hand, the TLR4 KO mice maintained normal pregnancy despite the LPS injection, showing a normal fetus and placenta, which means no inflammatory response to LPS. (Fig. 2C'). The weight of the fetus and the fetus isolated from the uterus of the LPS-injected mice was significantly lower in the WT and ASC KO mice than in TLR4 KO mice ($p < 0.001$; Fig. 3A), and the placenta was significantly higher in the TLR4 KO mice ($p < 0.05$ and $p < 0.001$; Fig. 3B).

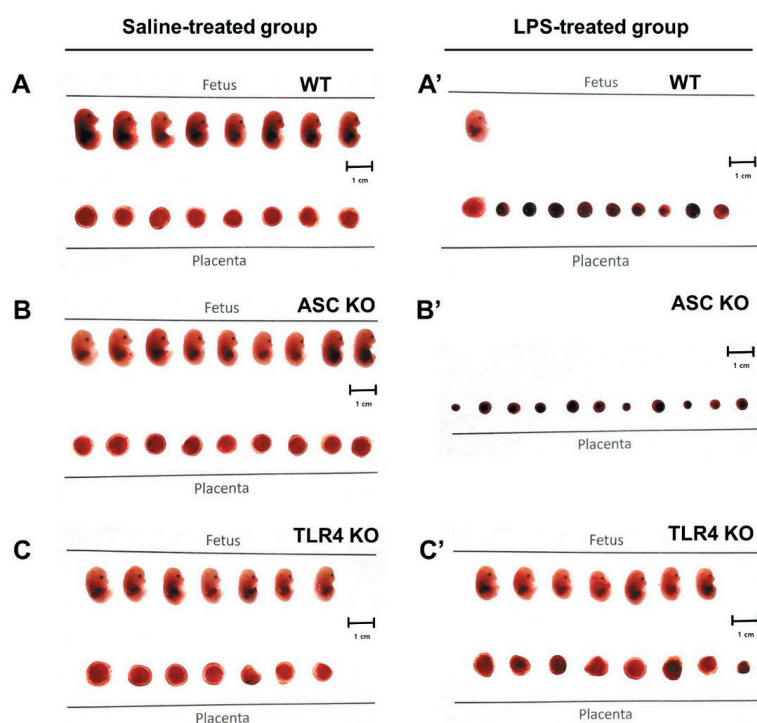


Fig. 2. Morphology of the fetus and placenta after sacrifice. Each mouse was injected with saline (A - C) or $30 \mu\text{g}\cdot\text{kg}^{-1}$ LPS (A' - C') on day 9.5 during pregnancy. The fetus and placenta were obtained on day 15.5. LPS, lipopolysaccharide; WT, wild type mice; ASC KO, apoptosis-associated speck-like protein containing a CARD knock out mice; TLR4 KO, toll-like receptor 4 knock out mice.

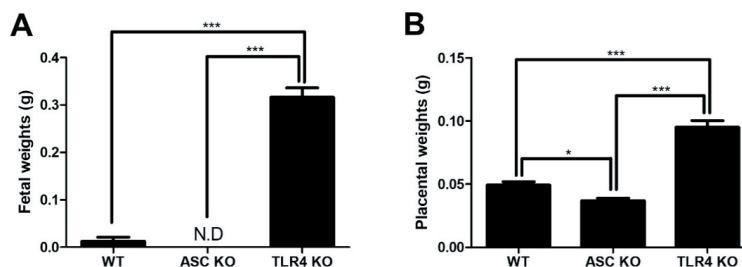


Fig. 3. Weight of fetus (A) and placenta (B) of mice injected with lipopolysaccharide (LPS). After dissection, the weight of each placenta and fetus were isolated from the uterus and measured. The data are expressed as the mean \pm standard error of the mean (SEM) of the samples. * $p < 0.5$, *** $p < 0.001$. WT, wild type mice; ASC KO, apoptosis-associated speck-like protein containing a CARD knock out mice; TLR4 KO, toll-like receptor 4 knock out mice; N.D, no data to display.

NLRP3 inflammasome can be activated in the absence of ASC on LPS-induced miscarriage

As shown in Fig. 4A, the saline injection produced a very low rate of fetus absorption, and there was no significant difference among the three kinds of mice. On the other hand, a higher absorption rate was observed in the WT and ASC KO mice injected with LPS compared to the TLR4 KO mice ($p < 0.001$; Fig. 4B). The results of the miscarriage experiment are expressed in detail in Table 1. When LPS was injected into the WT mice during pregnancy, miscarriage was effectively induced, including a larger number of absorbed fetuses. The ASC KO mice showed an absorbed fetus rate of 100% by an LPS injection, while the TLR4 KO mice showed a very low number and rate of fetus absorption after the LPS injection (Table 1).

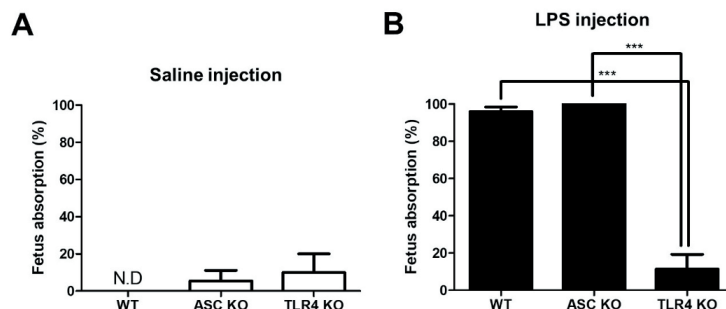


Fig. 4. LPS-induced miscarriage on mice. The mice were injected intraperitoneally with saline (A) or $30 \mu\text{g}\cdot\text{kg}^{-1}$ LPS (B) at 9.5 days of pregnancy to induce miscarriage. The data are expressed as the mean \pm standard error of the mean (SEM). *** $p < 0.001$. LPS, lipopolysaccharide; WT, wild type mice; ASC KO, apoptosis-associated speck-like protein containing a CARD knock out mice; TLR4 KO, toll-like receptor 4 knock out mice; N.D, no data to display.

Table 1. Comparison of the litter size and embryonic absorption rate in mice injected with lipopolysaccharide (LPS) or saline.

Treatment	Characteristics	No. of mice	No. of litter size	No. of absorbed fetus	Frequency of absorption (%)
Saline	WT	3	25	0	0.0
	ASC KO	3	24	1	4.2
	TLR4 KO	3	26	3	11.5
$30 \mu\text{g}\cdot\text{mL}^{-1}$ LPS	WT	5	48	46	95.8
	ASC KO	4	40	40	100.0
	TLR4 KO	4	32	3	9.4

WT, wild type; ASC, apoptosis-associated speck-like protein containing a CARD; KO, knock out; TLR4, toll-like receptor 4.

After dissecting the mice carefully, the placental sections were stained with hematoxylin and eosin for further observation (Fig. 5). Histological analysis of the placenta showed that the eosinophilic infiltration occurred on the placenta when LPS was treated during pregnancy, and partially tissue necrosis was observed in the placenta of the WT (Fig. 5A and A') and ASC KO (Fig. 5B and B'). On the other hand, the TLR4 KO mouse did not show a significant difference between the treatment of saline and LPS (Fig. 5C and C'). Consequently, when the function of TLR4 is nulled, the inflammatory response is not activated in LPS-induced miscarriage. By contrast, the ASC KO mice showed LPS-induced miscarriage, predicting that NLRP3 inflammasome activation occurred even when the ASC function was suppressed.

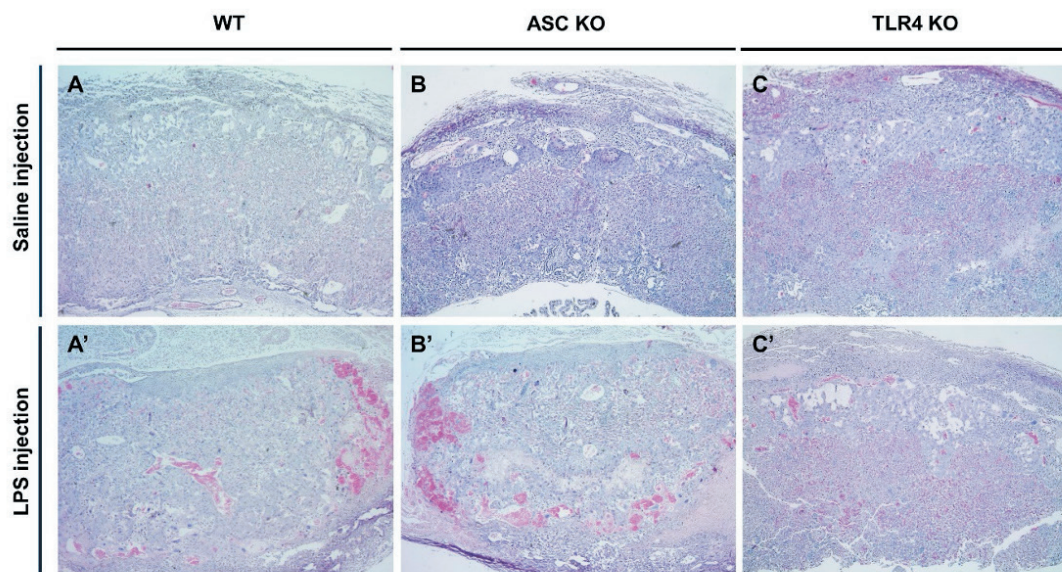


Fig. 5. Histology of the placenta. Each placenta was isolated from mice injected with saline or LPS, fixed and embedded by paraffin. Each placenta was derived from mice of WT (A and A'), ASC KO (B and B'), and TLR4 KO (C and C') injected with saline or LPS and observed under light microscope (x200 magnification). LPS, lipopolysaccharide; WT, wild type mice; ASC KO, apoptosis-associated speck-like protein containing a CARD knock out mice; TLR4 KO, toll-like receptor 4 knock out mice.

The human innate immune defense is based on the complement system. Specifically, it increasingly links to the roles during the gestation period of females (Denny et al., 2015). The innate immune system is the major defense that recognizes infections, initiates a defense against pathogen destruction, and induces tissue repair (Yang et al., 2019). According to McKay and Wong (1963), bacterial endotoxins induced the fetal resorption of mice. This study compared miscarriage and embryonic resorption in WT, ASC KO, and TLR4 KO mice after injecting $30 \mu\text{g}\cdot\text{kg}^{-1}$ LPS at 9.5 days of pregnancy. When the WT and ASC KO mice were injected with LPS, the frequency of miscarriage was similar, whereas TLR4-KO showed a significantly lower absorption rate (Fig. 2 - 4).

The NLRP3 inflammasome has a two-step activation pathway, including priming and activation. First, inflammatory stimuli, such as TLR4 agonists, induce NF- κ B mediated NLRP3 and pro-IL-1 β expression in the priming step, followed by activation triggered by PAMPs and DAMPs (danger-associated molecular patterns) by promoting the assembly of NLRP3, which induces the secretion of caspase-1-mediated IL-1 β and IL-18 and pyroptosis (Yang et al., 2019). On the other hand, non-canonical inflammasome activation may identify gram-negative bacteria (Yang et al., 2019). Cytosolic LPS plays a role in activating non-canonical inflammation without reaching the priming step. Therefore, pro-caspase is not cleaved but leads to pyroptosis. Moreover, caspase-1 and IL- β are secreted by NLRP3 activation (Yang et al., 2015). Consequently, ASC is not

necessarily progressed, and non-canonical inflammasome activation can lead to innate immunity, resulting in LPS-induced miscarriage (Schmid-Burgk et al., 2015). Hence, ASC KO mice were not resistant to LPS-induced miscarriage.

Denny et al. (2015) reported that the administration of LPS reduced the density of placental spongiotrophoblast in WT mice. Spongiotrophoblasts provide structural support to the placenta and produce angiogenic and anti-angiogenic factors that regulate the vasculature of the maternofetal interface (Carpentier et al., 2011). Morphological and histological analysis showed that depleted placentas, including eosinophilic infiltration, were observed in WT and ASC KO mice treated with LPS, compared to those treated with saline. In the case of TLR KO mice, however, there was no morphological difference between LPS or saline injection (Fig. 2 and 5). Therefore, the TLR4 KO mice did not show an immune response to LPS, which did not adversely affect the placenta and fetus.

Conclusion

Little is known about the effect of LPS on pregnancy. Hence, this study examined the difference in immune response on WT, ASC KO, and TLR4 KO mice injected with LPS during pregnancy. As a result, the WT and ASC KO mice showed higher rates of fetus resorption and abortion, while the TLR4 KO mice were resistant to LPS and maintained a normal pregnancy period. Thus, the ASC adaptor may not be directly related to the LPS-induced miscarriage. Further study will be needed to clarify the mechanism of miscarriage by LPS.

Conflict of Interests

No potential conflict of interest relevant to this article was reported.

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