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The macrophage pattern recognition scavenger receptors SR-A and CD36 protect against microbial induced pregnancy loss

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Abstract

Objectives and design—Microbial products can act via stress-induced signaling cascades to link dysregulated endogenous microbiota to immune activation (e.g., macrophages) and pregnancy loss. Our previous studies demonstrated that mice deficient in the macrophage pattern recognition scavenger receptors, SR-A and CD36, are more susceptible to inflammatory complications including gut leakiness and experimental colitis. We hypothesized that bacterial penetration of the maternal mucosal surfaces and replication in embryonic fluids compromise the fetal status and can result in miscarriage.

Materials and methods—Eighty pregnant ICR and SR-A/CD36-deficient mice were injected via tail vein or intraperitoneally with commensal bacteria (*Streptococcus cricetus* and/or *Actinobacillus* sp.) or sham controls. Dams were monitored daily for physical distress, pain and abortion.

Results—Dams injected with single dose bacterial inoculum did not develop clinical symptoms. Day old pups injected with bacteria developed internal focal abscesses, lost weight but recovered after 1 week. Dams receiving a second bacterial inoculum delivered dead fetuses. However, SR-A/CD36-deficient dams demonstrated 100% fetal death via aborted fetuses, and significant up-regulation of the proinflammatory markers (IL-6, serum Amyloid A) 24–74 h after single inoculum.

Conclusions—These data indicate that macrophage scavenger receptors are required for the fetal protection against microbial attack and support that maternal transfer of innate immunity contributes to this protection.

Keywords

Reproductive loss; Gut permeability; *Streptococcus*; *Actinobacillus*; SR-A/CD36^{-/-} mice

Introduction

Spontaneous miscarriage caused by stressors occurs in 15–20% of all pregnancies [1]. Microbial products can act on a stress-induced signaling cascade to link a dysregulated endogenous microbiota to immune activation (e.g., macrophages) and pregnancy loss. Microbial endotoxemia caused by lipopolysaccharide (LPS) has been postulated to play an important role in complicated pregnancies by bacterial infections. When exposed to LPS, or other noxious stimuli, macrophages release a variety of mediators of inflammation, including IL-6 and TNF α [2–4]. The stress signaling cascade exploits the presence of LPS as a danger signal via Toll-like receptor (TLR)4 [1–5]. LPS affects the state of feto-maternal tolerance during gestation [6, 7]. Reactive oxygen species (ROS) generated during chronic inflammation (e.g., inflammatory bowel disease) are important stimuli for macrophage activation and the induction of various cytokines (i.e., IL-6, TNF α) [2, 8]. ROS have been associated with LPS-induced developmental toxicity including embryonic resorption, intra-uterine fetal death, growth retardation, and preterm delivery, although the mechanisms of action remain unclear [9, 10].

The (nonopsonic) pattern recognition scavenger receptors (e.g., SR-A, CD36, TLR) are mainly expressed on the surface of the lamina propria macrophage (M ϕ) forming the first line of innate immune defense [11–13]. These pattern recognition receptors enable M ϕ to identify microorganisms and to facilitate the engulfment initiating intracellular signaling.

A 2001 outbreak of mare reproductive loss syndrome (MRLS) in Kentucky caused thousands of equine abortions, and estimated US \$500 million in economic loss. Equine mares appeared to develop MRLS after ingesting eastern tent caterpillars (*Malacosoma americanum*) [14]. The *Streptococcus* sp. and *Actinobacillus* sp., normally present in the oral cavity and digestive tract, have been isolated in field and experimental caterpillar-induced abortions, but not directly from caterpillars [15]. Therefore, microbial release from mares' gut and replication in the fetal fluids is likely to compromise the fetus and cause abortions. Our prior studies demonstrated that mice deficient in pattern recognition scavenger markers, SRA and CD36, are more susceptible to inflammatory complications, including gut permeability and experimental colitis [11]. We hypothesized that the accompanying bacterial penetration in the absence of these receptors, and replication in the embryonic fluids could result in compromised fetal status and miscarriages, such as that noted in MRLS.

Methods

Bacterial growth

An *Actinobacillus* sp. isolated from lung specimens and gastric content obtained from an aborted equine fetus was inoculated onto blood agar plates and incubated at 37°C for 18–24 h according the standard methods. *Streptococcus cricetus* (*Strep. cricetus*) originally from the oral cavity was grown at 37°C in tryptic soy broth without dextrose (Difco) and harvested by centrifugation at the end of the exponential phase of growth. Control animals received sham (saline) injection.

Murine infection model

Seventy 3-day programmed pregnant (10–12-week old) ICR mice were purchased from Harlan Inc. (Indianapolis, IN). Animals were housed individually in micro-isolator cages in a pathogen free environment maintained at 22°C with a 12:12-h light:dark cycle at VA Medical Center animal facility and the University of Kentucky Medical Center. Mice were fed irradiated rodent chow and sterilized drinking water ad libitum. One week after acclimation, at day 10 of pregnancy, dams were weighed and ear-punched for appropriate identification. They were separated into eight groups of 5–10 dams per group, and injected with 100 µl bacteria (1×10^4 to 1×10^8 CFU) via tail vein or intraperitoneally (i.p.) using a 0.5 ml insulin syringe. Dams were examined daily four times (early morning until 10 pm) for physical appearance for pain and distress, vaginal discharge, abortion and to detect early delivery or aborted fetuses. The experiment was terminated 1–2 weeks after initiation of the experiment. In order to study the direct effect of these bacteria on the pups' health, 1-day old pups were weighed and injected i.p. with 25 µl of bacteria (1×10^3 CFU).

This investigation was approved by ACORP-IACUC at VA Medical Center and UK Medical Center which are American Association of Accreditation of Laboratory Animal Care (AAALAC) certified.

SR-A/CD36 deficient dams

Scavenger receptor-A deficient mice (SRA^{-/-}) breeding pairs originally were provided by Dr. MF Linton (Vanderbilt University Medical Center, Nashville, TN). CD36-deficient mice (CD36^{-/-}) were donated by Dr. RL Silverstein (Weill Medical College of Cornell University, New York, NY). Double SRA/CD36 deficient mice were crossbred in our animal facility. Animals were maintained in the pathogen-free environment in the Lexington Veteran Administration Hospital Animal Facility. Ten days pregnant mice from the breeding colony were injected with *Strep. cricetus* or sham, as mentioned above.

Statistical analysis

Results are expressed as mean $\pm$ SEM unless otherwise stated. Data was evaluated with ANOVA followed by appropriate post hoc test using GraphPad Instat version 3 for Windows (GraphPad Software, San Diego, CA). Statistical significance was set at $p < 0.05$.

Results

Ten-day-programmed pregnant dams injected i.v. or i.p. with a single low dose of bacteria did not develop any clinical symptoms and there were no significant differences noted between the number and the weight of live pups delivered by these groups ($p > 0.05$, NS). There were sporadic cases of aborted fetuses that correlated with the highest single dose of *Strep. cricetus* and *Actinobacillus* sp. bacterial inocula leading to smaller numbers of pups, but did not reach statistical significant (data not shown).

Dams that received a second bacterial inoculum (2 days after the first injection) aborted or delivered dead fetuses ($p < 0.05$). There was a correlation between the number of live pups delivered from dams injected i.v. with bacteria compared with sham controls ($p < 0.05$). Moreover, the number of pups delivered from dams injected i.v. was markedly lower than the sham control or i.p. injected dams (Fig. 1).

In contrast, dams deficient in the macrophage scavenger markers (SR-A/CD36^{-/-}) demonstrated 100% fetal death and aborted 24–74 h post injection with a single dose of bacterial inoculate. Some dams developed more progressive disease leading to intrauterine mummified fetuses (Fig. 2). Aborting dams showed significant up-regulation of inflammatory markers, including elevated serum concentration of the pro-inflammatory cytokine, IL-6 (Fig. 3a, $p < 0.001$), and acute phase protein, serum amyloid A (Fig. 3b, $p < 0.0001$), compared with sham control dams.

Day old pups when injected i.p. with 1×10^3 CFU *Strep. cricetus*/0.25 μ l, all developed internal focal abscesses and granulomatous nodules with necrotizing centers in the abdominal cavity and organs including pancreas (Fig. 4a), liver (Fig. 4b), intestine (Fig. 4c), kidneys and heart (not shown), and lost significant weight ($p < 0.05$, data not shown) but recovered and showed normal growth after 1 week.

Discussion

In 2001 in Kentucky a hyperacute abortion syndrome termed the mare reproductive loss syndrome (MRLS) appeared in association with exposure to an epidemic of eastern tent caterpillars, *M. americanum* [14]. Approximately 15% of mares exhibited mild colitic symptoms, abdominal straining, and low fever, a few days before outbreak of early fetal loss [16]. Although the precise etiologic pathogenesis of MRLS has not been elucidated, evidence strongly supports that exposure of pregnant mares following incidental ingestion of large numbers of eastern tent caterpillars being linked to the pregnancy termination [17]. It has been predicted that the caterpillars' setal fragments resulted in intestinal irritation, ulcerative lesions, and increased permeability for translocation of the gut microbiota and/or their toxic metabolites into the blood stream and septic reaction, triggering abortion and fetal loss. A large number of the thousands of aborted fetuses exhibited bacterial infections during the outbreak [14] that may have caused endometritis resulting in the abortions and fetal death.

This report provides some supporting evidence that bacterial species representing a sizeable proportion of the isolated infections, i.e., *Streptococcus* sp., *Actinobacillus* sp., in the MRLS

could initiate a similar process in a murine pregnancy infection model. We identified that the fetal effects could be elicited following bacterial access via direct hematogenous spread (i.e., i.v. injection), or secondarily after intraperitoneal challenge.

No significant differences were noted whether the gram-positive oral *Strep. cricetus* or the gram-negative equine *Actinobacillus* sp. was used for the infectious challenge. This would suggest that multiple molecular mechanisms could be contributing to this adverse impact on fetal viability. Generally, the LPS from other *Actinobacillus* sp. (including *Aggregatibacter actinomycetemcomitans*, an oral species) has been shown to demonstrate potent endotoxic activities [18, 19] and activate cells via engagement of TLR4 receptors [19]. While not demonstrated for this particular equine species, members of the *Actinobacillus* genera from human and animal species have been shown to produce an RTX toxin that is leukotoxic [20, 21] and a toxin related to the cytolethal-distending toxin of *Campylobacter jejuni*, which is intimately linked to the virulence of this pathogen [22]. Additional studies will be required to determine if these virulence components have a role in MRLS. In contrast, the *Strep. cricetus* would be predicted to act primarily as a ligand for TLR2 via its content of lipoteichoic acid [23] or alter host cell responses through peptidoglycan engagement of the cytosolic sensors of the nucleotide-binding oligomerization family (Nod1, Nod2; 24). These receptors contain a caspase recruitment domain (CARD) linked to a nucleotide-binding domain, which regulate apoptosis and/or NF- κ B activation [25]. Importantly, these receptors are important in bacterial infections, signaling innate immune and likely adaptive immune response systems [26, 27]. Interestingly, pattern recognition scavenger receptor deficient mice (SR-A, CD36) develop an enhanced susceptibility with impaired clearance of microbial agents [28], severe colitis [11], and increased mortality in response to LPS and endotoxemia [29].

Thus, our findings with this murine system support a model in which embryos are protected against microbial infections invading through the transplacental barrier through innate immune mechanisms. The data indicate that macrophage scavenger receptors (SR-A/CD36) are crucial for this recognition and are required for fetal protection against microbial attack. The results also provide some potential molecular explanations for not only the MRLS disease, but potentially other bacterial infections that are fetotoxic. These findings warrant further molecular mechanistic studies of scavenger receptor function in fetal development, and related reproductive loss syndrome.

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Fig. 1.
Number of live pups delivered from dams injected with i.p. or i.v. with *Strep. cricetus* and *Actinobacillus sp.* bacteria compared with sham controls ($n = 8$)

Fig. 2. Mummified fetus shielded inside uteri (*red dark arrows*), bacterial colonies (*white arrow*) and necrotic tissue (*enlarged*) from SRA/CD36 deficient dams ($n = 6$) (color figure online)

Fig. 3. IL-6 and Serum amyloid A (SAA) protein levels in sera from control (sham) versus *Strep. cricetus* and *Actinobacillus* sp. bacterial induced aborted dams. SAA was increased (14-fold) in aborted dams in response to bacteria ($n = 6$)

Fig. 4. H & E stained sections from pancreas (a), liver (b), and intestine (c) from live pups injected i.p. with *Strep. cricetus*. Arrows show necrosis (narrow black arrow) infiltration of inflammatory cells (white-open arrow) and abscesses (red dark-open arrow) (color figure online)