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Acute acalculous cholecystitis-like phenotype in scavenger receptor A knockout mice

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Abstract

Background—Sepsis is a major health problem in the United States that affects more than three-quarters of a million people every year. Previous studies have shown that *scavenger receptor A* (*Sra*), also known as *macrophage scavenger receptor 1* (*Msr1*), is a modifier of interleukin 10 (IL-10) expression after injection of bacterial lipopolysaccharide (LPS). Therefore, we investigated the response to sepsis in *Sra* knock out mice.

Materials and methods—C57BL/6J (B6) (n= 88) and *Sra* (−/−) mice (n= 88) were subjected to cecal ligation and puncture (CLP) using 18G or 16G needles, sham operation, or non-operated controls. At the end, mice were autopsied for the determination of abnormalities after the procedure. Cytokine gene expression was examined in lung and liver samples by quantitative RT-PCR (qRT-PCR), and circulating cholesterol levels were also measured.

Results—*Sra* (−/−) mice displayed an enlargement of the gallbladder after CLP that was not detected in sham or non-operated mice or in B6 mice (wild-type) after CLP. The enlarged gallbladder resembles a condition of acute acalculous cholecystitis observed in humans. *Sra* (−/−) mice presented high cholesterol levels in circulation as opposed to wild type B6 mice. Moreover, *Sra* (−/−) mice exhibited a reduction in IL-10 mRNA levels in lungs compared to wild-type B6 mice after CLP.

Conclusions—The development of acute acalculous cholecystitis may be the combination of pre-existing conditions, such as hypercholesterolemia associated with a defect in *Sra* (*Msr1*) and a robust inflammation induced by sepsis.

Keywords

acute acalculous cholecystitis; sepsis; gallbladder; inflammation; hypercholesterolemia; *Msr1*; *Sra*

INTRODUCTION

Sepsis is an important clinical condition observed after the occurrence of a variety of insults from infection to trauma. An estimated 750,000 cases of sepsis per year are observed in the United States alone, with a mortality rate of 30-50% (1, 2). Moreover, the incidence of sepsis and the number of deaths associated with the disease is increasing (3). The cost of

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treating septic patients is a major health care burden and is estimated to approach 17 billion dollars per year (2, 4). Currently, therapy for sepsis remains mainly supportive, and there are no predictors for this condition. Sepsis is usually followed by the development of septic shock, multiple organ failure, and death. The etiology of these conditions has been associated with an uncontrolled inflammatory process, which is mounted by a massive production of inflammatory cytokines (5-7). The inability of the organism to control this cytokine storm is likely responsible for the mortality observed after septic shock (5, 8). We have previously performed a genetic analysis for phenotypic differences in the inflammatory response induced by LPS in mice in the search for potential markers of sepsis (9, 10). In this regard, *scavenger receptor A (Sra)*, also known as *macrophage scavenger receptor 1 (Msr1)*, was identified as a candidate gene based on differences in LPS-induced plasma levels of interleukin 10 (IL-10) between two mouse strains (10). Polymorphisms in *Msr1*, which were previously described (11), correlated with different expression levels of LPS-induced IL-10 in various mouse strains (10).

Sra is a multi-ligand cell surface glycoprotein primarily expressed on macrophages, which belong to a larger family of scavenger receptors (12). This receptor was initially discovered as a binding protein for modified lipoproteins, such as acetylated or oxidized LDL (13-14). Sra is also involved in cell adhesion (16, 17) and clearance of bacteria (18) and apoptotic cells (19). Sra has long been postulated to play a role in the innate immune response due to its interaction with a wide range of inflammatory agents as well as its expression on macrophages (20, 21). In addition, inbred strains of mice lacking Sra showed increased susceptibility to *Listeria monocytogenes*, herpes simplex virus, and malaria infections (22, 23). Similarly, Sra ($-/-$) mice were reported to be more susceptible to LPS if they were primed by injection of bacillus Calmette-Guerin (24). In contrast, other studies have shown that Sra ($-/-$) mice are protected from an LPS challenge (25, 26). Therefore, the role of Sra in inflammation and injury remains to be fully understood. In this study, we investigated the contribution of Sra in the response to sepsis in an animal model. We used cecal ligation and puncture (CLP), which is currently the gold standard animal model of sepsis. This model is composed of two events: cecum ligation, which results in a necrotic focus, and perforation, which causes bacteria and feces to spill out into the peritoneum, rapidly activating resident macrophages and triggering the infiltration of neutrophils (27). Our results indicated the presence of an enlarged gallbladder in Sra ($-/-$) mice very rapidly after CLP, which resembled the human condition of acute acalculous cholecystitis.

MATERIALS AND METHODS

Mice

Sra ($-/-$) mice, which were backcrossed over six generations to B6 mice, were originally obtained as a gift from Dr. Mason Freeman. These animals were bred in our colony at UCSD and have been certified to be helicobacter free. In addition, a colony of C57BL/6/J (B6) mice was maintained in the same environment as Sra ($-/-$) mice and used as a wild-type control. In other experiments, B6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Both strains of mice were fed with the same regular chow diet. All procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of California, San Diego School of Medicine.

Experimental animal model of sepsis

Sepsis was induced by CLP as previously described. Mice were NPO for 16 h before the procedure. Mice were anesthetized with isoflurane, and, under sterile conditions, a 2 cm incision was made in the lower abdominal region, and the cecum was exposed. The distal

portion of the cecum was ligated 1.5 cm from the end with a 4-0 silk suture and punctured once with either an 18- or 16-gauge needle. The cecum was replaced in the peritoneal cavity and squeezed to place a small portion of its content (bacteria and feces) into the peritoneum. Then, the peritoneal wall and skin were closed with double sutures. Mice were resuscitated with a 1 ml subcutaneous injection of sterile saline (0.9%). As a control, mice were sham operated as described above, except that the cecum was neither ligated nor perforated. Non-operated mice were also used as controls. After the procedure, mice had access to water and food at libitum.

Cytokine analysis using quantitative real-time PCR

Cytokine message levels were measured by quantitative real-time RT-PCR (qRT-PCR). Tissues were harvested, flash frozen in liquid nitrogen, and stored in -80°C at the time of sacrifice. Samples were then resuspended and homogenized in 1 ml of TRIZOL. Total RNA was purified using the TRIZOL protocol. RNA was then DNase treated to remove genomic DNA, and a cDNA library was created from 2 μg of total RNA using the Applied Biosystems (ABI) high capacity cDNA synthesis kit and amplified using an ABI 7500 fast qRT-PCR machine. Standards for each gene to be analyzed (GAPDH, TNF- α , IL-6, and IL-10) were prepared after PCR amplification using primers from Applied Biosystems, Inc. PCR amplified fragments were then subcloned into a pGEM-T easy vector from PROMEGA. A standard curve using different concentrations of these standards was obtained in parallel for each determination. Results for each sample were quantitated by comparison with the standard curve and expressed by mRNA copy numbers. The results for each sample were normalized by content of GAPDH used as a marker for the total amount of mRNA in the preparation. Results are expressed as normalized mRNA levels in arbitrary units.

Cholesterol analysis

Approximately 500 μl of blood was drawn from mice via cardiac puncture using a 16-gauge needle and placed in EDTA-coated tubes on ice. Tubes were spun down at $5000 \times g$ for 5 minutes, and plasma was collected from the supernatant, placed into fresh tubes, and stored at -20°C until further analysis. Cholesterol levels were performed by the hematology core facility at the University of California, San Diego.

Statistical analysis

All data were analyzed using GraphPad Prism software (GraphPad Prism Software, San Diego, CA). Significance was analyzed using a Student's t-test or one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. A p value of < 0.05 was considered statistically significant.

RESULTS

Sra (−/−) mice developed an acute acalculous cholecystitis-like phenotype after CLP

While performing autopsies of mice after CLP, we noticed that Sra (−/−) mice showed enlarged and darkened gallbladders after 20 h of CLP, which resembled the human condition of acute acalculous cholecystitis. This phenotype was not observed in Sra (−/−) mice after sham-operation nor in non-operated animals (Fig. 1A-C), suggesting that this enlarged gallbladder was not a pre-existing condition, but induced by CLP. B6 (wild-type) mice did not display the enlargement of the gallbladder after CLP (Fig. 1D). The phenotype was confirmed by performing CLP on Sra (−/−) or wild-type (B6) mice after two variants of the CLP model, puncture with a 16G or an 18G needle (summarized in Table 1). The incidence of acute acalculous cholecystitis-like phenotype in B6 mice (wild-type) was

reduced (20–27%) in comparison with Sra ($-/-$) mice (47–60%) after 20 or 72 h of CLP 16G or 18G. However, differences between B6 and Sra ($-/-$) mice were only statistically significant after 72 h of CLP 16G. Moreover, the incidence of acute acalculous cholecystitis-like phenotype was not observed in other mouse strains, such as A/J mice. To gain an insight into the acute acalculous cholecystitis-like phenotype, histological analysis of tissue from Sra ($-/-$) and B6 mice was performed. At 20 h post CLP 16G, there was a noticeable difference in the appearance of the gallbladders between knockout and wild-type mice. Sra ($-/-$) mice showed increased thickening of the gallbladder wall after CLP, corresponding to edema. These observations were not made in B6 mice after CLP or in non-operated controls. In addition, more inflammatory cells were observed within the gallbladders of knockout versus wild-type mice (Fig. 2A–D). These observations confirm the development of cholecystitis in mice lacking Sra.

CLP- induced IL-10 expression levels in the lungs were reduced in Sra ($-/-$) mice

Since we previously showed that B6 mice lacking *Sra* (*Msr1*) displayed reduced levels of IL-10 in plasma following injection of LPS in comparison with B6 mice (10), we investigated whether this prior observation could also be observed after CLP and correlated with the development of ACC. IL-10 mRNA levels (qRT-PCR) were measured in lung samples obtained from Sra ($-/-$) or B6 (wild-type) mice after 6 and 20 h of CLP with an 18G needle, and the results were compared with non-operated mice ($t = 0$). We observed significantly lower lung IL-10 mRNA levels at 20 h, but not at 6 h after CLP-18G in Sra ($-/-$) as opposed to B6 mice (Fig 3A). In contrast, no differences between Sra ($-/-$) and B6 mice were observed for TNF- α or IL-6 mRNA levels (Fig. 3B and C), which is consistent with prior observations after injection of LPS (10). Cytokine mRNA levels were significantly reduced in sham-operated animals in comparison with CLP for both strains (data not shown). Similarly, Sra ($-/-$) mice showed reduced lung IL-10 mRNA levels in comparison with B6 mice after 20 h of CLP-16G (Fig. 3D), whereas TNF- α or IL-6 did not display any significant difference between knockout and wild-type mice. On the contrary, no statistical differences for any cytokine mRNA levels were observed in the liver after CLP in Sra ($-/-$) in comparison with B6 mice (data not shown). To investigate whether the incidence of ACC could be due to reduced IL-10 levels, IL-10 ($-/-$) mice with a B6 genetic background were subjected to CLP (16G). The presence of an enlarged gallbladder was not observed in these animals.

A possible explanation for the differences in IL-10 levels between Sra ($-/-$) and B6 (wild-type) mice may be due to overcompensation of other receptors involved in the innate immune response. In fact, Sra has been postulated to associate with Toll-like receptor 4 (Tlr4) during the transduction of the LPS signal (28). We analyzed expression levels of Tlr4 and CD14 in the lungs after 20 h of CLP-18G by qRT-PCR. No differences were observed in these receptors between Sra ($-/-$) and B6 mice after CLP or in non-operated animals (Fig. 4A and B). Similarly, no differences in Tlr4 and CD14 surface expression were observed in peritoneal macrophages isolated from non-operated Sra ($-/-$) and B6 mice (not shown). Thus, the difference in IL-10 expression is not due to an alteration of the response of macrophages to gram-negative components.

Sra ($-/-$) mice showed elevated cholesterol levels in comparison with B6 mice

We investigated whether the presence of *Sra* influences cholesterol levels after CLP and could correlate with the enlargement of the gallbladder. Mice were fasted for 16 h, subjected to CLP, and plasma samples were harvested at 6 or 20 h after CLP. Sra ($-/-$) mice showed significantly higher total cholesterol levels than B6 mice in the absence of CLP. This profile did not change after CLP, remaining elevated in Sra ($-/-$) mice in comparison with B6 mice (Fig. 5).

DISCUSSION

The incidence of sepsis and septic shock are major health problems at both the level of mortality and the economic impact on health care costs (1-4). Therapy for sepsis is largely supportive, and there are no current predictors for the incidence of this condition. Sepsis and septic shock are likely the products of an uncontrolled inflammatory response, which is probably modulated by multiple confounding factors (6, 7). In fact, we have shown that genetic background and sex are modifiers of the inflammatory response and mortality associated with sepsis (30, 31) and endotoxemia (32, 33). Based on phenotypic differences in the response to LPS by two mouse strains, we have mapped several loci containing modifier genes of the inflammatory response (9, 10). These modifier genes, if properly validated, could become potential predictors for the incidence of sepsis and septic shock in humans. *Sra*, also known as *Msr1*, emerged as a candidate gene as the result of a screening based on differences in LPS-induced IL-10 plasma levels in mice (10). Various polymorphisms in *Sra* have been reported among several mouse strains (11). Indeed, mouse strains that shared a common haplotype for *Sra* displayed similar levels of LPS-induced IL-10 (10). Moreover, the role of *Sra* as a modifier of IL-10 levels was demonstrated in *Sra* ($-/-$) mice that showed reduced levels of this cytokine after injection of LPS compared to B6 (wild-type) mice (10). To validate the potential impact of *Sra* as a candidate gene in sepsis, we investigated its role in an experimental animal model for this condition, CLP.

We observed reduced IL-10 mRNA levels in the lungs of *Sra* ($-/-$) mice in comparison with wild-type mice after CLP. In contrast, TNF- α and IL-6 levels were not affected. These observations echo our prior studies indicating a specific reduction in plasma levels of IL-10 (10), but not TNF- α or IL-6 in *Sra* ($-/-$) as opposed to B6 mice after LPS injection. *Sra* has been proposed to interact with Tlr4, modulating the response to LPS (28). In fact, Tlr4 plays a fundamental role during sepsis (34). Since Tlr4 is involved in the induction of TNF- α , IL-6, and IL-10, we could assume that *Sra* may only modulate part of the pathway activated by Tlr4. Recently, it has been reported that Tlr4 can differentially activate the TIRAP-MyD88 and the TRAM-TRIF pathways depending on the subcellular localization of the receptor. Thus, the TIRAP-MyD88 pathway is activated when Tlr4 is on the plasma membrane, whereas the TRAM-TRIF pathway is turned on after translocation of Tlr4 to early endosomes (35). Since the TRAM-TRIF pathway is involved in IL-10 production, it is possible that *Sra* may play a role in modulating Tlr4 endocytosis. We also investigated whether deletion of *Sra* could result in an alteration of Tlr4 expression in the lungs. We did not observe any change in Tlr4 expression between *Sra* ($-/-$) and B6 mice. The expression of Tlr4 in the lungs was not statistically different between mice after 20 h of CLP and non-operated mice. Other studies have shown an increase in Tlr4 in lungs after CLP (36). The discrepancy between our studies and others could be due to the variant of the CLP model or the mouse strains that were used.

An unexpected observation from our studies was that *Sra* ($-/-$) mice developed gallbladder hypertrophy very rapidly after CLP. The CLP-induced enlargement of the gallbladder is similar to the human condition known as acute acalculous cholecystitis. Cholecystitis is an inflammation of the gallbladder and can occur with (calculous) or without (acalculous) associated gallstones. Cholecystitis occurring in an acute setting without the presence of gallstones is referred to as acute acalculous cholecystitis. It is characterized by bile stasis and ischemia leading to inflammation of the gallbladder wall (37). Although acute acalculous cholecystitis is a rare disorder, it is normally associated with a higher mortality rate (10%) compared with calculous cholecystitis (1%) (27). Previous studies have also shown a correlation between the development of acute acalculous cholecystitis and trauma (37-41), and it is commonly observed in multiple organ failure (40). Also, an association between peritonitis and gallbladder smooth muscle contractility has been reported in guinea

pigs (43) and opossum (44). Histological data suggest that the pathology of acute acalculous cholecystitis in critically ill patients is a result of systemic inflammation (45). An enlargement of the gallbladder and biliary sludge has been presented in ultrasonographic analyses of septic patients (42). The occurrence of acute acalculous cholecystitis has been associated with an ischemic insult or an impairment of gallbladder emptying (46). However, the incidence of acute acalculous cholecystitis has not been correlated with a particular protein. The development of gallbladder enlargement after CLP was not observed in non-operated or sham-operated *Sra* ($-/-$) mice, suggesting that it was not a precondition. The incidence of acute acalculous cholecystitis after CLP was higher in *Sra* ($-/-$) mice in comparison with B6 mice after 72 h CLP-16G and was never observed in other strains, such as A/J mice. Gross examination of gallbladder contents did not reveal the presence of stones, but rather a larger accumulation of bile. The development of acute acalculous cholecystitis-like syndrome in *Sra* ($-/-$) mice after CLP was not associated with reduced IL-10 levels, since this condition was not observed in IL-10 ($-/-$) mice after CLP. A potential precondition that could play a role in the development of acute acalculous cholecystitis-like syndrome in *Sra* ($-/-$) mice is elevated cholesterol levels, which were observed in the knockout mice in comparison with wild-type mice. In our studies, cholesterol levels remained elevated during sepsis induced by CLP, suggesting that it may be the initial levels rather than changes in the sterol content during sepsis that is likely responsible for the development of acute acalculous cholecystitis-like syndrome. This idea is supported by previous animal studies, which have demonstrated that excess cholesterol can contribute to gallbladder stasis by affecting calcium channel activity (48). This condition could be further aggravated by the robust inflammatory response induced by CLP, increasing gallbladder dismotility and possibly increasing bile excretion leading to the appearance of biliary sludge. The role of *Sra* in this process could be restricted to a chronic hyperlipidemic state, an involvement in gallbladder motility, or an alteration of the balance among inflammatory mediators, or a combination of all three following the appearance of the acute acalculous cholecystitis-like phenotype. The incidence of acute acalculous cholecystitis in human patients may be associated with a particular polymorphism of *SRA* in humans. Polymorphisms of *SRA* have been linked with increased incidence of prostate cancer (49, 50). In addition, the development of acute acalculous cholecystitis may be exacerbated by the presence of hypercholesterolemia in patients. Thus, it is possible that the incidence of acalculous cholecystitis in human trauma patients may be related to two pre-existing conditions, *SRA* polymorphisms and hypercholesterolemia, which is triggered by a robust inflammatory response after injury. Finally, *Sra* may emerge as a candidate gene for the incidence of sepsis if properly validated in human genetic studies.

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FIG. 1.

Sra (-/-) mice developed an acute acalculous cholecystitis-like phenotype after CLP. Sra (-/-) mice were fasted for 16 h before the procedure. Anesthetized mice were subjected to CLP (1.5 cm ligation) and puncture with 16G needle. Animals were euthanized at 20 h after CLP or sham operation, and gallbladders were exposed. A, Sra (-/-) after CLP; B, Sra (-/-) sham operated; C, Sra (-/-) non-operated; D, B6 after CLP. Representative picture of 15-17 animals. Arrows indicate the gallbladders. Notice the enlarged and darkened gallbladder in Sra (-/-) after CLP.

FIG. 2.

Histology of gallbladder in Sra (-/-) mice displayed the presence of edema. B6 mice (A, B) or Sra (-/-) (C, D) mice were fasted for 16 h before the procedure. Anesthetized mice were subjected to CLP (1.5 cm ligation) and puncture with 16G needle. Animals were euthanized at 20 h after CLP, and gallbladders and liver samples were collected for histological analysis. Representative picture of five animals analyzed in parallel.

FIG. 3.

Changes in lung cytokine mRNA levels in Sra (-/-) mice after CLP. Sra (-/-) (n=20) or B6 (n=20) mice were fasted for 16 h before the procedure. Anesthetized mice were subjected to CLP (1.5 cm ligation) and puncture with 18G (n=10) (A-C) or 16G (n=10) (D) needle. At different time points after CLP (6, 20 h, n=5 for each time point for each strain) or non-operated controls (t=0, n=3), lungs were harvested and total RNA was extracted. Cytokine mRNA levels were measured by qRT-PCR and levels determined by comparison with a standard curve. Data is presented as means $\pm$ standard errors of the mean. Significance was analyzed using a Student's t-test for each time point between Sra (-/-) or B6 mice. * p value of < 0.05.

FIG. 4.

Tlr4 and CD14 mRNA levels in Sra (-/-) mice after CLP. Sra (-/-) (n=5) or B6 (n= 5) mice were fasted for 16 h before the procedure. Anesthetized mice were subjected to CLP (1.5 cm ligation) and puncture with 18G needle. Lungs were harvested at 20 h after CLP or in non-operated control mice (n=3), and total RNA was extracted. A, Tlr4 and B, CD14 mRNA levels were measured by qRT-PCR and levels determined by comparison with a standard curve. Data is presented as means $\pm$ standard errors of the mean. Significance was analyzed using a Student's t-test for each time point between Sra (-/-) vs. B6 mice.

FIG. 5.

Cholesterol levels were elevated in Sra (-/-) mice. Sra (-/-) (n=9) or B6 (n=9) mice were fasted for 16 h before the procedure. Anesthetized mice were subjected to CLP (1.5 cm ligation) and puncture with 16G needle or were non-operated (NO; B6 n=6; Sra^{-/-} n=4). Total cholesterol was measured in plasma samples from NO mice, 6 or 20 h after CLP. Significance was analyzed using a One-way ANOVA followed by Newman-Keuls Multiple Comparison Test for each time point ($p < 0.0001$). Notice that cholesterol levels are elevated in non-operated Sra (-/-) mice without further change after CLP.

Incidence of acute acalculous cholecystitis-like phenotype after CLP in Sra (-/-) and B6 mice. Sra (-/-) or B6 mice were fasted for 16 h before the procedure. Anesthetized mice were subjected to CLP (1.5 cm ligation) and puncture with 16G or 18G needle. Animals were euthanized at 20 h (CLP-16G and 18G) or 72 (CLP-16G) after CLP, and the incidence of enlarged gallbladders was observed. The data is presented as total number of mice displaying acute acalculous cholecystitis-like phenotype (AAC) of the total number of observed animals. Statistical analysis was performed by Fisher's exact test, with two-tailed P value

Table 1

Strain	CLP	Time (h)	Total	AAC	% of total
Sra (-/-)	16G	20	17	8	47
B6	16G	20	15	3	20
Sra (-/-)	16G	72	10	7	70*
B6	16G	72	10	0	0
Sra (-/-)	18G	20	15	9	60
B6	18G	20	15	4	27

* P = 0.0031 for Sra (-/-) vs. B6 at 72 h after CLP-16G.