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Sulfatide Attenuates Experimental *Staphylococcus aureus* Sepsis through a CD1d-Dependent Pathway

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Natural killer T (NKT) lymphocytes are implicated in the early response to microbial infection. Further, sulfatide, a myelin self-glycosphingolipid, activates a type II NKT cell subset and can modulate disease in murine models. We examined the role of NKT cells and the effect of sulfatide treatment in a murine model of *Staphylococcus aureus* sepsis. The lack of CD1d-restricted NKT cells did not alter survival after a lethal inoculum of *S. aureus*. In contrast, sulfatide treatment significantly improved the survival rate of mice with *S. aureus* sepsis, accompanied by decreased levels of tumor necrosis factor alpha and interleukin-6 in the blood. The protective effect of sulfatide treatment depended on CD1d but not on type I NKT cells, suggesting that activation of type II NKT cells by sulfatide has beneficial effects on the outcome of *S. aureus* sepsis in this model.

Bacterial sepsis is a leading cause of death in hospital intensive care units (1). The main pathogen responsible for those infections is *Staphylococcus aureus* (2). Due to the limited efficacy of available treatments, mortality in complicated staphylococcal sepsis exceeds 50% (3). An additional challenge is posed by the increasing antibiotic resistance of *S. aureus* and spread of highly virulent methicillin-resistant strains (4). This makes staphylococcal sepsis a major health care challenge and urges a search for better treatment alternatives.

One of the hallmarks of sepsis is a deregulated immune response to infection (5). It is characterized by an early acute phase with an intense inflammatory response to the disseminated bacteria, with systemic elevation of proinflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α), followed by an immunosuppressed state that causes an inability to clear the primary infection and increased risk of secondary infections. Severe sepsis can lead to disseminated intravascular coagulation (DIC), multiorgan failure, and death (6). Natural killer T (NKT) cells are a subset of T lymphocytes restricted by the CD1d glycoprotein, a major histocompatibility complex (MHC) class I-like molecule (7, 8). Unlike most T cells, NKT cells do not recognize protein antigens but instead recognize lipid and glycolipid antigens presented on CD1d. Upon activation, they rapidly secrete vast quantities of cytokines to modify immune responses, acting as a bridge between innate and adaptive immunity (9–11). NKT cells contribute to the early immune response to a broad range of pathogens (12). Upon microbial infection, NKT cells can be activated in a direct manner by microbial lipids presented on CD1d or by the increased presentation on CD1d of stimulatory self-lipids in pathogen-associated molecular patterns activated in antigen-presenting cells (APC). In addition, APC-derived cytokines, such as IL-12 and IL-18, strongly enhance activation of NKT cells, even in the absence of CD1d. NKT cells are divided into two types (13): type I NKT cells (also known as invariant NKT, or iNKT, cells) express an invariant Vα14-Jα18 (in mice) or Vα24-Jα18 (in humans) α-chain of the T-cell receptor (TCR), whereas type II NKT cells use a diverse TCR repertoire. The two types of NKT cells have been shown to display different, or even opposite, activities in immune responses (8). A subset of type II NKT cells recognizes sulfatide (a self-glycosphingolipid derived from myelin) presented on CD1d (14–16). Treatment with native sulfatide was shown to modulate different diseases in murine models, providing protection from experimental autoimmune encephalitis, experimental hepatitis, and hepatic ischemic reperfusion injury and causing anergy in type I NKT cells (15, 17, 18).

Due to their contribution to microbial immunity and their rapid response to activation, NKT cells were proposed to have a role in sepsis and endotoxic shock (19). Initial studies pointed to a detrimental role of NKT cells in sepsis by magnifying damage and increasing mortality (20–24). However, those studies either did not discriminate between type I and type II NKT cells (22–24) or focused exclusively on type I cells (20, 21). Information about the relative activities of the two types of NKT cells in sepsis therefore is not available. Moreover, previous studies used models of Gram-negative septic shock (20, 22) and polymicrobial, predominantly Gram-negative sepsis (21, 23, 24). A significant proportion of hospital cases of sepsis is due to Gram-positive cocci, which induce a different inflammatory response from that of Gram-negative bacteria (25). The function of NKT cells in various milder infections is dependent on the type of infecting agent; thus, NKT cells play a beneficial role in some infections (26, 27) and are detrimental in others (11). Therefore, observations from Gram-negative sepsis models cannot be extrapolated to *S. aureus* sepsis.

In the present study, we explored the role of NKT cells and sulfatide treatment to activate type II NKT cells in an established mouse sepsis model that closely resembles human *S. aureus* sepsis

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(28). We found that the presence of NKT cells did not have a significant impact on mortality in this sepsis model. Activation of type II NKT cells with sulfatide exerted a protective effect associated with a decrease in the systemic levels of proinflammatory cytokines.

MATERIALS AND METHODS

Mice. Female C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). *CD1d*$^{-/-}$ (29) and *Jα18*$^{-/-}$ (30) mice (backcrossed with C57BL/6 for 17 and 11 generations, respectively) and their respective heterozygous littermates were bred at the Experimental Biomedicine Animal Unit, University of Gothenburg. During experiments, mice were housed in the animal facility of the Department of Rheumatology and Inflammation Research, Sahlgrenska Academy, University of Gothenburg, under standard light and temperature conditions and fed standard laboratory chow and water *ad libitum*. Mice were used for experimental infection at the age of 7 to 15 weeks. The study was approved by the Animal Research Ethical Committee of Gothenburg, and proper animal experimentation guidelines were followed.

Sulfatide preparation and treatment. Native sulfatide, prepared from pig brain as described before (31), had a purity of >95% as determined by thin-layer chromatography and mass spectrometry. Sulfatide was dissolved at a 10× final concentration in phosphate-buffered saline (PBS), sonicated for 10 min, heated at 80°C for 2 min, and diluted to a final concentration in PBS preheated to 80°C. Sulfatide was diluted at the day of injection and kept at 37°C until use. Sulfatide was given to mice according to two treatment protocols: the standard treatment protocol entailed the animals receiving intraperitoneal (i.p.) injection of 25 nmol sulfatide in 200 μl PBS or the same volume of PBS 1 h before and 3 days after bacterial inoculation, and the late treatment protocol included the mice receiving an i.p. injection of 25 nmol sulfatide in 200 μl PBS or the same volume of PBS on day 3 after bacterial inoculation.

Staphylococcal sepsis induction. In all experiments, the gender- and age-matched mice were inoculated intravenously (i.v.) into the tail vein with the TSST-1-producing *S. aureus* LS-1 strain in 0.2 ml of PBS. The LS-1 strain was isolated from a spontaneously arthritic NZB/W mouse (32) and has been used previously to study staphylococcal sepsis in mice (33, 34). In most experiments, a lethal dose of bacteria (1.5×10^8 to 3×10^8 CFU/mouse) was inoculated, while a lower dose of bacteria (4×10^7 CFU/mouse) was inoculated in a limited number of experiments as indicated. Weight loss and mortality of animals were monitored daily for 14 days. When a mouse was judged too ill to survive until the next time point, it was sacrificed by cervical dislocation and considered dead due to sepsis. In some experiments, mice were sacrificed on days 1 and 3 and blood was collected for bacteriologic examination and cytokine analysis. The kidneys, liver, and spleen were aseptically excised for bacteriologic examination and flow cytometry analysis of immune cells, and plasma was analyzed for levels of plasmin activity and fibrinogen.

Bacteriologic examination. The liver and kidneys of the mice were removed, homogenized, and diluted serially in PBS. The homogenates and blood were transferred to agar plates containing 5% (vol/vol) horse blood. Bacteria were grown for 24 h and quantified as CFU per organ or volume of blood.

Blood sample preparation and analysis. Blood samples were collected from mice into EDTA-containing tubes. Platelet counts were analyzed consecutively using standard laboratory techniques. The collected blood samples were centrifuged at 800 × *g* for 20 min, and plasma was aliquoted and stored in a −70°C freezer until further use. Plasmin activity was determined by hydrolysis of the plasmin-specific substrate S-2251 (H-D-Val-Leu-Lys-pNA.2HCL) as described previously (35). The levels of fibrinogen and plasminogen activator inhibitor-1 (PAI-1) in the plasma samples were measured using a mouse fibrinogen immunoperoxidase assay and a mouse PAI-1 total antigen assay (Innovative Research), respectively. The levels of TNF-α and IL-6 in plasma were determined using a DuoSet ELISA Development kit (R&D Systems Europe, Ltd.).

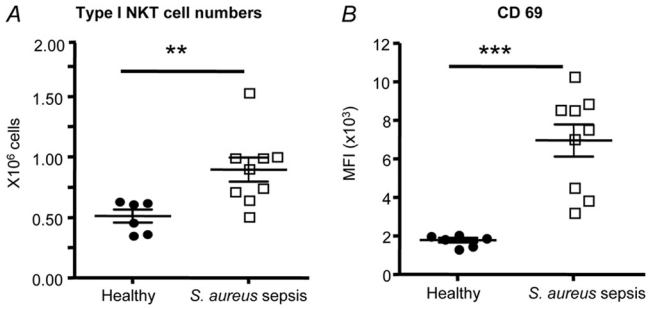

FIG 1 *S. aureus* infection activated type I natural killer T (NKT) cells. Mice were infected with *S. aureus* LS-1 (4×10^7 to 2×10^8 CFU/mouse), and 3 days later spleen cells were isolated, stained, and analyzed by flow cytometry. (A) Absolute numbers of type I NKT cells (PBS-57/CD1d tetramer positive, TCRβ positive, CD19 negative) and (B) mean fluorescence intensity (MFI) of CD69 expression on type I NKT cells in spleens of noninfected (healthy) and *S. aureus*-infected mice. Data are pooled from two separate experiments, and each symbol represents the value from one mouse. Statistical evaluations were performed using the Mann-Whitney U test. **, $P < 0.01$; ***, $P < 0.001$.

Assessment of the influence of sulfatide on *S. aureus* growth *in vitro*. *S. aureus* LS-1 (6×10^3/ml in tryptic soy broth) at 37°C was incubated with sulfatide (0, 12.5, and 125 nmol/ml). At specific time intervals, samples of the bacterial mixtures (0.1 ml) were spread on horse blood agar for CFU counts. The experiment was performed in triplicates for each sulfatide concentration.

Flow cytometry. For staining of type I NKT cells, spleen cells were incubated with 2.4G2 Fc block (15 min, 4°C), followed by staining with allophycocyanin-conjugated CD1d tetramers loaded with PBS-57 (termed PBD-57/CD1d; provided by the NIH tetramer facility) for 30 min at room temperature. After washing, the antibodies fluorescein isothiocyanate (FITC)-CD19 (clone ID3), phycoerythrin (PE)-TCRβ (clone H57-597), and PE-Cy7-CD69 (clone H1.2F3) were added, and the cells were incubated for 15 to 20 min at 4°C. Type I NKT cells were gated as CD19$^-$ TCRβ$^+$ PBS-57/CD1d tetramer$^+$. Alternatively, the cells were first stained with PE-conjugated PBS-57/CD1d tetramers followed by allophycocyanin-NK1.1 (clone PK136), FITC-TCRβ (clone H57-597), peridinin chlorophyll protein-B220 (clone RA3-6B2), and PE-Cy7-CD69 (clone H1.2F3), and gating was done for B220$^-$ TCRβ$^+$ PBS-57/CD1d tetramer$^+$ cells (type I NKT cells) and for B220$^-$ TCRβ$^+$ NK1.1$^+$ PBS-57/CD1d tetramer$^-$ cells. Samples were analyzed with an LSRII fluorescence-activated cell sorter (FACS) using CellQuest software (BD Biosciences).

Statistical analysis. The statistical significance of the results was assessed using the two-tailed Mann-Whitney U test, the chi-square test, and the log-rank survival test. The results are reported as the medians and interquartile ranges (IQR) or the means ± standard errors of the means (SEM).

RESULTS

NKT cells did not significantly affect the survival of mice with *S. aureus* sepsis. We investigated whether NKT cells are activated in the *S. aureus* sepsis model using flow cytometry. Type I NKT cells demonstrated an upregulation of CD69 expression in the spleen (Fig. 1) and liver (data not shown) day 3 after infection, and the number of type I NKT cells was increased 2-fold compared to that of noninfected mice (Fig. 1A). Type II NKT cells cannot be identified with certainty by surface markers (8), but type II NKT cells can express NK1.1 (8); thus, the population of TCRβ$^+$ NK1.1$^+$ cells that is negative for the type I NKT cell-specific PBS-57/CD1d tetramer should contain a proportion of type II NKT cells. Preliminary data also support that TCRβ$^+$ NK1.1$^+$ PBS-57/CD1d

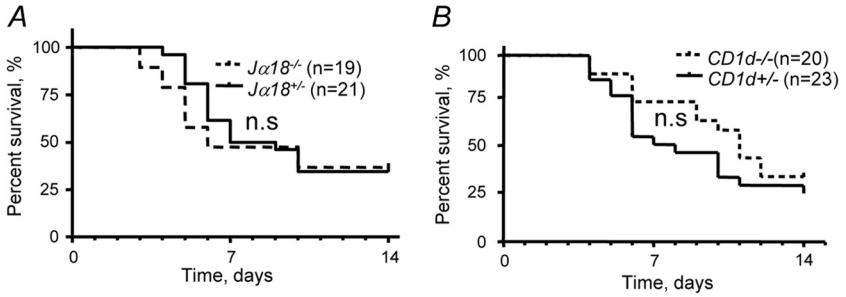

FIG 2 Impact of CD1d-restricted type I and type II NKT cells in experimental *S. aureus* sepsis. (A) *Ja18*$^{-/-}$ mice ($n = 19$) and their heterozygous littermates ($n = 21$), as well as (B) *CD1d*$^{-/-}$ mice ($n = 20$) and their heterozygous littermates ($n = 23$), were inoculated with *S. aureus* LS-1 (1.5×10^8 to 2×10^8 CFU/mouse). Mice were monitored for survival over a period of 14 days. Data from two separate experiments were pooled in both A and B. Statistical evaluations were performed using the Kaplan-Meier log-rank test.

tetramer-negative cells are activated by the infection, as indicated by a 2- to 3-fold upregulation of CD69. Thus, NKT cells were activated and had accumulated in these organs already by day 3 as a result of the infection and could play a role in the early antibacterial immune response. To address whether NKT cells influence the pathogenesis of *S. aureus* sepsis, we first studied the course of disease in Jα18-deficient mice lacking type I NKT cells. The survival curve in response to a lethal inoculum of *S. aureus* of Jα18-deficient mice was identical to that of littermate control mice (Fig. 2A). We next infected CD1d-deficient mice lacking all NKT cells with *S. aureus*. The overall mortalities were similar in CD1d-deficient and control groups at the endpoint of the experiments (Fig. 2B). This demonstrates that neither type I nor type II CD1d-restricted NKT cells significantly altered survival after a lethal inoculum of *S. aureus*.

Sulfatide treatment increased survival in *S. aureus* sepsis. To test the effect of sulfatide treatment in staphylococcal sepsis, mice inoculated with *S. aureus* were treated with sulfatide 1 h before and 3 days after infection. The lower dose of *S. aureus* (4×10^7 CFU/mouse) caused moderate sepsis with 40% mortality on day 14 (Fig. 3A), while a high dose (2×10^8 to 3×10^8 CFU/mouse) resulted in 100% mortality already on day 10 (Fig. 3B). Treatment with sulfatide significantly protected mice from both moderate ($P = 0.04$) (Fig. 3A) and severe sepsis ($P = 0.0007$) (Fig. 3B).

To test if one dose of sulfatide was sufficient to ameliorate sepsis, mice with severe sepsis were treated with a single sulfatide injection on day 3. No difference in mortality between groups was observed (Fig. 3C), demonstrating that a single injection at the late time point was not enough to elicit a therapeutic effect.

To exclude a direct bacteriostatic effect by sulfatide, we added sulfatide to nutrient broth at concentrations similar to or exceeding 10-fold, which are the estimated concentrations present in mice undergoing sulfatide treatment. The addition of sulfatide had no impact on bacterial growth (data not shown).

The protective effect of sulfatide treatment was dependent on the presence of CD1d but not on type I NKT cells. To elucidate if the protective effect of sulfatide is mediated through NKT cells, *CD1d*$^{-/-}$ mice were infected with a high dose of *S. aureus* and received sulfatide therapy on day 1 and day 3 as described above. All CD1d-deficient mice developed severe sepsis, and approximately 70% of them had died in both groups by day 14. No beneficial effect of sulfatide on the survival of *CD1d*$^{-/-}$ mice was observed (Fig. 4A), suggesting that CD1d-restricted NKT cells were required for the effect of sulfatide in wild-type (WT) mice. To investigate whether type I NKT cells played any role in the beneficial effects of sulfatide on sepsis, we tested *Jα18*$^{-/-}$ mice that harbor type II but not type I NKT cells. In contrast to the findings for CD1d-deficient mice, there was significant improvement in the survival of septic *Jα18*$^{-/-}$ mice receiving sulfatide compared to that of septic *Jα18*$^{-/-}$ mice receiving PBS ($P = 0.02$) (Fig. 4B). Taken together, these findings suggest that the protective effect of sulfatide in *S. aureus* sepsis was mediated by type II NKT cells and did not require the presence of type I NKT cells.

Sulfatide treatment downregulated systemic inflammation. A cytokine storm induced by the bacteria mediates lethality in the early stage of sepsis (36). Despite similar bacterial counts in blood, liver, and kidneys (Fig. 5A), on day 3 septic mice receiving sulfatide had significantly lower TNF-α levels than controls ($P =$

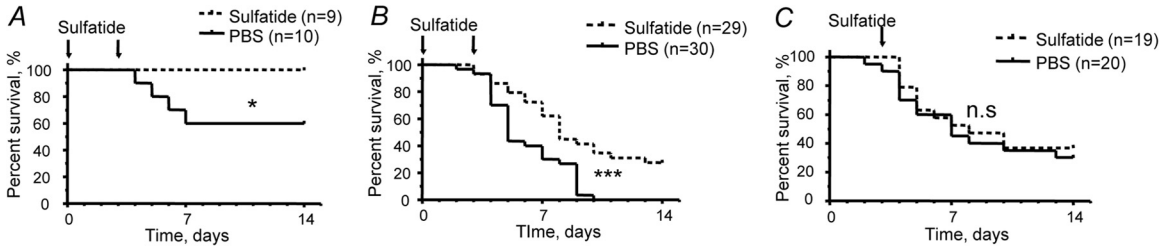

FIG 3 Sulfatide treatment ameliorated *S. aureus* sepsis in mice. C57BL/6 mice inoculated with 4×10^7 CFU/mouse (one experiment) (A), 2×10^8 to 3×10^8 CFU/mouse (pool of 3 experiments) (B), or 1.5×10^8 to 3×10^8 CFU/mouse (pool of 3 experiments) (C) of *S. aureus* LS-1 received i.p. injections of sulfatide (dotted line) or of the same volume of PBS (solid line) 1 h before and 3 days after bacterial inoculation (A and B) or only on day 3 after infection (C). The mice were monitored for survival over a period of 14 days. Statistical evaluations were performed using the Kaplan-Meier log-rank test. n.s., not significant; *, $P < 0.05$; ***, $P < 0.001$.

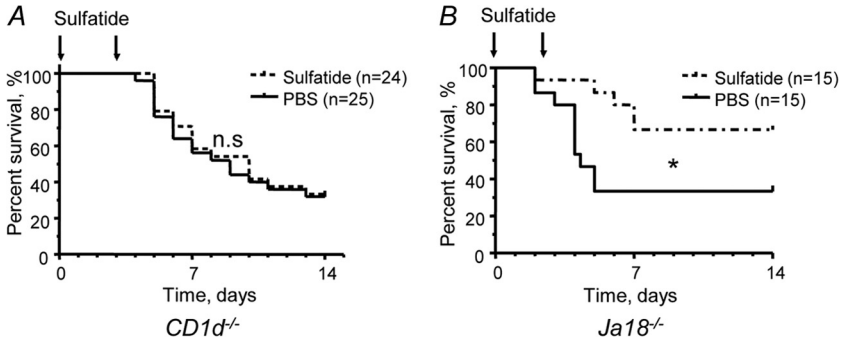

FIG 4 The protective effect of sulfatide treatment was dependent on the presence of CD1d but not on type I NKT cells. (A) *CD1d*$^{-/-}$ mice and (B) *Ja18*$^{-/-}$ mice inoculated with *S. aureus* LS-1 (1.5×10^8 to 2×10^8 CFU/mouse) received i.p. injections of sulfatide (dotted line) or the same volume of PBS (solid line) 1 h before and 3 days after bacterial inoculation. The mice were monitored for survival over a period of 14 days. The data from two separate experiments were pooled in both A and B. Statistical evaluations were performed using the Kaplan-Meier log-rank test. n.s., not significant; *, $P < 0.05$.

0.04) (Fig. 5B). A distinct reduction in IL-6 levels was observed already on day 1 ($P = 0.01$) and became more apparent on day 3 ($P = 0.008$) in the plasma of mice that received sulfatide compared to that of mice injected with PBS (Fig. 5C). PAI-1 acts as an acute-phase protein during acute inflammation. The elevated PAI-1 levels during sepsis were not significantly altered by sulfatide treatment (Fig. 5D). Thus, sulfatide activation of type II NKT cells resulted in decreased systemic levels of proinflammatory cytokines.

Effects of sulfatide on hemostatic markers in *S. aureus* sepsis. Thrombopenia and impaired fibrinolysis are hallmarks of pathogenesis in septic shock, and the dysregulated coagulation/fibrinolysis results in DIC in severe sepsis (6). Sulfatide treatment significantly increased the platelet counts ($P < 0.01$) (Fig. 6A), suggesting that sulfatide treatment moderates the development of DIC in *S. aureus* sepsis. There was a tendency of increased plasmin activity and low levels of fibrinogen in the sulfatide-treated group compared to controls, although the differences were not significant (Fig. 6B and C). We conclude that sulfatide treatment suppressed systemic inflammation and may moderate the development of DIC in mice with *S. aureus* sepsis.

DISCUSSION

In this study, we demonstrated that sulfatide treatment significantly improves the survival rate of mice with *S. aureus* lethal sepsis, being accompanied by decreased levels of TNF-α and IL-6 in the blood. Importantly, the protective effect of sulfatide treat-

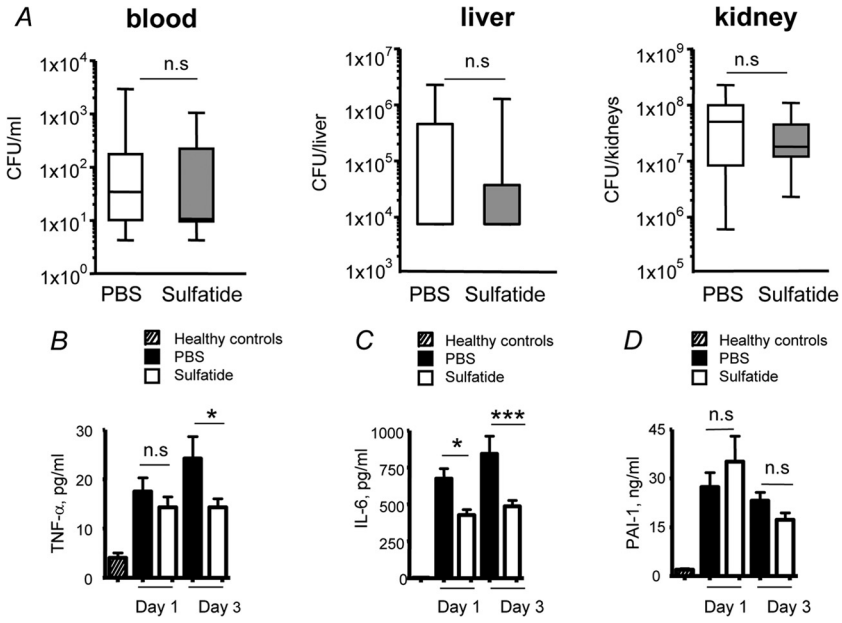

FIG 5 Sulfatide treatment downregulated systemic inflammation. C57BL/6 mice were inoculated with *S. aureus* LS-1 (4×10^7 to 2×10^8 CFU/mouse), and sulfatide was injected i.p. into mice 1 h before the bacterial inoculation (sulfatide). Infected control mice were injected with the same volume of phosphate-buffered saline (PBS). Blood, kidneys, and livers were collected 1 and 3 days after the bacterial inoculation. (A) Persistence of *S. aureus* in blood, liver, and kidneys 3 days after infection. The levels of TNF-α (B), IL-6 (C), and plasminogen activator inhibitor type 1 (PAI-1) (D) in the blood on day 1 and day 3 after bacterial inoculation are shown. Noninfected mice were used as controls in panels B to D (healthy controls). Statistical evaluations were performed using the Mann-Whitney U test. n.s., not significant; *, $P < 0.05$; ***, $P < 0.001$. Data have been pooled from three separate experiments ($n = 15$ to 16/group).

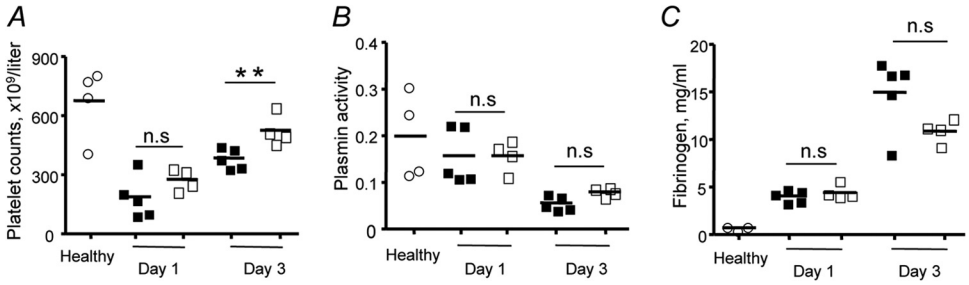

FIG 6 Effects of sulfatide on hemostatic markers in *S. aureus* sepsis. C57BL/6 mice were inoculated with *S. aureus* LS-1 (2×10^8 CFU/mouse). Sulfatide was injected i.p. into mice 1 h before the bacterial inoculation. Mice injected with the same volume of phosphate-buffered saline (PBS) served as controls. Blood was collected 1 and 3 days after the bacterial inoculation. The platelet counts (A), plasmin activity levels (B), and fibrinogen levels (C) in blood from mice with *S. aureus* sepsis on day 1 and day 3 after bacterial inoculation are shown. Each symbol represents the value from one mouse. Open circles (○) represent values from noninfected (healthy) mice; closed squares (■) represent values from septic mice receiving PBS; open squares (□) represent values from septic mice receiving sulfatide treatment. Statistical evaluations were performed using the Mann-Whitney U test. n.s., not significant; **, $P < 0.01$. Data are from one representative experiment of three performed ($n = 4$ to 5 mice/group).

ment was dependent on CD1d expression but not on type I NKT cells, suggesting a role for type II NKT cells.

Increasing evidence indicates that the immune response in the course of sepsis consists of an initial hyperreactive phase and a latent phase with reduced host defense (36). The hyperreactive phase is characterized by a potentially fatal cytokine storm induced by activated monocytes, macrophages, and other immune cells. Previous studies are consistent with a role for NKT cells in the early response to microbial infection, demonstrating that type I NKT cells can contribute to lipopolysaccharide (LPS)-induced endotoxic shock and Gram-negative sepsis (19). Mice lacking type I NKT cells had lower levels of circulating gamma interferon (IFN-γ) and TNF-α and increased survival in the generalized Shwartzman reaction (20). Studies of polymicrobial septic shock provide divergent data; Jα18-deficient C57BL/6J mice lacking type I NKT cells showed an increased survival level and decreased levels of proinflammatory cytokines in the serum (21), while another study found that CD1d-deficient mice lacking all NKT cells did not differ from WT mice in septic mortality and induction of proinflammatory cytokines (37). In the *S. aureus* model of sepsis studied here, despite the activation of type I NKT cells by the bacterial injection, there was no significant difference in mortality rate in mice lacking type I NKT cells ($J\alpha 18^{-/-}$) or in CD1d-deficient mice compared to their heterozygote littermates. We therefore conclude that while type I NKT cells may promote endotoxic shock and polymicrobial sepsis, neither type I nor type II NKT cells played a significant role in mortality in *S. aureus* sepsis.

In the present study, downregulation of the proinflammatory cytokines TNF-α and IL-6 by sulfatide treatment suggests that reduction of the initial hyperreactive phase of immune response is an important underlying mechanism for the protective effect of sulfatide in sepsis. Indeed, sulfatide has been reported to attenuate LPS-induced lung injury in rats (38) and to protect against endotoxin shock in mice with concomitant reduction in TNF-α production (39). However, a proper inflammatory response is crucial for the host to eliminate the microbes. TNF-α is known as a key early response cytokine involved in innate immunity against microbes. TNF-deficient mice are highly susceptible to *S. aureus* infection due to inefficient bacterial clearance (40, 41). Also, IL-6 deficiency leads to impaired immune responses against both viral and bacterial infection (42). Indeed, patients treated with TNF inhibitors or anti-IL-6 therapy are at an increased risk of developing certain infections (43–47). In the present study, sulfatide treatment efficiently downregulated expression of key cytokines, i.e., TNF-α and IL-6 in the cytokine storm during sepsis, but it did not prevent the immune response from clearing the microbes from different organs; there was even a tendency that sulfatide treatment decreased the bacterial load in kidneys on day 3 after bacterial inoculation. This indicates that sulfatide treatment was able to maintain an adequate immune response to the bacteria while preventing lethal levels of proinflammatory reactions. Sulfatide has also been described as a ligand for L-selectin (48), and in some of the studies mentioned above (38, 39) it was postulated that sulfatide-mediated inhibition of LPS-induced lung injury and endotoxic shock was due to sulfatide blocking the function of L-selectin. In the *S. aureus* sepsis model, we show that the beneficial effect of sulfatide depends on CD1d, suggesting that sulfatide activates CD1d-restricted sulfatide-specific type II NKT cells that ameliorate *S. aureus* sepsis. It is interesting that sulfatide-reactive CD1d-restricted T cells expressing TCRγδ were recently described in human peripheral blood lymphocytes (49). Sulfatide-reactive CD1d-restricted TCRγδ cells have not been described in mice, and the sulfatide-reactive T cells hitherto described in mice carry TCRαβ (8); however, we cannot exclude a contribution by sulfatide-specific, CD1d-restricted TCRγδ T cells in our study.

There are some studies that suggest a cross-talk between the two subsets of NKT cells; e.g., activation of type II NKT cells by sulfatide induces anergy in type I NKT cells, which in turn prevents experimental concanavalin A-induced hepatitis (18) and hepatic ischemic reperfusion injury (17). Type I NKT cells play a key role in both of these disease models; therefore, the induction of anergy in type I NKT cells by sulfatide treatment results in suppression of disease development. In the present study, sulfatide treatment significantly improved the outcome of *S. aureus* sepsis in $J\alpha 18^{-/-}$ mice but failed to do so in $CD1d^{-/-}$ mice, supporting the notion that the protective effect of sulfatide in *S. aureus* sepsis is mediated through CD1d-dependent type II NKT cells without the involvement of type I NKT cells. The independence of type I NKT cells for the effect of sulfatide in *S. aureus* sepsis was consistent with the demonstration that type I NKT cells did not have a detrimental role in this model. Thus, sulfatide modulates the immune responses of other cells than type I NKT cells, as has also been established in a tumor model in which sulfatide was shown to increase lung metastasis by suppressing tumor immunity both in

WT mice and mice lacking type I NKT cells but not in CD1d-deficient mice (50). The mechanism underlying the immunomodulatory role of sulfatide-activated type II NKT cells is not known; however, an anti-inflammatory effect is further supported by the prevention of both experimental autoimmune encephalomyelitis and type 1 diabetes by sulfatide administration, which is associated with decreased IFN-γ and increased IL-10 production (15).

High expression of proinflammatory cytokines during severe sepsis induces a shift in the hemostatic balance toward coagulation, leading to systemic activation of the coagulation system and subsequent fibrin deposition (51, 52). At the same time, the fibrinolysis is markedly impaired by the release of PAI-1 (53, 54). The most extreme manifestation of such a change is DIC leading to multiple-organ failure and death. Early inhibition of activated fibrinolysis and high levels of D-dimer are known to correlate with the fatal outcomes of some infectious diseases (55, 56). In the present study, distinctly decreased plasmin activities and reduced platelet counts in the blood indicate an impaired fibrinolysis and platelet consumption in the *S. aureus* sepsis model. Significantly higher thrombocyte counts in sulfatide-treated mice suggest improved clinical signs of DIC by the treatment, which might explain improved overall survival of *S. aureus* sepsis.

The lessons of history from clinical trials of sepsis teach us that promising results from animal studies cannot necessarily be easily translated into the clinical setting. In the present study, the single late dose of sulfatide failed to enhance survival when it was given on day 3, when infection symptoms debuted, suggesting that future clinical application of sulfatide in *S. aureus* sepsis based on these results is limited. However, the time window of sulfatide administration sustaining an efficient protection against *S. aureus* sepsis should be further explored in future studies to see whether a successful therapeutic treatment schedule can be achieved. As a first step toward this goal, this study for the first time demonstrated that sulfatide treatment significantly improved the overall outcome of experimental *S. aureus* sepsis by downregulating the hyperactivation of the inflammatory system and simultaneously maintaining an adequate immune response to limit bacterial growth. Our results support a model in which the protective effect of sulfatide is mediated through type II NKT cells.

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