# Sulfatide attenuates experimental *Staphylococcal aureus* sepsis through a CD1d dependent pathway

Jakub Kwiecinski[1], Sara Rhost[2], Linda Löfbom[2], Maria Blomqvist[3], Jan Eric Månsson[3], Susanna L. Cardell[2] and Tao Jin[1]

1. Department of Rheumatology and Inflammation Research, Sahlgrenska Academy at University of Gothenburg, S-41346 Göteborg, Sweden
2. Department of Microbiology and Immunology, Institute of Biomedicine, Sahlgrenska Academy at University of Gothenburg, S-405 30 Göteborg, Sweden
3. Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, Sahlgrenska Academy at University of Gothenburg, S-431 41 Mölndal, Sweden.

**Running title:** Sulfatide attenuates *S. aureus* sepsis

**Address for correspondence**:
Tao Jin, MD, Ph.D
Department of Rheumatology and Inflammation Research,
Guldhedsgatan 10A, S-413 46 Göteborg, Sweden
FAX: 46-31-823925 Phone: 46-31-3426475
E-mail: tao.jin@rheuma.gu.se

*** We do not have any commercial associations that might pose a conflict of interest.**

Word count: Abstract 135; Manuscript: 3669

Jakub Kwiecinski and Sara Rhost contributed equally to this work. Also, Dr. Jin and Prof. Cardell contributed equally to the study design and data interpretation.

**Funding**

This work was supported by the Göteborg Medical Society, the Swedish Medical Society, the Rune and Ulla Amlövs Foundation, the Åke-Wiberg Foundation, the Thölens and Kristlers Family Foundation, the Tore Nilsons Foundation, the Swedish Rheumatism Association, the Göteborg Rheumatism Association, the Strategic Research Center for Mucosal Immunobiology and Vaccines (MIVAC), the Swedish Cancer Foundation [CAN2010/793], the Swedish Research Council [K2010-57X-14063-10-3], LUA-ALF Göteborg [ALF-GBG-151251] to SC, and the W and M Lundgrens Foundation to SR. SR was financed by a PhD project grant from the Sahlgrenska Academy, University of Gothenburg.

**Acknowledgements**

We thank the NIH tetramer facility for providing PBS-57 loaded CD1d-tetramers.

## *Abstract*

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. 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 TNF-α and IL-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.



**Key words**: iNKT cells, type II NKT cells, sulfatide, *Staphylococcus aureus*, sepsis

## INTRODUCTION

Bacterial sepsis is a leading cause of death in hospital intensive care units (2). The main pathogen responsible for those infections is *Staphylococcus aureus* (*S. aureus*) (41). Due to limited efficacy of available treatments, mortality in complicated staphylococcal sepsis exceeds 50% (31). Additional challenge is posed by increasing antibiotic resistance of *S. aureus* and spread of highly virulent methicillin-resistant strains (20). This makes staphylococcal sepsis a major healthcare challenge and urges a search for better treatment alternatives.

One of the hallmarks of sepsis is a deregulated immune response to infection (25). It is characterized by an early acute phase with an intense inflammatory response to the disseminated bacteria, with systemic elevation of pro-inflammatory cytokines such as IL-6 and TNF-α, followed by an immunosuppressed state causing an inability to clear the primary infection and increased risk of secondary infections. Severe sepsis can lead to disseminated intravascular coagulation (DIC), multi-organ failure and death (35). Natural killer T (NKT) cells are a subset of T lymphocytes restricted by the CD1d glycoprotein, an MHC class I-like molecule (7, 45). Unlike most T cells, NKT cells do not recognize protein antigens, but instead recognize lipid and glycolipid antigens presented on CD1d, and upon activation rapidly secrete vast quantities of cytokines to modify immune responses, being a bridge between innate and adaptive immunity(6, 9, 49). NKT cells contribute to the early immune response to a broad range of pathogens (55). 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 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 (19): type I NKT cells (also known as invariant NKT cells, 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 (45). A subset of type II NKT cells recognizes sulfatide (a self-glycosphingolipid derived from myelin) presented on CD1d (10, 28, 40). Treatment with native sulfatide was shown to modulate different diseases in murine models, providing protection from experimental autoimmune encephalitis, experimental hepatitis, hepatic ischemic reperfusion injury and causing anergy in type I NKT cells (3, 21, 28).

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 (34). Initial studies pointed to a detrimental role of NKT cells in sepsis by magnifying damage and increasing mortality (15, 23, 38, 44, 48). However, those studies either did not discriminate between type I and type II NKT cells (38, 44, 48) or focused exclusively on type I cells (15, 23). Information about the relative activities of the two types of NKT cells in sepsis is therefore not available. Moreover, previous studies used models of gram-negative septic shock (15, 38) and polymicrobial, predominantly gram-negative sepsis (23, 44, 48). A significant proportion of hospital cases of sepsis is due to gram-positive cocci, which induce a different inflammatory response compared to gram negative bacteria (50). The function of NKT cells in various milder infections is dependent on the type of infecting agent, and thus NKT cells play a beneficial role in some infections (36, 37) and detrimental in others (49). Therefore, observations from gram-negative sepsis models cannot be extrapolated to the *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 (53). We found that the presence of NKT cells did not have a

1 significant impact on mortality in this sepsis model. Activation of type II NKT cells with
2 sulfatide exerted a protective effect associated with decrease in the systemic levels of pro-
3 inflammatory cytokines.
4

## MATERIALS AND METHODS

### Mice

Female C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). *CD1d*$^{-/-}$ (12) and *Jα18*$^{-/-}$ (13) 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 at 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 (47), had a purity of >95% determined by thin layer chromatography and mass spectrometry. Sulfatide was dissolved at 10 times final concentration in phosphate buffered saline (PBS), sonicated for 10 minutes followed by heating at 80°C for two minutes, and diluted to 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: 1) Standard treatment protocol - the animals received intraperitoneal (i.p.) injection of 25 nmol sulfatide in 200 $\mu$l PBS or the same volume of PBS one hour before and 3 days after bacterial inoculation; 2) Late treatment protocol - the mice received i.p. injection of 25 nmol sulfatide in 200 $\mu$l 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 (11), and has been used previously to study the staphylococcal sepsis in mice (17, 30). In most experiments, a lethal dose of bacteria ($1.5\text{-}3x10^8$ cfu/mouse) was inoculated, while a lower dose of bacteria ($4x10^7$ cfu/mouse) was inoculated in a limited number of experiments as indicated. Weight loss and mortality of animals were followed 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 day 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% (v/v) horse blood. Bacteria were grown for 24 h and quantified as CFUs 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 specific plasmin substrate S-2251 (H-D-Val-Leu-Lys-pNA.2HCL) as described previously (29). 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, USA), respectively. The levels of TNF-α and IL-6 in plasma were determined using a DuoSet ELISA Development Kit (*R&D* Systems Europe, Ltd).

**Assessment of the influence of sulfatide on *S. aureus* growth *in vitro***

*S. aureus* LS-1 ($6x10^3$/ml in TSB) at 37°C was incubated with sulfatide (0, 12.5, 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 minutes, 4°C) followed by staining with allophycocyanin-conjugated, CD1d-tetramers loaded with PBS-57 (provided by the NIH tetramer facility) for 30 minutes at room temperature. After washing, the following antibodies were added: FITC-CD19 (clone ID3), PE-TCRβ (clone H57-597) and PE-Cy7-CD69 (clone H1.2F3), and the cells were incubated for 15-20 minutes at 4°C. Type I NKT cells were gated as $CD19^-$ $TCR\beta^+$ 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), PerCP-B220 (clone RA3-6B2), PE-Cy7-CD69 (clone H1.2F3), and gating was done for $B220^-$ $TCR\beta^+$ PBS-57/CD1d-$tetramer^+$ cells (type I NKT cells), and for $B220^-$ $TCR\beta^+$ $NK1.1^+$ PBS-57/CD1d-$tetramer^-$ cells. Samples were analyzed with a FACS LSRII 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 logrank survival test. The results are reported as the median and interquartile range (IQR) or the mean ± the standard error of the mean (SEM).

## RESULTS

### NKT cells did not impact significantly on 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 two-fold compared to non-infected mice (Fig.1A). Type II NKT cells cannot be identified with certainty by surface markers (45), but type II NKT cells can express NK1.1 (45) and thus the population of $TCR\beta^+$ $NK1.1^+$ cells that is negative for the type I NKT cell specific PBS57-CD1d-tetramer should contain a proportion of type II NKT cells. Preliminary data support that also $TCR\beta^+$ $NK1.1^+$ PBS57-CD1d-tetramer negative cells are activated by the infection, indicated by a 2-3 fold upregulation of CD69. Thus, NKT cells were activated and had accumulated in these organs already day 3 as a result of the infection and could potentially play a role in the early anti-bacterial 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. Jα18 deficient mice showed an identical survial curve in response to a lethal inoculum of *S. aureus* when compared to littermate control mice (Fig.2A). We next infected CD1d deficient mice lacking all NKT cells with *S. aureus*. The overall mortality was 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 hour before and 3 days after infection. The lower dose of *S. aureus* ($4x10^7$ cfu/mouse) caused moderate sepsis with 40% mortality on day 14

(Fig.3A), while a high dose (2-3x$10^8$ 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, 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 the sulfatide therapy day 1 and day 3 as before. 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 mice. To investigate whether type I NKT cells played any role for the beneficial effects of sulfatide on sepsis, we tested *Jα18*$^{-/-}$ mice that harbour type II but not type I NKT cells. In contrast to the findings in CD1d deficient mice, there was significant improvement in the survival of septic *Jα18*$^{-/-}$ mice receiving sulfatide, compared to septic *Jα18*$^{-/-}$ mice receiving PBS ($p$=0.02, Fig.4B). Taken together, this suggests 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 down-regulated systemic inflammation**

A cytokine storm induced by the bacteria mediates lethality in the early stage of sepsis (46). 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 ($p$=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 in comparison with 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 pro-inflammatory cytokines.

**The effects of sulfatide on hemostatic markers in *S. aureus* sepsis**

Thrombopenia and impaired fibrinolysis are hallmarks of the pathogenesis in septic shock, and the dysregulated coagulation/fibrinolysis result in DIC in severe sepsis (35). Sulfatide treatment significantly increased the platelet counts (Fig.6A, $p$<0.01), suggesting that sulfatide treatment moderates the development of DIC in *S. aureus* sepsis. There was a tendency of increased plasmin activity and lower levels of fibrinogen in the sulfatide treated group compared to controls, although the differences were not significant (Fig 6B-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 treatment 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 hyper-reactive phase and a latent phase with reduced host defense (46). The hyper-reactive 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 LPS induced endotoxic shock and gram negative sepsis (34). Mice lacking type I NKT cells had lower levels of circulating IFN-γ and TNF-α and increased survival in the generalized Shwartzman reaction (15). Studies of polymicrobial septic shock provide divergent data; Jα18-deficient C57BL/6J mice lacking type I NKT cells showed an increased survival, and decreased levels of pro-inflammatory cytokines in the serum (23), while another study found that CD1d-deficient mice lacking all NKT cells did not differ from WT mice in septic mortality and induction of pro-inflammatory cytokines (16). 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 ($J\alpha18^{-/-}$) nor 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 for mortality in *S. aureus* sepsis.

In the present study, down-regulation of the pro-inflammatory cytokines TNF-α and IL-6 by sulfatide treatment suggests that reduction of the initial hyper-reactive phase of immune response might be an important underlying mechanisms for the protective effect of sulfatide in sepsis. Indeed, sulfatide has been reported to attenuate LPS-induced lung injury in rats (51) and protect against endotoxin shock in mice with concomitant reduction in TNF-α production (22). 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 (24, 52). Also, IL-6 deficiency leads to impaired immune responses against both viral and bacterial infection (33). Indeed, patients treated with TNF inhibitors or anti IL-6 therapy are at an increased risk of developing certain infections (14, 18, 26, 32, 39). 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 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 (4), and in the studies mentioned above (22, 51), 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 to note that sulfatide reactive CD1d-restricted T cells expressing the γδ TCR were recently described in human peripheral blood lymphocytes (5). Sulfatide reactive CD1d-restricted TCRγδ cells have not been described in the mouse; the

sulfatide reactive T cells hitherto described in the mouse carry the TCRαβ (45), however, we cannot exclude a contribution by sulfatide specific, CD1d-restricted TCRγδ T cells in our study.

There are some studies that suggest a crosstalk 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 (21) and hepatic ischemic reperfusion injury (3). Type I NKT cells play a key role in both of these disease models, and 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 *Jα18*$^{-/-}$ 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 also 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 (1). 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, associated with a decreased IFN-γ and increased IL-10 production (28).

High expression of pro-inflammatory cytokines during severe sepsis induces a shift in the hemostatic balance towards coagulation, leading to systemic activation of the coagulation system and subsequent fibrin deposition (54, 56). At the same time, the fibrinolysis is

markedly impaired by the release of PAI-1 (8, 27). 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 (42, 43). 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 the history from clinical trials in sepsis teach us that promising results from animal studies can not 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 probably 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 therepeutic treatment schedule can be achieved. As a first step towards 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 simutaneously 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 cell.

**References:**


1. **Ambrosino, E., M. Terabe, R. C. Halder, J. Peng, S. Takaku, S. Miyake, T. Yamamura, V. Kumar, and J. A. Berzofsky. 2007. Cross-regulation between type I and type II NKT cells in regulating tumor immunity: a new immunoregulatory axis. J Immunol 179:5126-5136.**
2. **Angus, D. C., W. T. Linde-Zwirble, J. Lidicker, G. Clermont, J. Carcillo, and M. R. Pinsky. 2001. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Critical care medicine 29:1303-1310.**
3. **Arrenberg, P., I. Maricic, and V. Kumar. 2011. Sulfatide-mediated activation of type II natural killer T cells prevents hepatic ischemic reperfusion injury in mice. Gastroenterology 140:646-655.**
4. **Aruffo, A., W. Kolanus, G. Walz, P. Fredman, and B. Seed. 1991. CD62/P-selectin recognition of myeloid and tumor cell sulfatides. Cell 67:35-44.**
5. **Bai, L., D. Picard, B. Anderson, V. Chaudhary, A. Luoma, B. Jabri, E. J. Adams, P. B. Savage, and A. Bendelac. 2012. The majority of CD1d-sulfatide-specific T cells in human blood use a semiinvariant Vdelta1 TCR. Eur J Immunol 42:2505-2510.**
6. **Behar, S. M., and S. Cardell. 2000. Diverse CD1d-restricted T cells: diverse phenotypes, and diverse functions. Semin Immunol 12:551-560.**
7. **Bendelac, A., P. B. Savage, and L. Teyton. 2007. The biology of NKT cells. Annu Rev Immunol 25:297-336.**
8. **Bertozzi, P., B. Astedt, L. Zenzius, K. Lynch, F. LeMaire, W. Zapol, and H. A. Chapman, Jr. 1990. Depressed bronchoalveolar urokinase activity in patients with adult respiratory distress syndrome. N Engl J Med 322:890-897.**
9. **Berzins, S. P., M. J. Smyth, and A. G. Baxter. 2011. Presumed guilty: natural killer T cell defects and human disease. Nat Rev Immunol 11:131-142.**
10. **Blomqvist, M., S. Rhost, S. Teneberg, L. Lofbom, T. Osterbye, M. Brigl, J. E. Mansson, and S. L. Cardell. 2009. Multiple tissue-specific isoforms of sulfatide activate CD1d-restricted type II NKT cells. Eur J Immunol 39:1726-1735.**
11. **Bremell, T., S. Lange, L. Svensson, E. Jennische, K. Grondahl, H. Carlsten, and A. Tarkowski. 1990. Outbreak of spontaneous staphylococcal arthritis and osteitis in mice. Arthritis Rheum 33:1739-1744.**
12. **Chen, Y. H., N. M. Chiu, M. Mandal, N. Wang, and C. R. Wang. 1997. Impaired NK1+ T cell development and early IL-4 production in CD1-deficient mice. Immunity 6:459-467.**
13. **Cui, J., T. Shin, T. Kawano, H. Sato, E. Kondo, I. Toura, Y. Kaneko, H. Koseki, M. Kanno, and M. Taniguchi. 1997. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. Science 278:1623-1626.**
14. **Curtis, J. R., N. Patkar, A. Xie, C. Martin, J. J. Allison, M. Saag, D. Shatin, and K. G. Saag. 2007. Risk of serious bacterial infections among rheumatoid arthritis patients exposed to tumor necrosis factor alpha antagonists. Arthritis Rheum 56:1125-1133.**
15. **Dieli, F., G. Sireci, D. Russo, M. Taniguchi, J. Ivanyi, C. Fernandez, M. Troye-Blomberg, G. De Leo, and A. Salerno. 2000. Resistance of natural killer T cell-deficient mice to systemic Shwartzman reaction. J Exp Med 192:1645-1652.**

16. Etogo, A. O., J. Nunez, C. Y. Lin, T. E. Toliver-Kinsky, and E. R. Sherwood. 2008. NK but not CD1-restricted NKT cells facilitate systemic inflammation during polymicrobial intra-abdominal sepsis. J Immunol 180:6334-6345.
17. Fei, Y., W. Wang, J. Kwiecinski, E. Josefsson, R. Pullerits, I. M. Jonsson, M. Magnusson, and T. Jin. 2011. The combination of a tumor necrosis factor inhibitor and antibiotic alleviates staphylococcal arthritis and sepsis in mice. J Infect Dis 204:348-357.
18. Gardam, M. A., E. C. Keystone, R. Menzies, S. Manners, E. Skamene, R. Long, and D. C. Vinh. 2003. Anti-tumour necrosis factor agents and tuberculosis risk: mechanisms of action and clinical management. Lancet Infect Dis 3:148-155.
19. Godfrey, D. I., H. R. MacDonald, M. Kronenberg, M. J. Smyth, and L. Van Kaer. 2004. NKT cells: what's in a name? Nat Rev Immunol 4:231-237.
20. Gould, I. M., M. Z. David, S. Esposito, J. Garau, G. Lina, T. Mazzei, and G. Peters. 2012. New insights into meticillin-resistant Staphylococcus aureus (MRSA) pathogenesis, treatment and resistance. Int J Antimicrob Agents 39:96-104.
21. Halder, R. C., C. Aguilera, I. Maricic, and V. Kumar. 2007. Type II NKT cell-mediated anergy induction in type I NKT cells prevents inflammatory liver disease. J Clin Invest 117:2302-2312.
22. Higashi, H., Y. Suzuki, N. Mukaida, N. Takahashi, D. Miyamoto, and K. Matsushima. 1997. Intervention in endotoxin shock by sulfatide (I3SO3-GalCer) with a concomitant reduction in tumor necrosis factor alpha production. Infect Immun 65:1223-1227.
23. Hu, C. K., F. Venet, D. S. Heffernan, Y. L. Wang, B. Horner, X. Huang, C. S. Chung, S. H. Gregory, and A. Ayala. 2009. The role of hepatic invariant NKT cells in systemic/local inflammation and mortality during polymicrobial septic shock. J Immunol 182:2467-2475.
24. Hultgren, O., H. P. Eugster, J. D. Sedgwick, H. Korner, and A. Tarkowski. 1998. TNF/lymphotoxin-alpha double-mutant mice resist septic arthritis but display increased mortality in response to Staphylococcus aureus. J Immunol 161:5937-5942.
25. Huttunen, R., and J. Aittoniemi. 2011. New concepts in the pathogenesis, diagnosis and treatment of bacteremia and sepsis. J Infect 63:407-419.
26. Hyrich, K. L., A. J. Silman, K. D. Watson, and D. P. Symmons. 2004. Anti-tumour necrosis factor alpha therapy in rheumatoid arthritis: an update on safety. Ann Rheum Dis 63:1538-1543.
27. Idell, S., K. K. James, E. G. Levin, B. S. Schwartz, N. Manchanda, R. J. Maunder, T. R. Martin, J. McLarty, and D. S. Fair. 1989. Local abnormalities in coagulation and fibrinolytic pathways predispose to alveolar fibrin deposition in the adult respiratory distress syndrome. J Clin Invest 84:695-705.
28. Jahng, A., I. Maricic, C. Aguilera, S. Cardell, R. C. Halder, and V. Kumar. 2004. Prevention of autoimmunity by targeting a distinct, noninvariant CD1d-reactive T cell population reactive to sulfatide. J Exp Med 199:947-957.
29. Jin, T., M. Bokarewa, Y. Zhu, and A. Tarkowski. 2008. Staphylokinase reduces plasmin formation by endogenous plasminogen activators. Eur J Haematol 81:8-17.
30. Klak, M., N. Anakkala, W. Wang, S. Lange, I. M. Jonsson, A. Tarkowski, and T. Jin. 2010. Tranexamic acid, an inhibitor of plasminogen activation, aggravates staphylococcal septic arthritis and sepsis. Scand J Infect Dis 42:351-358.

**31.** **Klevens, R. M., M. A. Morrison, J. Nadle, S. Petit, K. Gershman, S. Ray, L. H. Harrison, R. Lynfield, G. Dumyati, J. M. Townes, A. S. Craig, E. R. Zell, G. E. Fosheim, L. K. McDougal, R. B. Carey, and S. K. Fridkin. 2007. Invasive methicillin-resistant Staphylococcus aureus infections in the United States. Jama 298:1763-1771.**

**32.** **Kling, A., T. Mjorndal, and S. Rantapaa-Dahlqvist. 2004. Sepsis as a possible adverse drug reaction in patients with rheumatoid arthritis treated with TNFalpha antagonists. J Clin Rheumatol 10:119-122.**

**33.** **Kopf, M., H. Baumann, G. Freer, M. Freudenberg, M. Lamers, T. Kishimoto, R. Zinkernagel, H. Bluethmann, and G. Kohler. 1994. Impaired immune and acute-phase responses in interleukin-6-deficient mice. Nature 368:339-342.**

**34.** **Leung, B., and H. W. Harris. 2011. NKT cells: the culprits of sepsis? J Surg Res 167:87-95.**

**35.** **Levi, M., and H. Ten Cate. 1999. Disseminated intravascular coagulation. N Engl J Med 341:586-592.**

**36.** **Lin, K. H., J. J. Liang, W. I. Huang, S. Y. Lin-Chu, C. Y. Su, Y. L. Lee, J. T. Jan, Y. L. Lin, Y. S. Cheng, and C. H. Wong. 2010. In vivo protection provided by a synthetic new alpha-galactosyl ceramide analog against bacterial and viral infections in murine models. Antimicrob Agents Chemother 54:4129-4136.**

**37.** **Minagawa, S., C. Ohyama, S. Hatakeyama, N. Tsuchiya, T. Kato, and T. Habuchi. 2005. Activation of natural killer T cells by alpha-galactosylceramide mediates clearance of bacteria in murine urinary tract infection. J Urol 173:2171-2174.**

**38.** **Ogasawara, K., K. Takeda, W. Hashimoto, M. Satoh, R. Okuyama, N. Yanai, M. Obinata, K. Kumagai, H. Takada, H. Hiraide, and S. Seki. 1998. Involvement of NK1+ T cells and their IFN-gamma production in the generalized Shwartzman reaction. J Immunol 160:3522-3527.**

**39.** **Ogata, A., T. Hirano, Y. Hishitani, and T. Tanaka. 2012. Safety and efficacy of tocilizumab for the treatment of rheumatoid arthritis. Clin Med Insights Arthritis Musculoskelet Disord 5:27-42.**

**40.** **Patel, O., D. G. Pellicci, S. Gras, M. L. Sandoval-Romero, A. P. Uldrich, T. Mallevaey, A. J. Clarke, J. Le Nours, A. Theodossis, S. L. Cardell, L. Gapin, D. I. Godfrey, and J. Rossjohn. 2012. Recognition of CD1d-sulfatide mediated by a type II natural killer T cell antigen receptor. Nat Immunol 13:857-863.**

**41.** **Pfaller, M. A., R. N. Jones, G. V. Doern, H. S. Sader, K. C. Kugler, and M. L. Beach. 1999. Survey of blood stream infections attributable to gram-positive cocci: frequency of occurrence and antimicrobial susceptibility of isolates collected in 1997 in the United States, Canada, and Latin America from the SENTRY Antimicrobial Surveillance Program. SENTRY Participants Group. Diagn Microbiol Infect Dis 33:283-297.**

**42.** **Querol-Ribelles, J. M., J. M. Tenias, E. Grau, J. M. Querol-Borras, J. L. Climent, E. Gomez, and I. Martinez. 2004. Plasma d-dimer levels correlate with outcomes in patients with community-acquired pneumonia. Chest 126:1087-1092.**

**43.** **Raaphorst, J., A. B. Johan Groeneveld, A. W. Bossink, and C. Erik Hack. 2001. Early inhibition of activated fibrinolysis predicts microbial infection, shock and mortality in febrile medical patients. Thromb Haemost 86:543-549.**

**44.** **Rhee, R. J., S. Carlton, J. L. Lomas, C. Lane, L. Brossay, W. G. Cioffi, and A. Ayala. 2003. Inhibition of CD1d activation suppresses septic mortality: a role for NK-T cells in septic immune dysfunction. J Surg Res 115:74-81.**

**45.** **Rhost, S., S. Sedimbi, N. Kadri, and S. L. Cardell. 2012. Immunomodulatory Type II Natural Killer T Lymphocytes in Health and Disease. Scand J Immunol 76:246-255.**

**46.** **Riedemann, N. C., R. F. Guo, and P. A. Ward. 2003. Novel strategies for the treatment of sepsis. Nat Med 9:517-524.**

**47.** **Rosengren, B., P. Fredman, J. E. Mansson, and L. Svennerholm. 1989. Lysosulfatide (galactosylsphingosine-3-O-sulfate) from metachromatic leukodystrophy and normal human brain. J Neurochem 52:1035-1041.**

**48.** **Sherwood, E. R., C. Y. Lin, W. Tao, C. A. Hartmann, J. E. Dujon, A. J. French, and T. K. Varma. 2003. Beta 2 microglobulin knockout mice are resistant to lethal intraabdominal sepsis. Am J Respir Crit Care Med 167:1641-1649.**

**49.** **Skold, M., and S. M. Behar. 2003. Role of CD1d-restricted NKT cells in microbial immunity. Infect Immun 71:5447-5455.**

**50.** **Skovbjerg, S., A. Martner, L. Hynsjo, C. Hessle, I. Olsen, F. E. Dewhirst, W. Tham, and A. E. Wold. 2010. Gram-positive and gram-negative bacteria induce different patterns of cytokine production in human mononuclear cells irrespective of taxonomic relatedness. J Interferon Cytokine Res 30:23-32.**

**51.** **Squadrito, F., G. Bagnato, D. Altavilla, M. Ferlito, G. M. Campo, G. Squadrito, G. Urna, A. Sardella, M. Arlotta, L. Minutoli, C. Quartarone, A. Saitta, and A. P. Caputi. 1999. Effect of sulfatide on acute lung injury during endotoxemia in rats. Life Sci 65:2541-2552.**

**52.** **Stenzel, W., S. Soltek, H. Miletic, M. M. Hermann, H. Korner, J. D. Sedgwick, D. Schluter, and M. Deckert. 2005. An essential role for tumor necrosis factor in the formation of experimental murine Staphylococcus aureus-induced brain abscess and clearance. J Neuropathol Exp Neurol 64:27-36.**

**53.** **Tarkowski, A., L. V. Collins, I. Gjertsson, O. H. Hultgren, I. M. Jonsson, E. Sakiniene, and M. Verdrengh. 2001. Model systems: modeling human staphylococcal arthritis and sepsis in the mouse. Trends Microbiol 9:321-326.**

**54.** **ten Cate, J. W., T. van der Poll, M. Levi, H. ten Cate, and S. J. van Deventer. 1997. Cytokines: triggers of clinical thrombotic disease. Thromb Haemost 78:415-419.**

**55.** **Tupin, E., Y. Kinjo, and M. Kronenberg. 2007. The unique role of natural killer T cells in the response to microorganisms. Nat Rev Microbiol 5:405-417.**

**56.** **van der Poll, T., H. R. Buller, H. ten Cate, C. H. Wortel, K. A. Bauer, S. J. van Deventer, C. E. Hack, H. P. Sauerwein, R. D. Rosenberg, and J. W. ten Cate. 1990. Activation of coagulation after administration of tumor necrosis factor to normal subjects. N Engl J Med 322:1622-1627.**

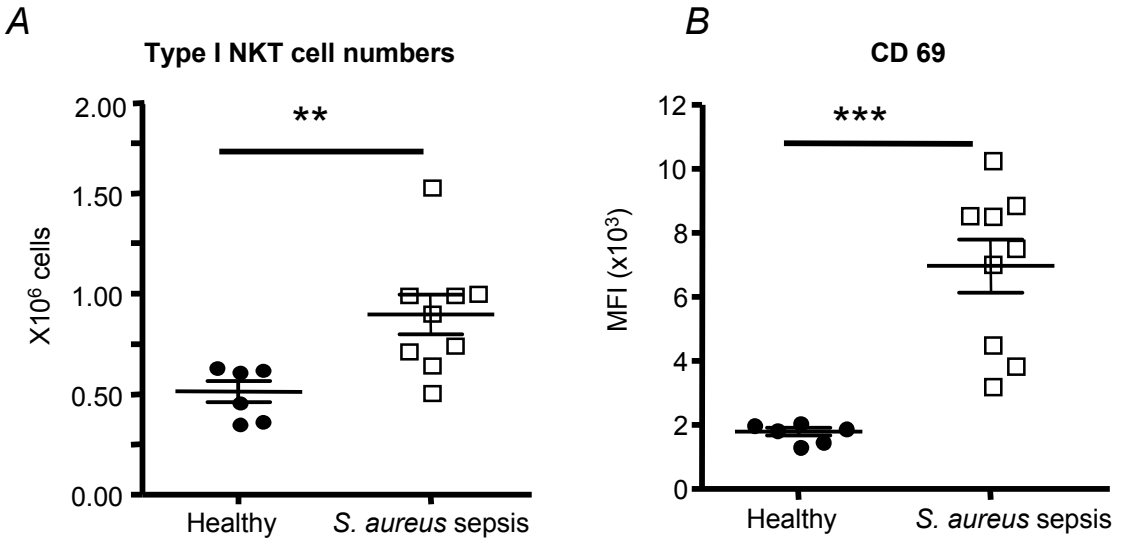


**Figure 1. *S. aureus* infection activated type I natural killer T (NKT) cells.** Mice were infected with *S. aureus* LS-1 ($4x10^7$ – $2x10^8$ cfu/mouse), and three days later spleen cells were isolated, stained and analysed by flow cytometry. A) Absolute numbers of type I NKT cells (PBS-57/CD1d tetramer positive, TCRβ positive, CD19 negative), and B) mean fluorescence intensity (MFI) of CD69 expression on type I NKT cells, in spleens of non-infected (Healthy mice) 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. ** = $p < 0.01$; *** = $p < 0.001$.

.

Figure 1

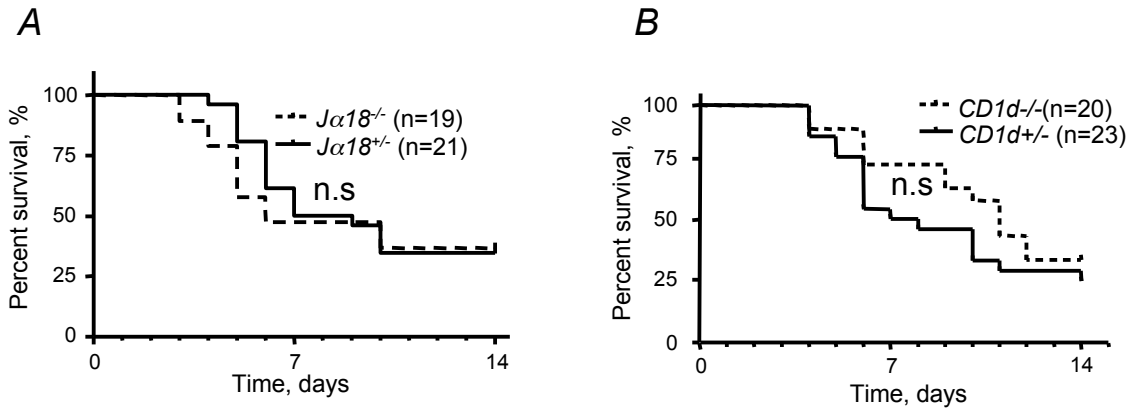


**Figure 2. The impact of CD1d restricted type I and type II natural killer T (NKT) cells in experimental *S. aureus* sepsis.** A) *Ja18*$^{-/-}$ mice (n=19) and their heterozygous littermates (n=21); and B) *CD1d*$^{-/-}$ mice (n=20) and their heterozygous littermates (n=23) were inoculated with *S. aureus* LS-1 (1.5-2x$10^8$ 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.

Figure 2

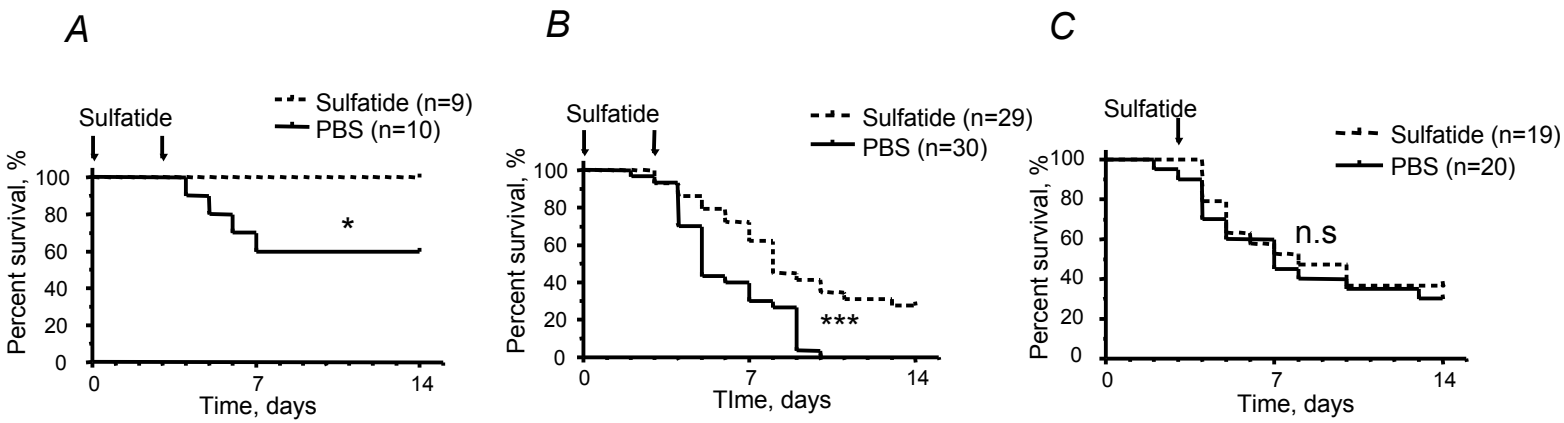


**Figure 3. Sulfatide treatment ameliorated *S. aureus* sepsis in mice.** C57BL/6 mice inoculated with A) $4x10^7$ cfu/mouse (one experiment); B) $2\text{-}3x10^8$ cfu/mouse (pool of 3 experiments); C) $1.5\text{-}3x10^8$ cfu/mouse (pool of 3 experiments) of *S. aureus* LS-1 received i. p. injections of sulfatide (Sulfatide, dotted line), or with same volume of PBS (PBS, solid line), one hour 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; * = $p < 0.05$; *** = $p < 0.001$.

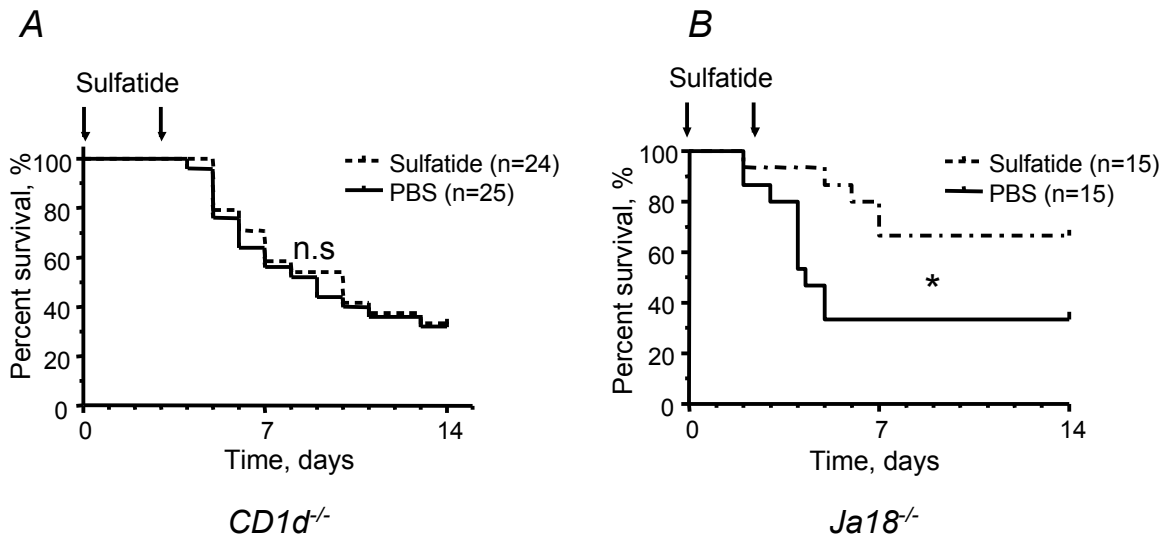


**Figure 4. Protective effect of sulfatide treatment was dependent on presence of CD1d, but not on type I NKT cells.** A) *CD1d*$^{-/-}$ mice; and B) *Ja18*$^{-/-}$ mice inoculated with *S. aureus* LS-1 (1.5-2x$10^8$ cfu/mouse) received i. p. injections of sulfatide (Sulfatide, dotted line), or with same volume of PBS (PBS, solid line) one hour 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.

Figure 4

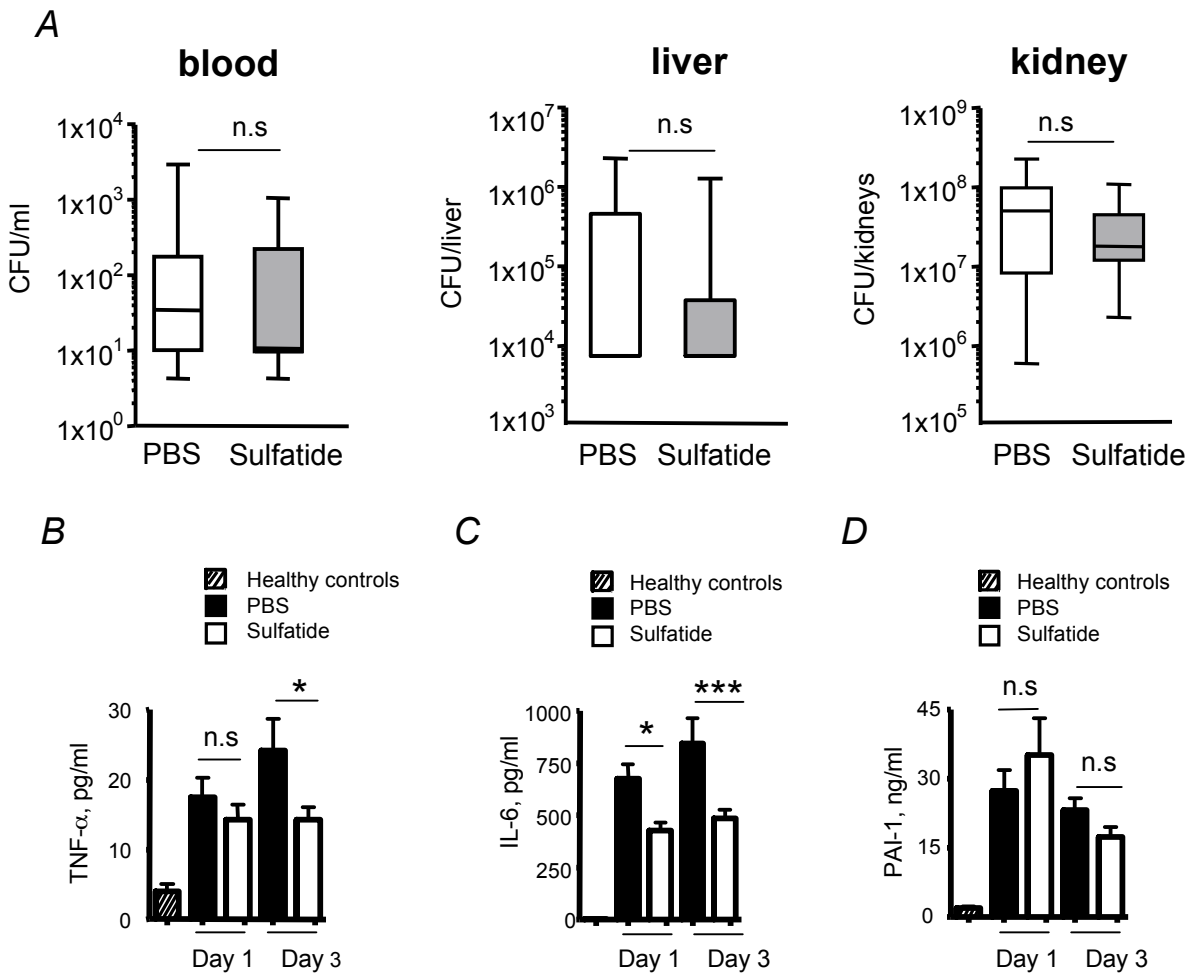


**Figure 5. Sulfatide treatment downregulated systemic inflammation.** C57BL/6 mice were inoculated with *S. aureus* LS-1 ($4x10^7$ – $2x10^8$ cfu/mouse), and sulfatide was injected i. p. into mice one hour before the bacterial inoculation (Sulfatide). Infected control mice were injected with the same volume of phosphate buffered saline (PBS). Blood, kidneys, and livers were collected one and three days after the bacterial inoculation. A) Persistence of *S. aureus* in blood, liver, and kidneys 3 days after infection; B) The levels of TNF-α; C) IL-6; and D) plasminogen activator inhibitor type 1 (PAI-1) in the blood on day 1 and day 3 after bacterial inoculation. Non-infected mice were used as controls in B-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=15-16/group).

Figure 5

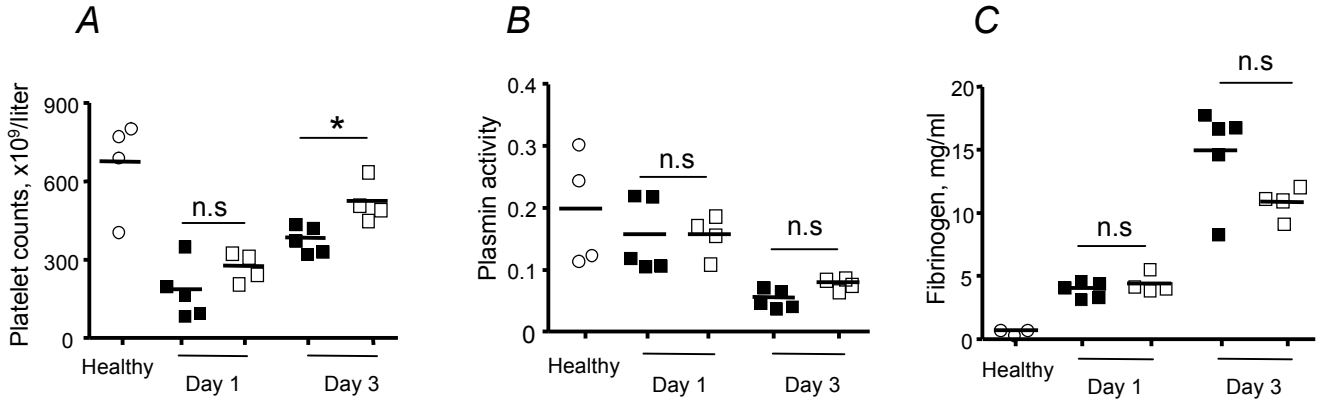


**Figure 6. The effects of sulfatide on hemostatic markers in *S. aureus* sepsis.** C57BL/6 mice were inoculated with *S. aureus* LS-1 ($2x10^8$ cfu/mouse). Sulfatide was injected i. p. to mice 1 hour before the bacterial inoculation. Mice injected with the same volume of phosphate buffered saline (PBS) served as controls. Blood was collected on both 1 and 3 days after the bacterial inoculation. A) The platelet counts, B) plasmin activity levels, and C) fibrinogen levels in blood from mice with *S. aureus* sepsis on day 1 and day 3 after bacterial inoculation. Each symbol represents the value from one mouse. Open circles (○) represent values from non-infected (Healthy) mice; closed squares (■) represent values from septic mice receiving PBS; open squares (□) respresent values from septic mice receiving sulfatide treatment. Statistical evaluations were performed using the Mann–Whitney *U*-test. n.s. = not significant; *= $p<0.05$. Data are from one representative experiment of three performed (n=4-5 mice/group).