amygdala (Fig. 3B). These data are consistent with previous studies in which genetic knockdown of hippocampal BDNF impaired fear extinction (17).

If the hippocampus is the source of IL BDNF, then increasing the available supply of hippocampal BDNF should have similar effects. We took advantage of the fact that BDNF infusions increase BDNF levels in effenter targets (18). There were three treatment groups in this experiment. After conditioning, one group received a hippocampal infusion of BDNF immediately after a saline infusion into the IL mPFC [SAL(IL) + BDNF(Hipp)]. A second group also received a hippocampal BDNF infusion, but this was preceded by infusion of a BDNF-inactivating antibody into the IL mPFC [anti-BDNF(IL) + BDNF(Hipp)] to test the hypothesis that Hipp-applied BDNF works via the IL mPFC. A control group received SAL infusions into both structures [SAL(IL) + SAL(Hipp)].

Similar to its effect on the IL mPFC, BDNF infused into the hippocampus reduced fear, as measured by both freezing [main effect of drug F(2,21) = 4.715, P = 0.020, post hoc P = 0.013 comparing SAL(IL) + SAL(Hipp) to SAL(IL) + BDNF(Hipp)] (Fig. 3C) and conditioned suppression of food seeking (Fig. S4). The effect of hippocampal BDNF could be prevented by coadministration of a BDNF-inactivating antibody in the IL mPFC [P = 0.461 comparing SAL(IL) + SAL(Hipp) to Anti-BDNF(IL) + BDNF(Hipp)], which suggests that the IL mPFC is the primary site of action for hippocampal BDNF.

We were able to pharmacologically induce extinction with a single infusion of BDNF into the hippocampal-infralimbic pathway, a key projection for extinction memory. This effect was not a facilitation of extinction, as no extinction training was required. We have adopted the term “BDNF-extinction” to parallel the term “BDNF-LTP” used to describe BDNF induction of hippocampal LTP in the absence of electrical stimulation (19). Extinction potentiates the hippocampal-prefrontal pathway, and disrupting this potentiation disrupts extinction recall (20). Our results provide further support for the importance of this pathway in extinction and extend these findings by identifying BDNF as a key molecular mediator.

In our experiments, BDNF-extinction required NMDA receptors, which are also necessary for extinction-related bursting in IL neurons (8). Because BDNF facilitates NMDA receptor currents (11, 12), exogenously applied BDNF may simulate extinction by inducing bursting in the IL mPFC. Additionally, BDNF-extinction may involve IL targets, such as intercalated (21) or basolateral amygdala (9, 15) neurons, which also participate in extinction.

Because the behavioral effects of BDNF were observed only when BDNF was infused after conditioning, it is possible that BDNF treatment may lead to partial reversal of conditioning-induced changes. Conditioning induces a rapid reduction in hippocampal BDNF, which reverts in 2 days (22). Extinction failure then may arise from a delayed normalization of BDNF levels after conditioning. If so, application of BDNF to the hippocampus (or to the IL mPFC) may work to reduce fear by restoring BDNF to preconditioning levels and/or reversing condition-inducing reductions in IL excitability (23).

Recall of extinction in healthy human subjects activates the ventromedial PFC and hippocampus (24), both of which are deficient in posttraumatic stress disorder (25). A single-nucleotide polymorphism in the gene encoding human BDNF (Val66Met) results in extinction impairment (26) and decreases the release of BDNF from hippocampal neurons (27). Pharmacotherapies that increase hippocampal BDNF may prove to be efficacious treatments for fear disorders characterized by extinction impairments. BDNF-extinction is complementary to reconsolidation blockade, in which pharmacological agents are used to eliminate the original fear memory (7). Both approaches represent potentially powerful strategies to treat anxiety disorders by manipulating traumatic memories within fear circuits.

**References and Notes**

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**Supporting Online Material**

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**Materials and Methods**

Figs. S1 to S5

**References**

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**SphK1 Regulates Proinflammatory Responses Associated with Endotoxin and Polymicrobial Sepsis**

Padmam Puneet, Celestial T. Yap, Lingkai Wong, Lam Yulin, Dow Rhoon Koh, Shabbir Moochhala, Josef Pfeilschifter, Andrea Huwiler, Alirio J. Melendez*<sup>x</sup>.

During sepsis, activation of phagocytes leads to the overproduction of proinflammatory cytokines, causing systemic inflammation. Despite substantial information regarding the underlying molecular mechanisms that lead to sepsis, several elements in the pathway remain to be elucidated. We found that the enzyme sphingosine kinase 1 (SphK1) is up-regulated in stimulated human phagocytes and in peritoneal phagocytes of patients with severe sepsis. Blockade of SphK1 inhibited phagocyte production of endotoxin-induced proinflammatory cytokines. We observed protection against sepsis in mice treated with a specific SphK1 inhibitor that was enhanced by treatment with a broad-spectrum antibiotic. These results demonstrated a critical role for SphK1 in endotoxin signaling and sepsis-induced inflammatory responses and suggest that inhibition of SphK1 is a potential therapy for septic shock.

The incidence of sepsis, and death from septic shock, has increased over the past few decades (1, 2). During sepsis, the host’s innate immune response to bacterial infection is primarily mediated by neutrophils and monocyes/macrophages (3). These cells express pattern-recognition receptors (PRRs) that bind conserved molecular structures shared by groups of microorganisms (3). Upon stimulation, PRRs initiate inflammatory signaling pathways leading to secretion of proinflammatory mediators, which promote the elimination of infectious agents and the...
induction of tissue repair. Excessive production of inflammatory mediators, however, can induce septic shock by triggering a systemic inflammation, resulting in vascular leakage, tissue damage, multiple organ failure, and death (4, 5).

Several proinflammatory stimuli, including anaphylatoxin C5a, tumor necrosis factor-α (TNF-α), and immune complexes, activate sphingosine kinase 1 (SphK1) on human neutrophils and macrophages, and blockade of SphK1 inhibits several proinflammatory responses triggered by these stimuli (6–12). SphKs are intracellular signaling enzymes that generate the lipid mediator sphingosine-1-phosphate (S1P) (13). The ability of SphK1 to mediate the secretion of proinflammatory mediators triggered by complement cascade anaphylatoxins (C5a) (9, 10) prompted us to investigate its role in inflammation caused by endotoxin and sepsis. We determined SphK1 protein and mRNA expression on human neutrophils and macrophages that were derived from peripheral blood and incubated in vitro with heat-inactivated Gram-positive bacteria, Gram-negative bacteria, mycobacteria, or with bacterial cell-wall components (14). SphK1 is constitutively expressed by most cells; however, SphK1 expression was strongly up-regulated by extracellular bacteria and lipopolysaccharide (LPS) (Fig. 1A) and bacterial lipoprotein (BLP) (fig. S1). In contrast, intracellular bacteria or the bacterial product, mycolic acid, had no effect (fig. S1). SphK1 was rapidly activated upon stimulation of human macrophages with LPS (Fig. 1B) or BLP (fig. S2). Silencing SphK1 by means of small interfering RNA (siRNA) (Fig. 1B), or inhibiting SphK1 with the specific SphK1 inhibitor 5c (fig. S3) (15), showed that SphK1 was required to activate the transcription factor nuclear factor κB (NF-κB) (Fig. 1C) and to induce the secretion of proinflammatory cytokines [TNF-α, interleukin-1β (IL-1β), and IL-6] and the proinflammatory protein high-mobility group protein B1 (HMGB1), but not type I interferon-α (IFNα) or IFNβ (Fig. 1D).

Fig. 1. Bacterial products increase human SphK1 expression and function in vitro. (A) Flow cytometric analysis of intracellular SphK1 expression after incubation of human macrophages and neutrophils with LPS and untreated control cells. Isotype control indicates staining of cells with an isotype control antibody. (B) SphK1 expression in human macrophages (top) pretreated with the siRNA-SphK1, scrambled siRNA (siRNA-Scramb.), or with vehicle control (Control PBS). SphK activity in human macrophages (bottom) in cells pretreated with vehicle control (PBS), siRNA-SphK1, siRNA-Scramb., or the SphK1-inhibitor 5c. (C) NF-κB activity in human macrophages stimulated as in (B). (D) Cytokine and HMGB1 production in untreated, nonstimulated human macrophages (Basal) or in human macrophages stimulated as in (B). (E and F) Flow cytometric analysis of SphK1 expression in purified (E) macrophages and (F) neutrophils from patients’ aseptic systemic inflammatory response syndrome (SIRS) from poly-microbial sepsis, and of healthy controls. Isotype control indicates staining of cells with an isotype control antibody. Data shown are representative from 30 septic patients and 15 healthy samples. (G) LPS-mediated cytokines and HMGB1 release by macrophages from septic patients after 16 hours under the indicated stimulation conditions. Basal indicates untreated, unstimulated cells; PBS was used as a vehicle control for 5c; and the cytokines and HMGB1 levels were measured by means of enzyme-linked immunosorbent assay (ELISA). (H) NF-κB activity in macrophages from septic patients was measured by means of p65 binding to specific DNA templates in an ELISA format. Data are shown as means ± SD (n = 4 separate experiments). Student’s t test P values (*P < 0.05 and **P < 0.01) are compared with LPS-induced control macrophages (LPS+PBS).
Inhibition of SphK1 protects against LPS-induced endotoxic shock in mice. (A) Survival curves for LPS-induced death in mice pretreated with vehicle alone (LPS+PBS), SphK1-siRNA, or scrambled siRNA. As a negative control, mice were pretreated and challenged with vehicle alone (PBS). (B) Serum cytokines or (C) HMGB1 from LPS-injected mice pretreated under the indicated conditions, measured 24 hours after LPS challenge. (D) Immune-cell infiltration and tissue damage in the lungs and liver, 24 hours after LPS challenge, in mice pretreated under the indicated conditions. Immune cells were detected by means of haematoxylin and eosin staining; the magnification is ×10. Scale bar, 30 µm. (E) Survival curves for LPS-induced death in the indicated mouse strains in the presence or absence of pretreatment with 2 mg/kg of 5c. (F) LPS-triggered secretion of cytokines and HMGB1 from mouse macrophages from the indicated mouse strains in the presence or absence of pretreatment with 10 µM 5c. Data points correspond to the mean ± SD of three independent experiments (six mice per treatment group; a total of 18 mice per condition were used). Student's t test P values (**P < 0.01) are compared with LPS-induced control (LPS+PBS).
We next studied the role of SphK1 in cells from patients with sepsis. We enrolled 30 patients with severe sepsis from different sources for this study. The main diagnoses included systemic Staphylococcus aureus infection in 12 (40%) patients, intra-abdominal infection in 12 (40%) patients, and trauma in six (20%) patients. Characteristics of the patients and the 15 healthy volunteer controls are summarized in table S1.

SphK1 expression was increased on infiltrating neutrophils and macrophages isolated from the peritoneal cavity of patients with septic shock (Fig. 1, E and F). Moreover, treating peritoneal macrophages with 5c blocked the LPS-triggered production of TNF-α, IL-1β, IL-6, monocyte chemotactic protein-1 (MCP-1), and HMGB1 but not type I interferons (Fig. 1G). LPS-triggered NF-κB activation was also inhibited in patient-derived macrophages that had been pretreated with 5c (Fig. 1H).

We next decided to establish whether PKCδ was indeed activated by LPS or BLP in macrophages and whether PKCδ played any role in the activation of NF-κB. We silenced PRKCD expression by pretreating cells with a PRKCD-validated siRNA (PKCδ-siRNA) and analyzed the siRNA specificity on the expression of other PKC isoforms (Fig. S5A), inlcuding PKCe, which is suggested to be involved in LPS-induced IKK and NF-κB activation in monocytic cell lines (16). PKCδ-siRNA, but not control siRNA, largely inhibited LPS and BLP-triggered PKCδ activation (Fig. 2B). Furthermore, in cells pretreated with the PKCδ-siRNA, LPS- and BLP-triggered NF-κB activation was substantially inhibited (Fig. 2C). siRNA-PKCδ-treated cells exhibited reduced phosphorylation of IKKα, IKKβ, and IKKγ and impaired IKKβ co-immunoprecipitation with IκBα, IκBβ, and p65 (fig. S5B), providing further evidence that PKCδ may act downstream of SphK1 in human macrophages to trigger activation of NF-κB.

We then investigated how SphK1 regulates PKCδ activation. We found that the LPS- and BLP-triggered PKCδ activation was inhibited in cells pretreated with the SphK1-siRNA or 5c (Fig. 2D). Moreover, in in vitro experiments in which we used recombinant PKCδ, we found that PKCδ can be activated by S1P (Fig. 2E). Taken together, these data suggest that LPS and BLP sequentially activate SphK1 and PKCδ, which then leads to the activation of IKK and NF-κB and subsequent generation and release of proinflammatory molecules.

To assess the direct involvement of SphK1 in inflammatory responses, we tested whether in vivo silencing of Sphk1 by means of siRNA or blocking SphK1 activity with 5c reduced systemic inflammation and lethal shock in mouse models of sepsis. A synthetic specific siRNA against SphK1 can silence SphK1 expression in vivo (17, 18), and we confirmed this in our system (fig. S5C). We then injected mice intraperitoneally with a lethal dose of Sphk1 antisense oligonucleotides.
LPS in order to induce shock. Lethality was monitored over time and compared with mice that had been pretreated with saline alone (PBS), scrambled siRNA, or siRNA against SphK1 before LPS administration. We observed 100% survival of LPS-injected mice pretreated with siRNA-SphK1 (Fig. 3A).

Analysis of blood samples taken from mice pretreated with the siRNA-SphK1 after LPS administration showed a significant reduction of the plasma concentrations of TNF-α, IL-1β, IL-6, MCP1 (Fig. 3B), and HMGB1 (Fig. 3C), whereas the amounts of IFNα and IFNβ remained unchanged. We also observed a reduction in the total immune-cell infiltration into the liver and lungs of mice in which SphK1 had been silenced (Fig. 3D). To further study the protection provided by the blockade of SphK1, groups of mice were injected with increasing doses of 5c 30 min before the systemic injection of LPS. Survival was dose-dependent, ranging from no protection at 0.1 mg/kg to reaching 100% survival at 2 mg/kg (Fig. S6). Taken together, these results show that siRNA-silencing of SPHK1 or SphK1 inhibition protects mice from endotoxic shock.

It has been reported that SphK1-null mice exhibit normal inflammatory cell recruitment during thioglycollate-induced peritonitis and in a collagen-induced arthritis (CIA) model of rheumatoid arthritis (19). In contrast and consistent with in vitro data (6–12), in vivo siRNA silencing of SphK1 in adult mice blocks C5a-induced acute peritonitis and chronic inflammation in the CIA model (17, 18), demonstrating that SphK1 is indeed required for acute and chronic inflammation. Thus, the phenotype of the mice in which SphK1 had been knocked out may be due to adaptive functional redundancy occurring during embryonic development (20, 21), necessitated by the fundamental roles of SphK1s in cellular responses. Consistent with previous reports showing no protection from inflammatory stimuli (18), neither SphK1−/− nor SphK2−/− mice were protected from LPS-induced death and cellular responses (Fig. 3, E and F). When we treated both SphK1−/− and SphK2−/− mice with 5c, only the mice lacking SphK2 were protected (Fig. 3, E and F). These data demonstrate that SphK1 is indeed a critical player in endotoxic shock and confirm that 5c is a SphK1-specific inhibitor.

Experimental endotoxic shock reproduces human sepsis only in part because it does not involve the replication and dissemination of bacteria. Under these conditions, blockade of SphK1 signaling could be deleterious by impairing the capacity of the immune system to fight infections, as observed for treatments with antibody to TNF-α (22). We therefore investigated whether blockade of SphK1 protects against septic shock in a model of polymicrobial peritonitis and sepsis caused by cecal ligation and puncture (CLP), a model that resembles human microbial sepsis (23). We found that mice pretreated with the siRNA-SphK1 or with 5c were protected against CLP-induced systemic inflammation and mortality as compared with a control siRNA and vehicle control (Fig. 4A). Histological examination of the lungs and liver showed a substantial reduction in inflammation and tissue damage (Fig. 4B) and lower amounts of inflammatory mediators (Fig. 4C and D). Inhibition of SphK1 did not compromise bacterial clearance; in fact, blood bacterial load was lower in mice pretreated with the SphK1 inhibitor or with the siRNA-SphK1 (Fig. 4E). These results suggest that blockade of SphK1 reduces inflammatory responses and may aid in control of bacterial infection. Use of SphK1−/− and SphK2−/− mice for the CLP model generated similar results as those for the endotoxin-mediated shock shown in Figs. 3, E and F (fig. S7).

To assess the therapeutic potential of SphK1 blockade in sepsis, we monitored whether blockade of SphK1 was still protective when 5c was administered 2, 6, 8, and 12 hours after the CLP procedure. 5c conferred 100% protection when applied 2 hours after CLP, 60% when applied 6 hours after CLP, 30% when applied 8 hours after CLP, and 10% when applied 12 hours after CLP (Fig. 4F). Thus, 5c is effective as a therapeutic for experimental sepsis.

We next treated CLP septic mice with 5c and a broad-spectrum antibiotic, co-amoxiclav, which is currently used to treat patients with sepsis. Administration of a broad-spectrum antibiotic is protective when administered before or shortly after the CLP procedure; however, the time-window for treatment opportunity is very narrow, and in the clinic, despite antibiotic treatment, a large number of patients with sepsis or septic shock still die. Co-amoxiclav alone increased survival by 50% when administered 6 hours after CLP and by 25% when administered 8 hours after CLP, and no survival was observed when it was administered 12 hours after CLP (Fig. 4G). Co-amoxiclav–mediated survival correlated with blood-bacterial clearance (fig. S8). The combination of 5c with co-amoxiclav enhanced survival to 100% when co-injected 6 hours after CLP, to 90% when co-injected 8 hours after CLP, and to 40% when the combination therapy was administered 12 hours after CLP (Fig. 4H). Thus, our data demonstrate a clear advantageous synergy with this combination therapy.

Studies in models of endotoxin-induced acute lung injury suggest that SIP and its receptors are critical players in maintaining the integrity of the vascular barrier function (24, 25). Our results suggest that, in the endotoxin and CLP shock models, elevated cytosolic SphK1 activity leads to an aberrant inflammatory response, resulting in multiple organ damage and death. Our results also suggest that blockade of SphK1 during septic shock does not interfere with the systemic SIP gradient that is required to maintain the integrity of the vascular barrier function.

SphK1 inhibition shows a clear potential therapeutic advantage against sepsis as compared with treatments with known anti-inflammatory agents, including TNF-α monoclonal antibodies and nitric oxide synthase inhibitors, which increase lethality potentially through suppression of the host’s ability to fight infection (27, 26). Indeed, bacterial clearance is enhanced when SphK1 is inhibited. Research efforts have recently been directed at finding a mechanism for limiting the excessive inflammation triggered by TLRs as a way of providing potential novel therapies to treat sepsis and other inflammatory disorders (27). Here, we provide such a mechanism by targeting SphK1. Thus, administration of SphK1 inhibitors—alone or in combination with antibiotics, after the initiation of sepsis—might offer a suitable new therapeutic tool for the treatment of septic shock and other microbe-mediated diseases in humans, in whom “out-of-control” inflammation often leads to fatal outcomes.

References and Notes
14. Materials and methods are available as supporting material on Science Online.
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Materials and Methods
Figs. S1 to S8
Table S1
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