

SEPSIS

Modulation of the sigma-1 receptor–IRE1 pathway is beneficial in preclinical models of inflammation and sepsis

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Sepsis is an often deadly complication of infection in which systemic inflammation damages the vasculature, leading to tissue hypoperfusion and multiple organ failure. Currently, the standard of care for sepsis is predominantly supportive, with few therapeutic options available. Because of increased sepsis incidence worldwide, there is an urgent need for discovery of novel therapeutic targets and development of new treatments. The recently discovered function of the endoplasmic reticulum (ER) in regulation of inflammation offers a potential avenue for sepsis control. Here, we identify the ER-resident protein sigma-1 receptor (S1R) as an essential inhibitor of cytokine production in a preclinical model of septic shock. Mice lacking S1R succumb quickly to hypercytokinemia induced by a sublethal challenge in two models of acute inflammation. Mechanistically, we find that S1R restricts the endonuclease activity of the ER stress sensor IRE1 and cytokine expression but does not inhibit the classical inflammatory signaling pathways. These findings could have substantial clinical implications, as we further find that fluvoxamine, an antidepressant therapeutic with high affinity for S1R, protects mice from lethal septic shock and dampens the inflammatory response in human blood leukocytes. Our data reveal the contribution of S1R to the restraint of the inflammatory response and place S1R as a possible therapeutic target to treat bacterial-derived inflammatory pathology.

INTRODUCTION

The endoplasmic reticulum (ER) is increasingly recognized as a powerful controller of inflammatory signaling (1, 2) and the response of immune cells to diverse stimuli (3, 4). Among the major ER stress sensors, inositol-requiring enzyme 1 α (IRE1) is selectively activated by the Toll-like receptor 4 (TLR4) ligand lipopolysaccharide (LPS) (3). IRE1 regulates inflammatory cytokine production via both its endonuclease activity and transcriptional regulation (2, 3) and the control of cellular signaling pathways (5). Given the considerable potential of IRE1 to modulate inflammation, there is interest in targeting IRE1 for therapeutic benefit (2, 6). However, caution should be applied, because IRE1 function is also critical during homeostasis, including in the liver and pancreas (7, 8). Therefore, to take full advantage of this potent inflammatory mediator, it is essential to identify alternative methods for targeting IRE1 signaling.

Sigma-1 receptor (S1R) is a ubiquitously expressed ER resident chaperone protein that associates with IRE1 during ER stress (9). S1R function is well described in the central nervous system (10), where it has been implicated in the regulation of neurodegenerative diseases (11, 12), cell fate control, and immune activity of microglia (13, 14). Targeting S1R has been reported to influence immune cells and cytokine production in vitro (15), with many well-tolerated S1R ligands currently in clinical use, placing S1R as an attractive therapeutic target (16).

In this work, we identify S1R as a critical regulator of IRE1-driven inflammation. S1R deficiency potently enhances inflammatory cytokine production in a manner dependent on IRE1 activity and reduces survival during models of hyperinflammation and septic shock in

mice. Conversely, forced expression of S1R can dampen the inflammatory response to LPS. Furthermore, we show that the S1R ligand fluvoxamine (FLV) can enhance survival in mouse models of inflammation and sepsis and can inhibit the inflammatory response in human peripheral blood cells. Collectively, our data show that S1R is uniquely poised to sensitively control IRE1 activity during inflammation.

RESULTS

S1R controls LPS-induced IRE1 activity in macrophages

S1R has been shown to interact with IRE1 under strong ER stress-inducing conditions (9). Given the role for IRE1 during the inflammatory response (2, 3), we wanted to test whether S1R participates in ER-mediated inflammation. We first used the BirA proximity ligation assay to test whether S1R interacts with IRE1 during LPS challenge in vitro. For this experiment, we used human embryonic kidney (HEK) 293 cells that express mTLR4/MD2/CD14 and therefore respond to LPS (17). Cells were transfected with S1R conjugated to the bifunctional ligase/repressor BirA or BirA alone as control, resulting in the biotinylation of proteins that are in close proximity to S1R (Fig. 1A) (18). We observed IRE1 biotinylation during homeostasis that was enhanced after LPS treatment (Fig. 1, B and C), indicating proximity and possible association (direct or indirect) between S1R and IRE1.

Upon activation with LPS, IRE1 endonuclease activity is triggered and splices the mRNA that encodes the transcription factor X-box binding protein 1 (XBP1) (Fig. 1D), resulting in expression of active XBP1 protein. We found increased LPS-induced XBP1 splicing in mouse bone marrow-derived macrophages (BMDMs) lacking S1R, indicating elevated inducible, but not basal, IRE1 endonuclease activity in S1R knockout (KO) macrophages (Fig. 1E). To confirm that XBP1 splicing was mediated by IRE1 endonuclease activity, the

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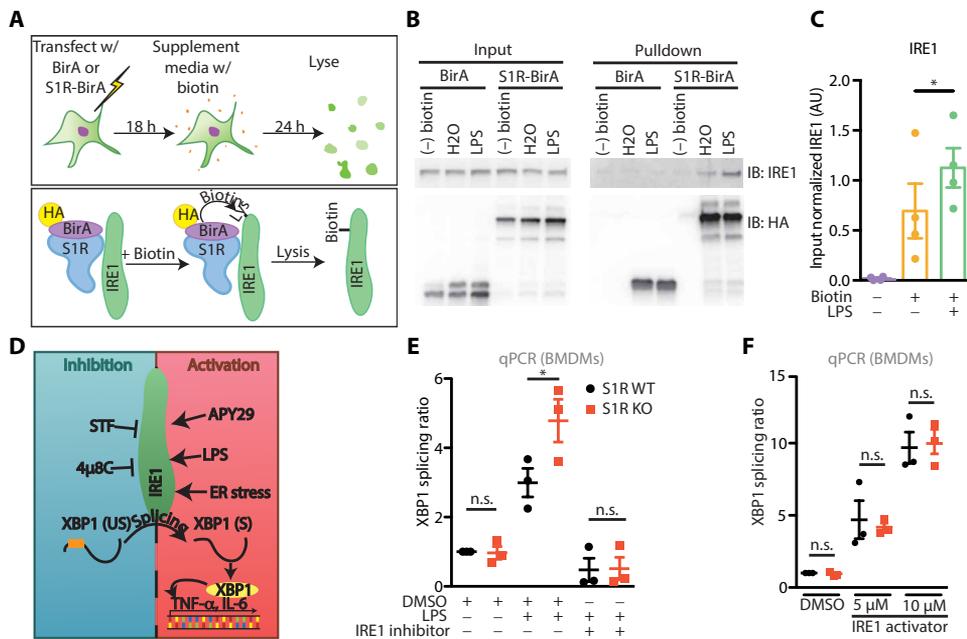


Fig. 1. S1R is an inhibitor of IRE1 during inflammation. (A) Experimental design and principle of proximity ligation assay. HA, hemagglutinin. (B) Western blots on input lysates and biotinylated (streptavidin pull-down) proximity ligation samples of HEK293 transfected with BirA or S1R-BirA and then stimulated for 24 hours with LPS (100 ng/ml) in the presence of 80 μM biotin. AU, arbitrary units. (C) Densitometric quantification of (B) [$N = 4$; $*P < 0.05$, repeated measures one-way analysis of variance (ANOVA) with post hoc Sidak test]. (D) Activity modulators of IRE1 and experimental parameters were used in the study. XBP1 (US), unspliced XBP1 transcript; XBP1 (S), spliced XBP1 transcript. (E) XBP1 splicing ratio [i.e., glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-normalized spliced XBP1 transcript/GAPDH-normalized unspliced XBP1 transcript] in S1R WT or KO BMDMs stimulated for 6 hours with dimethyl sulfoxide (DMSO), LPS (100 ng/ml), or LPS (100 ng/ml) + IRE1 inhibitor (5 μM 4μ8C) ($N = 3$; n.s., not significant; $*P < 0.05$, two-way ANOVA with post hoc Sidak test). (F) XBP1 splicing ratio in S1R WT or KO BMDMs stimulated with DMSO or IRE1 activator (5 μM or 10 μM APY29) for 6 hours ($N = 3$, each dot represents one individual experiment, two-way ANOVA with post hoc Sidak test).

selective IRE1 endonuclease inhibitor 4μ8C was tested (19). Treatment with 4μ8C abolished LPS-induced XBP1 splicing in both genotypes, ruling out IRE1-independent XBP1 splicing (Fig. 1E). We ruled out the presence of a larger pool of IRE1 in S1R KO cells by treating cells with APY29, which forces IRE1-dependent XBP1 splicing (20). In this IRE1 stimulation paradigm, XBP1 splicing amounts were equal in both genotypes (Fig. 1F), indicating that S1R KO affects IRE1 activity and not IRE1 protein abundance or substrate availability.

S1R critically regulates inflammatory cytokine production via IRE1

Because IRE1 activity is required for cytokine production (2, 3, 5), likely via XBP1-mediated transactivation of interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α), we next asked whether S1R deficiency alters macrophage cytokine expression upon exposure to LPS. We found that S1R KO BMDMs had elevated expression of IL-6 and pro-IL-1 β transcripts and secreted higher amounts of IL-6 protein, when compared to wild-type (WT) cells (Fig. 2, A and B, and fig. S1A). However, S1R deficiency does not result in a global increase of cytokine production, because the anti-inflammatory cytokine IL-10 expression was unaffected in S1R KO BMDMs (fig. S1B). Having established that deletion of S1R leads to an increased inflammatory response, we examined whether overexpression of S1R could be anti-inflammatory. We overexpressed S1R in HEK293 that

expresses mTLR4/MD2/CD14 and monitored expression of IL-8 after LPS treatment (17). Relative to control-transfected cells, overexpression of S1R resulted in a significant decrease in IL-8 production after LPS stimulation ($P < 0.05$; Fig. 2C). These data collectively suggest that overexpression of S1R can dampen inflammation, whereas S1R deficiency contributes to an enhanced inflammatory response.

We next tested whether IRE1 endonuclease activity is responsible for the increase in proinflammatory cytokine expression in S1R KO cells. Proinflammatory cytokines, including IL-6, are rapidly induced by LPS in mice and humans and correlate with poor prognosis in sepsis (21, 22). We treated WT and S1R KO BMDMs with LPS in the presence or absence of 4μ8C and analyzed IL-6 expression by quantitative polymerase chain reaction (qPCR). Inhibition of IRE1 endonuclease activity reduced IL-6 expression in KO cells to the amount observed in WT BMDMs (Fig. 2D). Because S1R is an ER-resident protein, we wanted to rule out that deletion of S1R might result in global ER dysfunction, which could lead to the observed increase in IRE1 activation. To test this, we performed an immunoblot for ER-resident proteins that become up-regulated during ER stress (23): protein kinase R-like endoplasmic

reticulum kinase (PERK), binding immunoglobulin protein (BiP), and protein disulfide isomerase (PDI). We found comparable amounts of protein expression of all three proteins in S1R KO BMDMs at baseline, with no change elicited by stimulation of BMDMs with LPS (fig. S1C). Therefore, we conclude that global ER stress does not drive IRE1 activity in S1R KO BMDMs. Cell surface expression of TLR4 was unaffected by S1R deletion, ruling out differential expression of the LPS receptor (fig. S2, A and B). Activation of NF- κ B (fig. S2C), ERK1/2, and JNK (fig. S2D) was identical between WT and S1R KO cells after LPS treatment. Last, when we tested selective pharmacologic inhibitors of NF- κ B, JNK, ERK1/2, and IRE1 for the ability to normalize LPS-induced IL-6 secretion in S1R KO BMDMs, only the IRE1 inhibitor was effective at blunting the augmented inflammatory response of S1R KO cells (Fig. 2E). Note that, although NF- κ B, JNK, and ERK1/2 inhibitors suppressed IL-6 production in both genotypes, S1R KO BMDMs still produced elevated IL-6 relative to WT BMDMs (Fig. 2E), indicating that these pathways are functional in S1R KO BMDMs. We also cultured primary lung fibroblasts from the S1R KO and WT mice and tested their response to LPS stimulation. Fibroblasts also presented with enhanced LPS-induced XBP1 splicing and inflammatory cytokine production that can be corrected by IRE1 inhibition (fig. S3, A and B). Together, these findings indicate that IRE1 signaling is selectively perturbed in S1R KO cells and that the proinflammatory effects of S1R deletion likely depend on the endonuclease activity of IRE1.

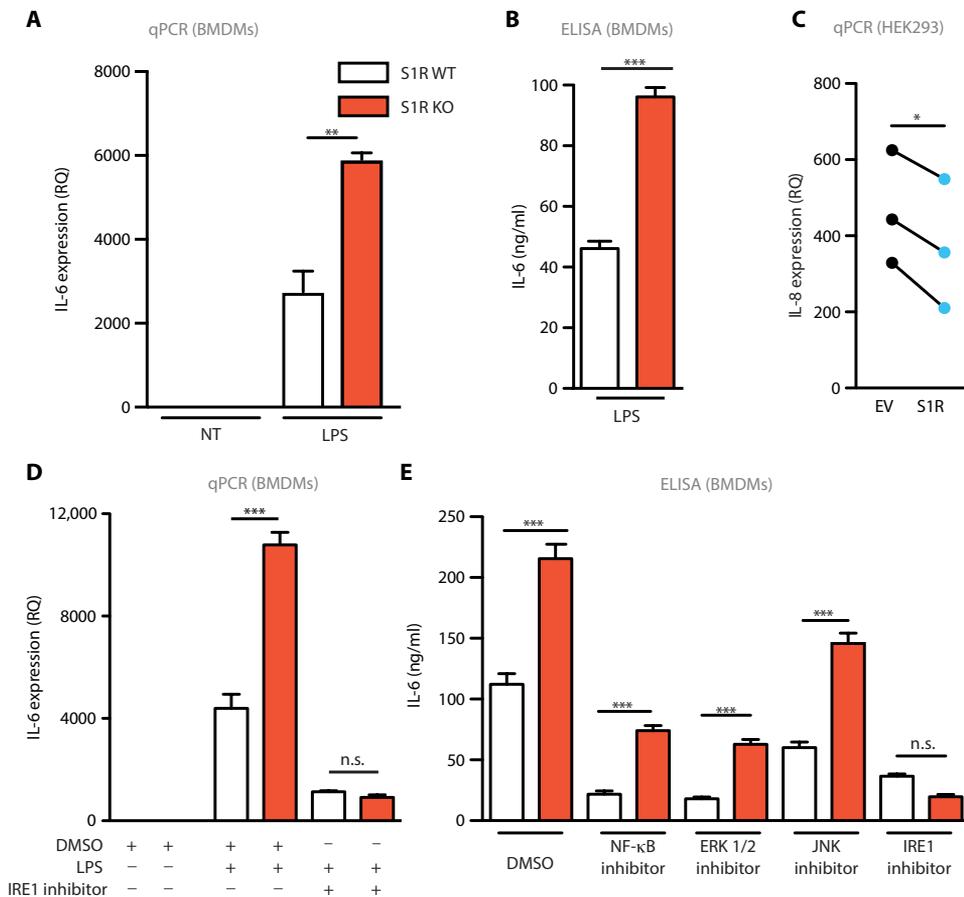


Fig. 2. S1R controls the production of inflammatory cytokines by inhibiting IRE1. (A) Relative quantity (RQ) of IL-6 was determined by qPCR on S1R WT or KO BMDMs stimulated for 6 hours with vehicle (NT) or LPS (100 ng/ml; representative of $N = 4$ independent experiments is shown; $***P < 0.01$, two-way ANOVA with post hoc Tukey test). (B) IL-6 enzyme-linked immunosorbent assay (ELISA) on supernatant from BMDMs stimulated for 6 hours with LPS (1 μ g/ml; $n = 1$, representative of $N = 4$ independent experiments is shown; $***P < 0.001$, t test). (C) qPCR on TLR4-MD2-CD14-expressing HEK293 cells that were transfected with empty vector (EV) or S1R and stimulated for 6 hours with LPS (100 ng/ml; $N = 3$, each dot pair represents one independent experiment; $*P < 0.05$, paired t test). (D) qPCR on BMDMs stimulated for 6 hours with vehicle (DMSO), LPS (100 ng/ml), or LPS (100 ng/ml) + IRE1 inhibitor (5 μ M 4 μ 8C) ($n = 1$, representative of $N = 3$ independent experiments is shown; $***P < 0.001$, two-way ANOVA with post hoc Tukey test). (E) IL-6 ELISA on supernatant from BMDMs stimulated for 6 hours with LPS (1 μ g/ml) with either <1% DMSO, nuclear factor κ B (NF- κ B) inhibitor (20 μ M JSH-23), mitogen-activated protein kinase (ERK) inhibitor (20 μ M PD98059), c-Jun N-terminal kinase (JNK) inhibitor (1 μ M SP600125), or IRE1 inhibitor (5 μ M 4 μ 8C) ($n = 1$, representative of $N = 3$ independent experiments is shown; $***P < 0.001$, two-way ANOVA with post hoc Tukey test).

S1R-deficient mice display increased mortality in sublethal models of sepsis

To test the function of S1R *in vivo*, we subjected S1R KO mice to LPS injection, an animal model to study the inflammatory response to endotoxin (24). S1R KO mice and WT littermate controls were injected with a sublethal dose of LPS (5 mg/kg), and survival was monitored for 6 days (Fig. 3A). WT animals experienced very low mortality (9%), whereas 62% of S1R KO mice succumbed to LPS-induced death (Fig. 3B), suggesting that S1R potently inhibits systemic inflammation. We next analyzed the concentration of proinflammatory cytokines TNF- α and IL-6 in serum at their reported peak expression, because these cytokines have been extensively shown to correlate with LPS-induced mortality (21, 25). Peak serum TNF- α and IL-6 were significantly increased in LPS-challenged S1R KO mice,

when compared to controls ($P < 0.05$; Fig. 3, C and D), whereas neither cytokine was detectable in the serum of unchallenged mice. To test whether the increase in TNF- α and IL-6 in S1R KO mice was due to baseline differences in the composition of immune cells, we performed an immunophenotyping analysis of blood (fig. S4), the peritoneal cavity (fig. S5), and immune organs (spleen and lymph nodes; fig. S6). Our flow cytometry analyses revealed no significant differences in the innate and adaptive cell numbers and frequency, suggesting that S1R-deficient mice do not have an overt immune defect.

Although LPS injection is a convenient model for the study of endotoxin-mediated inflammation, the use of a single pathogen-associated molecular pattern does not fully recapitulate the biological complexity of sepsis. Therefore, we tested S1R KO mice in fecal-induced peritonitis (FIP; Fig. 3E), a model of sepsis that involves injection of fecal material containing live bacteria (26). Similar to our LPS challenge finding, WT mice receiving a sublethal dose of fecal slurry (1 g/kg of body weight) did not succumb to septic shock induced by FIP, whereas S1R KO mice experienced significant mortality ($P < 0.05$; Fig. 3E). This increased mortality correlated with increased serum IL-6 ($P < 0.05$; Fig. 3F) and significantly lowered core body temperature in S1R KO mice ($P < 0.01$, Fig. 3G). S1R deficiency was also associated with elevated markers of organ failure, as revealed by serum chemistry analysis performed 24 hours after the initiation of septic shock. Two indicators of impaired liver function [ALT ($P < 0.05$; Fig. 3H) and AST ($P < 0.001$; Fig. 3I)], an indicator of kidney dysfunction [creatinine ($P < 0.001$; Fig. 3J)], and an indicator of heart dysfunction [CK

($P < 0.01$, Fig. 3K)] were all significantly elevated in S1R-deficient animals in both LPS and FIP models. Together, our data demonstrate increased susceptibility to models of sepsis and inflammation in S1R deficiency, characterized by elevated cytokines and multiorgan dysfunction.

S1R activation and IRE1 inhibition are protective in an animal model of inflammation

To test whether LPS-challenged S1R KO mice have increased IRE1 activity, we first examined XBP1 splicing in the liver, a key organ in the pathological progression of sepsis. LPS-challenged S1R KO mice had increased hepatic XBP1 splicing when compared to WT mice (Fig. 4A). This finding suggests that similar IRE1-dependent inflammatory mechanisms we identified in cultured macrophages may be

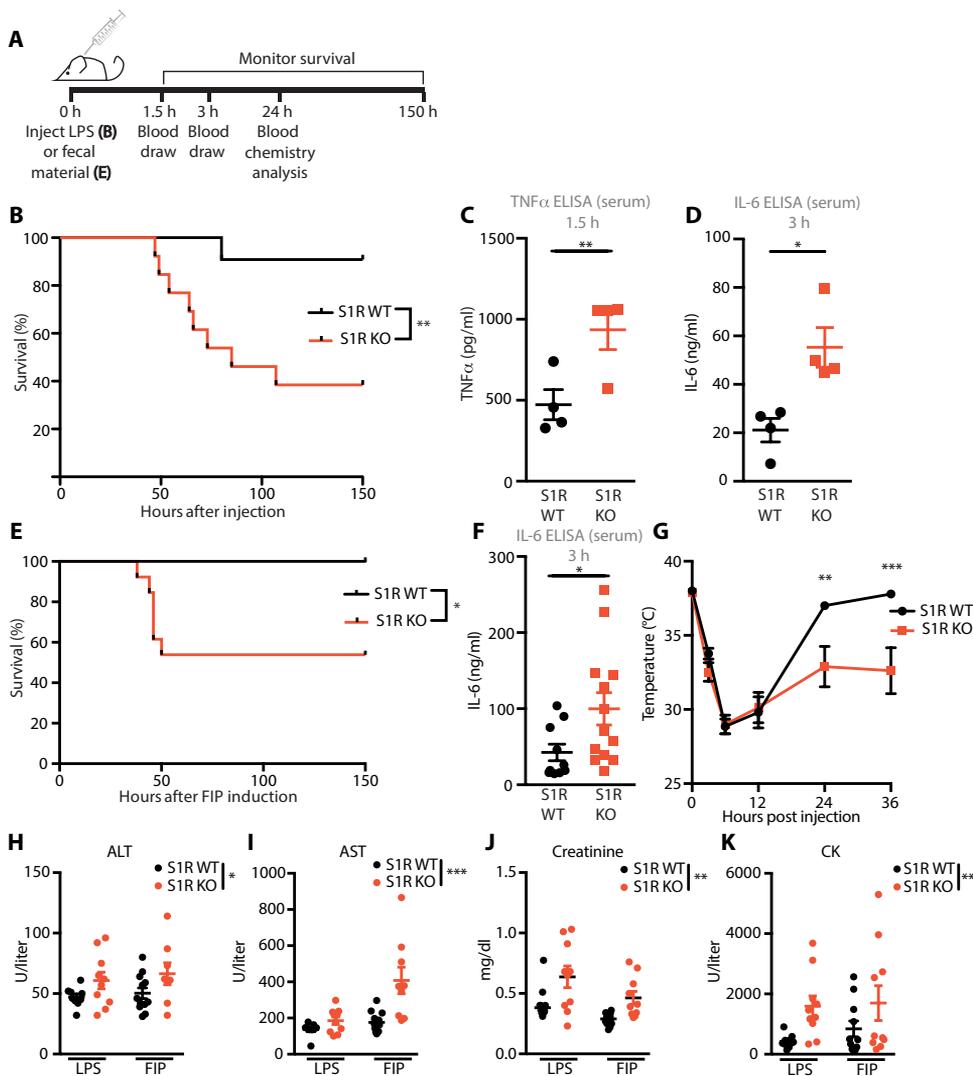


Fig. 3. S1R is protective in murine models of inflammation and septic shock. (A) Experimental design. (B) Survival curve of WT and S1R KO mice after LPS (5 mg/kg) administration ($n = 11$ to 13 mice per group; $**P < 0.01$, log-rank test). (C) ELISA for TNF- α in serum 1.5 hours after LPS injection (each dot represents one mouse; $**P < 0.01$, t test). (D) ELISA for IL-6 in serum collected 3 hours after LPS injection (each dot represents one mouse; $*P < 0.05$, t test). (E) Survival curve of WT and S1R KO mice after administration of fecal content ($n = 10$ to 13 mice per group, fecal slurry = 1 g/kg; $*P < 0.05$, log-rank test). (F) ELISA for IL-6 in serum collected 3 hours after fecal slurry injection (each dot represents one mouse; $*P < 0.05$, t test). (G) Rectal temperature of animals presented in (E) ($n = 10$ to 13 mice per group; $**P < 0.01$, $***P < 0.001$, two-way repeated measures ANOVA with post hoc Sidak test). (H to K) Mice were injected with LPS (5 mg/kg) or fecal slurry (1 mg/kg), and 24 hours later, serum was analyzed for amount of (H) alanine aminotransferase (ALT), (I) aspartate aminotransferase (AST), (J) creatinine, (K) and creatine kinase (CK) (each dot represents one mouse, $n = 10$ to 12 per group; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, two-way ANOVA; outliers have been removed from visualization and are available in data file S1).

at work in vivo. If increased IRE1 activity is responsible for reduced survival of S1R KO mice during LPS challenge, then IRE1 inhibition should protect S1R KO mice subjected to LPS challenge (Fig. 4B). Because of the reported short half-life of the IRE1 inhibitor 4 μ 8C in vivo (19), we selected instead to use STF-083010 (herein referred to as STF), an effective IRE1 inhibitor (fig. S7A) that has been used in in vivo studies (19, 27). Again, LPS-challenged S1R KO mice that received vehicle control experienced rapid mortality (Fig. 4C). STF administration (30 mg/kg at 0 and 24 hours) spared S1R KO mice

from LPS-induced mortality (Fig. 4C), whereas it did not significantly affect the survival of WT mice.

The finding that an IRE1 inhibitor rescues S1R KO mice in a model of endotoxemia is in agreement with our hypothesis that cytokine production and LPS-induced mortality in S1R KO mice require excessive IRE1 endonuclease activity. Further supporting this hypothesis, we detected a significantly higher IL-6 after 3 hours in the peritoneal exudate in LPS-challenged vehicle-treated S1R KO mice compared to WT controls, which was corrected by STF treatment ($P < 0.05$; Fig. 4D). IL-6 in the serum after STF treatment was not significantly different (fig. S7B). In this treatment paradigm, we noted that vehicle (Kolliphor) treatment significantly increased LPS-induced IL-6 in the serum when compared to LPS alone ($P < 0.01$; fig. S7C). Because Kolliphor exacerbates LPS-induced inflammation, a lower dose of LPS was selected than in other experiments. Injection of Kolliphor alone did not result in a detectable concentration of serum IL-6, nor did it cause any mortality in WT or KO mice, suggesting that vehicle treatment exacerbates LPS-induced inflammation but is not inflammatory on its own (fig. S7D). Collectively, these findings suggest that cytokine production and LPS-induced mortality in S1R KO mice require excessive IRE1 endonuclease activity. We next aimed to directly assess whether S1R function might be manipulated for benefit in an in vivo inflammatory context. We selected FLV, an antidepressant drug with low-nanomolar affinity for S1R, which has also been reported to have anti-inflammatory properties (28). To elicit higher mortality in WT mice, we selected a higher dose of LPS (6 mg/kg) for this experiment and first administered FLV (20 mg/kg) at the same time as LPS (Fig. 4B). FLV treatment significantly protected WT mice from mortality and reduced serum IL-6, whereas, as expected, no significant effect was observed in S1R KO animals ($P <$

0.05; Fig. 4, E and F). These results indicate that the anti-inflammatory effect of FLV is mediated by S1R.

Therapeutic administration of a S1R ligand is beneficial in preclinical models of sepsis and inflammation

We next tested whether FLV could be therapeutically administered to protect C57BL/6J from LPS administration or ongoing FIP sepsis model. FLV was administered as indicated in Fig. 5A (90 min after LPS challenge) and Fig. 5B (30 min after FIP induction), after animals

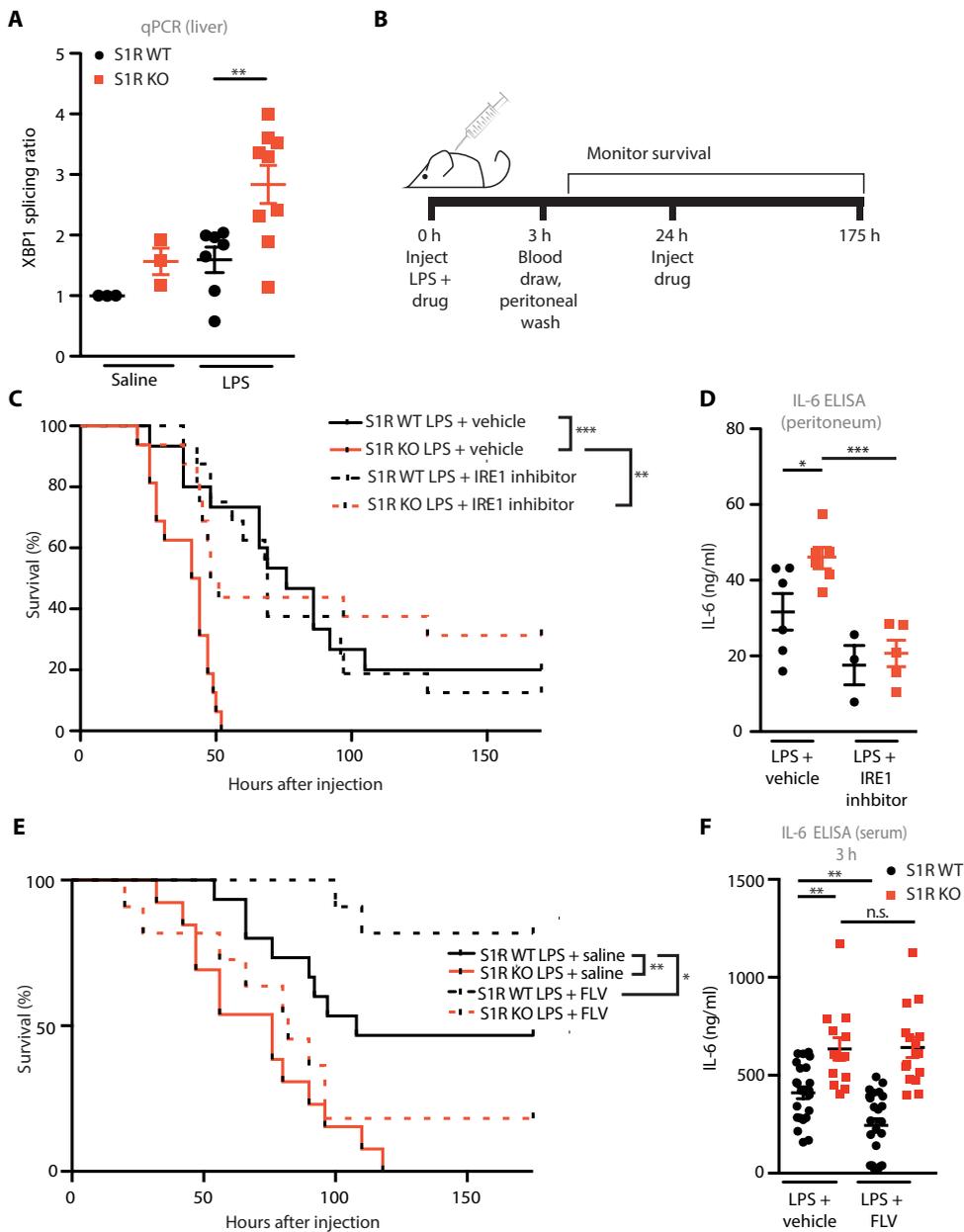


Fig. 4. Pharmacological modulation of S1R and IRE1 function in sepsis models. (A) XBP1 splicing ratio from liver homogenate of mice challenged with LPS (5 mg/kg) for 3 hours. Data shown are ratio of XBP1 spliced transcript/XBP1 unspliced transcript (each dot represents one mouse; $^{**}P < 0.01$, two-way ANOVA with post hoc Sidak test). (B) Experimental design. (C) Survival curve of WT and S1R KO mice treated with vehicle (33% Kolliphor in saline) or STF (30 mg/kg) after administration of LPS (2 mg/kg) as indicated in (B) ($n = 15$ to 16 mice per group; $^{**}P < 0.01$, $^{***}P < 0.001$, log-rank test). (D) IL-6 peritoneal exudate 3 hours after LPS injection in mice (each dot represents one mouse; $^{*}P < 0.05$, $^{***}P < 0.001$, two-way ANOVA with post hoc Sidak test). (E) Survival curve of WT and S1R KO mice treated with vehicle or FLV (20 mg/kg) after administration of LPS (6 mg/kg) as indicated in (E) ($n = 13$ to 17 mice per group; $^{*}P < 0.05$, $^{**}P < 0.01$, log-rank test). (F) Serum IL-6 ELISA 3 hours after LPS injection in mice challenged as shown in (E) (each dot represents one mouse; $^{**}P < 0.01$, two-way ANOVA with post hoc Sidak test; genotype-treatment interaction: $^{*}P < 0.05$, two-way ANOVA).

presented with a significant sickness behavior characterized by a decrease in body temperature ($P < 0.001$; Fig. 5, C and D) and a clinical presentation of sepsis signs ($P < 0.01$; Fig. 5, E and F). Therapeutic administration of FLV improved the clinical score (Fig. 5, E and F) and the temperature (Fig. 5, G and H) of challenged animals.

cells in numerous contexts (1, 30). However, little is yet known about the factors that modulate the extent of IRE1 signaling during inflammation. Here, we identify S1R as a regulator of IRE1 endonuclease function during LPS-induced inflammation (fig. S9). S1R and IRE1 may associate both basally and after LPS stimulation, suggesting that

The treatment also significantly enhanced survival in both animal models ($P < 0.01$; Fig. 5, I and J). FLV treatment was also beneficial in the FIP model when administered at an even later time point after FIP induction (90 min instead of 30 min; Fig. 5, K and L). To directly compare the effectiveness of FLV to the currently available therapeutics, we also administered ceftriaxone (CRO), an antibiotic currently used as a standard of care for sepsis patients (29), 90 min after FIP induction. FLV administration was as efficacious in enhancing survival as CRO (100 mg/kg; Fig. 5L), and the combination of FLV and CRO did not further improve survival when compared to single treatment (Fig. 5L).

FLV is anti-inflammatory in human cells

To assess whether targeting S1R can dampen inflammation in human cells, heparinized peripheral blood from healthy donors was stimulated ex vivo with LPS (10 ng/ml) in the presence or absence of FLV (20 μ M), and the production of inflammatory mediators was measured by multiplex analysis. FLV significantly reduced LPS-induced IL-6 ($P < 0.01$; Fig. 6A), IL-1 β ($P < 0.05$; Fig. 6B), and IL-12 p40 ($P < 0.01$; Fig. 6C) and decreased IL-8 (Fig. 6D) production in cells from all donors analyzed. These data indicate that the anti-inflammatory action of this S1R ligand is likely conserved across species. Modulation of S1R during LPS treatment was limited to a subset of inflammatory mediators (fig. S8) and was not the result of global cytokine suppression. Together, our data show that FLV can influence the inflammatory response in murine and human cells in a S1R-dependent manner and suggest that therapeutic exploitation of S1R targeting might hold promise for the control of inflammatory insults.

DISCUSSION

The ER stress sensing protein IRE1 α (and the closely related protein IRE1 β) is able to powerfully affect the inflammatory behavior of both immune and nonimmune

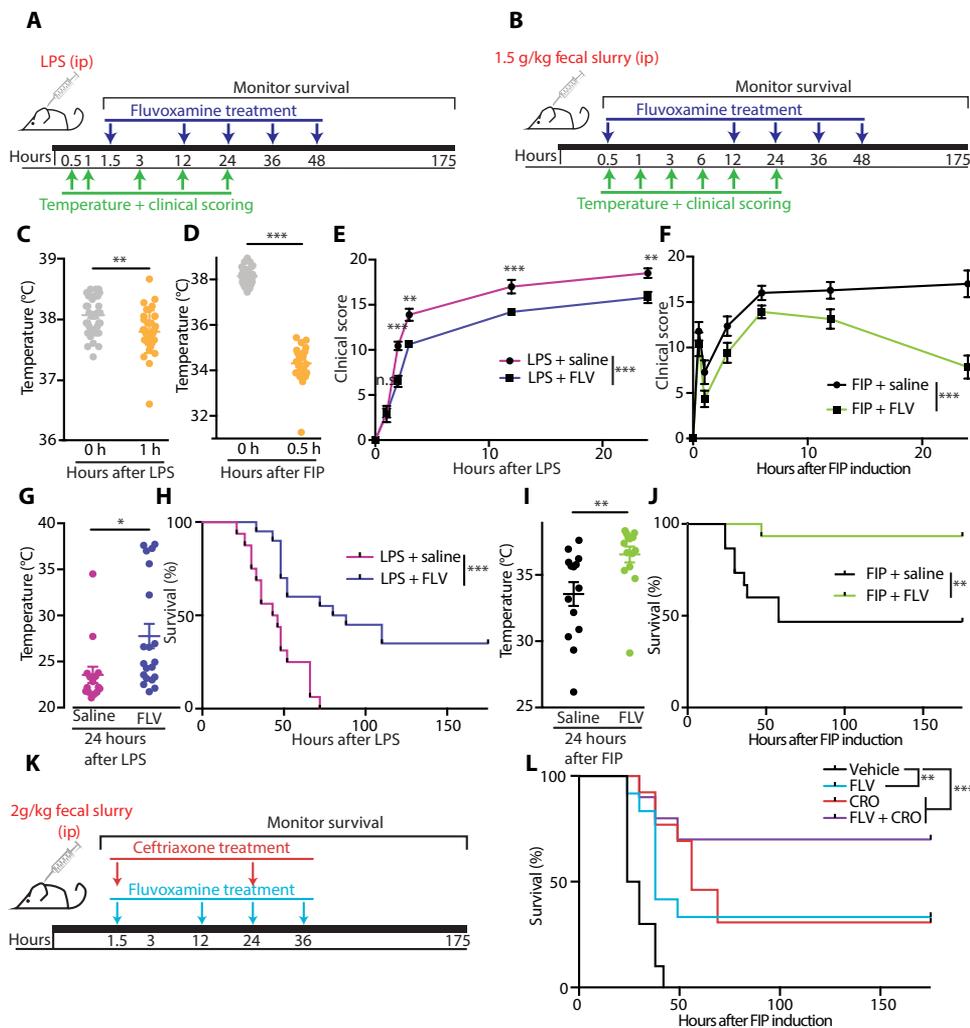


Fig. 5. Therapeutic administration of the S1R agonist FLV is protective during models of inflammation and sepsis. (A and B) Experimental design for (A) LPS challenge or (B) FIP with therapeutic S1R agonist treatment. Intraperitoneally, ip. (C) Rectal temperature of mice measured immediately before LPS injection and 1 hour after (each dot represents one mouse; $**P < 0.01$, paired *t* test). (D) Rectal temperature of mice measured immediately before FIP induction and 0.5 hour after (each dot represents one mouse; $***P < 0.001$, paired *t* test). (E) Clinical score, expressed as total murine sepsis score, of mice treated as in (A) ($**P < 0.01$, $***P < 0.001$, repeated measures two-way ANOVA with post hoc Sidak test). (F) Clinical score, expressed as total murine sepsis score, of mice treated as in (B) ($n = 14$ mice per group; $***P < 0.001$, two-way ANOVA). (G) Rectal temperatures of mice 24 hours after intraperitoneal LPS injection, treated with saline vehicle or FLV as indicated in (A) (each dot represents one mouse; $*P < 0.05$, *t* test). (H) Survival curve of mice challenged with LPS (6 mg/kg) and given therapeutic FLV or saline as indicated in (A) ($n = 16$ to 20; $***P < 0.001$, log-rank test). (I) Rectal temperatures of mice 24 hours after FIP induction, treated with saline vehicle or FLV as indicated in (B) (each dot represents one mouse; $**P < 0.01$, *t* test). (J) Survival curve of mice challenged with fecal slurry (1.5 g/kg) and given therapeutic FLV or saline as indicated in (I) ($n = 14$ mice per group; $**P < 0.01$, log-rank test). (K) Experimental design for FIP challenge with FLV and the antibiotic CRO treatment. (L) Survival of C57/Bl6 mice treated with FLV (20 mg/kg, ip) and/or CRO (100 mg/kg, subcutaneously) after administration of fecal slurry at 2 g/kg ($n = 10$ to 12 mice per group; $**P < 0.01$, $***P < 0.001$, log-rank test).

S1R is uniquely poised to sensitively control IRE1 activity. This finding is particularly exciting, because the physiological and pathophysiological relevance of IRE1 is well established (30). However, direct therapeutic targeting of IRE1 has been met with substantial challenges (7, 8), and alternative routes toward IRE1 signaling modulation are sought after. We propose that S1R, which can be targeted by several drugs that are already in clinical use, might represent one such option.

One important caveat of our study is that, although preclinical models of septic shock are convenient for the discovery of new therapeutic treatments for sepsis, they incompletely replicate human sepsis, and translational efficacy of preclinical findings in human patients is difficult to predict. There are likely many factors contributing to this challenge, including diversity of predisposition and underlying physiological features, heterogeneous pathophysiology, and variability of causative infectious agents (31). Clinically defining such a broad array of processes as one syndrome, sepsis, complicates the application of novel therapeutic approaches. Work to more effectively design clinical sepsis studies to properly apply preclinical findings is an active area of the sepsis field (32). At the same time, novel preclinical sepsis models and methods that may allow for experimentation in a wider range of conditions associated with sepsis are arising (33). Despite these challenges, preclinical sepsis models have shown promise, and more precise classification of sepsis conditions may lead to efficacious application of interventions that have been identified in animal models.

Here, we show that S1R deficiency appears to selectively enhance activity of IRE1 and does not influence other inflammatory pathways, including NF- κ B, JNK, and ERK. The ability of the S1R-IRE1 interaction to influence immune and non-immune cell activity may prove to be of importance in inflammatory and degenerative diseases in which S1R and IRE1 dysfunction have been implicated, including Alzheimer's disease (34, 35) and amyotrophic lateral sclerosis (11, 36). In addition, although our study focused on XBP1 splicing as an indicator of IRE1 endonuclease activity, IRE1 can also cleave other RNA species (in a process called regulated IRE1-dependent decay or RIDD), which may drive inflammation as well (1). Some of our observed findings may be a result of RIDD and may not depend on XBP1.

Another potential mechanism is that S1R may be altering calcium signaling, because S1R has been shown to modulate the conductivity of inositol triphosphate receptor (37). However, several lines of evidence suggest that changes in calcium signaling do not produce the observed inflammatory effects. First, we show that the cytosolic inflammatory signaling proteins NF- κ B, JNK, and ERK1/2, all of which are sensitive to changes in calcium, are not affected by S1R deletion. In addition, calcium dysregulation might inhibit protein folding, but

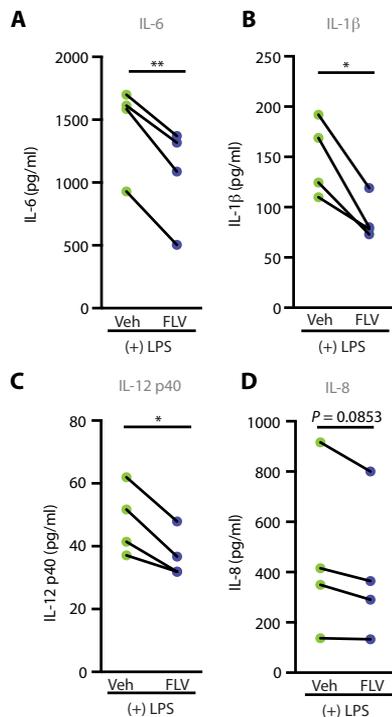


Fig. 6. The S1R agonist FLV is anti-inflammatory in human cells. (A to D) Multiplex ELISA on serum from human blood. Heparinized whole blood was stimulated ex vivo with LPS (10 ng/ml) and vehicle (RPMI 1640) or 20 μ M FLV for 4 hours ($n = 4$, each dot pair represents serum from one participant; * $P < 0.05$, ** $P < 0.01$, paired t test).

we do not observe changes in ER chaperone abundance basally or after LPS stimulation of S1R KO BMDMs, again suggesting that S1R does not strongly perturb calcium homeostasis in mammalian cells. Further studies examining calcium flux in S1R KO BMDMs might reveal additional important features of S1R function, such as during prolonged ER stress, but calcium flux does not appear to be central to the pathway described in this study.

One limitation of our study is that we have not fully elucidated the mechanism by which S1R controls IRE1. Although we have demonstrated with our proximity ligation approach that S1R and IRE1 were in close proximity and that interaction was further promoted upon LPS stimulation, our study did not address whether intermediates were necessary for the formation of the putative S1R-IRE1 complex. Our attempts at coimmunoprecipitation did not convincingly demonstrate a strong association between endogenous S1R and IRE1, unlike what has been described with overexpressed proteins (9). This suggests that the interaction might be transient and/or require intermediates. Furthermore, our study did not explore what are the signaling events that control S1R activity in the context of inflammation. To date, only one endogenous S1R ligand has been identified, *N,N*-dimethyltryptamine (DMT), a tryptophan metabolite known for its psychedelic activity (38). Limited evidence suggests that DMT-S1R interactions can influence the activation of immune cells (15). Perhaps understanding the interaction of S1R and its endogenous ligands that are regulated by inflammation could help answer this open question.

Although we found that the anti-inflammatory action of FLV depends on S1R in our animal models of sepsis and inflammation, there is still much to be learned about FLV and other selective serotonin

reuptake inhibitors (SSRIs) in inflammation. Some SSRIs that do not have affinity for S1R also have reported anti-inflammatory properties, linking serotonin signaling in immune cells to inflammation (39). Furthermore, it remains unknown whether the requirement for S1R in FLV efficacy is due to direct binding of FLV to S1R. Nonetheless, it will be important to consider S1R when studying SSRIs in inflammation.

MATERIALS AND METHODS

Study design

The goal of our study was to identify the role of S1R during LPS-mediated inflammation. Using animal models of inflammation and sepsis, we demonstrated that S1R is an inhibitor of cytokine production. We elucidated the mechanism by which S1R controls the inflammatory response via IRE1 with primary BMDMs and HEK293. Using pharmacological inhibitors, we used two in vivo models of sepsis to validate our in vitro findings showing the mechanism of action of S1R and IRE1. We lastly confirm our results obtained with FLV using human blood samples. In all experiments, animals were randomly assigned to treatment groups, and researchers were blinded during treatment and data collection. Group and sample size for each experiment are indicated in each figure legend. No statistical methods were used to predetermine sample sizes for in vitro experiments. Sample sizes for in vivo and ex vivo experiments were predetermined using G*Power, with $1 - \beta \geq 0.85$. Post hoc power calculations were performed on in vitro studies (except where representative data are shown) using G*Power to ensure that $1 - \beta \geq 0.85$. Primary data are reported in data file S1.

Mice

C57BL/6J (8 weeks old) was purchased from the Jackson Laboratory. The S1R KO mouse strain was acquired from the Mutant Mouse Resource and Research Centers and bred to C57BL/6J at the University of Virginia to generate WT and KO mice used in the study (40, 41). All animal experiments were approved and complied with regulations of the Institutional Animal Care and Use Committee at University of Virginia (no. 3918).

Tissue culture conditions and reagents

HEK293 mTLR4/MD2/CD14 (InvivoGen, catalog no. 293-mtlr4md2cd14), primary lung fibroblasts, and BMDMs were isolated and maintained as described (42, 43). Cells and animals were treated with LPS (Sigma, product no. L4391), 4 μ 8C (Tocris, catalog no. 4479), APY-29 (Medchem Express, catalog no. HY-17537), PD98059 (Medchem Express, catalog no. HY-12028), JSH-23 (Medchem Express, catalog no. HY-13982), SP600125 (Medchem Express, catalog no. HY-12041), STF-083010 (Medchem Express, catalog no. HY-15845), FLV (Medchem Express, catalog no. HY-B0103A), and CRO (Hospira, National Drug Codes code 0409-7337-01), as described in the text.

LPS challenge

In vivo LPS challenge was performed on adult mice (8 to 12 weeks of age). LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich, product no. L2630) was injected intraperitoneally, as described in the text. STF-083010 (Medchem Express, catalog no. HY-15845) was resuspended in 33% Kolliphor-EL (Sigma-Aldrich, product no. C5135) and administered intraperitoneally at 30 mg/kg immediately after and again 24 hours after LPS injection. FLV was resuspended in saline and administered at 20 mg/kg as indicated in the text. Blood for

serum ELISA was collected from facial vein at the predicted peak serum concentration of TNF- α and IL-6 (21).

Fecal-induced peritonitis

Fecal material was isolated from the caecum of age- and sex-matched WT animals coming from the University of Virginia vivarium for Fig. 3 or from the Jackson Laboratory for Fig. 5, resuspended in saline, and passed through a 70- μ M strainer to remove large particles. The slurry was prepared fresh for each experiment and administered intraperitoneally. Core body temperature was measured, and mice were scored with murine sepsis severity scale by two independent, blinded researchers (26). Blood for serum ELISA was collected from facial vein at 3 hours after FIP induction. FLV in saline was administered intraperitoneally at a dose of 20 mg/kg at the same time as FIP, 30 min later, or 90 min later, as indicated in schematic figure panels, and CRO in saline was given at a dose of 100 mg/kg subcutaneously as indicated in the text.

Serum preparation

Serum was collected 24 hours after injection of LPS or fecal slurry. Serum chemistry analysis was performed by Comparative Clinical Pathology Services LLC. ELISA was performed on serum as described below.

Enzyme-linked immunosorbent assay

ELISA for IL-6 and TNF- α were performed as previously described (25). Antibodies used were as follows: anti-mouse IL-6 MP5-20F3 (0.5 μ g/ml; BioLegend, catalog no. 504501), biotin anti-mouse IL-6 MP5-32C11 (1 μ g/ml; BioLegend, catalog no. 504601), anti-mouse TNF- α (0.5 μ g/ml; R&D systems, catalog no. AF-410-NA), and biotin anti-mouse TNF- α (0.25 μ g/ml; R&D systems, catalog no. BAF410).

Peritoneal exudates collection

Peritoneal cavities content were collected 3 hours after LPS injection in phosphate-buffered saline + 5 mM EDTA and then centrifuged to pellet cells. Supernatants were collected for ELISA, and cells were washed and stored as previously described (44).

Western blot

Protein extraction and Western blot were performed as previously described (44). Antibodies were used according to manufacturer's instruction: actin (1:5000; Sigma-Aldrich, catalog no. A2228), BiP (1:1000; BD Biosciences, catalog no. 610798), total ERK1/2 [1:1000; Cell Signaling Technology (CST), 9102], phospho-ERK1/2, (1:1000; CST, 4370), total IRE1 α (1:1000; CST, 3294), total JNK (1:1000; CST, 9252), phospho-JNK (1:1000; CST, 9251), total p65 NF- κ B (1:1000; CST, 8242), phospho p65 NF- κ B (1:1000; CST, 3033), PDI (1:1000; Abcam, ab2792), and total PERK (1:1000; CST, 3192). Linear level adjustments were applied to entire images to enhance visualization.

Cloning and transfection

Plasmids used were as follows: mammalian gene collection Mouse Sigmar1 cDNA (GE Life Sciences, MMM1013-202768624) and pcDNA3.1 multiple cloning site (MCS)-BirA(R118G) HA (Addgene plasmid no. 36047) (18). The S1R-BirA HA construct was generated by cloning murine S1R open reading frame upstream of BirA into pcDNA3.1 MCS-BirA HA. HEK293 mTLR4/MD2/CD14 were transfected using X-tremeGENE HP transfection reagent (Roche, 06366244001) according to the manufacturer's instructions.

Proximity biotinylation

Culture medium was supplemented with 80 μ M biotin (Research Products International, B40040) and LPS 18 hours after transfection. Biotinylated proteins were purified as described (44).

cDNA synthesis and qPCR

Total RNA was extracted using an ISOLATE II RNA kit (Bioline, BIO-52073), and cDNA synthesis was performed with the SensiFAST cDNA synthesis kit (Bioline, BIO-65054). TaqMan probes were obtained from Thermo Fisher [GAPDH, Mm99999915_g1; IL-6, Mm00446190_m1; pro-IL-1 β , Mm00434228_m1; and IL10, Mm004396]. Primers for the detection of XBP1, IL-8, and actin were previously published (45–47). qPCR was performed as described previously (44).

Flow cytometry

Flow cytometric analyses were performed as described (48). The following antibodies were used: TLR4/MD-2 Complex APC (Thermo Fisher Scientific, catalog no. 17-9924-82), F4/80 Antigen PE (Thermo Fisher Scientific, catalog no. 12-4801-80), F4/80 Antigen PE Cy7 (Thermo Fisher Scientific, catalog no. 25-4801-82), CD11b PE Cy7 (BioLegend, catalog no. 101215), CD11b eFluor 450 (Thermo Fisher Scientific, catalog no. 48-0112-82), CD11b APC (Thermo Fisher Scientific, catalog no. 17-0112-82), CD45 PerCP Cy5.5 (Thermo Fisher Scientific, catalog no. 45-0451-82), CD45 APC (Thermo Fisher Scientific, catalog no. 17-0451-82), Ly6G APC Cy7 (Tonbo Biosciences, catalog no. 25-1276), CD19 PE Cy7 (Thermo Fisher Scientific, catalog no. 25-0193-82), CD4 eFluor 450 (Thermo Fisher Scientific, catalog no. 48-0042-82), T cell receptor β (Thermo Fisher Scientific, catalog no. 12-5961-83), CD8 Alexa Fluor 488 (Thermo Fisher Scientific, catalog no. 53-0081-82), CD11c PerCP Cy5.5 (Thermo Fisher Scientific, catalog no. 45-0114-82), CD115 APC (Thermo Fisher Scientific, catalog no. 17-1152-82), Fc Block (Thermo Fisher Scientific, catalog no. 14-9161-71), and a Zombie Aqua Fixable Viability kit (BioLegend, catalog no. 423101).

Human whole-blood stimulation

Study participants were healthy adults (ages 18 to 45). The study was approved by the Institutional Review Board at the University of Virginia (no. 13166), and all participants signed an informed consent before enrollment. Blood was collected into a heparinized vacuum tube and then stimulated with LPS (10 ng/ml) \pm 20 μ M FLV for 4 hours, as described (49). Cytokine concentrations were determined by multiplex analysis.

Data analysis and statistics

Data are represented as means \pm SEM. Densitometry was performed using ImageJ software. Statistical analyses, as indicated in each figure legend, were performed using GraphPad Prism 6. All *t* tests were two-tailed. Robust regression and Outlier removal analysis was used to identify outliers, with *Q* = 1%, and outliers identified by this method were excluded from analysis. The D'Agostino and Pearson omnibus normality test was used to assess normality of data sets. Power analyses were performed with G*Power 3.1.

SUPPLEMENTARY MATERIALS

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Fig. S1. S1R deletion influences a subset of LPS-induced processes without causing global perturbation.

Fig. S2. Canonical TLR4 signaling in macrophages is unperturbed by S1R deletion.

Fig. S3. Gating strategy and quantification of immunophenotyping on blood.

Fig. S4. Gating strategy and quantification of immunophenotyping on peritoneal contents.

Fig. S5. Gating strategy and quantification of immunophenotyping on spleen and lymph node.
 Fig. S6. S1R controls inflammation in primary fibroblasts.
 Fig. S7. STF affects cytokine production in vitro and in vivo.
 Fig. S8. Anti-inflammatory effect of FLV does not globally suppress cytokine production from human blood.
 Fig. S9. Proposed mechanism of action of S1R during LPS-mediated inflammatory response.
 Data file S1. Primary data (Excel file).

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and A.G. are inventors on U.S. Provisional patent application no. 62/618,741 submitted by the University of Virginia entitled “Compositions and Methods for Regulating Inflammation.” The authors declare that they have no other competing interests. **Data and materials availability:** All data related to this study can be found in the paper and the Supplementary Materials.

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Modulation of the sigma-1 receptor–IRE1 pathway is beneficial in preclinical models of inflammation and sepsis

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Sigma-1 receptor subdues systemic inflammation

Systemic inflammation can be lethal, as in the case of septic shock. Rosen *et al.* hypothesized that the endoplasmic reticulum, now understood to affect inflammation, could be an untapped therapeutic target. They found that mice lacking the endoplasmic reticulum sigma-1 receptor had exacerbated responses to LPS or fecal slurry. The antidepressant fluvoxamine can bind sigma-1 and acts as an agonist. Therapeutic treatment of mice in the two inflammatory models revealed that fluvoxamine lowered inflammatory cytokine production and improved survival. Their results suggest that repurposing fluvoxamine to enhance sigma-1 activity may be beneficial for treating sepsis.

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