Sigma-1 Receptor and the Control of Inflammation

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A Dissertation presented to the Graduate Faculty of the
University of Virginia in Candidacy for the Degree of
Doctor of Philosophy

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May 2019

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Are you satisfied with it? With *this* world?

Chapter 1
What is known about sepsis, and what remains to be known

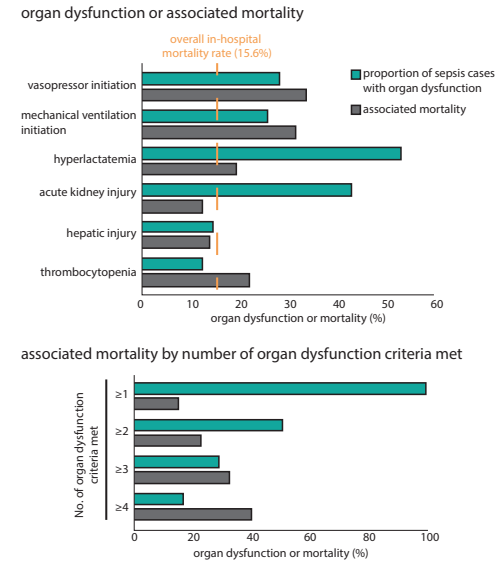
Sepsis overview

Sepsis is a heterogeneous syndrome of immune system dysfunction, vascular pathology, and organ failure that arises due to severe infection. It is a challenging clinical entity because it can be caused by a huge array of pathogens and often occurs comorbidly with other disease. Presently, best practices for improving outcomes in sepsis rely on hemodynamic support and antibiotic treatment, and no targeted therapeutics have been approved for clinical use in sepsis. Clinical course is unpredictable and can look very different from patient to patient, and many sepsis patients experience a hyperinflammatory state followed by an immunosuppressed state, with the transition being difficult to detect rapidly for appropriate pharmacologic modulation. Advances are being made in understanding sepsis and in efficiently testing novel therapeutic approaches tailored to patients' needs with good temporal resolution. At the same time, important discoveries about immune system function have been made utilizing animal models of sepsis, which may yet lead to the development of new sepsis therapies.

Sepsis epidemiology

In the United States, sepsis occurs in approximately 300-400 out of every 100,000 people, and the incidence has been rising over the last several decades, although there is some evidence that sepsis incidence is beginning to stabilize[1–4]. Potential explanations for increased rates of sepsis include increasing clinical awareness and formal diagnosis, aging populations, rise of antibiotic-resistant bacteria, and medical advances, such as transplantation, that carry a high risk of infection and sepsis[5]. Mortality from sepsis in wealthy countries, including the US, has been declining, but this is due to changes in clinical care protocols, not application of novel therapeutics[6]. In high-income countries, sepsis tends to arise in relation to other hospital procedures, often because of respiratory infections (Fig. 1A[7])[8]. In contrast, in low- and middle-income countries, sepsis arising from community-acquired infection is usually the result of skin or gastrointestinal infections or septic abortion[9].

A



B

Organism	Western Europe	Eastern Europe	Central/ South America	North America	Oceania
Staphylococcus aureus/MRSA	20/9	22/10	19/11	27/18	28/9
Staphylococcus epidermidis	11	12	9	12	8
Streptococcus pneumoniae	5	5	3	4	3
VSE	9	10	2	5	4
VRE	4	5	2	5	5
Escheria coli	17	15	14	14	13
Enterobacter spp	7	8	9	8	3
Klebsiella spp	10	21	16	9	12
Pseudomonas spp	17	29	26	13	15
Acinetobacter spp	6	17	14	4	4
ESBL producing GNR	2	2	3	0	0
Anaerobes	5	3	1	8	3
Candida spp	19	19	13	18	13
Aspergillus spp	2	0	0	3	2
Parasites	1	1	1	1	1

Figure 1: Factors associated with sepsis and sepsis mortality. A) Organ dysfunction and mortality in sepsis. Top: failures of different organs contribute differentially to mortality risk in sepsis. Bottom: involvement of more organs in organ failure correlates with increased mortality in sepsis. **B)** Rates of identified causative organisms in sepsis around the world. Adapted from Rhee et al. and Gotts & Matthay.

The incidence and mortality of sepsis have a bimodal distribution, with infants and adults over 60 being the most susceptible[10]. Comorbid conditions also increase sepsis incidence and mortality and include kidney disease, diabetes, and cancer[11,12]. Genetic characteristics that do not cause overt disease may also contribute to sepsis incidence and mortality, as patients with a mutation in TLR4, an extracellular receptor for lipopolysaccharide (LPS), a component of gram-negative bacterial walls, are more likely to develop gram-negative sepsis[13]. Evidence suggests that mitochondrial DNA haplogroup may also affect sepsis survival by influencing the interplay of cyclooxygenase and citrate synthase during inflammation[14]. These are only two of many candidate genes that may influence sepsis vulnerability, and this is an area of active study. Many other potential polymorphisms have been implicated in sepsis, ranging from LPS coreceptors and cytokines to coagulation factors and beyond[15]. A better understanding of how naturally occurring diversity influences sepsis may lead to the development of novel therapeutics.

Sepsis can be caused by any infectious agent, including bacteria, fungi, viruses, and parasites (Fig. 1B[16]). Historically, gram-negative bacteria have been the primary causative agents in sepsis, but sepsis due to gram-positive bacteria is becoming more common as sepsis arises more due to surgical procedures and hospital stays[10]. Gram-negative and gram-positive bacteria now contribute approximately equally to sepsis

cases, with fungi accounting for 5-20% of cases[17,18]. Viral and parasitic sepsis are less commonly studied. The most common causative organisms in sepsis are *Staphylococcus aureus* and *Pseudomonas*, each of which were identified in 20% of patient isolates in an examination of 13,796 patients from 75 countries[17]. In this study, infection with *Pseudomonas*, *Enterococcus*, or *Acinetobacter* species was associated with higher risk of death, emphasizing how causative pathogens can contribute to clinical variability[17]. Pathogen diversity remains a clinical challenge in sepsis treatment, as pathogen species is usually unknown until long after presentation of symptoms and may never be uncovered in some patients[7].

Sepsis pathophysiology

Sepsis begins when molecular pattern recognition receptors (PRRs) recognize the presence of a pathogen and/or tissue damage within a sterile tissue. PRRs recognize two subsets of molecular patterns, both of which drive inflammation during sepsis. One subset recognized consists of pathogen-associated molecular patterns (PAMPs), which are essential features of pathogens, such as LPS, flagellin, and dsRNA. The other subset recognized is damage-associated molecular patterns (DAMPs), also known as alarmins, molecules that are released when host tissue is injured. DAMPs include heat shock proteins (HSPs), including ER-resident HSPs, as well as genomic and mitochondrial DNA and HMGB1. In infection, PAMPs and DAMPs are initially detected by neutrophils and innate lymphoid cells (ILCs)[19]. Both neutrophils and ILCs secrete cytokines to activate additional immune cascades and can produce reactive oxygen species (ROS) to destroy pathogens. Neutrophils take direct action against pathogens, containing them by producing neutrophil extracellular traps or attacking pathogen populations by phagocytosis[20]. Macrophages and dendritic cells (DCs) are rapidly recruited as secondary responders in infection. Both of these cell types can produce cytokines and ROS and can phagocytose pathogen. Macrophages are of particular interest in sepsis as they are powerful controllers of cytokine cascades. DCs act as the major bridge to the adaptive immune system, activating B cells to produce antibodies and T cells to produce

cytokines and perform cytotoxic functions. In addition to professional immune cell activity, mesenchymal cells also produce inflammatory cytokines and ROS in infection and sepsis. High induction of these inflammatory factors can make blood vessels leaky, and the resulting failures of circulation lead to tissue dysfunction and organ damage.

In addition to their immunomodulatory functions at the site of infection, cytokines induce the liver to produce acute phase proteins. Acute phase proteins modulate coagulation, and can drive disseminated intravascular coagulation, which is a cause of mortality in sepsis[21]. An important subset of acute phase proteins, complement proteins, are produced in large quantities by the liver during sepsis. Complement can be activated by three mechanisms: antigen-bound antibody (classical pathway), spontaneous hydrolysis of C3, the first step in the complement pathway (alternative pathway), and binding to mannose on pathogen surfaces (lectin pathway). The alternative pathway, which is always running at a low level, acts first in sepsis patients and higher levels of early alternative complement activation may be protective, while the classical and lectin pathways are activated more slowly[22]. Complement is a potent anti-pathogen system, enhancing phagocytosis by opsonization, recruiting professional phagocytes like macrophages or neutrophils, and directly forming pores in pathogen membranes, and is therefore of significant interest for development of sepsis therapeutics. Inhibition of C5a, a chemotactic agent and anaphylatoxin produced by the complement cascade, has shown promise in rodent and primate models of sepsis[23,24].

Contrasting with the highly inflammatory state that typically occurs in early sepsis, critically ill sepsis patients with a high degree of organ failure tend to have few immune cells. In particular, B and T cells become massively depleted in patients dying of sepsis and organ failure, due to caspase-9 dependent apoptosis[25]. Populations of DCs, which interact extensively with B and T cells, also shrink in the spleens of critically ill sepsis patients, though macrophages appear to be spared from sepsis-driven cell death[26]. Death in these inflammatory anti-pathogen immune cells are accompanied by proportional increases in regulatory T cells (Tregs), which can inhibit B and T cells and DCs. Higher levels of a CD39 expressing subset of Tregs

correlate with higher mortality in sepsis patients[27]. Suppression of immune activity, whether by Tregs, lymphocyte exhaustion, metabolic depletion, or potentially other unknown mechanisms, allows for resurgence of pathogen populations, driving mortality in sepsis. At the same time, some Tregs are required for resolution of sepsis in rodents[28]. This delicate balance in Treg activity typifies the challenge of developing treatments for sepsis.

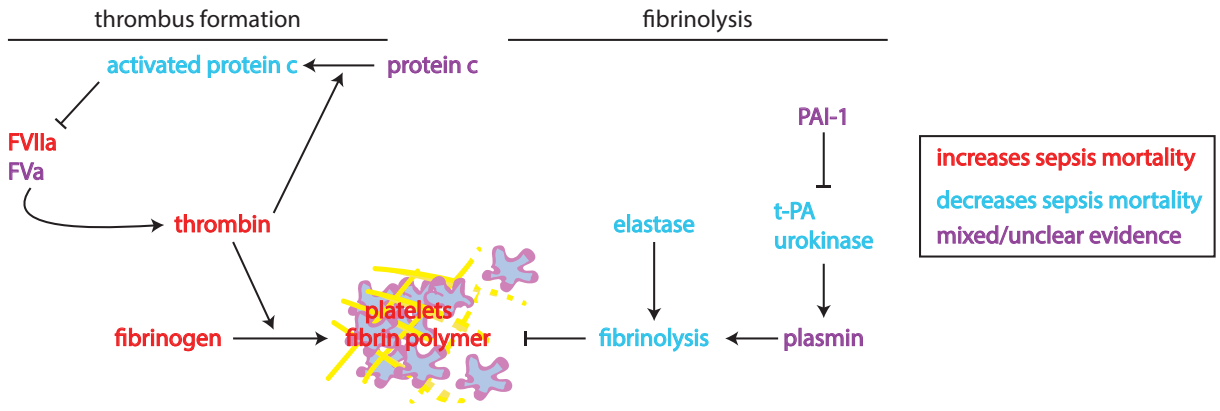


Figure 2: clotting pathways in sepsis

In addition to failures of the immune system, sepsis is very frequently characterized by coagulopathy, ranging from relatively mild clotting to disseminated intravascular coagulation, a dangerous complication of sepsis that occurs in about 35% of patients (Fig. 2[29–34])[35]. Coagulation in sepsis is mediated in large part by activation of protein C. Protein C typically circulates in its inactive form, and is activated by thrombin and thrombomodulin on the surface of epithelial cells. Activated protein C (APC) is a vitamin K dependent serine protease that cleaves coagulation factors FVa and FVIIa, which has the effect of suppressing thrombus formation[36,37]. Because activation of protein C requires cleavage by thrombin, which is a product of the coagulation cascade, APC acts as a negative feedback regulator, limiting excessive coagulation. Indeed, sepsis patients with higher levels of APC, or those whose levels rise more over time, have better survival than sepsis patients with low APC[38,39]. For this reason, recombinant APC was pursued as a potential sepsis therapeutic, and an initial clinical trial showed a 6.8% reduction in 28-day mortality,

but this promising result was not replicated in later studies[40]. Coagulation and inflammation are not independent pathways in sepsis, and APC does not act independently of inflammation: APC inhibits nuclear translocation of the essential inflammatory transcription factor NF-κB, and APC can be inhibited, in turn, by the pro-inflammatory cytokine TNFα[41,42].

In addition to clot formation, coagulopathies in sepsis are also driven by changes in fibrinolysis. During coagulation, thrombin, activated by coagulation factors, cleaves fibrinogen to create the fibrin polymers in clots, whereas in fibrinolysis, fibrin polymers are degraded, depending on the conversion of plasminogen to plasmin. Plasminogen is produced by the liver and incorporates in this inactive form into clots. Tissue plasminogen activator (t-PA) and urokinase cleave plasminogen into the active plasmin, which then breaks down fibrin polymers. In sepsis, levels of plasminogen activator inhibitor (PAI-1), an inhibitor of t-PA and urokinase that suppresses fibrinolysis, are significantly elevated, as PAI-1 is an acute-phase protein produced by the liver in response to injury. PAI-1 is higher in patients with very severe pathology, and a 2018 meta-analysis found that PAI-1 correlates negatively with survival in sepsis[43,44]. However, another study showed a protective influence of PAI-1 in a rodent model severe gram-negative pneumonia, emphasizing how important it is to understand sepsis heterogeneity in order to develop and deliver appropriate therapeutics[45]. Plasminogen processing is not the only mechanism by which clots can be degraded. Neutrophils produce elastase, which can also break down fibrin under conditions of sepsis-induced disseminated intravascular coagulation. Low levels of neutrophil-derived elastase were associated with higher mortality in sepsis patients with this severe coagulopathy[46].

In summation, tissue dysfunction in sepsis is driven by a mechanism that is incompletely understood but is currently believed to depend on three distinct, yet interlocking, pathobiological features: inflammation, coagulation, and fibrinolysis. These mechanisms converge to create a state where tissues become filled with metabolic waste and damaged protein components that they are unable to efficiently clear.

Preclinical models of sepsis

As for many human diseases and syndromes, there are no animal models that completely recapitulate the clinical features of human sepsis. The heterogeneous nature of sepsis in humans complicates both clinical recognition and preclinical modeling of the syndrome. In humans, the features and severity of sepsis depend on numerous factors, including the source of initial infection, the underlying biological vulnerabilities of sepsis patients, and the organ systems affected. Nonetheless, several models exist that reproduce bacterial-derived cytokine storm that is characteristic of sepsis in humans, as well as resulting vasculature and organ dysfunction causing death. Preclinical animal models have led to the discovery of several promising novel therapeutic approaches to sepsis [47]. Although presently none of these preclinical findings have led to clinical advances, this may be due in part to the heterogeneity of the sepsis syndrome. An active area of clinical research is in better characterizing distinct sepsis populations in order to apply novel therapeutics appropriately to patient populations most likely to benefit from them.

Bacterial-derived toxins are known to drive pathology in sepsis[18]. The most commonly used toxemia model of sepsis is endotoxin (or LPS) challenge. In endotoxin challenge, LPS, a component of gram-negative bacterial walls, is administered systemically to animals, typically as a single dose, though multiple dosing is sometimes used to model diffuse intravascular coagulation, known as the generalized Shwartzman reaction[48,49]. TLR4, the receptor for LPS, is expressed both on professional immune cells and non-immune cell types, and the effects of LPS are due to action in both of these categories of cells[50]. LPS may be given intravenously or intraperitoneally, and the kinetics of cytokine induction in mice are similar across routes of administration[51]. Administration of LPS results in a well-characterized pattern of events, the severity of which varies with dose. As early as 1 hour after exposure, LPS rapidly induces neutrophilia and lymphopenia[52]. Neutrophils infiltrate the liver and lung after systemic LPS, and may infiltrate the brain, although it is likely that neutrophils exclusively remain in the brain vasculature instead of entering the parenchyma[53–55]. Serum levels of pro-inflammatory cytokines IL-1β, TNFα, and IL-6 peak sharply around

1.5-3 hours after LPS[56,57]. The response of anti-inflammatory cytokine IL-10 is more gradual and is sustained for at least 8 hours[56]. These are all multifunctional cytokines that act on numerous cell types, including lymphoid, myeloid, and non-hematopoietic cells, and their production has varied and sometimes contradictory consequences, which makes them difficult therapeutic targets, though they may be instrumental in developing prognoses[58]. Corticosterone, a stress hormone, rises rapidly and remains elevated for at least 12 hours after LPS administration[59].

The effects of LPS have been extensively studied, and this firm foundation allows for potential innovation drawing on previous knowledge. Furthermore, low doses of LPS can be administered safely to human subjects, allowing for direct comparisons between human and animal models to be made. In this way, it is a powerful tool for understanding the overlap between rodent (or other animal) and human immune systems. Systemic LPS robustly induces cytokine storm, hypotension, and lactic acidosis. It also lacks inter-experiment variability caused by use of live bacteria. However, it does not reflect the balance between inflammation and bacterial invasion that makes sepsis such a challenging pathology. Anti-cytokine therapeutics have been tested, based in part on LPS challenge findings, but have not proven therapeutically beneficial in human sepsis trials[60,61].

In addition to toxemia models, there is a second category of animal models that can be used to study sepsis. These sepsis models utilize live bacteria instead of their toxic components to drive inflammation. Studies focusing on septic mortality driven by certain pathogens can be performed by administering that pathogen to a variety of sites, whether to the skin, the peritoneum, or other compartments, as desired. *E. coli* strains are commonly used in sepsis models. Alternatively, polymicrobial models, usually involving introduction of fecal contents to the peritoneum, can be used. Cecal ligation and puncture (CLP) has proven a powerful tool for preclinical sepsis studies[62]. In CLP, a 1-2 cm incision is made in the abdomen of an animal (usually mouse or rat) and the cecum is extruded. The cecum is tightly ligated and a large gauge needle is used to perforate the cecum. A small amount of cecal content is squeezed into

the peritoneum. After the wound is surgically closed, warmed saline is injected to precipitate the hyperdynamic phase. Severity of CLP can be modulated by changing the length of cecum that is ligated, with longer lengths causing more severe pathology. CLP consists of a cytokine storm (hyperdynamic) phase followed by an anti-inflammatory (hypodynamic) phase, similar to what is often seen in human patients[62]. Indeed, CLP studies have recapitulated failure of therapeutics, like IL-1 blockade, that appeared promising in LPS challenge but subsequently failed in humans[63].

CLP replicates several essential features of sepsis, including live pathogen and immune dysfunction beyond hyperinflammation, and can be highly reproducible when performed correctly. Furthermore, the surgical injury that occurs in CLP echoes the fact that many sepsis patients in high-income countries acquire sepsis after surgical procedures. One significant drawback of the CLP model is that it relies on the mouse's own microbiome to induce illness, and the proportions and species of gut bacteria can vary significantly depending on an array of factors that are still being investigated. It is clear that genetic background, including potential mutations that would be introduced to study the role of proteins in sepsis, as well as lived experience, can influence the populations of bacteria that can be found in the cecum of animals including humans[64–66]. One strategy to avoid this complication is direct injection of fecal material, by a method called fecal induced peritonitis (FIP). In this system, cecal material (or sometimes whole gut fecal contents) are isolated from donor mice and pooled such that every mouse receives the same proportions of bacteria that precipitate the polymicrobial sepsis. Like CLP, FIP can induce both hyperinflammatory and immunosuppressive pathology, and represents an alternative when surgical recovery and/or microbe composition may differ between experimental groups.

Though clinical trials have not yet been optimized for maximal translational efficiency, animal models of sepsis are important for understanding human inflammation across a variety of contexts. For example, LPS challenge was used to identify TNFα, a cytokine that influences the pathobiology of diseases as diverse as cancer, arthritis, and Alzheimer's disease[67]. Although there are still substantial clinical and

preclinical challenges in sepsis therapeutic development, findings arising from sepsis model studies have already had significant translational impact.

Defining sepsis: an ongoing challenge

Sepsis describes a constellation of damage to organs and vasculature resulting from dysfunctional host response to infection. It is a condition that has been a leading cause of human death throughout history. The oldest reported recording of sepsis comes from Egypt in 1700 B.C.E., though this papyrus is believed to be a copy of a document dating to 3000 B.C.E.[68]. In ancient Egypt, sepsis was understood to be caused by mixing of intestinal contents with blood vasculature, a definition that is reflected in the modern understanding of human sepsis as well as in preclinical animal models[68,69]. Over centuries, knowledge of sepsis has developed and its known causes have expanded to include more sources of infection, but even modern clinicians struggle to optimize its definition.

Defining sepsis as a distinct entity is an important clinical pursuit because the available supportive interventions become less efficacious as disease progresses[70]. The first modern consensus definitions of sepsis, developed in 1991, utilized systemic inflammatory response syndrome (SIRS) criteria, which include temperature outside 36-38 °C, tachycardia, tachypnea, and abnormalities in white blood cells, alongside presumed or confirmed infection[71]. The SIRS criteria had poor specificity, being reflective of many different inflammatory processes that may not be related to infection. Moreover, thresholds for identifying sepsis that rely on SIRS criteria excluded 1 in 8 patients who had similar sepsis-like clinical courses to patients who did meet the criteria[72]. Most recently, sepsis was clinically defined using the Sepsis-3 consensus, which aimed to reflect an expanded, more complicated understanding of sepsis than previous models, which were believed to focus excessively on inflammatory aspects without sufficiently addressing organ failure[73]. Sepsis-3, which conceptualizes sepsis as including both inflammation and coagulopathy, represents a significant advancement as the first consensus definition to be built on clinical data instead of expert

opinion alone. The sepsis-3 criteria utilize the sequential organ failure assessment (SOFA) scale, which measures the function of renal, cardiovascular, respiratory, and nervous systems, as well as accounting for the effect of clinical interventions that may have already been applied[73]. Importantly, sepsis-3 criteria rely on an increase in SOFA score instead of an absolute number, which helps to account for the differing underlying pathologies that sepsis patients may be experiencing as a baseline condition, unrelated to sepsis. The sepsis-3 definition of sepsis is *life-threatening organ dysfunction caused by a dysregulated host response to infection*, and is meant to encompass the current understanding of sepsis as potentially characterized by a wide range of system failures, including both inappropriately excessive inflammatory activity and dysfunctionally suppressed immune activation.

The sepsis-3 definition and criteria are not settled clinical practice. Although the sepsis-3 definition aims to emphasize the urgent need for treatment, requiring organ dysfunction to be present to constitute sepsis inherently means that the syndrome must be quite severe before it can be clinically recognized. Requiring organ failure to be present to determine sepsis means that sepsis patients, who are known to benefit significantly from early intervention, may experience unnecessary delays in treatment[74]. Indeed, sepsis-3 has been criticized for being difficult to implement and esoteric outside of ICU settings[75]. Discussion of clinical criteria for sepsis is ongoing in these and many other areas, such as the importance of hyperlactatemia in the absence of organ failure for recognizing sepsis risk early[75,76]. New clinical definitions, reflecting an ever more nuanced understanding of sepsis, are likely to be forthcoming.

Chapter 2
The endoplasmic reticulum is more than a trafficking hub

Overview: the many functions of the endoplasmic reticulum

The endoplasmic reticulum (ER) transports a heavy load of secreted and membrane-bound proteins, as well as being a site of lipid biosynthesis and membrane maintenance. At the same time, it contains multiple mechanisms for monitoring and responding to cellular perturbations, including proteostatic, metabolic, and inflammatory disruptions. The action of the ER has profound effects on physiology and pathology across an array of systems.

The endoplasmic reticulum in the secretory pathway

Though it is a powerful organelle for cellular signaling and integration of extracellular information, the ER is primarily known for its role in the secretory pathway. Proteins destined for membranes or the extracellular space transit the secretory pathway, a specialized protein synthesis, modification, and trafficking system that serves approximately 30% of the proteome. Most secreted proteins are cotranslationally translocated. In eukaryotes, nascent proteins are targeted to the endoplasmic reticulum when the signal recognition particle (SRP) recognizes and binds to a N-terminal signal sequence on the nascent peptide. SRP arrests translation until it is proximal to its receptor, SR, and the nascent peptide can be inserted into ER. Insertion occurs via the translocon, a complex that spans the ER membrane and functions to move proteins from the cytosolic compartment into the ER. The translocon consists of a channel formed by the Sec61 heterotrimer and is closely associated with peptide modification machinery including signal peptidases and oligosaccharyl transferase, as well as the translocating chain associated membrane protein (TRAM), and the translocon-associated protein complex (TRAP), whose functions are unclear but may be involved in glycosylation or initiation of translocation in a subset of secreted proteins[77,78]. Sec61 contains a weak seam that exposes translocating proteins to the hydrophobic lipid membrane, allowing proteins with suitable transmembrane domains to partition into the lipid phase[79]. Translocation requires a driving force to move nascent proteins through the passive Sec61 pore. In

cotranslational translocation, it is GTP hydrolysis in the ribosome itself that provides the impulse to move the protein through the transmembrane channel[80].

Multiple mechanisms for post-translational translocation of proteins have been identified. These are more common in yeast and bacteria, and usually involve secreted proteins without transmembrane domains. Post-translationally translocating proteins can enter Sec61 channels associated with tetrameric Sec62/Sec63. Post-translational translocation can be driven by the ER-resident chaperone BiP, which acts as a molecular ratchet, binding to the ER luminal segments of the protein to prohibit backwards movement through the channel[81]. Another subset of secreted proteins that tend to be post-translationally translocated are tail anchored proteins whose transmembrane sequences are translated late in the process, allowing them to evade early detection by SRP. Tail anchored proteins are usually inserted into the ER membrane by the TCR40/GET system[82].

Once the nascent peptide is inside the ER lumen, it must acquire its proper tertiary conformation and glycosylation status before being exported. Much of the ER machinery is dedicated to ensuring that proteins are properly folded and glycosylated, and malfunctions in this machinery are a primary cause of ER stress in naturally-occurring pathologies as well as in experimentally induced ER stress. The first step in this quality assurance process is the co-translational, *en bloc* transfer by oligosaccharyltransferase of a precursor oligosaccharide from a lipid to an asparagine on the nascent peptide. The consensus sequence for this transfer is asparagine, followed by X (any amino acid except proline), then serine, threonine, or rarely cysteine[83,84].

The structure of the attached oligosaccharide forms the basis for ER quality control and permitting export to the golgi apparatus. One protein folding mechanism, the calnexin/calreticulin cycle, takes advantage of the oligosaccharide status of proteins to monitor folding status. Shortly after transfer to an asparagine residue, glucosidase I and glucosidase II remove two glucose molecules from the oligosaccharide. Monoglucosylated glycoproteins are bound by calnexin (CNX) and calreticulin (CRT),

calcium-binding lectin proteins. CNX is a membrane-bound protein, while CRT is a soluble protein with ~39% sequence identity[85]. The target repertoires of CNX and CRT are generally distinct, though there appears to be some overlap, and the preference of CNX or CRT for specific targets appears to be affected by their localization within the ER[86,87]. CNX and CRT, in conjunction with the thiol oxidoreductase ERp57, are the primary facilitators of protein folding in the ER. CNX and CRT have affinity for monoglucosylated glycoproteins, and associate with misfolded monoglucosylated proteins to facilitate their proper folding. Glucosidase II can remove the final glucose from these proteins, releasing them from CNX/CRT. If the protein is properly folded, it proceeds through the secretory pathway at this point, whether by the action of lectin transporters or bulk flow. However, if the protein still possesses disordered regions, these will be recognized by UGGT1, which adds a glucose back onto the oligosaccharide, again allowing for association with CNX/CRT. As the protein remains in this loop, the terminal mannose is removed, inhibiting UGGT1 action and reentry to the CNX/CRT cycle[88]. If proteins are intractably misfolded, removal of mannose causes the misfolded protein to be targeted for retrotranslocation and degradation.

Other chaperones besides CNX and CRT can facilitate protein folding. Of these, the most abundant in the ER is immunoglobulin heavy chain binding protein (BiP). BiP is so named for its association with immunoglobulin heavy chain, which occurs only in the ER and not with the secreted immunoglobulin[89]. BiP is an ATP-binding chaperone with a C terminal domain that has affinity for hydrophobic sequences. Hydrolysis of ATP by BiP causes a conformational change that results in release of the substrate, but BiP acts as a chaperone and not an active foldase[90]. Like CNX and CRT, BiP has calcium-binding ability and participates in ER calcium buffering[91]. Whether the folding of any given protein is likely to be facilitated by CNX/CRT or BiP depends on the placement of the oligosaccharide: When the glycan occurs within 50 residues of the N terminus, the protein is likely to be folded by CNX/CRT, and glycans occurring later increase the chance that the protein will be folded by BiP[92]. In addition to its chaperone activity, BiP is notable for its role in maintaining ER membrane integrity by sealing the ER luminal side of the translocon

when translocation is not actively occurring and early in translocation[93]. This function of BiP is important for maintaining electrochemical gradient and redox balance across the ER membrane[94]. In addition to its capacity in protein folding, BiP associates with ER stress sensors to modulate their activity.

The protein trafficking functions of the ER and its signaling activity cannot be dissociated from each other. This is particularly true in the context of ER stress, when the amount of unfolded proteins in the ER lumen exceed the ability of the ER to effectively fold and secrete them. The demands of the secretory pathway feed onto ER stress signaling, and ER stress signaling, in turn, can influence the activity of secretory machinery.

Perturbing ER function precipitates the unfolded protein response

ER stress, defined as a state in which the load of unfolded proteins in the ER exceeds its ability to effectively fold and secrete those proteins, can be precipitated by diverse stimuli, including pathogens, metabolic state changes, and protein aggregates. ER stress is not inherently detrimental to cells, as it occurs as part of the regular physiology of highly secretory organs[95,96]. However, ER stress is also a feature of many human diseases, and modulation of ER stress signaling represents a rich potential field for the development of novel therapeutics, including for pathological states such as prion disease, for which there are currently no therapeutic options[97,98].

In experimental settings, ER stress can be induced by interfering with protein secretion mechanisms directly. Two of the most common experimental ER stress inducers are tunicamycin and thapsigargin, and much of what is known about ER stress comes from studies that utilize these molecules. Tunicamycin is an antibiotic and inhibitor of GlcNAc phosphotransferase, an enzyme involved in forming the precursor oligosaccharide that is transferred by oligosaccharyl transferase to nascent proteins exiting the transolocon. Preventing *en bloc* oligosaccharide transfer to proteins entering the ER lumen prevents proteins from passing sugar-dependent quality control mechanisms, so they fail to be transported to the

golgi apparatus and accumulate in the ER, activating ER stress sensors. Thapsigargin is an inhibitor of sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA), whose application depletes calcium from the ER, as leaked or released calcium cannot be restored. SERCA inhibition results in suppression of calcium-dependent chaperones, including CNX and CRT, ultimately causing an accumulation of unfolded proteins in the ER lumen[99]. One drawback of using these molecules is that they produce much more severe ER dysfunction than is typically observed in physiological systems, but their ability to robustly and reproducibly induce ER signaling makes them powerful tools for understanding how the ER responds to stress. ER stress is sensed by three ubiquitously expressed proteins in the ER membrane, inositol-requiring enzyme 1 (IRE1), protein kinase R-like ER kinase (PERK), and activating transcription factor 6 (ATF6). Upon activation, each of these ER stress sensors signal through defined pathways to allow cells to respond transcriptionally and translationally to changes in the state of the ER.

Upon activation by ER stress, these sensors orchestrate a signaling pattern that constitutes the ER unfolded protein response (UPR). The fundamental role of the UPR is the management of ER stress. The UPR occurs in two phases, the adaptive UPR and the terminal UPR. In the adaptive phase, the ER attempts to resolve ER stress using signals that suppress the load of nascent proteins being imported and/or by increasing the capacity of the ER to export proteins, whether for secretion or degradation via ER quality control mechanisms. However, with prolonged or very severe ER stress, the terminal UPR predominates, characterized by multiple mechanisms of apoptosis induction. These include loss of ER membrane integrity by insertion of the pro-apoptotic Bax/Bak pore, activation of pro-apoptotic gene programs by JNK, and/or cleavage of caspase-12[100,101,102]. Dysfunction of ER stress signaling can lead to inappropriate induction of apoptosis, which drives tissue damage in a wide array of diseases, including Alzheimer's disease and ALS[103,104,].

While the UPR serves as a useful framework for understanding the activity of IRE1, PERK, and ATF6, signaling through these molecules has been identified as affecting pathological cellular processes in

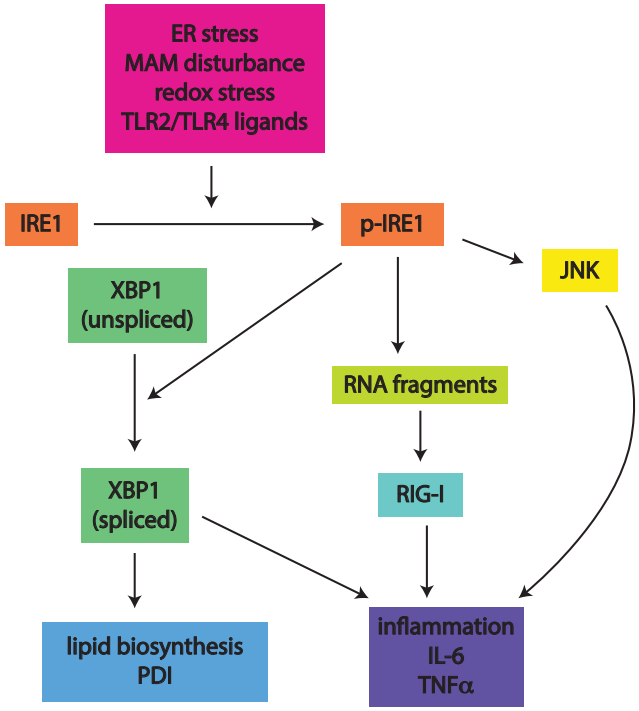


Figure 1: IRE1 signaling pathways.

numerous human diseases and models thereof. It is important to note that this signaling is sometimes activated under conditions, like redox dysregulation, that do not have a large effect on protein secretion capacity and can therefore occur in the absence of overt ER stress or the UPR.

IRE1

Of the three ER stress sensors, IRE1 is evolutionarily the oldest, as it is the only one expressed in yeast. IRE1 is so named for its role in myo-inositol synthesis in yeast[105]. There are two IRE1 paralogues, termed IRE1α and IRE1β. IRE1α is ubiquitously expressed, whereas IRE1β expression is restricted to cells of the intestinal epithelium[106,107]. IRE1α and IRE1β have overlapping and similar functions. Both have RNase domains that can splice mRNA for the transcription factor XBP1 and attenuate translation. However, IRE1α is more efficient at XBP1 splicing, while the primary function of IRE1β appears to be degradation of 28s ribosomal RNA, suppressing translation[108,109].

IRE1 is a type-1 transmembrane protein, with a luminal domain that contains its dimerization interface, while its cytosolic side is responsible for effector functions[110]. The cytosolic side of IRE1 includes a Ser/Thr kinase domain and an RNase domain, which form the platform for its signaling capacities. The kinase domain of IRE1 can act as a scaffold for other cytosolic signaling proteins[111]. Upon activation, the RNase domain of IRE1 has two functions: the structure-specific cleavage of mRNA for the transcription factor XBP1 and the degradation of a host of other mRNAs though a process called regulated IRE1-

dependent decay (RIDD). The mechanism by which IRE1 becomes activated is incompletely understood, though it may be influenced by lipid perturbations, local changes in redox state, and binding of unfolded proteins to its luminal domain[110,112,113,114,115]. One hypothesis holds that IRE1 is typically maintained in its inactive state by the ER chaperone BiP, and that ER stress activates IRE1 as unfolded proteins compete for BiP binding, causing IRE1 to become disinhibited[116,117]. However, although the association of BiP with IRE1 is reported to affect its activity level, BiP modulation alone may be insufficient for IRE1 activation[118,119].

Upon activation, human IRE1 forms dimers and higher-order oligomers, allowing for *trans* autophosphorylation. This autophosphorylation is a prerequisite for its endonuclease functions[120]. Human IRE1 molecules associate in a "face to face" orientation, and extensive contact along the interface is required for phosphorylation of Ser724, a residue in the kinase activation segment of IRE1[121]. Ser724 phosphorylation is required for induction of XBP1 splicing[121,122]. Although S724 is the best characterized human IRE1 phosphorylation site, several other phosphorylation sites have been identified, such as S726 and S729 in the kinase activation loop, as well as distal sites in the C terminus and cytosolic linker domain[123]. Phosphorylation of these sites may determine the functionality of IRE1. For example, phosphorylation of S729 is essential for RIDD induction in B cells[124]. However, the significance of phosphorylation outside of the kinase activation loop are yet unknown[122]. The phosphorylation status of IRE1 is also an essential negative regulator of IRE1 endonuclease activity, as dephosphorylation of IRE1 is required for attenuation of HAC1 (i.e. XBP1 homologue) splicing in yeast during prolonged ER stress[125].

In addition to its kinase-dependent RNase activity, the cytosolic domain of IRE1 can signal to the rest of the cell through cJUN NH_2-terminal kinases (JNK). Upon activation, IRE1 can induce JNK activity, a process which requires functionality of the IRE1 kinase domain[111]. Activation of JNK by IRE1 depends on the adaptor protein TRAF2, which is well established as an essential mediator of JNK activation[111,126]. TRAF2-IRE1 association, in turn, depends on ubiquitination of IRE1 by the E3 ligase CHIP at K828[127]. JNK is

recognized as a potent driver of cell death pursuant to ER stress, and IRE1-dependent JNK activation is one of the ways ER dysfunction can precipitate apoptosis[111,128].

Mutations in the IRE1 pathway confer susceptibility to colitis in humans. Mutations in XBP1 were identified in patients with colitis[129]. XBP1 mutations, including nonsense mutations in the hinge region between the bZIP and transactivation domains of the active XBP1 protein, and a mutation shortly upstream of the XBP1 splice site, have been identified[129]. Overexpressing these human mutants in XBP1 knockout MEFs results in abnormal XBP1-related transcriptional activity during ER stress[129]. It is unknown whether mutations in IRE1 are linked to human disease, but the centrality of IRE1 in mammalian development and in the stress response across evolution may make even slight perturbations in its function impermissible for human life.

PERK

PERK is an ER membrane anchored eIF2α kinase that is not found in yeast, but is thought to exist in all metazoans. PERK shares a similar structure to IRE1, with a luminal domain that is structurally and functionally similar to the IRE1 luminal domain[110,116]. Like IRE1, PERK activation occurs alongside BiP dissociation and oligomerization of PERK ER luminal domains. PERK *trans* autophosphorylates and assembles into back-to-back homodimers, possibly aided by binding of unfolded proteins to MHC-like grooves in the PERK luminal domains. This causes PERK monomers to "line up" and bringing the activation loop of one monomer in proximity to Thr980 on the adjacent monomer, allowing Thr980 phosphorylation, a process that is required for signaling

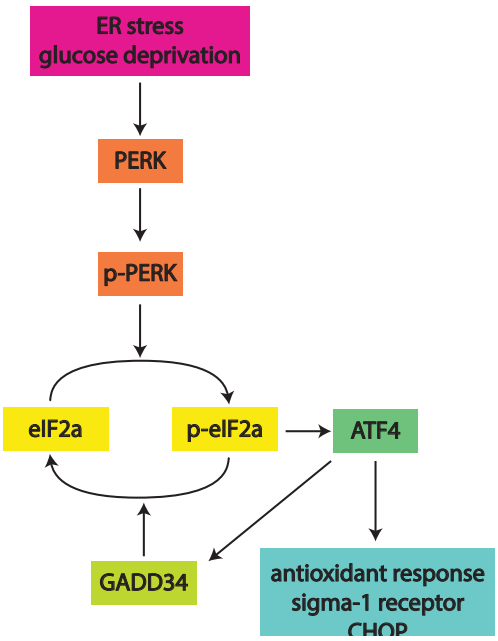


Figure 2: PERK signaling pathway.

to the rest of the cell[130]. Phosphorylated PERK is then able to phosphorylate eIF2α. eIF2α is a part of the eukaryotic initiation complex 2, which is responsible for loading initiator Met-tRNA into the ribosomal start site[131]. Upon phosphorylation, eIF2α binds to eIF2β, inhibiting the exchange of GDP for GTP. GTP is required for eIF2β function and initiation of translation. This translational inhibition is not restricted to ER-targeted proteins, and eIF2α phosphorylation is a central feature of the integrated stress response, which can also originate in the cytosol or nucleus[132]. Global translational inhibition depresses the rate at which new proteins can be made and translocated into the ER, and may allow for resources to be diverted from other cellular compartments towards the ER.

While the translational inhibition caused by eIF2α phosphorylation results in suppression of most protein translation, some transcripts are favored under conditions of eIF2α phosphorylation. Notable among these is the transcription factor ATF4. The 5' UTR of ATF4 contains two upstream ORFs. Under non-stressed conditions, uORF1 is recognized and facilitates translational reinitiation at the decoy uORF2, but when eIF2α is phosphorylated, reinitiation takes more time, bypassing uORF2 and occurring instead at the ATF4 coding region[133]. Production of the active ATF4 transcription factor results in the induction of ER-associated gene programs, as well as those associated with autophagy [134].

One well-studied target of ATF4 is C/EBP homologous protein (CHOP), a key mediator of ER-driven apoptosis. CHOP was identified by its role in the adaptive response to nutrient deprivation, and is recognized as sensitizing cells *in vitro* and *in vivo* to apoptosis during ER stress[135,136]. CHOP can form heterodimers with other C/EBP family proteins, but CHOP has substitutions of proline for basic residues in the DNA-binding zipper region, which can suppress the activity of C/EBPs bound to CHOP[137]. C/EBPs play an adaptive role in responding to stress, and inhibition of their function by CHOP is thought to be a driver of apoptosis during cellular stress. However, deletion of C/EBPβ, a major CHOP partner, protects cells from ER stress induced death in a way that is similar to deletion of CHOP itself, indicating that CHOP has a more complex relationship with C/EBP family proteins than simple inhibition[136]. CHOP can also cooperate with

AP-1 complex proteins, potentially enhancing their apoptotic activity[138]. The apoptosis-inducing interaction of AP-1 and CHOP during ER stress remains incompletely understood, but one mechanism by which this might occur is through enhancing production of the apoptotic mediators PUMA and Bax[139].

Mutation of PERK in humans causes Wolcott-Rallison syndrome, which is characterized by early-onset, non-autoimmune diabetes, typically appearing by six months of age, as well as growth defects. Identified mutations in patients with Wolcott-Rallison syndrome are mostly predicted to cause truncation of PERK, though missense mutations and an intronic mutation have been identified[140]. The effect of PERK disruption in humans is very similar to the phenotype observed in PERK knockout mice, with early postnatal diabetes and pancreatic islet degeneration, skeletal dysplasia, and growth retardation[141]. PERK is not required for beta cell function in the adult pancreatic islet, but rather seems to play a role in fetal and early postnatal beta cell differentiation and proliferation[142].

ATF6

ATF6 is a type-II membrane protein with two isoforms, ATF6α and ATF6β, both of which are ubiquitously expressed. While deletion of either ATF6 genes alone has little effect on physiology, deletion of both ATF6 isoforms is embryonically lethal[143]. Initially identified as part of a large family of basic leucine zipper transcription factors recognizing a consensus element, ATF6 is relatively understudied compared to IRE1 and PERK, owing to a smaller array of molecules available to specifically modulate ATF6 and the fact that many of its transcriptional targets overlap with XBP1[144]. ATF6 is unlike the other two major ER stress sensors, IRE1 and PERK, in that it does not remain in the ER membrane to orchestrate signaling through the cytosol to communicate ER dysfunction to the rest of the cell. Instead, ATF6 itself is a transcription factor that loses

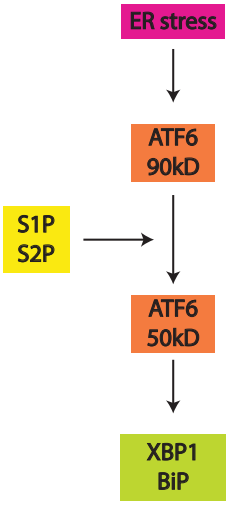


Figure 3: ATF6 signaling pathway.

its ER localization during ER stress. ATF6 is typically retained in the ER in an unusually stable complex with BiP, which is actively disassembled during ER stress[145]. Dissociation of BiP from ATF6 allows ATF6 to progress through the secretory pathway to the golgi apparatus, where it encounters two proteases, site-1 protease and site-2 protease[146,147]. These proteolytic events liberate a 50kD fragment into the cytosol, which then transits to the nucleus. ATF6 induces gene programs associated with ER function, notably the highly abundant ER chaperone BiP and mRNA for the transcription factor XBP1, which is processed as part of IRE1 signaling[143]. 40% of identified ATF6 targets are involved in ER quality control, a further 20% are other ER-resident proteins, and the remainder serve a diverse array of functions[148]. ATF6 can form heterodimers with related CREB proteins, which may influence its transcriptional repertoire[149].

XBP1

XBP1 is a transcription factor whose activation by IRE1 leads to the induction of numerous gene programs, including those associated with lipid biogenesis, cell survival, and immunity. During ER stress, XBP1 transcript is induced by ATF6, and the mRNA is cleaved by IRE1 as described above[150]. After cleavage by IRE1, XBP1 (or, in yeast, its homologue HAC1) mRNA is ligated by the tRNA ligase Trl1 in yeast and the tRNA ligase RtcB (with its cofactor archease) in mammalian cells[151,152]. Most work on XBP1 focuses on the protein produced by transcript that has been spliced by IRE1. However, there is some evidence that the short-lived protein product of the unspliced XBP1 transcript may affect cellular functions including

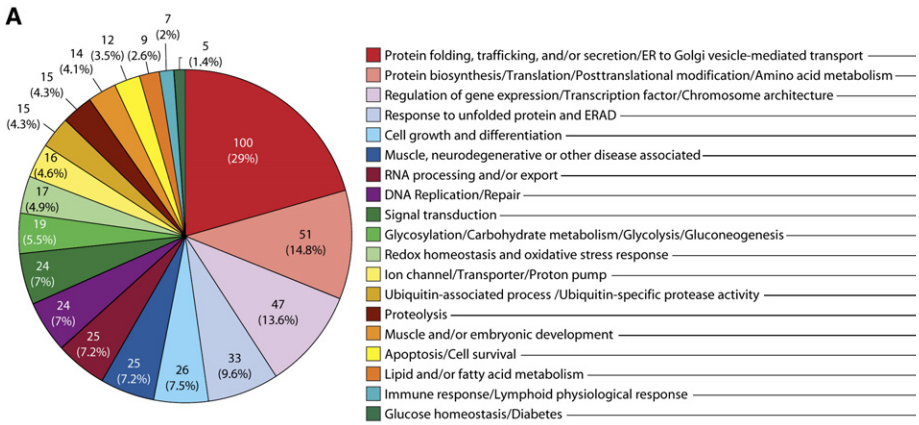


Figure 4: Summary of XBP1 transcriptional targets. XBP1 target genes were identified by ChIP, and the distribution of gene ontology (GO) terms is shown, reflecting the diversity of processes XBP1 can transcriptionally alter. From Acosta-Alvear et al.

survival and proliferation as well[153,154]. Notably, unspliced XBP1 protein is upregulated during the recovery phase of ER stress, and forms complexes with spliced XBP1 (XBP1s) that are rapidly targeted for proteasomal degradation[155].

As a key transcriptional executor of the UPR, XBP1 has control over an array of adaptive gene programs. The mechanism by which XBP1 modulates transcription is incompletely understood, but some known XBP1 gene targets lack the ER stress response elements (ERSE) and unfolded protein response elements (UPRE) involved in the activity of other ER-associated transcription factors[156]. XBP1 seems to be able to utilize a ACGT core sequence in addition to its ability to associate with ERSE and UPRE regions[157,158]. XBP1s gene targets identified by chromatin immunoprecipitation assays are diverse, encompassing not only strict ER-related functionality but also apoptosis, embryonic development, and immunity (summarized in Fig. 4[158]). XBP1s targets associated with ER function notably control protein quality control/degradation and lipid biosynthesis. Unlike ATF4 and ATF6, XBP1s is not noted for its role in upregulating ER chaperones, although it can induce PDI expression. XBP1s is required for the function of numerous cell types, usually those that experience high secretory demand, including antibody-secreting plasma cells and Paneth cells in the intestines[159,95]. The features of the transcriptional programs induced by XBP1 may be influenced by cell type and the precipitating stimulus, which is an active area of research in the XBP1 field[160].

RIDD

In addition to its role in processing XBP1 mRNA, IRE1 is also able to cleave RNAs it encounters, leading to their degradation. The molecular mechanism of RIDD requires the IRE1 endonuclease domain, but is distinct from the XBP1 splicing mechanism. *In vitro* experiments showed that splicing of radiolabeled XBP1 mRNA was competitively inhibited by addition of cold XBP1 mRNA, but noncompetitively inhibited by addition of cold mRNA for insulin, a RIDD target[161]. Conversely, cold XBP1 did not compete with degradation of radiolabeled insulin mRNA, indicating distinct mechanisms for RIDD and XBP1 splicing[161]. The

oligomerization states of IRE1 during XBP1 splicing and RIDD have been of interest. Some studies have argued that XBP1 splicing requires high-order IRE1 oligomerization, while RIDD does not, though others report IRE1 dimers splicing XBP1[161,162,163]. In one example, activation of IRE1 using quercetin, which results in formation of IRE1 dimers, but not higher-order oligomers, can drive RIDD that is insensitive to inhibition by the endonuclease inhibitor STF 083010, which robustly inhibits XBP1 splicing[161]. While the specific requirements are still being elucidated, it nonetheless seems clear that XBP1 splicing and RIDD activation are separable activities of the IRE1 endonuclease.

IRE1 may be able to recognize RIDD substrates by a putative consensus element, consisting of a CUGCAG sequence and a stem-loop structure, in mammalian cells, but subsequent work showed that an ER-targeting sequence can be enough to sensitize mRNAs to RIDD during ER stress[164,165]. In experimental settings, a stem-loop forming CNGNNGN is sufficient to enable RNA degradation by IRE1, and this sequence is present in $tRNA_{Phe}$, which can be degraded by IRE1 endonuclease[166]. It has been speculated that $tRNA_{Phe}$ degradation may be another mechanism by which IRE1 can control translation rates, and this mechanism would not be restricted to ER-localized nascent proteins. Further complicating investigation of the role of IRE1 in mRNA degradation is the finding that IRE1 can degrade precursor miRNAs, which may indirectly lead to changes in mRNA suppression[167,168]. Intriguingly, RIDD appears to lie at the intersection of multiple methods of autoregulation of the IRE1 pathway itself. Deletion of XBP1 in cells drives RIDD via enhancing IRE1 phosphorylation, suggesting that crosstalk between these two endonuclease-dependent activities of IRE1 may be relevant in modulating IRE1 function, and furthermore, IRE1 can degrade its own mRNA by RIDD[120].

Despite ongoing investigation of the mechanisms behind RIDD, it is understood that during ER stress, RIDD serves as a mechanism for reducing the load of nascent peptides translocating into the ER lumen. RIDD accomplishes this by decreasing the availability of mRNA for ER-localized proteins. The mRNA fragments produced by RIDD can also act as signaling mediators, sensed by the viral RNA sensor RIG-I to

enhance NF-κB activity and inflammation[169]. Finally, RIDD can control cell fate decisions. Enforcing RIDD sensitizes cells to apoptosis in the absence of ER stress, and RIDD contributes to apoptosis in the presence of intractable ER stress[163]. Additionally, degradation of miR-17 by RIDD allows for accumulation of caspase-2 driven by ER stress, and the subsequent cleavage of caspase-2 results in Bax/Bak oligomerization and apoptosis[168].

Mitochondria-associated membrane of the ER

The mitochondria-associated membrane (MAM) is a specialized feature of the ER whose role is to coordinate functions integrating signals from the ER, the mitochondria, and the cytoplasm. The ER is littered with mitochondrial contact sites, and at points of interaction between these two organelles are specific tethering proteins that make up the MAM, as well as an array of proteins that do not specifically act in interorganellar crosstalk but are enriched in abundance at the MAM. Historically, the MAM has been recognized as an important suborganellar structure for buffering and transfer of calcium, an ion that is primarily contained in the ER and mitochondria and which is essential for their function[170]. Furthermore, by influencing ROS production, lipid transfer, and protein complex assembly (including autophagosome assembly), the MAM serves as a potent platform for coordinating signals between the ER and mitochondria[171]. ER-mitochondrial contact sites influence mitochondrial activities, including by driving apoptosis or directing mitochondrial fission[172].

The MAM is an important structure for coordinating inflammatory responses. For example, RIG-I, a molecule involved in antiviral immunity that can be activated by IRE1 endonuclease activity, is highly enriched at the MAM[173]. Moreover, the influence of the MAM over mitochondrial function makes it a powerful regulator of mitochondrial ROS production, which drives activation of the inflammatory transcription factor NF-κB. Mitochondrial ROS feeds back onto MAM proteins, including IRE1 and sigma-1

receptor, which can drive production of cytokines and antioxidants to influence the inflammatory balance of the tissue environment.

IRE1 and autophagy

One way the ER signals to other systems to influence cellular outcomes is by affecting autophagy. Autophagy can protect cells from apoptosis, and serves to alleviate ER stress by removing protein aggregates. The ER is intimately involved in autophagic processes, as the ER is believed to be the primary membrane source for autophagosomes. Importantly, autophagy may serve as a mechanism by which IRE1 can influence immune cell function, since autophagy mediators are well established as affecting innate immunity. While the contribution of ER stress sensors to autophagy are still contested, IRE1 seems to be uniquely in control of autophagy, at least in some cell types. MEFs lacking IRE1, but not PERK or ATF6, have deficient autophagy in response to ER stressors tunicamycin or thapsigargin, or in response to nutrient deprivation[174]. Control of autophagy by IRE1 seems to depend on its association with TRAF2 and JNK[175]. Though IRE1 is typically considered as a driver of autophagy, in some contexts, IRE1 activation appears to dampen autophagy. One example of this is in the livers of Ob/Ob mice, where increased IRE1 phosphorylation correlates with suppressed autophagy, or in mutant Huntingtin aggregation, where IRE1 overexpression reduces autophagic flux[176,177].

IRE1 profoundly influences physiology across species

In drosophila and mammals, IRE1 and XBP1 are essential proteins, both during development and in the maintenance of adult homeostasis. Deletion of IRE1β causes increased expression of UPR mediators in the gut and sensitizes mice to a dextran sodium sulfate colitis model[107]. IRE1α deletion results in embryonic lethality at day E12.5 in C57/Bl6 mice[159]. IRE1 is highly expressed in the mouse fetal liver and the placenta, and IRE1-dependent embryonic lethality has been attributed to both fetal liver hypoplasia

and dysfunctional placental vasculature[159,178]. XBP1 deletion results in embryonic lethality starting at day E12.5, and may be attributable to failure of blood cell development in dysfunctional fetal liver, since total blood cell counts in surviving XBP1-/- fetuses at day E14.5 are 20% of those seen in WT littermates[179]. Expressing XBP1 only in the fetal liver of otherwise XBP1-/- animals results in early postnatal lethality, driven by failure of the exocrine pancreas to produce sufficient levels of digestive enzymes, such as alpha-amylase and trypsin, with a minor contribution of dysfunctional salivary glands[180]. Surprisingly, deletion of IRE1α only in the embryo and not in the placenta allows for largely normal development of mice, with modest effects on metabolism including insulin and glucose levels and body size, histological abnormalities of the exocrine pancreas and salivary glands, and failure of plasma cell development from B cells and antibody secretion[181]. Very low levels of spliced XBP1 could detected in some of the tissues of these mice, which may contribute to the mild phenotype observed[181]. In drosophila, IRE1-dependent developmental lethality is driven by defects in the alimentary canal, and depends on XBP1 splicing as well as non-XBP1-related functions of IRE1[182]. IRE1 deletion is not lethal in yeast S. cerevisiae, except in growth conditions that cause ER stress[183]. Similarly, IRE1 is not required for *C. elegans* development, but deletion of both XBP1 and PERK results in synthetic lethality in these animals[184].

Cell type restricted deletion of IRE1 or XBP1 in a vast array of cell types has been shown to affect physiological outcomes. Deletion of IRE1 in Pomc neurons results in dysregulated glucose and insulin balance as well as dysfunctional fat beiging[185]. Deletion of XBP1 in neural cells with Nestin Cre impairs long-term potentiation and memory formation, independent of ER stress and due to loss of BDNF transactivation by XBP1 in the hippocampus[186]. Deletion of IRE1 in pancreatic islets suppresses insulin secretion and development of a typical diabetic phenotype, resulting from a lack of PDI transactivation by XBP1 that, in turn, results in improper disulfide bond formation in proinsulin[187]. Additionally, deletion of XBP1 profoundly affects lipid balance in numerous cell types. In the adult liver, XBP1 deletion results in hypolipidemia without causing ER stress or altering liver histology, and causing only modest decreases in serum albumin

and total protein[188]. This may be due to degradation of transcripts associated with cholesterol and triglyceride synthesis via RIDD[189]. *In vitro* differentiation of adipocytes is also significantly depressed by XBP1 deletion, as the XBP1 gene target and central adipogenesis mediator C/EBPβ is insufficiently induced when XBP1 is absent[190]. Additionally, IRE1 and XBP1 have significant roles in immune cell development and activity, as will be detailed below. The wide-ranging and profound effects of IRE1 and its downstream mediator XBP1 on mammalian biology position them as enticing potential mediators for numerous physiological and pathophysiological conditions, but complicate their potential as therapeutic targets.

IRE1 pathway in immune cell development

The IRE1 pathway, and in particular its downstream mediator XBP1, is of special interest in immune cells, where it is known to control numerous aspects of development and function. Indeed, XBP1 was initially identified by its role in regulating MHC II in B cells[191]. In B cells, total XBP1 levels are elevated by stimuli, such as CD40 stimulation, that induce plasma cell differentiation[96]. Early studies utilizing Rag2 blastocyst complementation identified a role for IRE1 in early in B cell development, as IRE1 deficient pro-B cells had suppressed VDJ recombination and BCR production[159]. However, these findings are not recapitulated in CD19-driven Cre recombinase systems, suggesting that the contribution of IRE1 to early B cell development may occur prior to CD19 expression. Furthermore, B cells that lack XBP1 fail to differentiate into antibody-secreting plasma cells *in vivo* in response to T cell independent and T cell dependent stimuli[96]. XBP1 is not required for expression of the plasma cell surface marker CD138, but XBP1 deficient plasma cells fail to expand the ability of their secretory pathway to accommodate higher demand[192]. XBP1 is not required for all types of B cell terminal differentiation, as long-lived memory B cells develop normally from XBP1 deficient B cell pools[192]. Curiously, although IRE1 and XBP1 are required for efficient antibody secretion, IRE1 is also able to act as a brake on IgM secretion via RIDD, and the different

mechanisms by which IRE1 performs XBP1 splicing and RIDD may serve as a pathway for modulating immunoglobulin production[193].

Spliced XBP1 is expressed in hematopoietic stem cells, with about 15% of total XBP1 spliced in LSK progenitors, then more highly expressed in common myeloid progenitors and granulocyte-macrophage progenitors (~30% and ~60%, respectively)[194]. Curiously, though many immune cell types expand their secretory capacity as they mature from progenitors, eosinophils and plasma cells are the only cell types that have been identified as requiring XBP1 for differentiation[194]. Although these two cell types belong to different hematopoietic lineages, they both require rapid bursts of secretory molecule production during differentiation[195,196].

XBP1 is also known to be important for functioning of immune cells after development. Immature dendritic cells have high basal XBP1 splicing levels, and deletion of XBP1 impairs their differentiation and survival[197]. In ovarian cancer, XBP1 integrates signals from the tumor microenvironment to control the antitumor action of DCs and T cells[198,199]. Either XBP1 deletion or IRE1 deletion suppresses cytokine production in response to the TLR4 ligand LPS in macrophages[98,200]. It is possible that TLR2/TLR4 signal transduction, which has been identified as depending on XBP1 splicing in macrophages, may play a role in the activation of other immune cell types that mature following ligation of these extracellular receptors.

Chapter 3
The elusive sigma-1 receptor

Sigma-1 receptor overview

Misunderstood from its first identification, sigma-1 receptor (S1R) is a non-opioid receptor that has been assigned an enormous repertoire of cellular functions, from controlling ion flux to chaperoning secreted proteins and beyond. Due to its distinct pharmacologic profile and affinity for readily available selective drugs, much of the research that has been conducted on S1R utilizes potentially confounding pharmacologic approaches, such as multiple drug treatment combinations, that have resulted in an internally inconsistent body of evidence as to its true molecular features. Despite the cacophony of findings describing S1R, it remains an alluring potential therapeutic target, owing to its seemingly limitless influence on disease processes, including neurodegeneration and inflammation.

Sigma-1 receptor, a non-opioid receptor

First identified in beagles in 1976, S1R was initially characterized as an opioid receptor. So named for its affinity for the drug SKF 10,047 (other opioid receptors identified in this study were μ, which binds morphine, and κ, which binds ketocyclazocine), agonism of S1R caused pupil dilation and manic hyperactivity in dogs, and this effect was antagonized by naltrexone, a classical opioid receptor antagonist[201]. S1R was further characterized in guinea pig brains by Tsung-Ping Su as a distinct pharmacologic entity whose affinity for SKF 10,047 was enhanced by the presence of sodium and lithium ions and depressed by manganese, magnesium, and calcium[202]. It was this study that initially proposed that S1R is in a distinct receptor family, presenting evidence that l-etorphine inaccessible sites (i.e., S1R) had a distinct ligand binding profile from μ receptors, κ receptors, dopaminergic receptors, benzomorphan receptors, and beta-adrenergic receptors. Su also noted two peculiar features of S1R. The first was Its affinity for a wide array of chemically distinct molecules, including those that were not known to affect psychosis, which had previously been a defining feature of putative S1R ligands. Secondly, Su puzzled over the enantiomeric preference of S1R in drug binding, where a racemic mixture of pentazocine was as potent

as d-pentazocine, while l-pentazocine was much less potent, contrary to the expected result where racemates have intermediate potency between enantiomers.

S1R was cloned in 1996 from guinea pig liver and conclusively characterized as 25kD protein, distinct from all other known families of mammalian proteins[203]. This study identified S1R as closely related to the yeast sterol isomerase ERG2, but expression of S1R cDNA in ERG2 deficient *S. cerevisiae* did not rescue sterol synthesis, indicating that the functionality of S1R has drifted over evolutionary time. The cloned S1R sequence was predicted to contain at least one transmembrane segment[204]. Subsequent studies produced conflicting conclusions about the membrane topology of S1R. Hydrophobicity analysis of S1R and sterol isomerases suggested three or four transmembrane domains[203]. Then, experiments utilizing a custom antibody and imaging techniques including confocal microscopy proposed a single transmembrane domain, with a membrane-anchored N terminus and a C terminus in the ER lumen[205]. However, hSI, a different protein with affinity for the S1R ligand SR31747A, was also predicted to have four transmembrane segments[205]. Later, domain accessibility experiments indicated a two-pass structure of S1R, with a short N terminal tail and a large C terminal domain, both on the intracellular side of frog oocytes[206]. Other studies suggested that the C terminus was inside the ER lumen, as it is not accessible to trypsin digestion in microsomal preparations[207]. This proposed ER luminal C terminus was thought to play key roles in the function of S1R, possessing chaperone activity and binding the ER chaperone BiP[208,209]. BiP binding was thought to modulate S1R activity and represented a potential molecular explanation for the effects of S1R ligands, since S1R agonists inhibited BiP binding and S1R antagonists promoted it[210].

Surprisingly, when the crystal structure of S1R was solved in 2016, S1R was reported to have only one transmembrane domain, with a very short N terminal tail in the ER lumen and a C terminus that is likely embedded partway into the ER membrane (Fig. 1[211])[211]. Additionally, it was predicted that S1R likely forms homotrimers, while previous work had suggested S1R exists in monomeric or dimeric form, in addition to higher order oligomers[212]. The crystal structure provided some clues as to how S1R is able to bind such a

wide array of ligands. S1R was crystallized with two chemically divergent molecules, 4-IBP and PD144418. Very little structural variation was observed when S1R bound either ligand. Furthermore, it was noted that the ligand binding pocket was highly occluded, and it remains unclear how ligands access the relevant site. The crystal structure did provide some clarity into the mechanism behind aggregation of an ALS-causing S1R mutant, as will be discussed later, but more work is needed to reconcile the crystal structure with the reported functions of S1R at the ER.

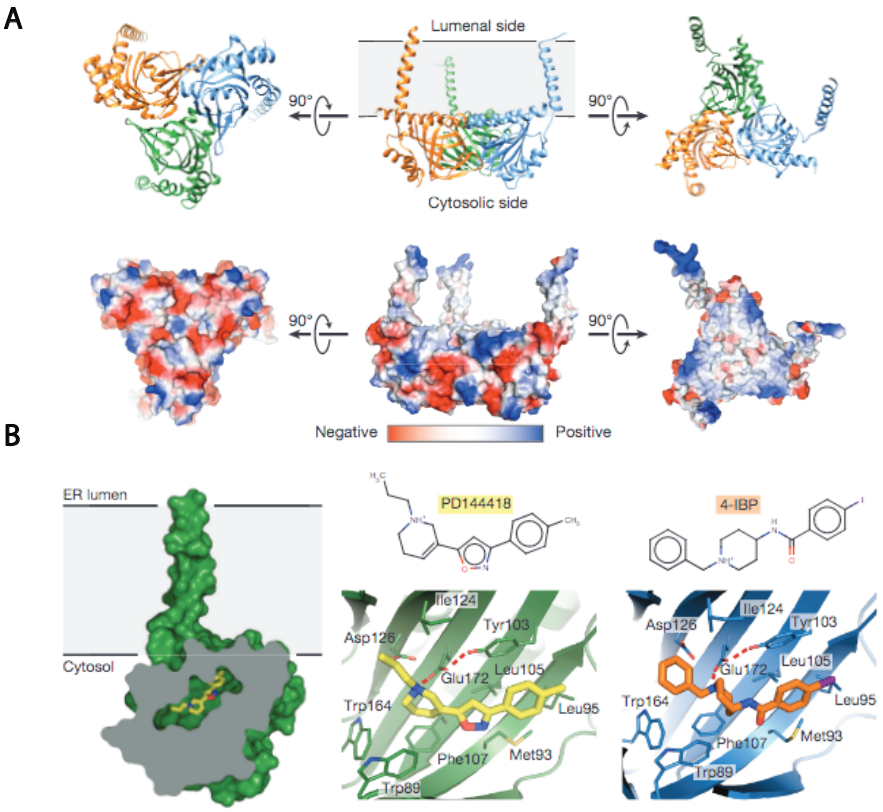


Figure 1: Crystal structure of S1R. A) Crystal structure of three S1R protomers in their trimeric configuration. **B)** Ligand binding pocket of S1R, showing the conformation of the drug binding pocket when bound to two structurally distinct drugs. Adapted from Schmidt et al.

Molecular functionality of sigma-1 receptor

Knowing the crystal structure of S1R may help to elucidate the molecular mechanisms behind the multitude of functions attributed to it. S1R is now known to be highly enriched at the mitochondria-associated membrane of the ER, but it has been reported to act at the nuclear envelope and the outer plasma membrane as well[213,214]. The C terminus of S1R is able to act as a chaperone *in vitro*, reducing the aggregation of heat shocked citrate synthase, as well as protecting the secreted proteins insulin and BDNF from aggregation[210]. Given that the crystal structure predicts the chaperone-competent S1R residues (116-

223) to be cytosolic, S1R may instead act to chaperone proteins on the cytosolic site of the ER membrane in living cells. S1R has been reported to stabilize ER membrane proteins, including the inositol triphosphate receptor (IP3R) and the ER stress sensor IRE1[210,215]. S1R is thought to prevent disordering and aggregation of these ER proteins, suppressing the rates of their degradation. S1R coimmunoprecipitates with IRE1 and alters the kinetics of IRE1 phosphorylation in response to the ER stressor thapsigargin[215]. Another possible mechanism by which S1R may control the stability of membrane proteins is by influencing ganglioside levels within lipid rafts, altering how membrane proteins organize within membranes[216].

S1R can affect conductance of ion channels. One mechanism by which S1R is believed to influence calcium flux is via association with inositol triphosphate receptor 3 (IP3R), and association with IP3R3 was one of the first proposed molecular interactions of S1R[217]. S1R was found to protect from degradation mature, but not nascent, IP3R, prolonging IP3R half-life at the MAM, enhancing calcium flux through IP3R[210]. In conflicting findings, knockdown of S1R in NSC34 cells increases bradykinin-induced calcium flux through IP3R, indicating that cellular context may significantly alter the effect that S1R has on IP3R conductivity[218,219]. In addition to its potential chaperone functionality at IP3R, S1R is believed to modulate the conductance of numerous other ion channels. S1R ligands (including both agonists and antagonists) reduced NMDA-induced calcium flux into the cytosol in cultured rat neurons[220,221]. S1R ligands potentiate cytosolic calcium increases driven by bradykinin-induced IP3R opening, though it is unclear how S1R ligands may affect rates of IP3R degradation[222]. At the ER of NG-108 cells, S1R forms a complex with IP3R type 3 and ankyrin B, an inhibitor of IP3R conductivity. Putative S1R agonists cause dissociation of ankyrin B from S1R, while a NE-100, a putative S1R antagonist, enforced their association[223]. Notably, S1R ligands themselves do not affect cytosolic calcium levels, but rather tune the response of IP3R to other signals[223]. In CHO cells, S1R is not believed to form stable complexes with IP3R, but depletion of ER calcium results in a transient interaction of S1R and IP3R in CHO cells that dissipates after 20 minutes[210]. S1R is believed to control the conductivity of other ions as well, via physical association with a variety of voltage-gated ion channels, including Kv1.4,

Kv1.5, and Nav1.5[224,225]. Atomic force microscopy showed ordered, symmetrical assembly of Nav1.5 and S1R and suggested a potential interaction of transmembrane segments, making it unlikely that the observed interaction was due to C terminal S1R chaperone activity[224]. It should be noted that many, though not all, studies on the association of S1R with ion channels utilize cancer cells, which are known to highly upregulate S1R and may not reproduce the functionality of S1R outside the context of cancer[226].

The ligand-binding capacity of S1R led to a search for what endogenous molecule/s might alter its activity *in vivo*. Several steroid hormones have been reported as having high to moderate (tens to hundreds nanomolar K_i) affinity for S1R, including progesterone and DHEA-S[227,228]. The physiological relevance of these findings were disputed, since these S1R ligands failed to affect conductance of potassium channels known to be affected by S1R *in vitro*[229]. However, these S1R-binding neurosteroids seemed to have influence over S1R *in vivo*. For example, DHEA enhances neurogenesis and supports fEPSPs in olfactory bulbectomized mice, and this is inhibited by the selective S1R antagonist NE-100[230]. The endogenous, short-lived hallucinogen N,N-dimethyltryptamine (DMT) has been proposed as the endogenous S1R ligand[231]. DMT chemically resembles many other S1R ligands and alters sodium current through Nav1.5 in a S1R-dependent fashion (Table 1[231–236])[231]. DMT also has a phenotypically relevant interaction with S1R *in vivo*: Mice lacking S1R expression experience a significantly blunted hyperlocomotion response to DMT compared to S1R-expressing controls[231]. Recently, the nutrient choline was reported as another endogenous S1R. Choline alters calcium flux through IP3R in a S1R-dependent fashion[232]. The Ki of choline at S1R s 525µM; this low-affinity interaction may be advantageous for finely tuning the response of S1R to choline in a context-dependent fashion, since the concentration of choline in blood is typically much lower (low µM), but can be higher (up to 1mM) at cholinergic synapses and was estimated to be ~900µM after GPCR stimulation in NG108-15 cells[232].

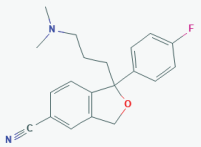
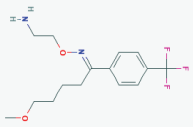
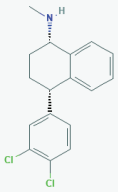
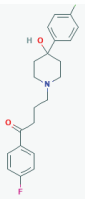
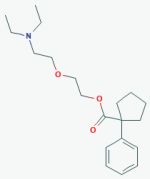
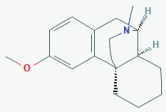
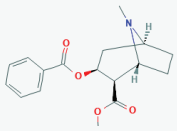
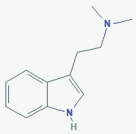
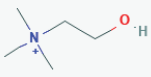
Molecule	2D structure	S1R Ki (nM)	Drug type
Citalopram		292	SSRI
Fluvoxamine		36	SSRI
Sertraline		57	SSRI
Haloperidol		0.9	typical antipsychotic
Carbetapentane		75	antitussive
Dextromethorphan		365	antitussive, sedative
Cocaine		2,000	stimulant; serotonin, norepinephrine, dompamine reuptake inhibitor
N, N dimethyltryptamine (DMT)		14,750 (K_D)	endogenous psychedelic tryptamine
Choline		525,000	nutrient

Table 1: Characteristics of selected S1R ligands. 2D structure source: PubChem.

S1R modulates oxidative stress

Given its positioning at the MAM, S1R is well-equipped to respond to redox stress. Indeed, one of the best-characterized activities of S1R is in controlling reactive oxygen species (ROS). S1R is reported to influence NF- κB during redox stress to affect cell survival. In CHO cells, knockdown of S1R raised basal p105 NF-κB, as well as raising p105 and nuclear p50 NF-κB induced by KCl challenge, while overexpression of S1R conversely depresses p50 production[237]. S1R suppresses NF-κB abundance to dampen H2O2-induced apoptosis via regulation of the anti-apoptotic mediator Bcl-2. This interaction may have relevance *in vivo*, since both p50 NF-κB and Bcl-2 were found to be depressed in the retina of 1 year old mice lacking S1R expression[238]. The effect on NF-κB and Bcl-2 may be related to S1R control of redox stress, since ROS tends to increase with age, but the redox status of the retinal tissue was not determined in this study. In retinal Muller glia, deletion of S1R strongly impairs redox balance, with depression of antioxidant proteins including SOD1, catalase, and NQO1[239]. S1R deletion in retinal glia also leads to decreased expression of the cysteine-glutamate exchanger, whose activity is important for production of glutathione, a major cellular antioxidant[239]. In the livers of young adult S1R knockout mice, markers of redox stress were upregulated, including oxidized glutathione[240]. Overexpression of S1R or treatment with S1R agonist (+)-pentazocine enhances production of antioxidants SOD1 and NQO1, and enhanced activity at the antioxidant response element, a transcriptional regulatory element associated with the production of many antioxidant proteins[240]. Microarray and qPCR analysis of cultured rat neurons found that knocking down S1R enhanced production of both antioxidant NQO1 and cytochrome p450, a producer of free radicals[241]. The mechanism by which S1R may affect antioxidant transcription remains unknown.

Redox status is intertwined with cellular survival, and one of the functions attributed to S1R is in controlling apoptosis. Indeed, superoxide correlates negatively with Bcl-2 in lymph node[242]. S1R can influence apoptosis rates of cultured cells, which may have to do with its influence over oxidative stress, but results are variable, suggesting that there is more to know about S1R in cell death decisions. For

example, knocking down S1R decreases viability of unchallenged HeLa cells, but not unchallenged primary rat optic nerve head astrocytes[243]. Similarly, twelve month old S1R knockout mice experience late onset retinal ganglion cell loss, but aged S1R knockout mice are not reported to have markedly increased apoptosis in other cell populations[243]. S1R-binding molecules are also believed to influence rates of apoptosis via S1R. One example of this is the selective S1R ligand (+)-pentazocine, which has been reported to protect cells from apoptosis due to insults including oxidative stress and excitotoxic stimuli, but (+)-pentazocine is also reported to induce apoptosis in a human uveal melanoma cell line[244,245,246]. A better understanding of the molecular functions of S1R may illuminate the mechanisms driving these discrepancies.

S1R is involved in ER stress

Although S1R is an ER resident that can chaperone secreted proteins, its role in ER stress is incompletely understood. Deletion of S1R increases BiP, a hallmark of ER stress, in the liver, but in the retina, deletion of S1R has no effect on abundance of BiP (nor IRE1α, PERK, or ATF6) in the whole retina or brain[247,248]. Unlike with deletion of ER stress mediators such as IRE1 and PERK, S1R knockout mice have no overt phenotype (they display a depressive-like phenotype upon behavioral testing), reflecting the fact that S1R deletion does not grossly disturb ER function, even in highly secretory tissues[249]. Nonetheless, deletion of S1R in the liver may result in detectable, yet manageable, amounts of ER stress resulting from the quotidian secretory demands of liver function. S1R deletion has also been reported to enhance eIF2α phosphorylation and CHOP abundance in motor neurons, though it is possible that this reflects the interaction of S1R with a different eIF2α kinase besides PERK[250]. Some studies indicate that knockdown of S1R may be sufficient to induce activation of ER stress mediators in neuronal cell lines, including phosphorylation of eIF2α and induction of chaperones BiP and HSP70[218,251]. However, this finding is not

universal and may depend on cell type or other conditions, because in IPAG-induced ER stress, knockdown of S1R was found to instead depress BiP induction and autophagy[252].

S1R has been shown to modulate IRE1 activation and endonuclease activity under conditions of severe ER stress, but it is unknown whether S1R can influence PERK or ATF6[215]. In Muller glia isolated from S1R knockout mice, ATF6 and its transcriptional target BiP are elevated, while expression of PERK, IRE1, and ATF4 are all depressed[248]. Curiously, CHOP, a primary transcriptional target of ATF4, is upregulated in these cells. In cultured RGC-5 cells challenged with the oxidative stressor xanthine:xanthine oxidase (X:XO), the S1R agonist (+)-pentazocine greatly suppressed transcriptional induction of ER stress factors BiP, PERK, IRE1, ATF6, and CHOP[246]. In this study, (+)-pentazocine had only a modest effect on ATF4, possibly reflecting the fact that S1R has variable control over activation of other eIF2α kinases through the integrated stress response, which could be activated by X:XO.

S1R may be able to affect its own transcription via an unknown mechanism. S1R abundance is decreased by cellular stressors, but S1R agonist treatments protect S1R from this stress-induced downregulation both in X:XO treated RGC-5 cells and in PG6.3 cells challenged with mutant huntingtin[248,253]. In contrast, S1R is upregulated in tunicamycin or thapsigargin treated HEK293 cells undergoing ER stress[254]. In this system, S1R was identified as being under the transcriptional control of ATF4[254]. Fluvoxamine, an SSRI with low-nanomolar affinity for S1R as well as serotonin transporter, induces ATF4 transcript and protein in Neuro2a cells, independently of PERK activation[255]. While it is uncertain how fluvoxamine feeds onto ATF4, fluvoxamine did not increase ATF4 abundance in S1R deficient MEFs, suggesting that the action of fluvoxamine at S1R, and not its other targets, is involved[255].

The role of S1R in inflammation

From the early days of its characterization, S1R has been proposed to control inflammatory cytokine production. The ligand SR31747, believed to be an allosteric modulator of S1R, suppresses LPS-induced production of the pro-inflammatory cytokines IL-6, TNFα, interferon gamma, and IL-1β in mice[256–258]. SR31747 had no effect on cultured murine peritoneal macrophages or human PMBCs stimulated with LPS, instead exerting its anti-inflammatory effect by increasing serum corticosterone and ACTH produced by the adrenal glands[258]. However, other studies found that SR31747 could dampen transcription of inflammatory mediators including M-CSF and interferon gamma in cultures of splenocytes from mice undergoing a model of graft versus host disease[259]. SR31747 not only reduced levels of pro-inflammatory molecules, but also increased LPS-induced IL-10 production in mouse serum and in splenocytes isolated from LPS-treated mice[256]. In contrast, SR31747 suppresses LPS-induced IL-10 production in the murine macrophage-like cell line RAW264.7[260]. This finding suggests that the effects of SR31747 may depend on cell type and the context in which LPS challenge occurs. Though SR31747 was an early influence on the field of S1R and inflammation, a multitude of chemically distinct S1R ligands are reported to affect inflammatory cytokine production.

Unlike what is reported for challenge with strong ER stressors, stimulation with LPS or the TLR3 ligand poly I:C does not affect S1R abundance in human dendritic cells[261]. Nonetheless, S1R knockdown has significant effects on cytokine production in these cells, enhancing TNFα and suppressing IL-10 secretion[261]. Deletion of S1R increases GFAP expression, a marker of astrocyte activation, in neuronal-glial cultures from fetal mouse brain, and alters astrocyte morphology[262]. Curiously, S1R knockout mice are protected from inflammatory hyperalgesia in a carrageenan model, though it is unclear whether inflammatory processes might affect this outcome, since S1R deletion also protects mice from developing hyperalgesia after sciatic nerve ligation[263,264].

Mutations in S1R cause neurodegenerative disease in humans

Human mutations in S1R cause motor neuron diseases, including juvenile amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration, and distal hereditary motor neuropathy[265–267]. The S1R mutation causing ALS, a homozygous E102Q substitution, was identified in a consanguineous Saudi Arabian family with six members who started showing signs of motor abnormality at 1-2 years of age[267]. In NSC34 neuronal cells overexpressing wild type or E102Q S1R, the mutated S1R formed detergent-resistant 50kD dimers that localized to low-density membrane fractions, compared to wild type S1R, which appeared to be 25kD and preferred higher density membrane fractions[267]. S1R aggregates more in lymphoblastoid cell lines established from patients with E102Q S1R compared to controls[251]. Additionally, NSC34 or neuro2A cells challenged with thapsigargin undergo higher rates of apoptosis when overexpressing E102Q S1R compared to overexpressing wild type S1R[267,268]. The toxicity of E102Q S1R has been attributed to both loss of function and gain of function, and perturbations in an enormous range of cellular processes have been identified as caused by E102Q S1R expression, including abnormal proteostasis, calcium flux, ER stress, and mitochondrial toxicity. Knockdown of S1R or overexpression of E102Q S1R in neuronal cell lines impairs autophagy, resulting in a buildup of substrates inside autophagosomes[218,251]. Vesicular transport generally may become dysfunctional in these cells, since transit of proteins from the ER to golgi apparatus is also impaired[218,251]. EM studies find that S1R knockdown or overexpression of E102Q S1R cause the ER to take on a bloated morphology and the golgi to become disorganized[219,251]. In contrast, studies on S1R knockout cells have not identified these same perturbations in organelle morphology, though unusually elongated mitochondria have been noted in S1R knockout cardiomyocytes and motor neurons[250,269]. This discrepancy could be because cells that experience long-term S1R deficiency may develop adaptive mechanisms to curtail organelle disorder.

Importantly, S1R may play a role forms of ALS that are not caused by a mutation in S1R itself. Mice carrying an ALS-causing mutation in SOD1 have decreased survival when they also lack expression of S1R,

compared to SOD1 mutants that express wild type S1R, and this may be due to control of S1R over motor neuron excitability[270]. Conversely, there may be therapeutic benefit in activating S1R, since the selective agonist PRE-084 increased survival and motor function in mice with an ALS-causing SOD1 mutation[271]. The functional benefits of S1R activation may be context dependent: A heterozygous mutation in the 3' UTR of S1R was found to increase S1R expression in human brain and lymphocytes, and this was associated with frontotemporal dementia and increased cytoplasmic TDP-43 inclusions in the hippocampus[272].

Mutations in S1R in multiple families have been found to cause distal hereditary motor neuropathy (dHMN), a wasting disease of motor neurons that is clinically related to ALS. One S1R mutation, a splice-site mutation resulting in in-frame deletion of 60 base pairs ($S1R^{31\text{-}50del}$), was identified in a consanguineous Chinese family[266]. Overexpression of $S1R^{31\text{-}50del}$ in HEK293 cells resulted in lower levels of S1R protein than overexpression of wild type S1R. $S1R^{31\text{-}50del}$, but not wild type S1R, formed nuclear aggregates and was targeted for degradation by ER-associated degradation machinery, though little else is known about why this mutation results in the muscle wasting observed in affected individuals[266]. One other individual, from a non-consanguineous Portuguese family, has also been identified as having dHMN caused by compound heterozygous mutations in S1R, both in exon 4 (one total deletion, one partial deletion), but the cellular and molecular effects of these mutations were not investigated[265]. Point mutations in S1R (E138Q and E150K) were identified in two Italian families[273]. When neuronal SH-SY5Y cells were transfected with either of these S1R mutants, the cells had higher rates of apoptosis basally and after oxidative or ER stress, abnormal subcellular localization of S1R, reduced bradykinin-induced calcium flux, and higher levels of autophagy, similar to what has been observed in the ALS-causing E102Q point mutation[273]. Finally, although it has not been definitively identified, S1R maps within the known dHMN-causing region in consanguineous Jordanian families[273,274].

The influence of S1R over motor coordination makes sense, since S1R is highly enriched in the motor neurons of the cerebellum and the ventral horn of the spinal cord, especially at the cervical

level[275,276]. Within motor neurons, S1R is localized to cholinergic synapses, where it may act to modulate potassium conductivity via association with Kv1.4 and/or Kv2.1, or may modulate muscarinic type 2 cholinergic receptors[275,277]. While S1R knockout mice do not have any overt locomotion deficits, they do have a lower latency to fall during rotarod testing than controls[275]. Surprisingly, S1R knockout mice are faster swimmers than control mice, and they have a noticeably different method of propulsion through the water that utilizes the tail[275]. The molecular interactions that underlie the toxicity of S1R mutations in motor neurons are still unknown. The crystal structure of S1R provided a hint about how E102Q mutation may drive S1R aggregation and mislocalization. E102 acts as a hydrogen bond acceptor for V36 and F37, and mutation to Q replaces one hydrogen bond acceptor with a donor, which is energetically unfavorable[211]. Because S1R is ubiquitously expressed throughout the body, it is an open question as to why S1R dysfunction has such a profound effect on motor neurons in particular.

Chapter 4
The paper!

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

Dorian A Rosen, Scott M. Seki, Anthony Fernández-Castañeda, Rebecca M. Beiter, Jacob D. Eccles, Judith A. Woodfolk, Alban Gaultier

ABSTRACT

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 1a (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 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.

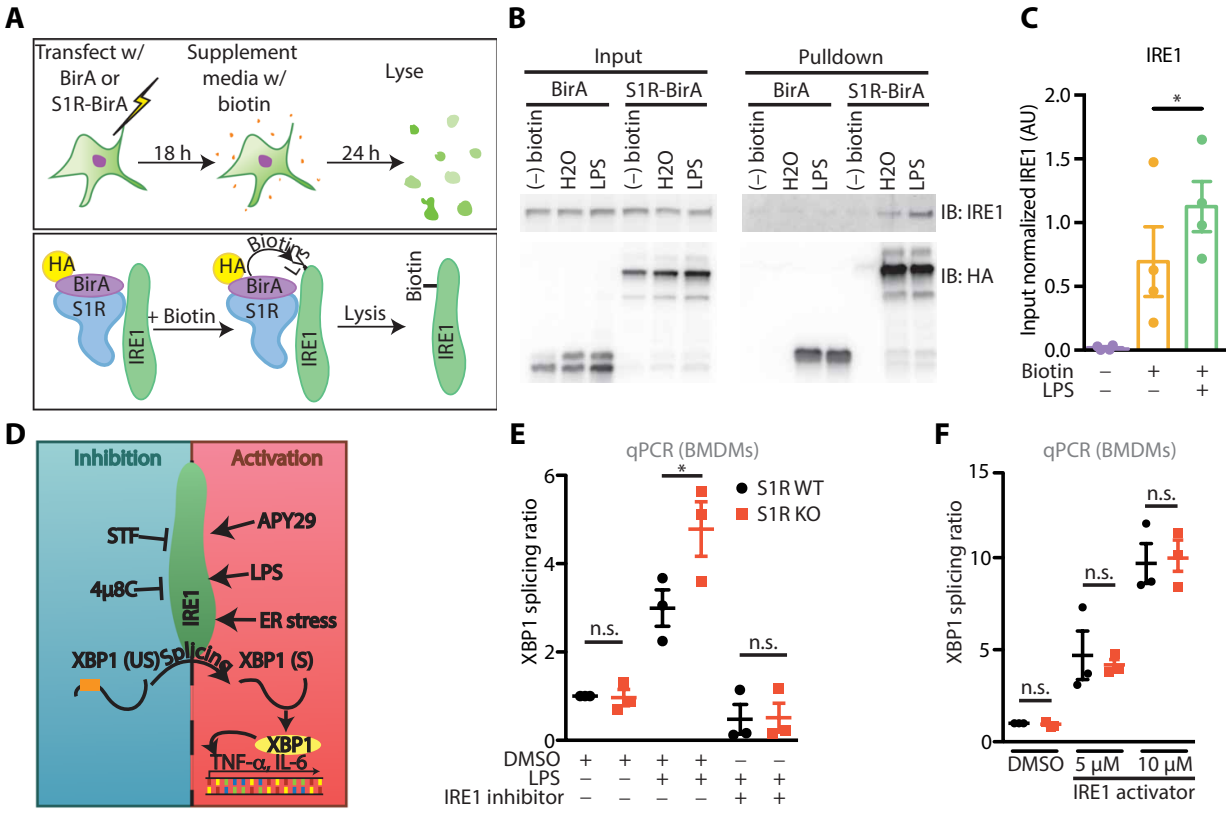


Fig. 1. S1R is an inhibitor of IRE1 during inflammation. (**A**) Experimental design and principle of proximity ligation assay. HA, hemagglutinin. (**B**) Western blots on input lysates and biotinylated (streptavidin pulldown) proximity ligation samples of HEK293 transfected with BirA or S1R-BirA and 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).

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–a (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-1β 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.

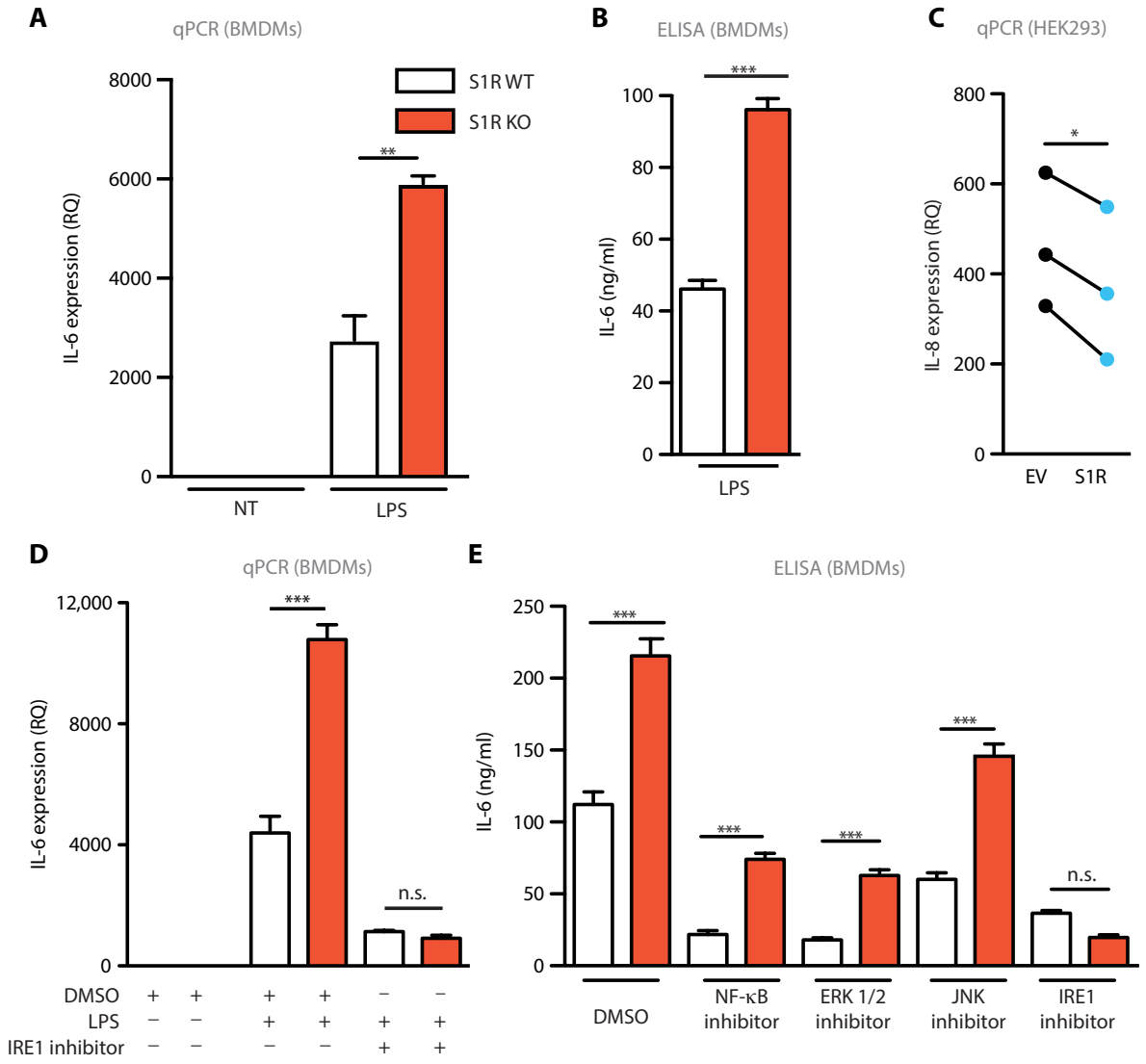


Fig. 2. S1R controls the production of inflammatory cytokines by inhibiting IRE1. (**A**) Relative quantity (RQ) of IL-6 was determined by qPCR on S1R WT or KO BMDMs stimulated for 6 hours with vehicle (NT) or LPS (100 ng/ml; representative of $N = 4$ independent experiments is shown; $^{**}P < 0.01$, 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 μg/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 μM 4μ8C) ($n = 1$, 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 μg/ml) with either <1% DMSO, nuclear factor κB (NF-κB) inhibitor (20 μM JSH-23), mitogen-activated protein kinase (ERK) inhibitor (20 μM PD98059), c-Jun N-terminal kinase (JNK) inhibitor (1 μM SP600125), or IRE1 inhibitor (5 μM 4μ8C) ($n = 1$, representative of $N = 3$ independent experiments is shown; $^{***}P < 0.001$, two-way ANOVA with post hoc Tukey test).

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 upregulated 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-κB (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-κB, 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-κB, 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.

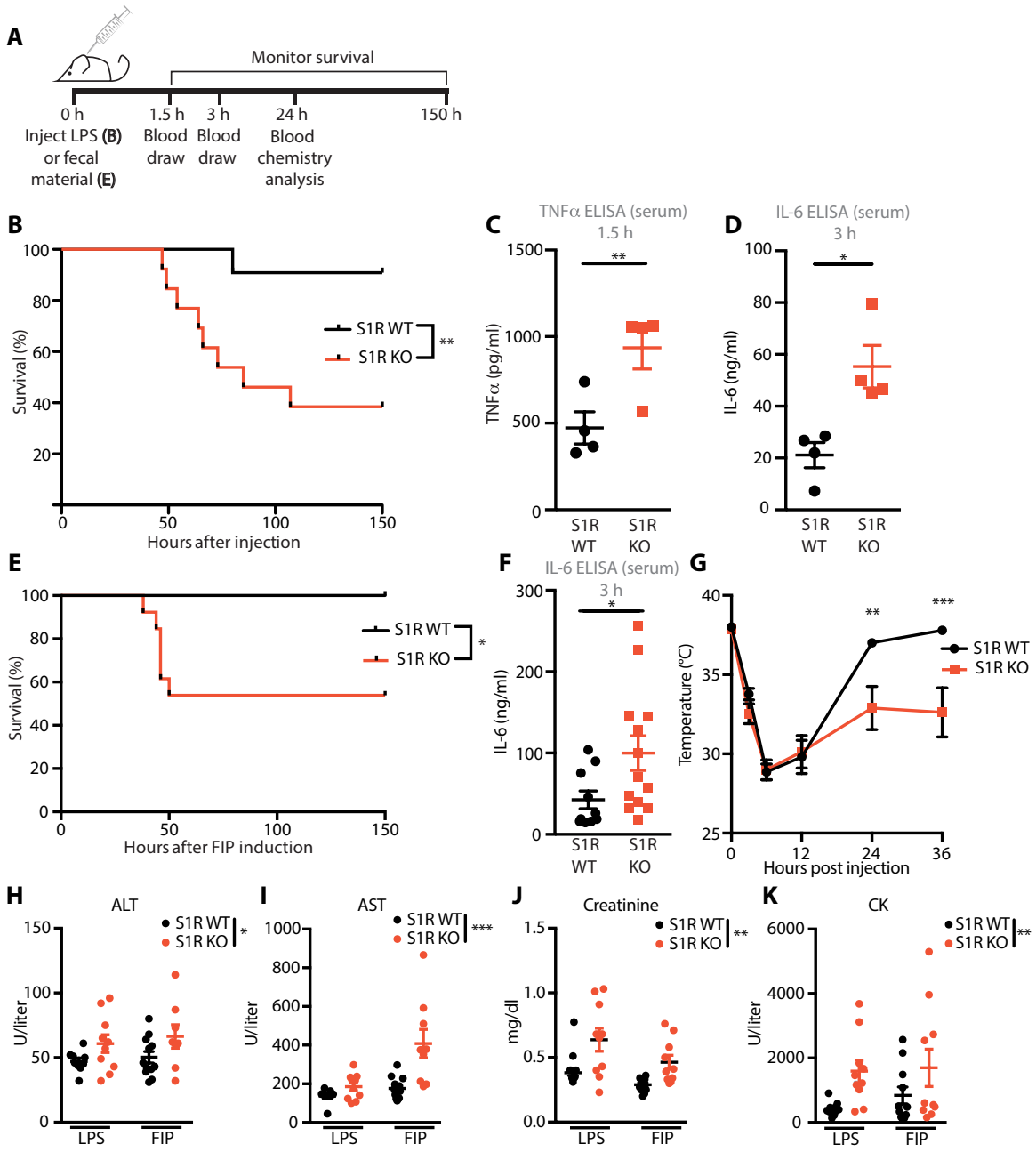


Fig. 3. S1R is protective in murine models of inflammation and septic shock. (**A**) Experimental design. (**B**) Survival curve of WT and S1R KO mice after LPS (5 mg/kg) administration (n = 11 to 13 mice per group; $^{**}P < 0.01$, log-rank test). (**C**) ELISA for TNF-α in serum 1.5 hours after LPS 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).

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.

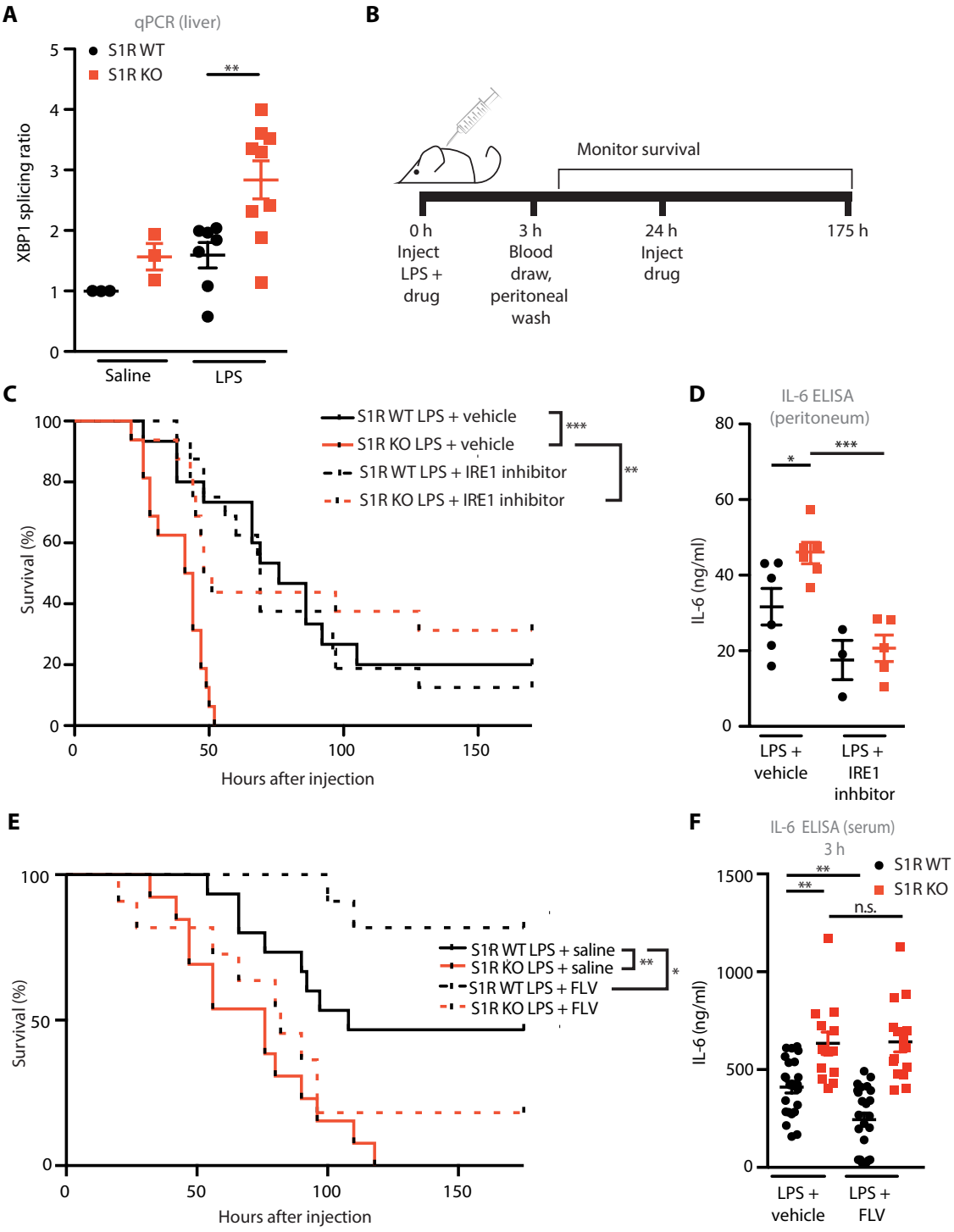


Fig. 4. Pharmacological modulation of S1R and IRE1 function in sepsis models. (**A**) XBP1 splicing ratio from liver homogenate of mice challenged with LPS (5 mg/kg) for 3 hours. Data shown are ratio of XBP1 spliced transcript/XBP1 unspliced transcript (each dot represents one mouse; $^{**}P < 0.01$, 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).

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), 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 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 4μ8C 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.

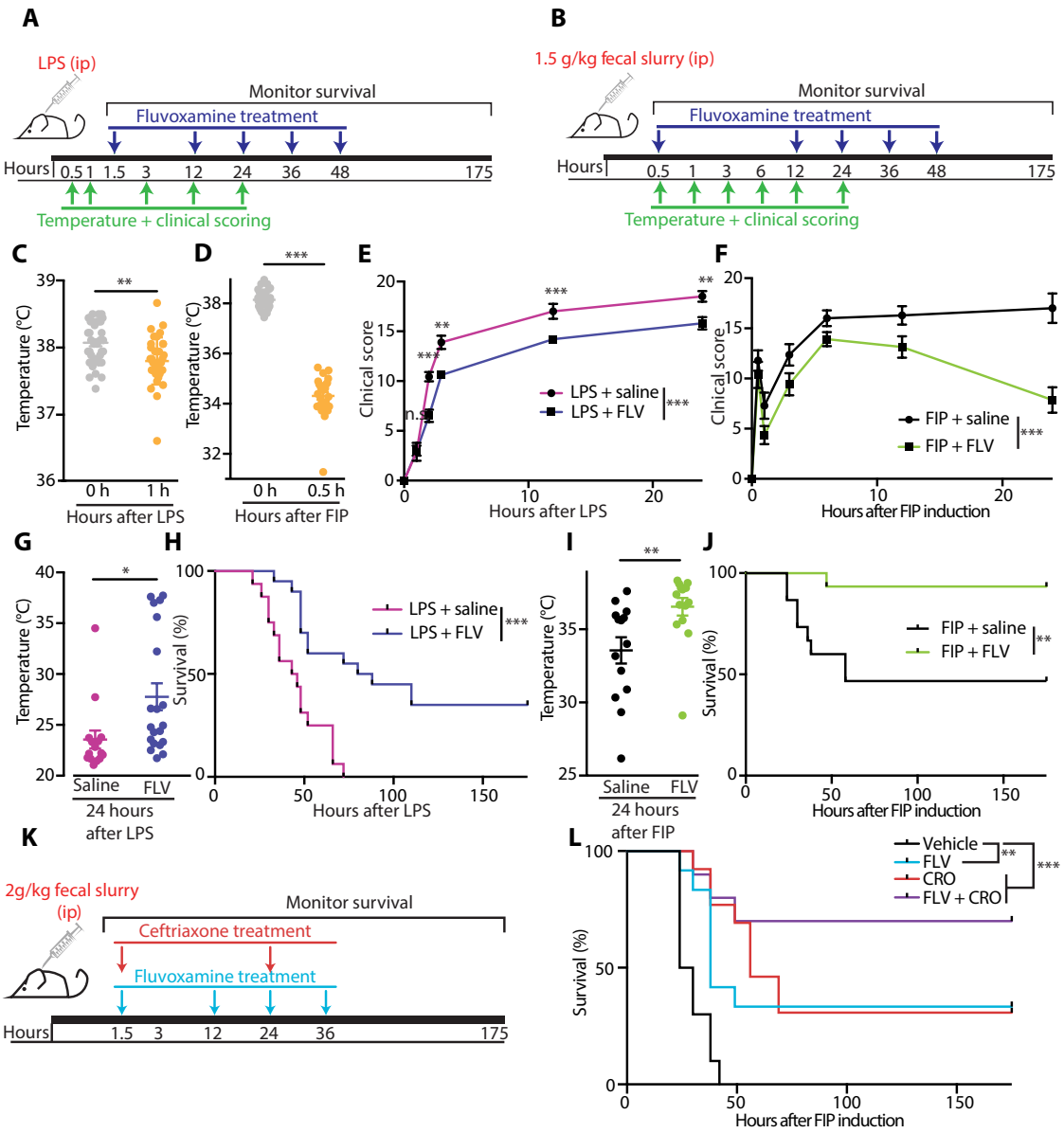


Fig. 5. Therapeutic administration of the S1R agonist FLV is protective during models of inflammation and sepsis. (**A** and **B**) Experimental design for (A) LPS challenge or (B) FIP with therapeutic S1R agonist treatment. Intraperitoneally, ip. (**C**) Rectal temperature of mice measured immediately 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 1.5 g/kg ($n = 10$ to 12 mice per group; $^{**}P < 0.01$, $^{***}P < 0.001$, log-rank test).

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 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. 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).

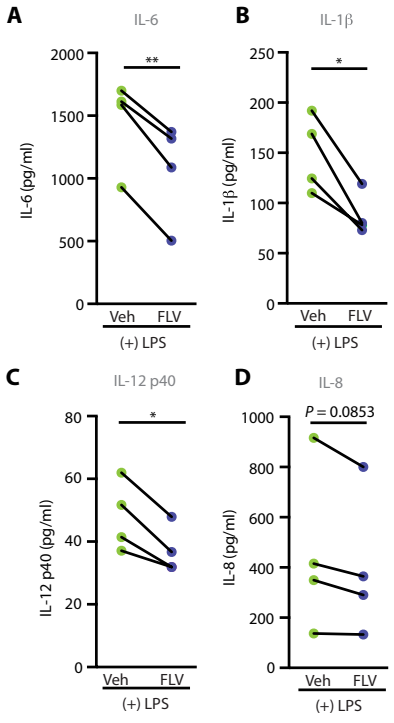


Fig. 6. The S1R agonist FLV is anti-inflammatory in human cells. (**A** to **D**) Multiplex ELISA on serum from human blood. Heparinized whole blood was stimulated ex vivo with LPS (10 ng/ml) and vehicle (RPMI 1640) or 20 μM FLV for 4 hours ($n = 4$, each dot pair represents serum from one participant;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

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 mM), and the production of inflammatory mediators was measured by multiplex

analysis. FLV significantly reduced LPS-induced IL-6 ($P < 0.01$; Fig. 6A), IL-1β ($P < 0.05$; 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 IRE1a (and the closely related protein IRE1b) is able to powerfully affect the inflammatory behavior of both immune and nonimmune 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 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-κB, 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-κB, 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 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 de-pends 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 $1 - b \geq 0.85$. Post hoc power calculations were performed on in vitro studies (except where representative data are shown) using G*Power to ensure that $1 - b \geq 0.85$. 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), 4μ8C (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-mM 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 mg/ml; BioLegend, catalog no. 504501), biotin anti-mouse IL-6 MP5-32C11 (1 mg/ml; BioLegend, catalog no. 504601), anti-mouse TNF-α (0.5 mg/ml; R&D systems, catalog no. AF-410-NA), and biotin anti-mouse TNF-α (0.25 mg/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 IRE1a (1:1000; CST, 3294), total JNK (1:1000; CST, 9252), phospho JNK (1:1000; CST, 9251), total p65 NF-κB (1:1000; CST, 8242), phospho p65 NF-κB (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 mM 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-1β, 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 b (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) ± 20 mM FLV for 4 hours, as described (*49*). Cytokine concentrations were determined by multiplex analysis.

Data analysis and statistics

Data are represented as means ± 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.

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Acknowledgments: We thank S. Arandjelovic and the BIG center (University of Virginia) for critical reading of the manuscript. Funding: The authors are supported by NIH grants R01 NS083542 and R21 NS101281 (to A.G.), the Owens Family Foundation (to A.G.), and T32 GM007055 (to D.A.R.). Author contributions: D.A.R. designed, performed, and analyzed the research and wrote the paper. S.M.S., A.F.-C., J.D.E., R.M.B., and J.A.W. performed the research. A.G. designed and analyzed the research and wrote the paper. Competing interests: D.A.R. 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.

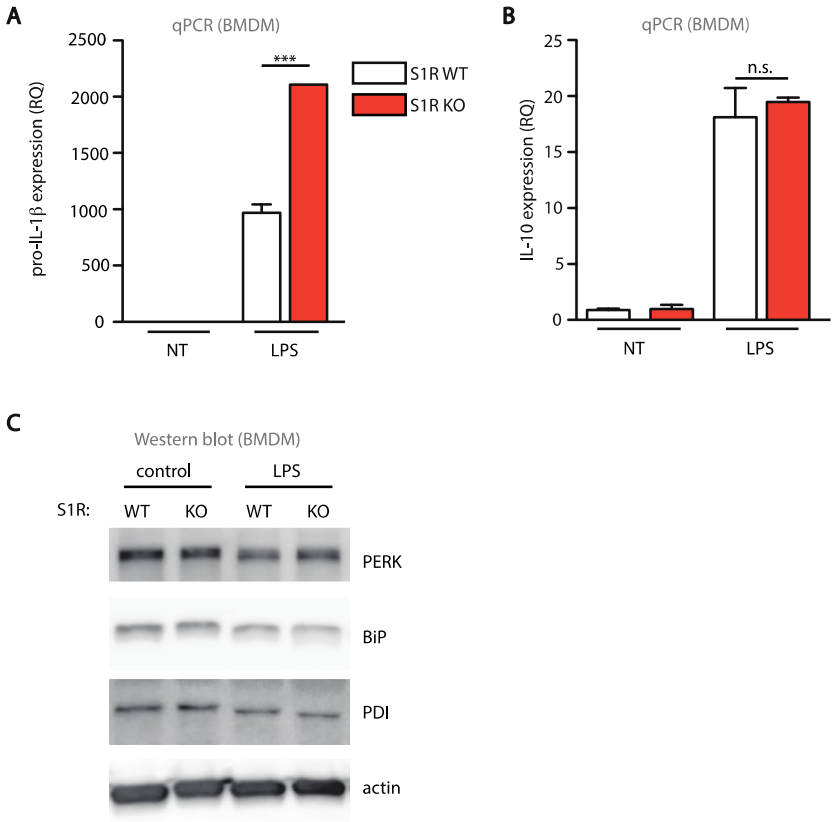

Figure S1. S1R deletion influences a subset of LPS-induced processes without causing global perturbation. (A-B) Relative quantity (RQ) of **(A)** pro-IL-1β or **(B)** IL-10 transcript by qPCR on BMDM stimulated for 6 hours with vehicle (NT) or 100ng/mL LPS (representative of n=3 independent experiment is shown, n.s. not significant, ***P<0.001, two-way ANOVA with post-hoc Tukey test). (**C**) BMDM were treated with vehicle (control) or 100ng/mL LPS for 6 hours and lysates were analyzed by immunoblotting for the indicated proteins (representative of n=3 independent experiments).

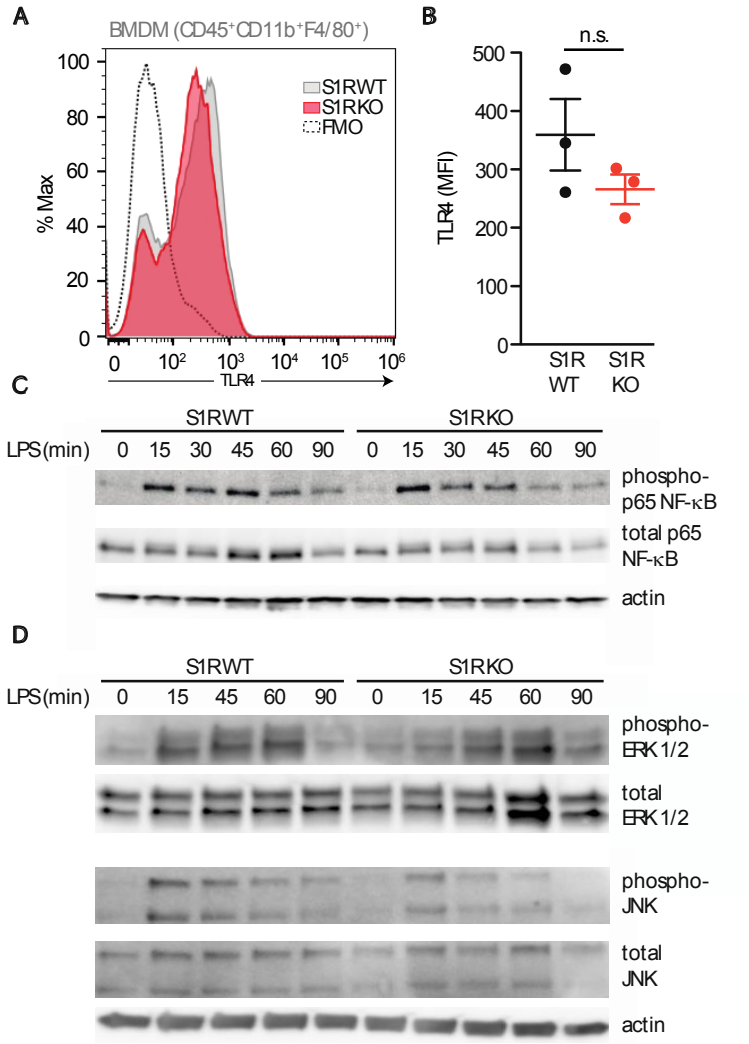

Figure S2. Canonical TLR4 signaling in macrophages is unperturbed by S1R deletion. (**A-B**) Flow cytometric assessment of surface TLR4 on $CD45^+$ $CD11b^+$ $F4/80^+$ BMDM (n=3 mice/group, each dot represents one mouse, t-test). (**C-D**) BMDM were treated with 100ng/mL LPS for indicated durations and lysates were analyzed by immunoblotting for the indicated proteins (n=1, representative of n=3 independent experiments).

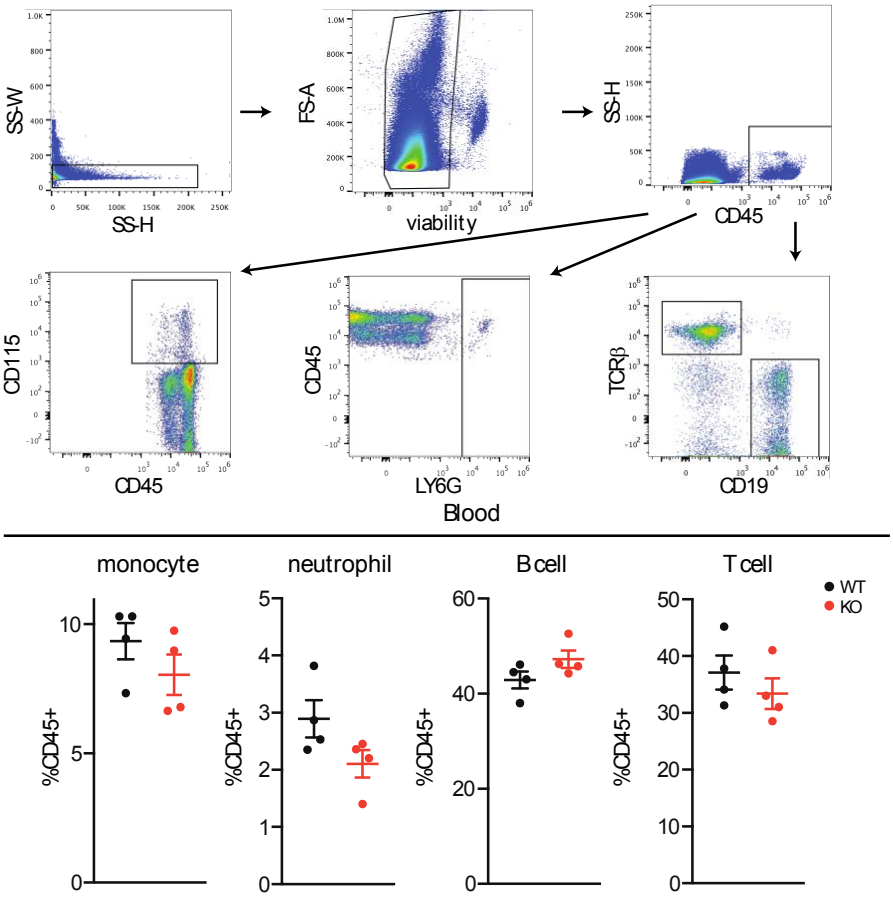

Figure S3. Gating strategy and quantification of immunophenotyping on blood. n=4, each dot represents data from one animal, all comparisons not significant, t-test.

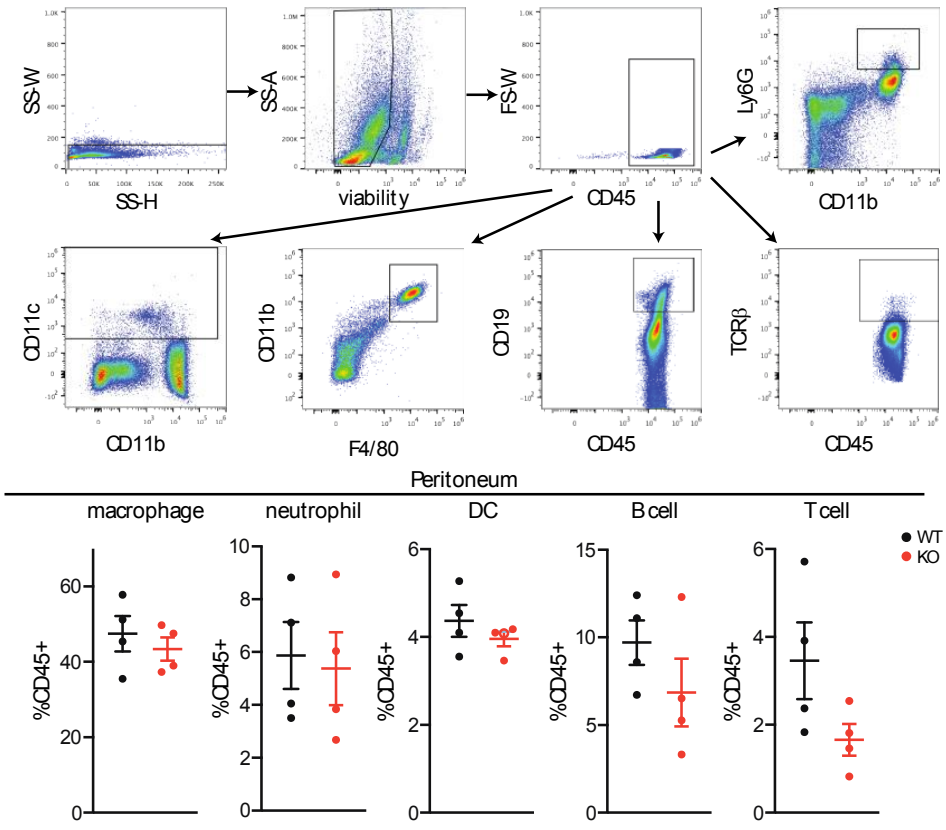

Figure S4. Gating strategy and quantification of immunophenotyping on peritoneal contents. $CD11c^{+}$ gate was drawn on CD11c FMO. n=4, each dot represents data from one animal, all comparisons not significant, t-test.

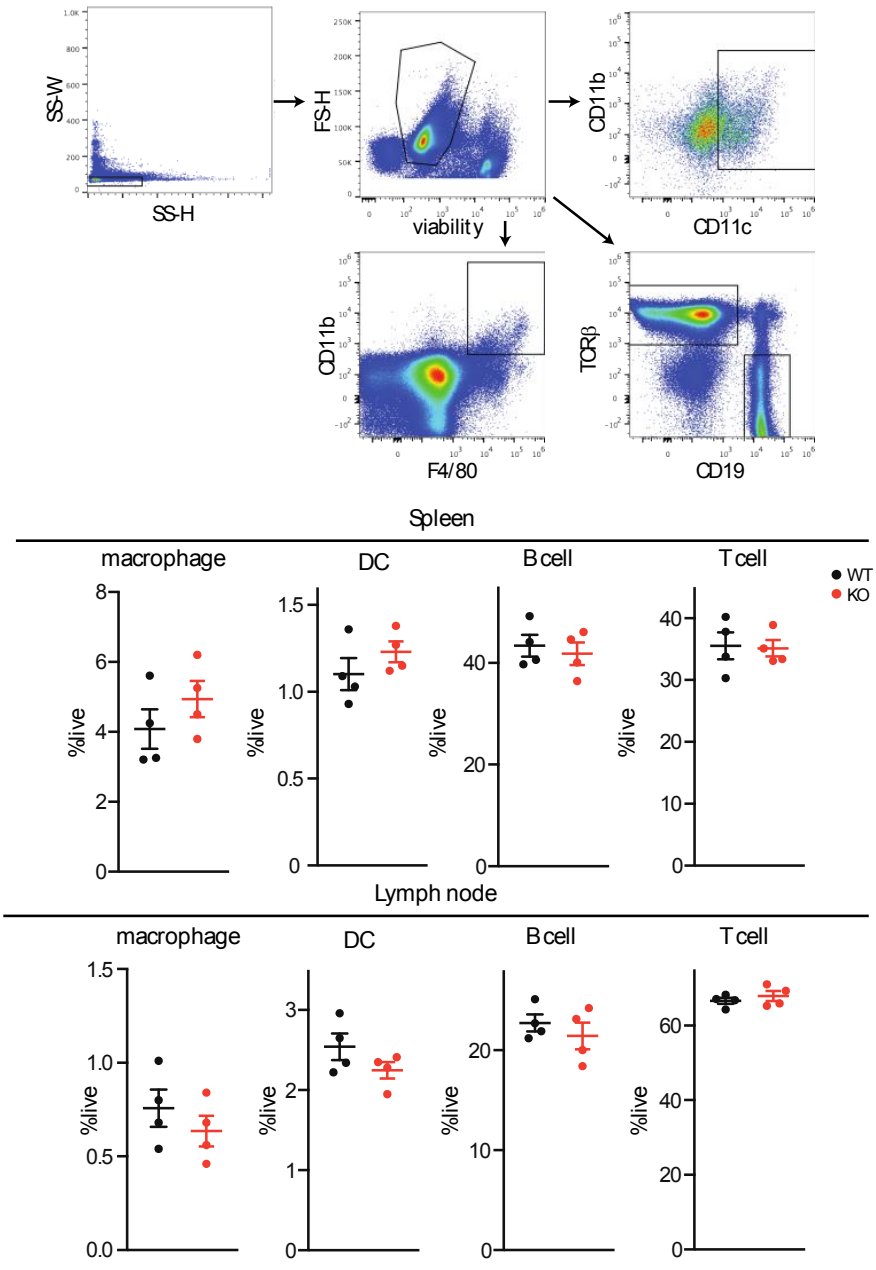

Figure S5. Gating strategy and quantification of immunophenotyping on spleen and lymph node. CD11c^{+} gate was drawn on CD11c FMO. n=4, each dot represents data from one animal, all comparisons not significant, t-test.

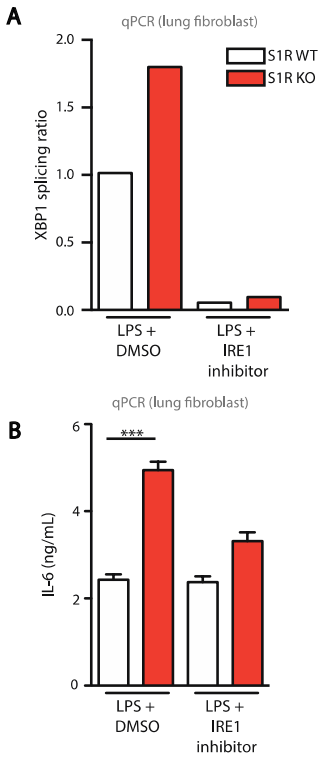

Figure S6. S1R controls inflammation in primary fibroblasts. (A) qPCR analysis for XBP1 splicing ratio (i.e. GAPDH-normalized spliced XBP1 transcript/GAPDH-normalized unspliced XBP1 transcript) in primary lung fibroblasts stimulated for 6 hours with 100ng/mL LPS and vehicle (<1% DMSO) or IRE1 inhibitor (5μM 4μ8C) (representative of n=3 independent experiments). **(B)** ELISA on supernatant from primary lung fibroblasts stimulated for 6 hours with 1μg/mL LPS and vehicle (<1% DMSO) or IRE1 inhibitor (5μM 4μ8C), normalized to total protein in well (representative of n=3 independent experiment is shown, ***P<0.001, two-way ANOVA with post-hoc Tukey test).

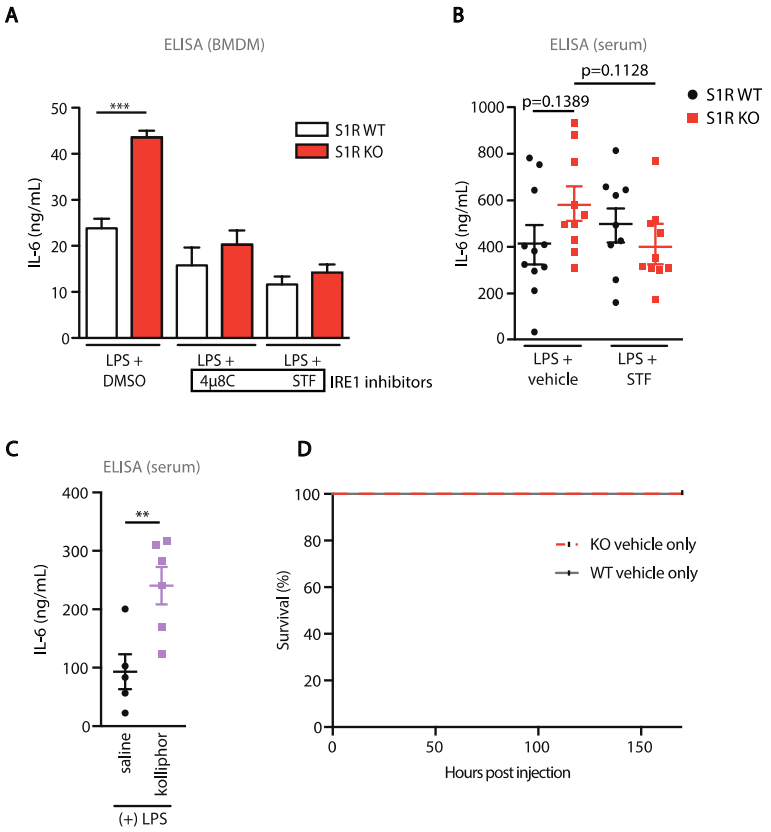

Figure S7. STF affects cytokine production in vitro and in vivo. (A) ELISA on supernatant from BMDM stimulated for 6 hours with 1μg/mL LPS and and vehicle (<1% DMSO) or two IRE1 inhibitors (5μM 4μ8C or 20μM STF), (representative of n=3 independent experiment is shown, ***P<0.001, two-way ANOVA with post-hoc Tukey test). **(B)** ELISA on serum from mice challenged with 2mg/kg LPS and 33% Kolliphor (vehicle) or 30mg/kg of the IRE1 inhibitor (STF) as in **(Fig. 3I)** (n=9-11 mice/group, each dot represents one mouse. Genotype-treatment interaction *P<0.05, two-way ANOVA). **(C)** ELISA for IL-6 in serum of WT mice treated with 9μL/g of 33% Kolliphor in saline (Kolliphor) or an equivalent volume of saline (n=5-6 mice/group, each dot represents one mouse, **P<0.001, t-test). **(D)** Saline + 33% Kolliphor, no LPS, controls, challenged as in **Fig. 4C** (n=6-7, n.s., log-rank test).

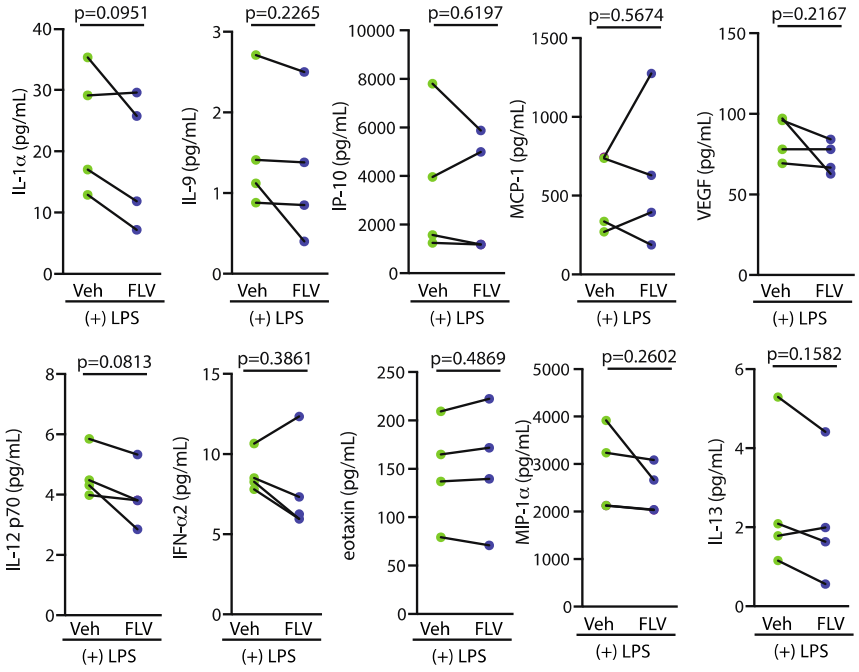

Figure S8. Anti-inflammatory effect of FLV does not globally suppress cytokine production from human blood. Multiplex ELISA on serum from heparinized human blood stimulated *ex vivo* with 10ng/mL LPS and vehicle (RPMI) or 20μM fluvoxamine for 4 hours (n=4, each dot pair represents serum from one participant, paired t-test).

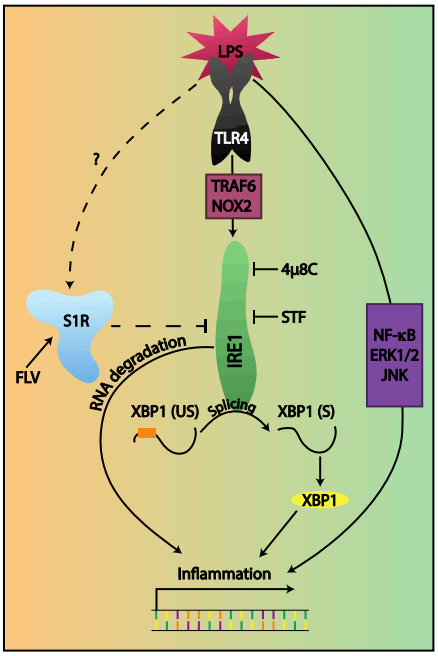

Figure S9. Proposed mechanism of action of S1R during LPS-mediated inflammatory response. FLV: fluvoxamine; XBP1 (US): Unspliced XBP1 transcript; XBP1 (S) Spliced XBP1 transcript.

Chapter 5
Additional findings: Loose ends and new beginnings

Molecular insights

Having observed that S1R influences production of pro-inflammatory cytokines via modulation of IRE1, we wanted to better understand the molecular underpinnings of this action of S1R. Using cells in culture, we observed how S1R acts under inflammatory stimuli. In cultured cells, including bone marrow derived macrophages (BMDM), treatment with the pro-inflammatory factors LPS and interferon gamma suppress S1R expression (Fig. 1). It is unclear how this transcriptional regulation occurs, since LPS increases the intranuclear abundance of the S1R transactivator ATF4 at the timepoints examined[1,2]. Nonetheless, the responsiveness of S1R to inflammatory stimuli is suggestive of its potential activity in inflammatory responses.

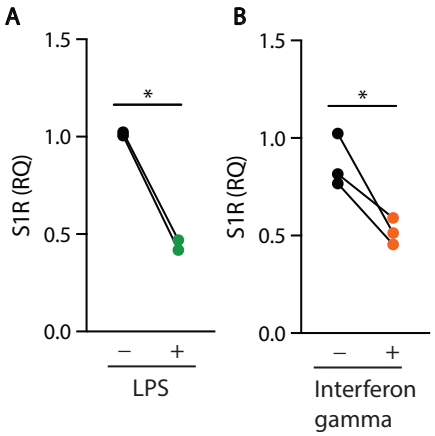

Figure 1: Inflammatory stimuli suppress S1R transcription. A) qPCR on BMDM stimulated for 6h with 100ng/mL LPS. **B)** qPCR on BMDM stimulated for 24h with 20ng/mL interferon gamma. Statistical analysis: t-test.

S1R may be able to control IRE1 abundance, as has been previously suggested[3]. While we were unable to consistently detect changes in IRE1 abundance in S1R KO BMDM compared to wild type, overexpression of S1R in HEK293 cells robustly suppresses total IRE1α compared to transfection with empty vector or MESD (Fig. 2A-B). The effect of S1R overexpression on IRE1 does not seem to be the simple result of overexpressing any ER protein, since overexpressing the low density lipoprotein receptor chaperone MESD had no effect on IRE1 compared to empty vector (not shown). An outstanding question is how S1R overexpression produces this

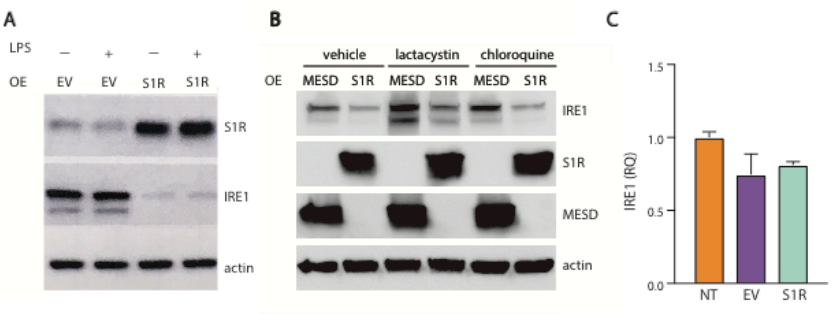

Figure 2: S1R overexpression reduces IRE1α abundance. A) Western blot on HEK293 cells transfected with IRE1α and empty vector or S1R, stimulated with 100ng/mL LPS for 24h. **B)** Western blot on HEK293 cells transfected with IRE1α and MESD or S1R stimulated. **C)** qPCR on NIH 3T3 cells not transfected or transfected with empty vector or S1R. Statistical analysis: **(C)** one-way ANOVA, all comparisons not significant.

effect on IRE1 abundance, since S1R overexpression has no effect on IRE1 transcript levels (Fig. 2C). Strangely, S1R-induced suppression of IRE1 doesn't seem to depend on traditional degradation pathways either, since inhibition of the proteasome (by lactacystin) or autophagy (by chloroquine) does not restore IRE1 to the amount seen in MESD-transfected cells (Fig. 2C). Further work on the interplay between the abundance of S1R and IRE1 could yield novel therapeutic opportunities: Enforcing high S1R abundance may be a potential method for dampening IRE1-driven inflammation.

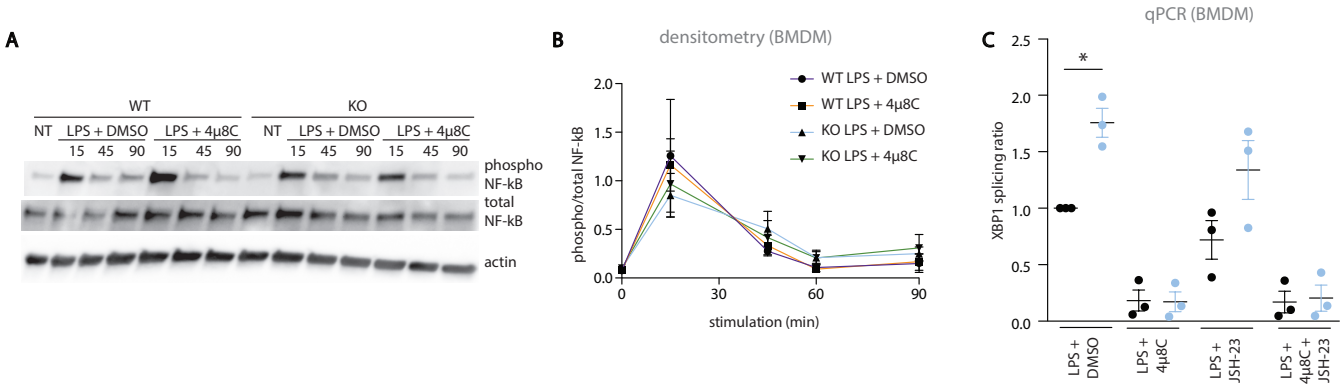

Figure 3: NF-kB and IRE1 do not appear to interact in our BMDM stimulation paradigm. A) Representative western blot on BMDM stimulated with 100ng/mL LPS for the number of minutes indicated. **B)** Densitometric quantification of actin-normalized phospho-NF-kB relative to actin-normalized total NF-kB, N=4 independent experiments. **C)** qPCR on BMDM stimulated with 100ng/mL LPS and 5μM 4μ8C or 20μM JSH-23 (NF-kB inhibitor) for 6h. Statistical analysis: **(B)** repeated measures two-way ANOVA, treatment not significant, **(C)** repeated measures one-way ANOVA.

While we have worked to elucidate the mechanisms by which S1R can affect inflammatory cytokine production, much remains unclear. For example, other groups have identified S1R as potentially influencing the phosphorylation of p65 NF-κB and ERK1/2, while we found that S1R deletion did not affect LPS-induced phosphorylation of these proteins (Ch. 4 Fig. S2)[4,5]. Indeed, the mechanism we identify, whereby S1R deletion leads to rampant IRE1 endonuclease activity and drives inflammatory cytokine production, seems wholly independent of p65 NF-κB phosphorylation. Inhibition of IRE1 endonuclease activity does not affect the amount or rate of NF-κB phosphorylation in LPS-treated BMDM (Fig. 3A-B). Conversely, inhibition of

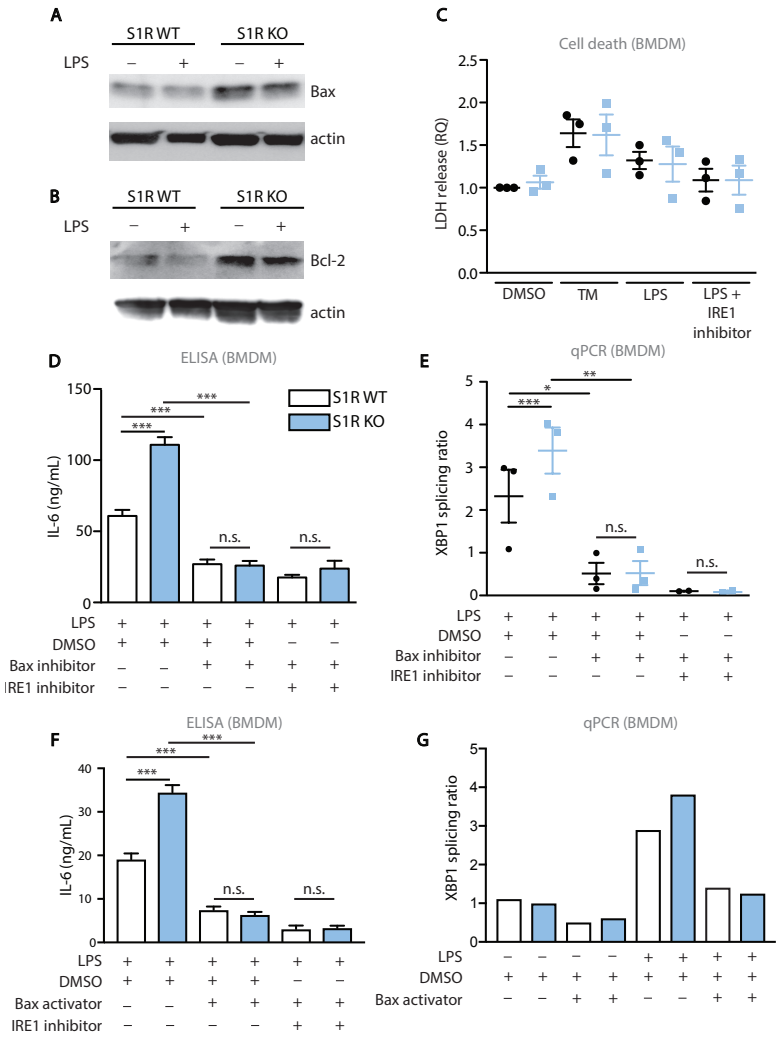

Figure 4: Bax is required for S1R anti-inflammatory function. A-B) Western blot on BMDM treated with vehicle (NT) or 100ng/mL LPS for 6h (representative of N=4). **C)** LDH release assay on BMDM treated for 6h with <1% DMSO, 5μM tunicamycin (TM), 100ng/mL LPS, or 100ng/mL LPS + IRE1 inhibitor (5μM 4μ8C). Values are % maximal LDH release, relative to WT DMSO treated within each independent replicate (N=3, each dot represents one independent experiment. **D)** IL-6 ELISA on supernatant from BMDM stimulated for 6h with 1μg/mL LPS and either vehicle (DMSO), Bax inhibitor peptide V5 (200μM BIPV5), or 200μM BIPV5 + IRE1 inhibitor (5μM 4μ8C) (representative of N= 3 independent experiments). **E)** qPCR on BMDM stimulated with 100ng/mL LPS and either vehicle (DMSO), Bax inhibitor peptide V5 (200μM BIPV5), or 200μM BIPV5 + IRE1 inhibitor (5μM 4μ8C) for 6h (N=2-3, each dot represents one independent experiment. **F)** IL-6 ELISA on supernatant from BMDM stimulated for 6h with 1μg/mL LPS and either vehicle (DMSO), Bax activator (30μM BAM7), or 30μM BAM7 + IRE1 inhibitor (5μM 4μ8C) (representative of N= 2 independent experiments). Statistical analysis: **(C-F)** two-way ANOVA.

NF-κB nuclear localization with the inhibitor JSH-23 modestly suppresses overall XBP1 splicing but does not return XBP1 splicing in S1R KO BMDM back to the levels seen in wild type controls (Fig. 3C). It is unclear why some of our findings do not align with published features of S1R, but it could due to cell type differences, the age of the mice whose cells were harvested, and/or stimulation conditions.

One potential mechanism by which S1R might be able to affect IRE1 endonuclease activity is via modulation of Bcl-2 family proteins. Bax and Bak have been shown to physically associate with IRE1 and potentiate ER stress induced XBP1 splicing[6]. S1R is known to influence the abundance of Bcl-2 family proteins, and we observe that deletion of S1R results in increased levels of Bax and Bcl-2 (Fig. 4A-B). Curiously, despite elevated Bax, an essential apoptotic effector, S1R KO BMDM do not undergo more

apoptosis either basally in culture or after challenge with LPS or the ER stressor tunicamycin (Fig. 4C). This may be because the increased Bcl-2 offsets the apoptotic influence of Bax. Bax seems to be important in the interplay of S1R and IRE1 during LPS-induced inflammation, since treatment with a cell-permeable Bax inhibitor peptide normalizes IL-6 secretion between S1R KO and WT BMDM (Fig. 4D). This inhibition of S1R-driven excessive inflammation correlates with a suppression of IRE1 endonuclease activity as measured by XBP1 splicing, suggesting that Bax, IRE1, and S1R may be interconnected in inflammation (Fig. 4E). However, although IRE1 was determined to be near S1R by proximity ligation assay Ch. 4 Fig. 1), Bax is not biotinylated by our S1R-BirA construct. Strangely, we find that treatment of BMDM with BAM7, a Bax-activating molecule, also inhibits S1R-dependent and S1R-independent cytokine production and XBP1 splicing (Fig. 4F-G). This may be because BAM7 affects the BH3 domain of Bax, a region that is important for its modulation of IRE1 activity[6,7]. More work is needed to fully understand how Bax, S1R, and IRE1 interact to influence XBP1 splicing and cytokine production. For example, Bax modulators may alter the proximity of S1R and IRE1, which could be examined by proximity biotinylation or immunoprecipitation assays.

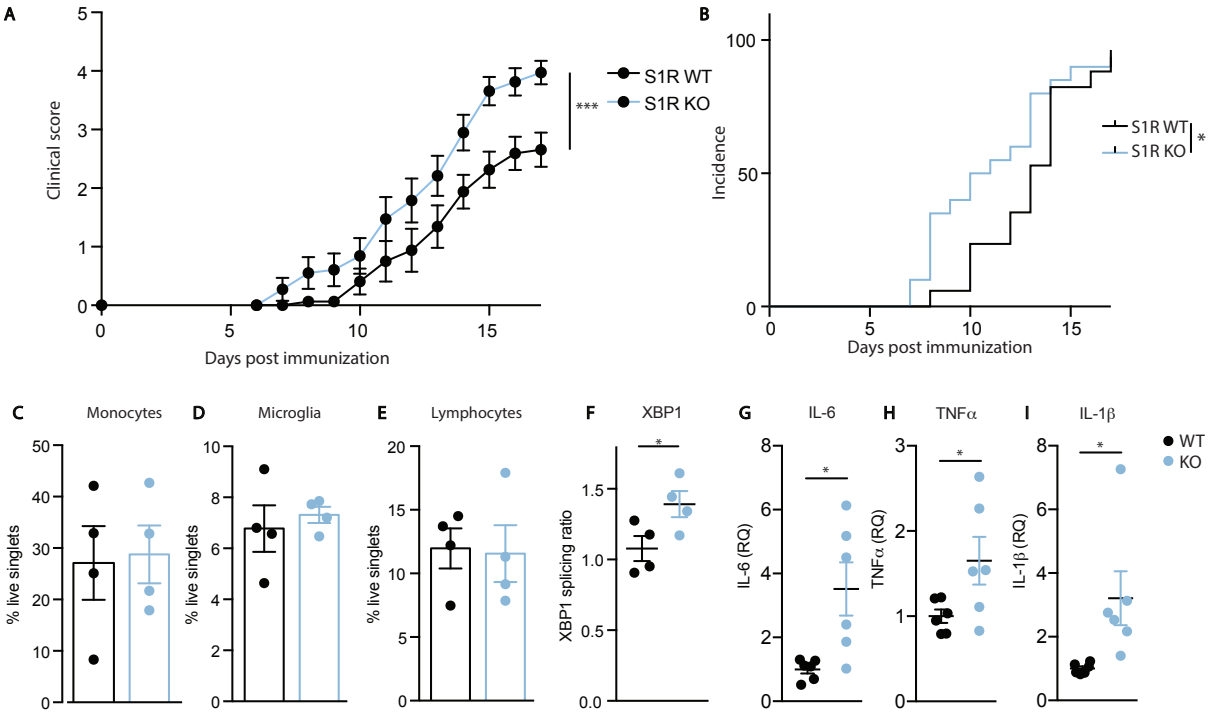

Figure 5: Deletion of S1R results in more severe pathology in EAE. A) Clinical assessment of WT and S1R KO mice. EAE was induced by immunizing mice with 50ng MOG_{35-55} and complete Freund's adjuvant. Higher clinical score indicates more severe paralysis (n=17-20 mice/group). **B)** Incidence of EAE clinical signs onset (n=17-20 mice/group). **C-E)** Quantification of infiltrating monocytes, resident microglia, and lymphocytes in the EAE spinal cord harvested on day 16 post immunization. Monocytes were gated on singlets, live, CD11b+, CD45high; microglia were gated on singlets, live, CD11b+, CD45mid; lymphocytes were gated on singlets, live, CD11b-, CD45+. **F)** qPCR for XBP1 splicing in EAE spinal cord harvested on day 16 post immunization. **G-I)** qPCR on EAE spinal cords harvested on day 16 post immunization. Statistical analysis: **(A)** Wilcoxon matched pairs test, **(B)** log-rank test,**(C-I)** t-test, each dot represents one mouse.

Signaling pathways and modulators of cytokine production may be shared across multiple inflammatory contexts. The role of S1R in controlling bacteria-induced inflammation raises the possibility that S1R may be involved in other kinds of inflammatory disease. To test this, we used experimental autoimmune encephalomyelitis (EAE), a model of the autoinflammatory demyelinating disease multiple sclerosis. In this model, animals (here mice) are immunized against myelin components, resulting in infiltration of immune cells into the spinal cord, causing demyelination and ascending flaccid paralysis. When S1R KO mice are subjected to this model, they experience a slightly earlier onset of clinical signs as well as more severe paralysis in the plateau phase (Fig. 5A-B). The severity of EAE is variable and depends

on factors not entirely under experimental control, such as the season at immunization; in replicates where the EAE is more severe overall, we observe that S1R KO mice spontaneously die[8]. More investigation is needed to understand why this occurs, since the animals appear grossly normal upon necropsy and usually did not appear distressed or severely paralyzed 24 hours before death, making dehydration an unlikely cause.

Preliminary findings indicate that some of the mechanisms we have observed in LPS-induced inflammation and sepsis models are conserved in EAE. Spinal cords harvested from mice in the early chronic phase of EAE (day 16 post immunization) have higher levels of the pro-inflammatory cytokine transcripts IL-6, IL-1β and TNFα, as well as a higher ratio of spliced to unspliced XBP1 (Fig. 5F-I). The increased levels of cytokine transcript did not correlate with any changes in immune cell populations in the spinal cord (Fig. 5C-E). This finding suggests that S1R may not affect cell infiltration, but the immune cells present have a more inflammatory character when lacking S1R, or it is possible that the observed effects on cytokine production come primarily from non-infiltrating cells of the spinal cord. More work is needed to understand how S1R influences autoimmune attack at the EAE spinal cord over the course of the disease.

The future of S1R in inflammatory diseases

We and others have shown that S1R can powerfully influence inflammatory mediator production by immune and non-immune cell types. This exciting finding adds to a growing body of evidence that signaling by ER resident proteins has significant impact on inflammatory diseases, including both acute conditions like bacterial infection, as well as in chronic autoimmune conditions like arthritis. ER stress exacerbates inflammation across a range of conditions, and the downregulation of S1R that occurs as a result of ER stress may be an important overlooked aspect of how ER dysfunction can drive inflammation. S1R is a highly druggable nexus of ER dysfunction, redox stress, and apoptosis, three cellular processes that combine to exacerbate outcomes in an array of human diseases, including Alzheimer's disease and cancers.

If we can better understand how S1R is working, it may be possible to disrupt disease processes using the S1R ligands that already exist and are approved for clinical use.

In continuing to investigate the role of S1R in sepsis, there are several important next steps. Because serotonin is also implicated in bacterial-derived inflammation and sepsis, we will cross S1R KO mice to mice lacking serotonin transporter (SERT), the other major target of fluvoxamine. We will compare the efficacy of fluvoxamine in S1R and SERT KO mice to wild type and S1R/SERT double KO mice. Because our data indicate that S1R significantly contributes to the therapeutic benefit of fluvoxamine in sepsis models, we anticipate that, although there may be some effect of SERT KO on fluvoxamine efficacy, SERT KO mice will nonetheless have some degree of anti-inflammatory response to fluvoxamine treatment. To further bolster our hypothesis that drugs' action at S1R can strongly contribute to anti-inflammatory benefits, we will test additional S1R ligands that are chemically distinct from fluvoxamine in sepsis models, ideally identifying S1R-dependent anti-inflammatory action of several drugs in clinical use, from an array of drug classes. Having identified a number of clinically-approved drugs that benefit sepsis models via S1R, we can then partner with clinical scientists and statisticians to conduct retrospective studies of sepsis patients taking the drugs identified. Understanding how S1R ligands interact with clinical course in human sepsis will be an important step towards implementing S1R-targeted sepsis therapeutics.

Appendix I
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Appendix II
Abbreviations + Glossary

ALS: Amyotrophic lateral sclerosis. Degenerative disease of motor neurons.
ANOVA: Analysis of variance. F-test, used for statistical analysis of sources of variation.
APC: Activated protein C. Blood factor involved in clotting.
ATF4: Activating transcription factor 4. Transcription factor induced by the integrated stress response and PERK activation.
ATF6: Activating transcription factor 6. Transcription factor induced by the UPR and ER stress sensor.
BAM7: Bax activator molecule 7. Selective Bax BH3-domain binding protein, triggers Bax oligomerization *in vitro*.
BCR: B cell receptor. Antigen-specific receptor on B cell outer surfaces, involved in B cell activation.
BMDM: Bone marrow derived macrophages. Macrophages differentiated in culture with macrophage colony stimulating factor from bone marrow hematopoietic stem cells. ~95% CD11b+ F4/80+.
C/EBP: CCAAT-enhancer binding protein. Family of transcription factors targeting CCAAT-box motif.
CHOP: C/EBP homologous protein. Transcription factor induced by ER stress, involved in ER-related apoptosis.
CLP: Cecal ligation and puncture. Model of polymicrobial sepsis.
CNX: Calnexin. Membrane-bound major ER-resident chaperone.
CREB: cAMP response element binding protein. Transcription factor targeting cAMP response element.
CRT: Calreticulin. Luminal ER-resident chaperone.
DAMP: Damage-associated molecular pattern. Molecules released in tissue damage that precipitate damage responses, which are often pro-inflammatory.
DC: Dendritic cell. Professional antigen-presenting cells.
dHMN: distal hereditary motor neuropathy. One of a group of motor neuron diseases resulting from mutations, characterized by motor neuron loss and muscle wasting.
DMT: N, N-dimethyltryptamine. Endogenous psychedelic compound produced mainly by the pineal gland.
EAE: Experimental autoimmune encephalomyelitis. Animal model of myelin-directed autoimmunity, characterized by immune cell infiltration into CNS tissues and destruction of myelin.
eIF2: Eukaryotic initiation factor 2. Protein required for initiation of translation.
ER: Endoplasmic reticulum. Organelle that is a site of protein trafficking, homeostatic maintenance, and threat responses.
ERG2: Sterol isomerase (Name from ERGosterol biosynthesis). Yeast homologue of S1R, involved in yeast ergosterol biosynthesis.
ERSE: ER stress response element. Cis-acting response element that appears in many genes regulated by ER stress.
fEPSP: Field excitatory postsynaptic potential. Measure of induced electrical activity within a brain region, often used to measure long-term potentiation.
FIP: Fecal-induced peritonitis. Model of polymicrobial sepsis.
HAC1: Transcriptional activator HAC1. Yeast homologue of XBP1.
hSI: Human sterol isomerase. Protein involved in sterol biosynthesis, related to S1R.
ICU: Intensive care unit. Medical ward for very ill patients, where many sepsis patients receive treatment.
IL-10: Interleukin 10. Cytokine with typically anti-inflammatory signaling capabilities.
IL-1β: Interleukin 1β. Cytokine with typically pro-inflammatory signaling capabilities.
IL-6: Interleukin 6. Cytokine with pro-inflammatory and anti-inflammatory signaling capabilities (context- and source-dependent).
ILC: Innate lymphoid cells. Innate immune cells arising from common lymphoid progenitor.
IP3R: Inositol triphosphate receptor. Ligand-gated calcium channel.
IRE1: Inositol-requiring enzyme 1. The eldest ER stress sensor.

JNK: c-Jun N-terminal kinase. Signaling kinase that responds to changes in the cellular environment, especially to drive apoptosis and/or inflammation.
LPS: Lipopolysaccharide. Component of gram-negative bacterial walls, ligand for TLR4, inflammatory stimulus commonly used *in vitro.*
LSK: Lineage marker -, Sca-1+, cKit+. Non-committed hematopoietic stem cell progenitors.
MAM: Mitochondria-associated membrane. ER membrane segments forming close contacts and interacting with mitochondria.
MEF: Mouse embryonic fibroblast. Cell lines derived from mouse embryo.
MESD: Mesoderm development LRP chaperone. Dedicated chaperone for low-density lipoprotein receptors, which are secreted proteins.
NF-κB: Nuclear factor kappa B. Transcription factor that responds to changes in the cellular environment, especially to drive apoptosis and/or inflammation.
NQO1: NADPH dehydrogenase quinone 1. Antioxidant enzyme.
PAI-1: Plasminogen activator inhibitor-1. Inhibits fibrinolysis.
PAMP: Pathogen-associated molecular pattern. Common components of pathogens, sensed by pattern recognition receptors to induce antimicrobial responses.
PDI: Protein disulfide isomerase. ER chaperone involved in disulfide bond formation.
PERK: Protein kinase R-like ER kinase. ER stress sensor whose output overlaps with the integrated stress response.
PMBC: Peripheral blood mononuclear cells. White blood cells including lymphoid and myeloid cells, excluding cell types with multi-lobed nuclei such as neutrophils, found in circulation.
PRR: Pattern recognition receptor. Receptors highly expressed in innate immune cells, important for early response to infection or tissue injury.
RIDD: Regulated IRE1-dependent decay. Process by which IRE1 cleaves RNAs for degradation.
RIG-I: Retinoid acid-inducible gene I. Intracellular pattern recognition receptor that senses viral RNA.
ROS: Reactive oxygen species. Oxygen-containing molecules, such as hydrogen peroxide and superoxide radicals, that can oxidize a range of molecules. ROS have adaptive, signaling roles as well as detrimental damage capability.
S1R: Sigma-1 receptor. A protein that so defies definition, I have spent thousands of words and years of my life striving to understand it.
SERCA: Sarco/endoplasmic reticulum Ca^{2+} ATPase. Maintains Ca^{2+} gradient across the ER membrane, which is important for proper protein folding.
SIRS: Systemic inflammatory response syndrome. Inflammatory response to severe injury or infection.
SOD1: Superoxide dismutase Cu-Zn. Enzyme involved in processing of free radical superoxide into oxygen or hydrogen peroxide. Mutations in SOD1 cause ALS.
SR: Signal recognition particle receptor. ER-localized protein involved in docking of nascent peptides to the translocon.
SRP: Signal recognition particle. Recognizes nascent peptides that need to transit the secretory pathway and participates in bringing them to the translocon for cotranslational translocation.
SSRI: Selective serotonin reuptake inhibitor. A class of antidepressant drugs with affinity for serotonin transporter.
TLR2: Toll-like receptor 2. PRR recognizing an array of pathogen-derived molecules, such as zymosan.
TLR3: Toll-like receptor 3. PRR recognizing dsRNA.
TLR4: Toll-like receptor 4. PRR recognizing an array of pathogen-derived and host-derived molecules including but not limited to LPS.
TNFα: Tumor necrosis factor alpha. Pro-inflammatory and pro-apoptotic cytokine.
TRAF2: TNF receptor-associated factor 2. Scaffold protein involved in activation of JNK and other signaling proteins.

TRAM: Translocating chain-associated membrane protein. Protein involved in translocation.
TRAP: Translocon-associated protein. Protein involved in translocation.
UGGT1: UDP-glucose glycoprotein glucosyltransferase. ER protein involved in calnexin/calreticulin cycle.
UPR: Unfolded protein response. Orchestrated response of ER to (usually severe) ER dysfunction.
UPRE: Unfolded protein response element. Cis-acting response element that appears in many genes regulated by ER stress.
VRE: Vancomycin resistant enterococci. Bacteria from the genus Enterococcus, that are resistant to treatment with vancomycin, considered an antibiotic of last resort in severe gram-positive bacterial infection.
VSE: Vancomycin susceptible enterococci. Bacteria from the genus Enterococcus, that are susceptible to treatment with vancomycin.
X:XO: Xanthine: xanthine oxidase. Xanthine oxidase is an enzyme that generates ROS from xanthine, when added to culture together these molecules constitute a method of experimentally inducing redox stress.
XBP1: X-box binding protein 1. Transcription factor involved in IRE1 signaling and the UPR.

Acknowledgments

I. The beginning

At the end the parashah, a genealogy
is given; names, unfamiliar as jumbled syllables,
are what remain of those who built the story.
My father reads these in a playful rhythm–
they become a blessing for what might be.

This was no torah portion! Still, by studying,
I become more than I was. I record
who stacked and sculpted science
beside me.

II. The birds

A shadow jay that casts no shadow
carries my spirit on galaxy wings.
I am in the wings, and the galaxy,
we dash among the chaos and ghosts, uncontained.

Fledgling flame with fuel to burn brighter
in the strength that consumes, and I am fire-polished
in the breath of a phoenix. Her path
leads into conflagration as drifting ash kisses my face.

Ravenous bright darkness, a stygian blue.
It's alien to want so much. Alien,
halcyon, the homeworld I will someday know.

White down is snowy soft, a hush upon the world,
bestowing the tornado in her talons, a power carefully given.

The source laid the stones to create a path for me.
This nest, my earth, its nurture and bounty, the safety
and permanence of glittering quartz deep in caves.
Creation I have come from, to return on murky days.

Fury and the sharp flash of a violet feather,
knowledge like danger turned kindly to me,
I stopped kneeling.

III. The fighting

Before dawn, the watchtower that aches to be unknown
carries my secrets upward, etched into the steps.

The general surveys the arena, watchful,
guiding moves, and I, insubordinate, yield not
to conferred authority, the markings of rank,
but to his wisdom, my steadied blade.

What exists of my body is owed to protection
and reflection of the incorporeal
upon my armor's surface. My wounds
are fused to the metal. My flesh now responds
to temperature, the clang and reverberation of steel.

The power: my cloak of a thousand souls.
They were my garrison, their blood and fear
unequal, surely, to the blessings I bought with their pain, but how
does the scale tilt?
As a very old book pays tribute to corpses,
I honor the dead who have no names.