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Role of Caveolin Proteins in Sepsis

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Abstract

Despite the progress in medical treatment sepsis remains one of the major causes of death in pediatric and elderly patients. Understanding signaling pathways associated with sepsis may be of key significance for designing more efficient therapeutic approaches which could alleviate sepsis outcome. Earlier studies suggested that cholesterol- and sphingolipid-rich lipid rafts and their morphologically distinct subset, caveolaecan be utilized by certain bacterial pathogens to enter and invade host cells. Moreover, there is also evidence that the expression levels of the major caveolar coat proteincaveolin-1 can be regulated by the major component of the outer membrane of Gram-negative bacteria,lipopolysaccharide (LPS) in various cell types involved in sepsis. In particular recent studies using caveolin-1 knockout mice and cells have revealed that caveolin-1 is directly involved in regulating numerous signalingpathways and functions in various cell types of the immune system and other cell types involved in sepsis. Moreover, the most recent report implies that in addition to extensively studied caveolin-1, caveolin-2 is also important in regulating LPS-induced sepsis and might possibly play an opposite role to caveolin-1 in regulating certain pro-inflammatory signaling pathways. The purpose of this review is to discuss these new exciting discoveries relatedto the specific role of caveolin-1 and the less studiedcaveolin-2in regulating signaling and outcome associated with sepsis induced by LPS and pathogenic bacteria at molecular, cellular and systemic levels.

Introduction

Despite an overwhelming increase in our knowledge on the pathogenesis of sepsis in recent years, severe sepsis, clinically defined as sepsis along with organ dysfunction [13], remains associated with an unacceptable high mortality ranging from 18 to 50% [4,37,71]. The process of sepsis is very complex and is initiated when the body responds to a local pathogen with a generalized, innate inflammatory response. The innate immune system is a highly evolutionarily conserved host defense mechanism against pathogens [9], although an alternative viewpoint suggests that this system evolved to respond to trauma and injury [41]. Innate immune responses to pathogens are initiated by pattern recognition receptors (PRRs) that recognize specific structures of microorganisms. At least four families of PRRs are recognized: Toll-like receptors (TLRs), nucleotide oligomerization domain leucine-rich repeat proteins, cytoplasmic caspase activation and recruiting domain helicases such as retinoic acid-inducible gene I–like helicases, and C type lectin receptors expressed on dendritic and myeloid cells [7,9,46,48]. Bacteria have molecular structures that are generally not shared with their host, common among related pathogens, and invariant. These molecular signatures are also expressed by nonpathogenic and commensal bacteria [22] and are now referred to as pathogen-associated molecular pattern (PAMP) molecules or microbial-associated molecular pattern molecules [7,46]. Inflammatory signals are transduced by a series of adaptor molecules that bind to the PRRs and protein kinases and phosphatases that control signal propagation in the cytoplasm, culminating either in the rapid, posttranscriptional, or posttranslational modulation of a variety of inflammatory mediators or in the activation of various transcription factors. These factors include nuclear factor-κB, (NF-κB), activator protein 1, members of the CCAAT enhancer-binding protein family, early growth response protein 1 (EGR-1), p53, and signal transducer and activator of transcription 1 (STAT1). These mechanisms have been the subject of considerable study and have been reviewed extensively elsewhere [46]. The major cell types involved in this initial response are monocytes, macrophages, and neutrophils. The latter cells once activated, release pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukins (ILs), caspase, proteases, leukotrienes. kinins, reactive oxygen species, and nitric oxide (NO) [47]. Subsequently, these severe inflammatory events lead to microvascular injury, thrombosis, loss of endothelial integrity, capillary leak, edema and tissue ischemia, which result in global tissue hypoxia and organ dysfunction [8].

Caveolae or "small caves" are specialized and morphologically distinct subset of cholesterol- and glycolsphingolipid-rich lipid rafts and were originally identified as 50-100 nm flask-shaped, non-clathrin coated invaginations of the plasma membrane [49-51,79]. These organelles are present in most mammalian cell types and tissues, and are particularly abundant in endothelial cells, adipocytes, and pneumocytes type I [3,17,54,63]. The originally described functions for caveolae included cholesterol transport [65], endocytosis [61], and potocytosis [3]. However, later studies have revealed that caveolae play a pivotal role in regulating cell signaling. This pivotal role of caveolae in signaling stems from the fact that these microdomains concentrate multiple membrane proteins and other components involved in transport and signal transduction [1,26,55,56]. Although a large body of evidence regarding the involvement of caveolae was originally gathered using pharmacological approaches targeting plasma membrane cholesterol, these approaches do not distinguish between a specific function of caveolae and other subsets of lipid rafts. Therefore, a significant advance in understanding the roles of caveolae was revealed by identification of the coat proteins of caveolae, caveolins, VIP21/caveolin-1 (Cav-1), caveolin-2 (Cav-2), and caveolin-3 (Cav-3) [21,30,60,68,74]. Cav-1 and -2 are ubiquitously co-expressed, while Cav-3 is muscle-specific

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[76]. In addition to caveolins, caveolae also contain adapter proteins, cavins [5,23]. Unlike caveolins, the research on cavin proteins is just beginning and to date, no data regarding possible involvement of cavin proteins in sepsis is available. In most cases, Cav-1 and Cav-2 tightly interact with each other and form hetero-oligomeric complexes within caveolae [10]. The interaction with Cav-1 is necessary for transport of Cav-2 to the cell surface [45,53]. In the absence of Cav-1, Cav-2 is degraded, and its expression is strikingly reduced [14,58]. Cav-1 expression is essential for the formation of caveolae, whereas the role of Cav-2 could vary depending on a cell and tissue type [18,33,59,67]. Caveolins, in particular the mostly studied Cav-1 play numerous important functions. In addition to being key structural proteins that organize caveolar structures, caveolin proteins are important in regulating various aspects of cell signaling and function [26,29,52,76]. Cav-1 and -2 are often expressed in various cells of the immune system [24]. This review will primarily focus on ubiquitously expressed Cav-1 and Cav-2 and the role which these proteins play in regulating often cell type-specific signaling associated with sepsis at molecular, cell, and systemic levels.

Pharmacological evidence supporting the role of lipid rafts/caveolae in pathogen infection and related signaling

Earlier studies using pharmacological approaches targeting both lipid raft and caveolar domains have revealed that these microdomains can be involved in regulating the function of the immune system. Large body of evidence accumulated suggesting that lipid rafts and caveolae are utilized by a number of bacterial pathogens including *Pseudomonas aeruginosa, Escherichia coli, Camylobacter jejuni, Chlamydia species, Mycobacteria species, Brucella species, Listeria monocytogenes, Shigella flexneri, Ehrlichia chaffeensis, Anaplasma phagocytophilum*, or Group A streptococci to enter into host cells (Reviewed in: [15,24,81,83]). These pioneering studies clearly suggested the involvement of lipid rafts and/or caveolar microdomains in regulating the signaling and function associated with host cell infection by numerous pathogens some of which are involved sepsis. However, majority of these studies utilized pharmacological tools such as cholesterol sequestering drugs and thus cannot distinguish between caveolae and the remaining subsets of lipid raft microdomains. Moreover, such pharmacological tools could not directly address the specific role of caveolin proteins. It is also important to remember that Cav-1 and Cav-2 may also reside out of lipid raft/caveolar microdomains [25] and thus drugs targeting lipid rafts through altering membrane cholesterol are not suitable for testing the functional significance of these caveolins pools. In recent years, a significant progress was made in developing more specific approaches manipulating the expression levels of each individual caveolin protein such as overexpression, siRNA and knockout (KO) mice. Especially, utilizing KO mice allowed testing the direct role of Cav-1 and most recently Cav-2 in regulating signaling and function in various mouse models of sepsis.

Cav-1 expression is regulated by bacterial lipopolysaccharide (LPS) in a cell type-specific manner

Evidence accumulated suggesting that depending on the cell type, LPS could induce or suppress the expression levels of Cav-1 (Summarized in Table 1). Initially, Lei et al. [35] observed that Cav-1 mRNA levels were higher in fibroblasts isolated from LPS-hyporesponsive mice compared to their counterparts with normal response to LPS. This data suggests that LPS could suppress Cav-1 transcription in fibroblasts. Similarly, in RAW264. 7 macrophage cell line, both Cav-1 mRNA and protein levels were also down-regulated by LPS. In contrast to fibroblasts and RAW264. 7 cells, in thioglycolate (TG)-elicited C3HeB/FeJ peritoneal macrophages, the expression levels of Cav-1 protein and mRNA were up-regulated in response to LPS stimulation in vitro. Importantly, this LPS-induced upregulation of Cav-1 appears to be TLR4-dependent because LPS did not trigger an up-regulation of Cav-1 in macrophages from the LPS-hyporesponsive TLR4 mutant C3H/HeJ mice [35]. Since activation of macrophages by bacterial products is a very important early event during sepsis, the LPS-induced upregulation of Cav-1 in primary macrophages indirectly suggests that Cav-1 could play a role in LPS-induced signaling and function in macrophages. The subsequent study from the same laboratory [36], has shown that increases in Cav-1 expression could be achieved by different types of LPS, LPS-mimetic taxol, and heat-killed *E. coli* and to a much lesser extent by zymosan, polysaccharide-peptidoglycan, and heat-killed *Staphylococcus aureus*. Moreover, *Rhodobacter sphaeroides* lipid A failed to induce Cav-1 expression in macrophages. These data suggest that expression of Cav-1 in response to LPS is only partly dependent upon lipid A. TNF-α only marginally induced Cav-1, suggesting that the ability of LPS to induce Cav-1 is not primarily mediated through an autocrine/paracrine mechanism involving TNF-α. Moreover, the LPS-induced upregulation of Cav-1 was not associated with changes in the expression levels of TLR4. Remarkably, Cav-1 localized to two subcellular compartments, associated with lipid rafts and with TLR4. This observation suggests that non-lipid raft pool of Cav-1 could possibly be involved in interaction and regulation of signaling associated with TLR4. Finally, using pharmacological inhibitors, they have also shown that LPS-induced upregulation of Cav-1 expression in murine macrophages in vitro was p38 MAPK- and proteasome-dependent [36]. Taken together, these studies suggest that Cav-1 expression levels are modulated by LPS and bacteria and that this modulation could possibly be involved in regulating signaling and function in macrophages during sepsis.

Cell type	mRNA	Protein	Additional comments	Reference
Mouse fibroblasts	↓			[35]
RAW264.7 macrophage cell line	↓	↓		[35]
Peritoneal macrophages from C3HeB/FeJ mice	↑	↑	p38 MAPK- and proteasome-dependent.	[35] [36]
Peritoneal macrophages from TLR4 mutant C3H/HeJ mice	NC	NC	Negative data suggests TLR4 dependence	[35]
B-lymphocytes from C57BL6 mice	↑	↑		[42]
Porcine kidney-15 (epithelial) cells	↑			[39]
Human lung microvascular endothelial cells	↑	↑	NF-κB-dependent	[69]
Mouse liver sinusoidal endothelial cells	↑	↑	ET-1-dependent	[27]

Table 1: Cell type-specific decrease (↓), increase (↑) or no change (NC) in Cav-1 expression by LPS *in vitr*.

In another study, using RT-PCR and immunoblotting approaches Medina et al. [42] showed that Cav-1 could also be induced at both mRNA and protein levels in B-lymphocytes treated with LPS. These results clearly indicate that there is a regulated expression of Cav-1 in primary B-lymphocytes activated with LPS. However, no further mechanistic insights as to how LPS induces Cav-1 in B-lymphocytes were provided. Of note, the maximum upregulation of Cav-1 in LPS-treated B-lymphocytes took place 72 hrs after adding LPS [42] as opposed to only 4-6 hrs in macrophages [35]. These differences in kinetics of response to LPS suggest that Cav-1 upregulation in macrophages might play a role in early stage of sepsis, while Cav-1 upregulation in B-lymphocytes might be relevant for later events.

Using RT-PCR approach, Liu et al. [39] have shown that LPS markedly induced the mRNA levels of Cav-1 in porcine kidney-15 epithelial cells. Interestingly, they also tested expression of Cav-1 in pigs infected with *Haemophilus parasuis* and determined decreased expression of Cav-1, particularly in seriously impaired organs such as the brain and lung. However, it is possible that the observed downregulation of Cav-1 was secondary to severity of injury in examined tissues. Therefore, further mechanistic studies examining possible changes in Cav-1 expression levels in infected cells will be necessary to determine if infection with pathogenic bacteria could specifically regulate expression levels of Cav-1.

Endothelial dysfunction and altered microvascular permeability are always accompanying sepsis, often leading to multiple organs failure [12]. Interestingly, experimental evidence suggests that LPS can also regulate Cav-1 expression levels in endothelial cells. Specifically, Tiruppathi et al. [69] demonstrated that LPS-induced concentration- and time-dependent increases in expression of Cav-1 mRNA and protein in human lung microvascular endothelial cells in vitro. Of note, the maximal upregulation of Cav-1 protein could be seen only 4 hrs after adding LPS, suggesting relevance of Cav-1 for early events associated with sepsis. This stimulating effect of LPS was NF-κB-dependent as evidenced by pharmacological inhibition with IKK-NBD peptide and siRNA knockdown of NF-κB subunit p65/RelA expression. Treatment with LPS also increased the number of caveolae in the apical and basal plasmalemma of endothelial cells as well as transendothelial albumin permeability. Importantly, the NF-κB-dependent upregulation of Cav-1, caveolae, and microvascular permeability could be recapitulated in lung tissue from mice challenged with a single intraperitoneal injection of LPS. Taken together, these data suggest that LPS induces NF-κB-dependent Cav-1 expression and caveolae in lung microvascular endothelial cells in vitro and in vivo. Moreover, LPS-induced upregulation of Cav-1 and caveolae in lung microvascular endothelial cells could contribute to LPS-induced increase in microvascular permeability. Given the fact that increased microvascular permeability plays an important role in sepsis, these data also suggest that upregulation of Cav-1 in microvascular endothelial cells by LPS might be an important signaling mechanism contributing to sepsis.

Consistent with [69], studies of Kamoun et al. [27] revealed that LPS treatment can also increase the expression levels of Cav-1 in liver sinusoidal endothelial cells (LSECs). Interestingly, endothelin 1 (ET-1) was involved in LPS-induced increase of Cav-1 expression, since antagonizing ET-1 effects and blocking its activation in LPS-pretreated LSECs decreased the LPS-induced overexpression of Cav-1. Moreover, treatment with ET-1 had the same effects on Cav-1 expression as LPS. The authors concluded that LPS-induced suppression of ET-1-mediated eNOS activation is ET-1-dependent and suggest a critical role of Cav-1 in eNOS induction inhibition under stress. Overall, the results of studies discussed in this paragraph suggest that LPS or bacterial pathogens regulate expression of Cav-1 in various cell types of the immune system and in endothelial cells. Such cell-type specific regulation of Cav-1 expression levels during sepsis could possibly be an important signaling mechanism contributing to sepsis outcome.

Genetic evidence supporting direct involvement of Cav-1 in regulating signaling and events associated with sepsis in vivo and in vitro

Generation of Cav-1 KO mice, allowed to test direct involvement of Cav-1 in various animal models of sepsis induced with LPS [11,20,44], infection with pathogenic bacterial strains [19,42,70,80,83], and cecal ligation and puncture (CLP) model of sepsis [16] (Summarized in Table 2). Some of the above mentioned studies also examined importance of Cav-1 in regulating phagocytic activity or pro-inflammatory responses in macrophages or neutrophils in vitro [19,42,70,80] (Included in Table 3).

Initially, using LPS-induced inflammation model of sepsis Garrean et al. [20] have observed decreased mortality in LPS-challenged Cav-1

Model of sepsis and/or infection with bacterial pathogen	Survival	Inflammatory response	Microvascular permeability in the lung	Bacterial burden (pathogen)	Genetic background	Reference
LPS (i.p.)	↓ ↓ NC	↑ Plasma levels of TNF-α and MIP-1α	↑ LPS-induced Kfc and edema ↑ LPS-induced (Evans Blue)		C57BL/6 x129 C57BL/6 x129? C57BL/6 x129? C57BL/6	[20] [44] [44] [11]
S. Typhimurium(i.v and p.o.)	↑	↓ Serum IL-6, TNF-α, IFN-γ		↓ Spleen and liver	C57BL/6	[42]
*P. aeruginosa*strain 27853 (intratracheal)	↓	↑ BAL IL-1β, MIP-2, TNF-α		↑ Lung	C57BL/6 x129	[84]
*P. aeruginosa*strains PAO1 or PAK (intranasal)	↑ ↑	↓BAL IL-1β, TNF-α, IL-6 ↓BAL IL-6, TNF-α, and IL-12 ↑ Phagocytic ability of macrophages in vivo		↓ Lung and spleen ↓ Lung, BAL, and serum	C57BL/6 x129 C57BL/6 x129	[19] [80]
E. coli (i.p.)		↑PlasmaIL-1β, TNF-α, IL-6			C57BL/6	[70]
CLP model of sepsis	↑	↓ Serum IL-6, TNF-α ↓Thymocyte apoptosis *in vivo*	NC Kfc and edema	↓ Spleen and liver	C57BL/6 x129	[16]

Table 2: Positive (↑) or negative (↓) regulation of major events associated with sepsis by Cav-1 in mice.

Cell type	Sepsis-associated response/signaling	Additional comments: (approach and/or mechanism)	Reference
Peritoneal macrophages from WT and Cav-1 KO mice	↑ Ability to phagocytize *E. coli* ↑ LPS-induced: expression of iNOS, production of NO, IL-1β, IL-6, TNF-α, activation of NF-κB	Reduced expression ofCD14, CD36, TLR4 and MyD88 in Cav-1 KO macrophages	[38] [70]
Neutrophils from WT and Cav-1 KO mice	↑ Ability to phagocytize *P. aeruginosa*		[19]
Mouse alveolar and peritoneal macrophages	↓LPS-induced TNF-α and IL-6 production	siRNA knockdown TLR4-dependent	[72] [73]
Peritoneal macrophages from WT and Cav-1 KO mice	↓LPS-induced CXCL1, CXCL10, CCL5, TNF-α, IL-6 production		[42]
RAW264.7 macrophage cell line	↓LPS-induced TNF-α and IL-6 production	Overexpression P38 MAPK-dependent	[73]
B-lymphocytes from WT and Cav-1 KO mice	↑ LPS-induced IgG_3 secretion		[43]
Lung endothelial cells from WT and Cav-1 KO mice	↑ LPS-induced: ICAM-1, TNF-α, iNOS	Mechanism: Loss of Cav-1 leads to hyperactivation of eNOS resulting in inactivating IRAK4 nitration and impaired TLR4-dependent signaling in Cav-1 KO mice	[44]
Liver sinusoidal endothelial cells from WT and Cav-1 KO mice	↑ LPS-induced eNOS inhibition	Cav-1 KO	[31]
Mouse alveolar type 1 (AT-1) cells	↑ LPS-induced: TNF-α and IL-6 production P38 MAPK and NF-κB activation	Overexpression	[40]

Table 3: Cell type-specific positive (↑) or negative (↓) regulation of sepsis-associated responses and signaling by Cav-1 *in vitro*.

KO as compared to WT mice. However, the authors used very high dose (125 mg/kg; i. p.) of LPS for mortality experiments, resulting in 87% death rate in WT mice within first 12 hrs. In contrast to mortality experiments, 12. 5-fold lower concentrations of LPS (10 mg/kg) were used for all other in vivo experiments in this study. Moreover, adhesion of WT polymorphonuclear neutrophils (PMN) to mouse lung endothelial cells after exposure to LPS was markedly reduced in endothelial cells isolated from Cav-1 KO mice as compared to WT counterparts. Furthermore, diminished ICAM-1 expression in Cav-1 KO mice coincided with the reduction in PMN binding to lung endothelial cells isolated from Cav-1 KO mice. In addition, lung PMN sequestration following LPS injection was significantly decreased in Cav-1 KO compared to WT lungs. They also evaluated pulmonary microvascular liquid permeability by measuring the capillary filtration coefficient (Kfc). Interestingly, LPS-induced increase in Kfc was only observed in WT lungs but not Cav-1 KO lungs. Moreover, the effect of LPS on Kfc in the isolated perfused mouse lung was significantly reduced in Cav-1 KO mice. In addition, significantly reduced wet-to-dry weight ratios were observed in Cav-1 KO relative to WT lungs were observed upon LPS administration. To address the mechanisms responsible for the reduced inflammation and injury in Cav-1 KO lungs, they also examined the role of NO since its plasma concentration was previously reported to be elevated in Cav-1 KO mice. Consistent with the negative role of Cav-1 in regulating eNOS activity, Cav-1 KO mouse lungs displayed significant increase in eNOS-derived NO production compared to WT. Moreover, Cav-1 KO lungs had suppressed NF-κB activity and decreased transcription of iNOS and ICAM-1. Co-treatment with the NO synthase inhibitor nitro-L-arginine prevented the suppression of NF-κB activity and restored lung PMN sequestration in Cav-1 KO mice challenged with LPS. Taken together, these data suggest that the ability of Cav-1 to inhibit eNOS-derived NO production is essential for NF-κB activation, followed by subsequent inflammatory response and injury in the lung upon LPS challenge.

In a follow up study from the same group, Mirza et al. [44] confirmed reduced mortality and diminished lung microvascular permeability in Cav-1 KO mice following treatment with LPS. In addition, they also observed reduced plasma levels of pro-inflammatory cytokines such as TNF-α and macrophage inflammatory protein 1-α (MIP-1-α) in Cav-1 KO mice compared to WT mice. Because eNOS is hyperactivated in Cav-1 KO mice, they examined the involvement of eNOS, using double KO mice with genetic deletions of Cav-1 and eNOS (Cav-1/eNOS KO). Interestingly, the plasma levels of pro-inflammatory cytokines and mortality rate induced by LPS in Cav-1/eNOS KO were comparable to WT mice, suggesting that the reduction in the plasma concentration of pro-inflammatory cytokines and reduced mortality observed in Cav-1 KO mice are eNOS-dependent. Moreover, activation of eNOS secondary to loss of Cav-1 resulted in decreased activation of nuclear NF-κB in response to LPS challenge, and thereby protected the animals from LPS-induced lung injury. They also determined that interleukin-1 receptor associated kinase (IRAK4), which is essential for TLR-induced NF-κB activation and innate immunity pathways, was nitrated in lung endothelial cells isolated from Cav-1 KO mice cells. Moreover, in vitro nitration of IRAK4 resulted in impairment of the kinase activity. In contrast to Cav-1 KO, Cav-1/eNOS KO endothelial cells displayed marked decrease of IRAK4 nitration, suggesting eNOS dependence [44]. Taken together, these data indicate that loss of Cav-1 reduces the innate immune response to LPS indirectly via eNOS hyperactivation and resultant IRAK4 nitration, leading to impairment of IRAK4 kinase activity, and alleviation of inflammatory lung injury.

In contrast to the two previously mentioned studies [20,44], de Almeida et al. [11], did not observe reduction in mortality of Cav-1 KO mice challenged with LPS (20 mg/kg; i. p.) relative to WT mice. However, consistent with the two previous studies, they determined reduced lung microvascular permeability in Cav-1 KO mice compared to WT mice. In addition, they detected decreased iNOS expression and NO production in Cav-1 KO intestinal tissue, but no alteration in intestinal permeability upon LPS challenge. The reduced expression of iNOS in Cav-1 KO was associated with a significant reduction of STAT-1 activation in intestinal tissue from Cav-1 KO mice. Specifically, immunoblotting data showed that intestinal tissue of Cav-1 KO mice had reduced phosphorylation of STAT-1 at tyrosine 701 relative to

WT mice. Taken together, these data suggest that Cav-1 expression promotes STAT-1 activation as well as iNOS expression and NO production in intestinal (most likely, epithelial) cells but does not affect the mortality in LPS-induced sepsis model. It is important to reconcile these dramatically different effects of Cav-1 loss on LPS-induced mortality observed by de Almeida et al. [11] with previously reported studies [20,44]. A much higher lethal dose of LPS (125 mg/kg; i. p.) resulting in 87% mortality in WT mice which was by Garrean et al. [20] could be a plausible explanation. However, studies of de Almeida et al. [11] and Mirza et al. [44] used identical dose of LPS (20 mg/kg; i. p.), and yet they observed dramatically different mortality rate. Specifically, in studies of Mirza et al. [44] only 10% WT mice survived first 60 hrs, while in studies of de Almeida et al. [11], 50% WT mice survived between 2 and 6 days of LPS challenge. The latter difference suggests that control WT mice used in studies by Mirza et al. [44] could be more sensitive to LPS than WT mice used by de Almeida et al. [11]. The most likely explanation is a different genetic background used in these studies. Specifically, studies by Garrean et al. [20] and likely by Mirza et al. [44] used B6129SF2/J (C57BL/6 x129) mice while studies by de Almeida et al. [11] used C57BL/6 mice. It is also possible that a protective role of Cav-1 against LPS-induced sepsis may depend on its severity. Additional studies using more standardized conditions and wider a range of LPS concentration will be required to more precisely determine the role of Cav-1 in LPS-induced signaling and sepsis outcome.

Several studies addressed the role of Cav-1 in host response to pathogenic bacteria. Initially, using Cav-1 KO approach, Medina et al. [42] examined the role of Cav-1 expression in *Salmonella* pathogenesis. They have observed that Cav-1 KO mice displayed a significant decrease in survival upon a challenged with *Salmonella enteric serovar Typhimurium (S. Typhimurium)*. Moreover, Cav-1 KO mice also had significantly higher bacterial burdens in the spleen and other tissues. Interestingly, infection of macrophages with *S. Typhimurium* in vitro did not result in significant differences in bacterial invasion between Cav-1 KO and WT macrophages, suggesting that different mechanism could be responsible in vivo. Moreover, Cav-1 KO mice displayed increased serum levels of pro-inflammatory cytokines such as IL-6, TNF-α, or IFN-γ. Also, serum levels of certain chemokines and nitric oxide were elevated in *S. Typhimurium*-infected Cav-1 KO compared to WT mice. Consistent with in vivo data, peritoneal macrophages isolated from Cav-1 KO mice displayed increased production of TNF-α and IL-6 as well as iNOS expression and NO production upon LPS challenge in vitro. Taken together, these data suggest that Cav-1 is a negative regulator of macrophage pro-inflammatory responses in vivo and in vitro. Interestingly, Cav-1 KO macrophages displayed reduction in STAT3 phosphorylation upon LPS challenge in vitro, suggesting that Cav-1 could mediate anti-inflammatory action via promoting activation of STAT3 signaling pathway. Despite increased pro-inflammatory responses, Cav-1 KO mice were unable to control the systemic infection of *S. Typhimurium*. The authors concluded that the increased production of toxic mediators from Cav-1 KO macrophages is likely to be responsible for the marked susceptibility of Cav-1 KO mice to *S. Typhimurium*.

Three groups have recently reported the results of studies involving infection of WT and Cav-1 KO mice with *Pseudomonas aeruginosa* [19,80,84]. Initially, Zaas et al. [84] compared intratracheal *Pseudomonas aeruginosa* infection in WT and Cav-1 KO mice to examine the role of Cav-1 in the pathogenesis of *Pseudomonas aeruginosa* pneumonia. Remarkably, WT mice rapidly succumbed to pneumonia and died within first 24 hrs. In contrast, Cav-1 KO mice were resistant to *Pseudomonas aeruginosa*. This data suggests that Cav-1facilitates infection with *Pseudomonas aeruginosa*. In contrast to studies mentioned above, Gadjeva et al. [19] observed different outcome of infection with *Pseudomonas aeruginosa*. Specifically, using the two models of acute (intranasal infection with *Pseudomonas aeruginosa* strains PAO1 or PAK) and chronic (oropharyngeal colonization and lung infection with *Pseudomonas aeruginosa*) infection, they observed that Cav-1 KO mice were more sensitive to *Pseudomonas aeruginosa* infection as compared to WT mice. This increased sensitivity in Cav-1 KO mice was manifested by increased mortality, higher bacterial burdens recovered from lungs and spleens, and elevated inflammatory responses (increased production of bronchoalveolar lavage (BAL) IL-1β, TNF-α, IL-6) as compared to WT mice. In the agreement with in vivo findings, Cav-1 KO neutrophils displayed reduced ability to phagocytize *Pseudomonas aeruginosa in vitro*. Moreover, *Pseudomonas aeruginosa* colonized Cav-1 KO mice much more efficiently than WT mice in a model of chronic infection. Taken together, these data suggest that Cav-1 plays a positive role in regulating innate host immunity to *Pseudomonas aeruginosa* infection in the setting of both acute pneumonia and chronic infections.

Consistent with studies of Gadjeva et al. [19], using a respiratory infection model Yuan et al. [80] confirmed importance of Cav-1 in host defense against *Pseudomonas aeruginosa*. Specifically, besides increased mortality, they also observed severe lung injury as well as systemic dissemination of the pathogen as compared with WT mice. Moreover, Cav-1 KO mice had increased levels of pro- inflammatory cytokines such as IL-6, TNF-α, and IL-12, reduced phagocytic ability of macrophages, and augmented superoxide release in the lung, liver, and kidney. Further studies determined that STAT3 and NF-κB were markedly activated in *Pseudomonas aeruginosa*-infected Cav-1 KO, relative to WT mice. Taken together, these data suggest that hyper-activation of STAT3/NF-κB pathway due to loss of Cav-1 could be accountable for exaggerated cytokine response, which likely contributes to increased mortality and disease progression. It is not entirely clear why the outcome of *Pseudomonas aeruginosa* infection was so dramatically different in the initially reported study [84]. The most likely explanation could be different and poorly characterized strain of *Pseudomonas aeruginosa* a single injection of which resulted in 100% death in WT mice within first 24 hrs. In contrast to Zaas et al. [84], studies of Gadjeva et al. [19] and Yuan et al. [80] used two well characterized strains (PAO1 and PAK), where all or over 50% WT mice survived first 24 hrs of infection [19,80]. As previously discussed for LPS-induced sepsis, it is possible that the pro- versus anti-survival role of Cav-1 in sepsis induced by infection with pathogenic bacteria depends on the severity of each individual sepsis model. Therefore, future studies examining relationship between pro-survival versus pro-death role of Cav-1 in sepsis models with various degree of severity could help to better understand the complex role of Cav-1 in sepsis.

Most recently, using intraperitoneal infection with *E. coli* sepsis model, Tsai et al. [70], have examined the role of Cav-1 in the response of macrophages and mice to *E. coli* or LPS exposure. Consistent with results of Medina et al. [42], Cav-1 KO peritoneal macrophages displayed reduced ability to phagocytize and to kill bacteria in vitro and

in vivo including peritoneal cavity, tissue, and plasma. These defects in anti-microbial function of macrophages were partly attributed to impaired expression of iNOS induced by *E. coli* or LPS. Moreover, Cav-1 KO macrophages had decreased expression of CD14, CD36, TLR4, as well as myeloid differentiation factor 88 (MyD88). NF-κB activation was also impaired in Cav-1 KO macrophages. Surprisingly, in contrast to other reports using pathogenic bacteria-induced sepsis [19,42,80], the production of pro-inflammatory cytokines in response to *E . coli* exposure was reduced in Cav-1 KO macrophages and mice [70]. At this point, it is unclear as to which factors could account for these differences. Taken together, these data suggest that Cav-1 regulates the expression levels of CD14, CD36, TLR4, MyD88 and is essential for the optimal innate immune response to bacterial infection. Interestingly, no survival data in mice infected with *E. coli* are reported in this study, therefore it is unknown if the final outcome of infection differs between Cav-1 KO and WT mice.

Unlike the above discussed models of sepsis induced by infection with specific strains of pathogenic bacteria, Feng et al. [16] applied a clinically relevant CLP model of sepsis to in Cav-1 KO mice. These studies demonstrated that Cav-1 KO mice were more sensitive to CLP-induced sepsis as compared to WT mice. The mortality rate was over two-fold higher in Cav-1 KO than in WT mice and this was associated with a more sustained increase in pro-inflammatory cytokines such as TNF-α, IL-6 accumulation in serum of Cav-1 KO mice. These data suggest that Cav-1 is a negative regulator of pro-inflammatory responses during CLP-induced sepsis. Moreover, Cav-1 KO mice displayed significantly elevated bacterial burdens in liver and spleen, suggesting that Cav-1 is required for an optimal innate immune response during sepsis. Furthermore, a 2-fold increase in thymocyte apoptosis was observed in Cav-1 KO compared with WT mice, suggesting that Cav-1 could act as anti-apoptotic factor and thereby promote thymocyte survival during sepsis. However, the exact mechanisms responsible for this anti-apoptotic function remain to be elucidated. Taken together, the results of these studies clearly indicate a protective role for Cav-1 in CLP-induced sepsis in mice via controlling inflammatory responses, reducing bacterial burdens, and thymocyte apoptosis. Overall, the final outcome of CLP-induced sepsis in Cav-1 KO mice is consistent with most studies using sepsis models involving infection with pathogenic bacterial strains [19,42,80], suggesting apparent similarities between these models. In contrast, the opposite outcome observed in the two studies involving LPS-induced sepsis models [20,44] suggests that relative to LPS, additional events associated with bacterial infection could be more important when sepsis is induced by live microbial pathogens. In particular, reduced phagocytic and killing ability of Cav-1 KO macrophages could account for increased mortality observed in Cav-1 KO mice infected with pathogenic bacteria or challenged with CLP-induced sepsis.

In addition to previously discussed data obtained using various in vivo sepsis models and concurrently obtained in vitro correlates, there are several other studies addressing the specific role of Cav-1 in regulating inflammatory signaling in various cell types in vitro including macrophages [38,72,73], B-lymphocytes [42], endothelial cells [31] and alveolar type 1 (AT-1) cells [40] (Summarized in Table 3).

Initially, using in vitro phagocytosis assays of both apoptotic thymocytes and *Escherichia coli* K-12 bioparticles, Li et al. [38] have observed reduced phagocytic ability of thioglycollate-elicited peritoneal macrophages from Cav-1 KO mice. This data is the first direct experimental evidence suggesting that Cav-1 could be essential for the optimal phagocytic activity of macrophages. Interestingly, they also observed an increase in the number of apoptotic cells in the thymus and spleen of Cav-1 KO mice, following whole-body gamma-irradiation. Therefore, they suggested that impaired macrophage phagocytosis in Cav-1 KO mice could have implications for altered innate immunity against pathogens, the regulation of inflammatory responses, and the development of autoimmune disease.

Wang et al. [73] showed that siRNA knockdown of Cav-1 expression in mouse alveolar and peritoneal macrophages increased LPS-induced pro-inflammatory cytokine TNF-α and IL-6 release but decreased anti-inflammatory cytokine IL-10 release. This data is consistent with other studies showing increased production of pro-inflammatory cytokines in Cav-1 KO macrophages treated with LPS in vitro [42] and with most studies involving Cav-1 KO mice infected with pathogenic bacteria [19,42,80] or subjected to CLP-induced sepsis [16]. In addition, Wang et al. [73] also showed that overexpression of Cav-1 in RAW264. 7 cells resulted in an opposite to siRNA effect on the aforementioned cytokines release. Further mechanistic experiments revealed that p38 MAPK phosphorylation was augmented by overexpressing Cav-1 in RAW264. 7 cells, and the anti-inflammatory effect of Cav-1 on LPS-induced cytokine production was significantly suppressed by p38 inhibitor, SB203580. In addition, Cav-1 overexpression failed to modulate LPS-induced cytokine production in peritoneal macrophages isolated from MKK3 KO mice. Moreover, LPS-induced activation of NF-κB and AP-1 were reduced in RAW264. 7 cells overexpressing Cav-1 and these reductions were reversed by treatment with p38 inhibitor. Altogether, these data suggest that Cav-1 inhibits LPS-induced pro-inflammatory cytokine production and stimulates anti-inflammatory cytokine production in mouse macrophages and that the MKK3/p38 MAPK pathway is necessary for anti-inflammatory effect of Cav-1 in macrophages. Subsequent mechanistic studies from the same laboratory [72] have demonstrated that Cav-1 directly interacts with TLR4 and thereby inhibits LPS-induced TNF-α and IL-6 production in mouse macrophages. Using mutation analysis approach, they showed that Cav-1 binding motif in TLR4 was critical for this interaction and for suppression of pro-inflammatory cytokines production. Taken together, these data suggest that Cav-1 suppresses LPS-induced pro-inflammatory signaling in mouse macrophages via a direct interaction with TLR4. In addition, they showed that Cav-1 is required for the anti-inflammatory effects of carbon monoxide (CO), a product of heme oxygenase-1 (HO-1) activity. Specifically, CO augmented the Cav-1/TLR4 interaction. LPS-treatment resulted in HO-1 translocation to caveolae via a p38 MAPK-dependent mechanism, and subsequent down-regulation of pro-inflammatory signaling. These results suggest that Cav-1 is also involved in anti-inflammatory function of HO-1/CO pathway.

In addition to the above discussed macrophages, B-lymphocytes have been recently shown to enhance early innate immune responses during bacterial sepsis [28]. Thus previously reported studies of Medina et al. [43] involving B-lymphocytes could also be relevant for sepsis. Specifically, they showed that Cav-1 KO mice displayed decreased serum levels of antibodies, and although these mice had a normal response to T cell-dependent antigens, they displayed a diminished response to both type I and type II T cell-independent antigens. Albeit Cav-1 KO B-lymphocytes did not show altered proliferation in

response to different stimuli, these cells had reduced IgG_3 secretion in vitro upon stimulation with LPS [43]. Altogether, these data suggest that Cav-1 plays a role in the development of T cell-independent immune responses.

Using LSECs isolated from WT and Cav-1 KO mice Kwok et al. [31] examined the role of Cav-1 in LPS-induced inhibition of ET-1-mediated activation in LSECs [31]. These studies revealed increased basal eNOS activity and loss of LPS-induced inhibition of ET-1-stimulated eNOS activity in Cav-1 KO LSECs. This loss of LPS inhibition resulted in an increase in ET-1-induced eNOS translocation to the plasma membrane and enhanced NO production in Cav-1 KO LSECs [31]. Taken together, these results suggest that Cav-1 upregulation is required for decreased eNOS activity in LSECs upon exposure to LPS. In addition, these data also imply that Cav-1-mediated inhibition of eNOS leading to LSEC dysfunction could worsen the outcome of LPS-induced sepsis via promoting liver damage.

Lv et al. [40] examined the effect of Cav-1 on the pro-inflammatory responses such as cytosolic phospholipase A2 (cPLA2), p38 MAPK and NF-κB in mouse lung AT-1 cells induced by LPS [40]. Specifically, they determined the levels of TNF-α, IL-6, cPLA2, p38 MAPK and NF-κB by ELISA, western blotting and EMSA in control and Cav-1 over-expressing AT-1 cells treated with LPS. Over-expression of Cav-1 increased the production of pro-inflammatory cytokines TNF-α and IL-6 and enhanced the expression of the cPLA2, p38 MAPK, and NF-κB [40]. These data suggest that Cav-1 enhances pro-inflammatory responses in AT-1 cells treated with LPS. Thus unlike most studies involving macrophages and mice infected with bacterial pathogens or subjected to CLP, where Cav-1 plays an anti-inflammatory role, Cav-1 appears to promote inflammatory response in AT-1 cells stimulated with LPS in vitro.

Role of Cav-2 in signaling and outcome of LPS-induced sepsis and host cell invasion with pathogenic bacteria

Although relative to Cav-1, the functional role of Cav-2 is less defined, recent studies have started to reveal a growing body of evidence suggesting that Cav-2 may regulate various processes in a tissue/cell-specific manner [6,11,32,34,58,62,64,66,67,75,77,78,82,84].

Remarkably, the most recent study reported dramatically increased mortality of Cav-2 KO mice upon intraperitoneal injection with LPS [11]. Specifically, 100% Cav-2 KO mice died within first 24 hrs after LPS injection (20 mg/kg; i. p.), while only 40% WT mice died within the same period of time. This augmented mortality of Cav-2 KO mice exposed to LPS was associated with increased intestinal injury and intestinal permeability, and correlated with enhanced expression of iNOS, production of NO and tyrosine 701 phosphorylation of STAT-1. Importantly, in contrast to Cav-2, Cav-1 KO mice did not display an altered intestinal permeability, and had decreased iNOS expression, NO production, and STAT-1 phosphorylation at tyrosine 701 compared to WT mice. Curiously, no significant differences in plasma levels of pro- and anti-inflammatory cytokines were observed between Cav-2 KO and WT mice. Taken together, these data suggest that Cav-2 plays a protective role in LPS-induced sepsis model. Moreover, the opposite changes in LPS-induced signaling in Cav-1 KO versus Cav-2 KO mice, suggest that Cav-2 may antagonize Cav-1 in regulating the signaling and outcome of sepsis induced by LPS (Summarized in Table 4). It is also possible that the delicate balance between the expression levels of Cav-1 and Cav-2 could regulate the outcome of sepsis induced by LPS. However, additional studies simultaneously examining possible changes in the expression levels of both Cav-1 and Cav-2 during LPS-induced or other models of sepsis in vivo and in vitro will be necessary to better understand this reciprocal role of both caveolin proteins in regulating signaling associated with sepsis.

Event/signaling	Cav-1 KO	Cav-2 KO
Survival	NC	↓
Lung permeability (Evans Blue)	↓	NC
Small intestinal (Colon and Ileum) permeability (Evans Blue)	NC	↑
Small intestinal (Ileum) tissue damage	NC?	↑
iNOS expression (Colon lysates)	↓	↑
NO production (Peritoneal lavage)	↓	↑
STAT-1 Y701 phosphorylation (Colon lysates)	↓	↑

Table 4: Antagonistic effect of Cav-1 and Cav-2 on the events and signaling associated with LPS-induced sepsis in mice. Relative increase (↑), decrease (↓), or no change (NC) in Cav-1 and Cav-2 KO compared to WT mice are shown based on the results of study by de Almeida et al. [11].

Besides the above discussed direct involvement in the outcome of LPS-induced sepsis in vivo, Cav-2 was shown to associate or even play a direct role in infection with several pathogenic bacteria. In one of such studies, Cav-2 facilitated infection of murine lung epithelial cell line MLE-12 with *Pseudomonas aeruginosa* and lipid raft targeting as well as tyrosine phosphorylation of Cav-2, followed by interaction with Csk and c-Src was essential for facilitating cell infection with *Pseudomonas aeruginosa* [82,84]. Specifically, siRNA-mediated knockdown of Cav-2 decreased [82], while overexpression of WT but not Y19/27F-Cav-2 increased the ability of *Pseudomonas aeruginosa* to invade MLE-12 cells. In addition, the siRNA knockdown of Cav-1 also resulted in reduction of *Pseudomonas aeruginosa* invasion. However, unlike Cav-2 siRNA which did not change Cav-1 expression level, Cav-1 siRNA reduced Cav-2 expression levels, indicating that Cav-2, not Cav-1 was directly responsible [82]. Taken together, these data suggest that Cav-2 regulates invasion of murine lung epithelial cells with *Pseudomonas aeruginosa*.

In another study, using siRNA approach, it was demonstrated that Cav-2, actin, E3 ubiquitin ligase, c-Cbl, and clathrin but not Cav-1 is involved in invasion of HeLa cells with another pathogen, *Rickettsia conorii* [6]. Because Cav-1 is required for lipid raft/caveolar targeting of Cav-2, the negative effect of Cav-1 siRNA could suggest that non-lipid raft/caveolar Cav-2 is responsible for facilitating invasion with this pathogen.

The role of non-caveolar Cav-2 was also suggested in chlamydial infection of various epithelial cell lines including HeLa and FRT cells, where Cav-2 associated with the chlamydial inclusion independently of Cav-1 [75. However, the functional significance of Cav-2 either in the uninfected cell or in the chlamydial developmental cycle, were not addressed in this study. Overall, there is a growing body of evidence suggesting that Cav-2 also plays a role in regulating some aspects of signaling associated with LPS-induced sepsis and invasion with pathogenic bacteria such as *Pseudomonas aeruginosa*.

Conclusion and Future Directions

Thanks to more specific experimental tools targeting Cav-1 such as KO and siRNA, our understanding of how Cav-1 regulate sepsis-associated signaling in vivo and in vitro has substantially increased

within recent years. In addition to Cav-1, most recent data suggest that Cav-2 emerges as another important player in LPS-induced sepsis and that this protein could potentially antagonize the role of Cav-1 in LPS-induced sepsis outcome in vivo (for more details see Table 3). However, there are many unanswered questions in this rapidly developing field. For example, what is the relative contribution of Cav-1 localized to caveolae versus other subcellular domains? How does Cav-1 regulate different events associated with sepsis in various tissues and cell types involved and/or affected by sepsis? What does determine the anti-inflammatory role of Cav-1 in certain cell types such as macrophages versus pro-inflammatory role in AT-1 cells? What are the specific factors responsible for the different outcome of sepsis induced by LPS, pathogenic bacteria or CLP in Cav-1 KO mice? Besides Cav-1, are there other direct targets of Cav-2 responsible for pro-survival role of Cav-2 in LPS-induced sepsis? Does Cav-2 regulate sepsis induced by pathogenic bacteria or CLP? Does Cav-2 play a role in controlling pro- and anti-inflammatory responses in macrophages and other relevant cell types? Do recently discovered adapter proteins of caveolae, cavins play any role in sepsis-associated signaling and outcome?

These and many other questions will need to be addressed first in order to achieve the level of understanding of the complex role of Cav-1 and -2 in regulating signaling in sepsis which could help to design more accurate therapeutic approaches alleviating sepsis outcome.

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