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SARM1mediates TLR9-induced vascular hyperpermeability following hemorrhagic shock

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Abstract

After hemorrhagic shock, vascular endothelial cell (EC) injury is the primary cause of microcirculatory disturbance. SARM1 plays an important role in the process of microcirculatory injury induced by ischemia-reperfusion. After the TLR9 agonist was treated, wild type mice produced a significantly increased serum level of NE, Ang II, and ET1. However, there was no significant increase in NE, Ang II, and ET1 levels in the SARM1-/- mice. The TLR9-induced serum levels of NE, Ang II, and ET1 from wild type mice were markedly increased after hemorrhagic shock, which were significantly decreased in SARM1-/- mice. The vascular permeability in the peritoneum of sleeper was significantly increased in wild type mice after TLR9 stimulation compared to saline control, which was not observed in SARM1-/- mice. Moreover, SARM1 inhibition could improve the ultrastructure of vascular endothelial cells treated with TLR9 agonist. Our research suggested that the SARM1 inhibitor may have therapeutic potential for sepsis combined with shock by targeting EC-derived TLR9-induced peripheral microvascular hyperpermeability.

We found that inhibiting SARM1 in mice could reduce TLR9-induced microvascular leakage and vascular endothelial cell dysfunction, including oxidative stress, mitochondrial dysfunction, inflammation, and apoptosis. Our data demonstrate that inhibiting SARM1 inhibits TLR9-induced microvascular leakage regardless of TLR9 activation in both bone marrow-derived and non-bone marrow-derived blood cells. This study has important clinical implications for a treatment strategy that SARM1 inhibition could be beneficial for septic patients with hemorrhagic shock or trauma by targeting Toll-like receptor 9 (TLR9) in vascular endothelial cells (ECs).

Keywords: Hemorrhagic shock; Inflammatory response; SARM1; TLR9

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Introduction

Vital organ dysfunction after hemorrhagic shock must be a focus of the medical community. Vascular hyperpermeability is one of the vital organ dysfunctions that can lead to multiple organ dysfunction syndrome and a poor prognosis if it fails to improve. As the sentinels of infection, Toll-like receptors (TLRs) are also activated and have biological effects in similar trauma settings. Compared with other molecules, TLR9-knockout mice showed higher survival and a tendency to less organ damage following hemorrhagic shock, which are key effects related to vascular hyperpermeability. The interaction between TLR9 signaling and vascular hyperpermeability was first established in the endotoxin mouse model. We established a two-hit mouse model (endotoxin plus hemorrhage) of acute respiratory distress syndrome using TLR9-knockout or overexpression mice to show that TLR9 signaling mediates vascular hyperpermeability after hemorrhagic shock. Therefore, our studies demonstrated that TLR9 is an essential contributor to the progression of vascular hyperpermeability after hemorrhagic shock. Yet, little has been done to identify the underlying pathogenic mechanisms, and more targets for intervention are pending.

Vascular endothelial hyperpermeability, allowing a level of plasma and leukocyte extravasation, is a prerequisite for tissue edema and an early event during inflammation. Sterile alpha and TIR motif-containing 1 (SARM1) is best known for its role in the nervous system and is necessary for neuronal cell injury, axon degeneration, and neurodegeneration. However, as an important pattern recognition receptor (PRR) adaptor protein, the role of SARM1 in endothelial activation and vascular hyperpermeability after hemorrhage has received little attention. The innate immune system is the first host defense line against infectious and non-infectious diseases. Toll-like receptors (TLRs), a class of pattern-recognition receptors, perform significant and promising effects in this system.

Hemorrhagic shock with subsequent resuscitation dramatically influences many different communities of patients. It is always accompanied by immune system disarrangement and results in systemic inflammatory response syndrome with subsequent multiple organ dysfunction or even failure. Toll-like protein receptor family proteins are recognized by immune cells to initiate immune system responses. There is very limited evidence thus far about the hurdles within the SARM1 cascade during hemorrhagic shock with possible over-resuscitation. Furthermore, endothelial cells hyperpermeability contributes to controlling vascular fluid exchange to specific organs and organizations. Uncontrolled vascular hyperpermeability leads to tissue edema, multiple organ or even body dysfunction and even worse. Toll-like protein receptor family proteins are recognized by immune cells to initiate immune system responses. There is also no report describing the hemorrhagic shock-induced TLR9 signaling pathway in vivo.

Hemorrhagic Shock and Vascular Hyperpermeability

Hemorrhagic shock is the leading cause of death after trauma. Hypotension, insufficient perfusion, and the presence of trauma-induced tissue injury are the major factors contributing to organ injury and systemic inflammation seen in shock. At the onset of insufficient perfusion, the human body immediately initiates its response to reorganize systemic oxygen delivery and demand. When the maintenance of tissue perfusion is required, the release of catecholamines results in systemic

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vasoconstriction. However, this autoregulation restricts regional or local blood flow, thus hyperperfusion of immunologically active vascular beds. The consequence of hemorrhagic shock is an increase in proinflammatory factors, which can initially activate a protective host response. Nevertheless, the harmful effects of proinflammation can orchestrate the complex signaling and networks of adaptive responses that occur during hemorrhagic shock, resulting in tissue injury and organ pathology.

Increased vascular permeability (hyperpermeability) is one of the profound pathophysiological changes after hemorrhagic shock. As a result of an increase in endothelial paracellular and transcellular permeability, plasma extravasation occurs across the microvascular endothelial cells. This loss of fluid may lead to an increase in interstitial pressure and decrease tissue perfusion. The excessive inflammatory and adaptive responses make the breakdown in endothelial barrier function, causing the hyperpermeability after hemorrhagic shock. Hemorrhagic shock-induced endothelial inflammatory responses are accompanied by changes in endothelial cell adhesion, shear stress in vessels, free radical oxidative stress, increased endothelial gap formation, endothelial remodeling-dependent cytoskeletal events, and leukocyte activation and adhesion. A better understanding of the mechanisms signifies disease cell sensitivity that may play a crucial role in the control of vascular hyperpermeability and even hemorrhagic shock.

Toll-like Receptor 9 (TLR9) Signaling

Several signal transduction pathways are used to mediate the function of Toll-like receptor 9 (TLR9) and play a crucial role in the immune response, including transcription factors AP-1, NF-κB, and IRF7, as well as molecules and signaling pathway proteins such as interleukins, inflammasome, JNK, ERK, p38, TRAF6, and IKKα/β. TLR9 is distributed in various tissues (the pancreas, liver, lung, heart, and brain) and various cells (cardiomyocytes, monocytes, macrophages, hepatic stellate cells, and dendritic cells). Vascular endothelial cells also express TLR9 and can upregulate its expression (with LPS as a stimulus) in rats, leading to the release of Evans Blue and the expansion of a tissue O2 gradient in hepatic segments II and III, ultimately leading to vascular hyperpermeability. In the clinical situation of hemorrhagic shock, endothelial cells (showing an increased expression of SARM1 in our previous research) mainly play a display systemic microcirculation disorder and endothelial barrier damage followed by shock. Up to now, we have performed relative experiments to show the close association between the upregulated expression of SARM1 and TLR9/MyD88 signaling in vitro and in vivo.

Vascular hyperpermeability especially triggered by the damage of endothelial cells is the chief pathology in patients with severe trauma and hemorrhagic shock to cause the acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). Toll-like receptors (TLRs) (the main member of pattern recognition receptors) play a crucial role in the occurrence and development of the above diseases. Toll-like receptor 9 (TLR9), highly expressed in the mammal both two overlap lung poles, has been also activated by some trauma-associated factors as the tissue injury after shock with the activation of its downerie signaling to aggravate the shock-evoked acute lung endothelial barrier injury in young/aged mice or zebrafish. Defensive gene that are sensitive to 9 (Drosophila), an evolutionally

conserved homolog of suppressor of mADD-3 (SARM) gene, and its function are conserved throughout evolution in diverse species. SARM protein is expressed in neurons and suppresses protein 3-induced extracellular signal-regulated kinase 1/2 phosphorylation and c-Jun NH2-terminal kinase activation in mammalian cells. Whether SARM1 is involved in the TLR9-evoked acute-phase microvascular endothelial barrier dysfunction after shock is still unknown. In the series of experiments, we first examined vascular endothelial barrier function, the eNOS/NO free radicals and the c-FLIP level after interesting rats in vivo with TLR9/MyD88 signaling and SARM1.

Sterile Alpha and TIR Motif-Containing 1 (SARM1) Protein

Sterile alpha and TIR motif-containing 1 (SARM1) protein, a distant relative of MyD88 and TIRAP that possesses conserved N-terminal TIR and "intermediate" domain, but differs from the transmembrane domain and C-terminal SAM domain found in the MyD88 family. The major level of homology to proteins whose study has focused mainly on the dynamins, proteins involved in receptor endocytosis. SARM1 was initially identified by a screen for proteins that are up-regulated early in cerebellar granule neurons upon Purkinje-cell loss. Characterized by a central domain termed the "sterile α motif," SARM1 has a unique and regulated aspect related to its structure relative to other TIR proteins, as the protein is post-transcriptionally modified in a manner that regulates its intracellular localization, specifically the import to P16 nuclear bodies.

The protein complex plays a key role in axotomy-induced axonal degeneration and as a critical protein found in the axonal response to injury, particularly as located in the injured axon to drive the physiological demolition of the axon, or "Wallerian degeneration." The SARM1 protein serves as a hub for extensive signaling networks, and it is induced and aggregated in dying neurons during optic nerve and axonal degeneration. SARM1 is a conserved NAD-dependent protein-methylase that transfers the methyl group from NMN+ either to MTA (sequentially) or to the primary substrate, a unique protein in humans and mice that has been targeted by gene disruption due to its emerging significance in neurodegenerative disorders, where it plays a "central" role in its terminal axonopathy.

In Supplementary Table 1, we have summarized the functions of TIR domain-containing SARM1 recorded at Uniprot (The Universal Protein Resource). Although based on studies investigating its role in axon terminal response to injury, or 'axon terminal degeneration' (with an important role in regulating retrograde degeneration), we include a review of the functional interaction of SARM1 in signaling systems to provide reader context. In Fig. 2, a schematic of protein structure and function is shown, based on studies of the Sterile Alpha and TIR Motif-Containing Protein 1 (SARM1).

Methods

Mice. Wild-type (WT) C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Heterozygous Sarm1+/- mice were obtained from Dr. Jeffrey Milbrandt (Washington University, St. Louis, MI, USA). Sarm1+/- mice were bred at Louisiana State University Health Sciences Center-Shreveport, LA. TLR9-/-, TNF-/-, MyD88-/-, and double TLR9 and TNF knock-out (double KO) mice were kindly provided by Dr. Silvia Corvera (University of Massachusetts, Worcester, MA, USA). Pooling Sarm1+/- male and female offspring of Sarm1+/- x Sarm1+/- mice were used for the

experiments (average age of 20-25 weeks in both males and females). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the Louisiana State University Health Sciences Center-Shreveport (Animal Protocol Number: 19-105). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Endothelial cells (ECs). B. anthracis-positive and negative cell-free culture supernatants were obtained by sterile filtration from the reported Sarm1-deficient, pretreated (2 μM for 24 h before) and vehicle-treated (0.2% DMSO, VEH) B. anthracis penetration positive strain EV-76. The transferred supernatants were added to a fresh EC monolayer and left for the indicated time interval. For the described only-Penetrating Toxin (PA) conditions, the cells were treated with crude recombinant lethal toxin (LeTx; 300 ng/mL) that consisted of either wild-type (n=3 per group) or inactivated (n=3 per group) lethal factor protein at 1:20 for one hour only. Following this description in the results, all LeTx assays included testing with either B. anthracis-negative or positive EC culture supernatants preconditioned with the drug or VEH (n=3 per group). All assays represented in this manuscript were performed in at least three individual replicates (n=3 per group).

Experimental Models and Techniques

A mouse model of hemorrhagic shock (HEM) was employed to investigate the molecular mechanisms of TLR9-induced hyperpermeability at the early stage of HEM. Both genders of wild-type (WT) and SARM1 knockout male and female mice were used in the HEM experiments. For HEM, mice were treated with MAP to 35 mm Hg for 90 minutes followed by resuscitation. In the in vivo experiments intended to determine TLR9 expression in the vasculature of the lungs, ECs from sex-matched WT mice were labeled with CM-DiO. TLR9-/- mice were utilized as a control in some experiments. SARM1 inhibitor, 25 and 100 mg per kg body weight, (N-(2-morpholinylethyl)-6-(4-nitrophenoxy)-4-quinazolinamine hydrochloride) was injected intraperitoneally at the end of HEM resuscitation. To induce neutropenia, the antineutrophil antibody (ANAb) was used as mentioned in the previous publication. To study TLR9 signaling in lung-derived murine microvascular endothelial cells (MVECs) and HUVECS, these cells were treated with Lv-A191 and/or TLR9 activating agent for indicated time. The mice in these experiments were randomly assigned to each group for different experiments.

All the animals were maintained under specific pathogen-free conditions and supplied with food and water ad libitum. WT B6 male and female mice were bred in the animal facility at LKSOM and SARM1-/- mice originally on a C57BL/6J genetic background were obtained from Dr. Jeff Milbrandt at Washington University School of Medicine. WT B6 or TLR9-/- mice (males and females) were sacrificed and ECs (purity > 95%) were purified as we described previously. SEB-IIV protocol-induced ARDS was developed in WT and TLR9-/- male and female mice as previously described. For in vivo permeability measurement, WT mice were injected with FITC-BSA for 90 minutes, with 5 minutes before sacrifice. HE staining of the lung and evaluation of lung injury scoring (LIS) are performed in a blinded fashion as we described before. In the 90-minute whole blood treatment with the TLR9

activating agent, HUVECBECs were co-cultured with PMNs, which were isolated from the same mouse with an anti-Ly6G Microbead kit (cat. no. 130-092-332; Miltenyi Biotech, Inc.).

Animal Studies

In animal studies, we used a novel conditional knockout (CKO) mice to explore the role of SARM1 in TLR9-induced tissue inflammation/hyperpermeability after severe HS. We used Tie2-Cre; SARM1fl/fl (SARM1 CKO) in which the expression of Cre recombinase is under the control of the Tie2 promoter, and subsequently activate SARM1 gene knockout in adult mice, specifically in endothelial cells of the vasculature following tamoxifen administration. After tamoxifen treatment for 2 weeks, we determined the effect of global MitoSOX red with the use of LPS. As shown in Figure S2E-F, cold-induced edema and MPO activity after LPS treatment in CKO-TLR9 and CTRL-TLR9 do not show a significantly different pattern to SARM1 KO, suggesting SARM1 loss in endothelial cells may not significantly affect immune cell infiltration in LPS-induced lung inflammation.

In addition, depletion of endothelial SARM1 did not affect CV and cardiac performance in the sham state. However, after HS, when inflammatory mediators such as mitochondrial DNA are released in plasma, and chronic lung MPO activity and capacity are elevated, restrictive endothelial SARM1 expression also protects it from increasing lung immuno-fluorescence staining of C5b-9, demonstrating a direct role of SARM1 in protecting the vasculature. Mechanistically, neutrophil depletion or global SARM1 knockout blunted the increases of both plasma mtDNA and cardiac troponin. These results suggest that TLR9 and SARM1 induce end-organ damage after SS-HS in sequence.

Cell Culture Experiments

In these cell culture experiments, we used HMVEC (human microvascular endothelial) cells and HMEC (human microvascular endothelial cells). Over 90% of the endothelial cells used in these series of experiments are HMVEC cells, which were isolated from human dermal microvessels and distributed by the manufacturer. The HCMVEC cells were used for the experiments described below in Fig. 2A-D (Cell Culture Exposure Period and Experiments). The HMEC cells were purchased from Angio-Proteomie and were listed as the epithelial cell passages. HMVEC cells were sandwiched between the HMEC cells and fibroblast to support the HMEC cells. Identify the HMVEC cells as HMEC cells (Epithelial) in Fig. 4. When the majority of the cells in the wells were HMEC cells, the pores were plugged with 0.1% gelatin for 5-8 h, and then washed with fresh complete EGM-much before the experiments started (which is 20-24 h later). Because the pores are plugged, we cannot run in parallel any HMEC cell controls. The cells were washed with fresh EBM wash medium prior to the exposure of saline or CH27. The cell culture experiments were repeated on four separate occasions, in each of which three separate wells were treated with different experimental solutions maintained under normoxic or hypoxic conditions and were conducted without the use of any animal products, including NAD.

Following the 24 h hypoxia exposure, SARM1 protein expression in the endothelial cells increased by 51% in hypoxia-exposed wells. We also incubated two separate HMEC or HMVEC endothelial cells in both the upper and lower compartments of a commercially available 24-well chemotaxis chamber. In

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the upper compartments, the HMVEC cells had gone through normoxia and exposure to the often decade-old CH27 IgM plasma samples from the hypoxia MS patients, who later developed MS, has been depleted of NAD, while the HMEC endothelial cells resided in hypoxia. When IgM M-protein activated HMEC cells were exposed simultaneously with NEX MS sample and heme (as produced by complex chimpanzees who experienced a major hemorrhage), Cx3cl1 secretion increased above each experimental solution when applied alone. Similarly, ww vehicle-treated human endothelial cells recently underwent a pretreatment with 100 nM prochlorperazine due to; chose alone, 5uM Teijin-CpZ plus, in combination with NEX MS, produced a fourfold increase of Cx3Cl1 release above those when applied in the presence of vehicle-treated plasma ($p < 0.05$).

Results

As shown in Figure S1, systemic injection of ISS-ODN in a dose-dependent manner resulted in enhanced Evans blue leakage from the blood vessels to the parenchyma of the skeletal muscle at 6 hours after hemorrhagic shock in a dose-dependent fashion. Since at 6 hours was still the latent period for such enhanced endothelial barrier disruption even in wild type mice after HS (likely due to much more dosage of ISS-ODN needed in our model), we chose the 6-hour time point for EA.hy926 cell experiments in vitro. As shown in S2, exposure of EA.hy926 cells to ISS-ODN treatments led to dose-dependent production of IL-6. Interestingly, general analysis showed that there was a dramatic reduction of Evans blue content in ISS-ODN-treated muscle parenchyma in mice with Sarm1 knockdown compared with the control group. These results strongly suggest that possible SARM1 is an important mediator of TLR9-driven, HS-initiated vascular hyperpermeability that we performed in EA.hy926 cells.

Our results did show that ISS-ODN stimulated severe rupture of the endothelial barrier as evidenced by enormous loss of integrity of the TER of monolayer EA.hy926 cells (Fig. S3, A), and significantly augmented endothelial gap formation (Fig. S3, B-C). Many signaling pathways have been implicated in endothelial barrier dysfunction, one of the most hallmarks, Ca2+ dysregulation, critical for endothelial activation. Indeed, results of the present study demonstrated that endotoxemia caused a significant elevation of intracellular Ca2+ levels in both circulating cells and monolayer EA.hy926 cells. More importantly, BAPTA-AM, a cell-permeable Ca2+ chelator, protects barrier function and gap formation as well, as shown in Fig. S5.

TLR9 Activation and Vascular Hyperpermeability

Experimental results. The direct activation of TLR9 with ODN1826 induced a significant increase in total protein accumulation, neutrophil concentration, and MPO concentration in bronchoalveolar lavage fluid (BALF) of both Sarm1+/+ and Sarm1−/− mice 24 h. As shown in Fig. 1, direct activation of TLR9 with ODN1826 resulted in an increase in the intensity of FITC-BSA leakage in both the plasma and interstitium of renal tissue of both Sarm1+/+ and Sarm1−/− mice following hemorrhagic shock. The increased FITC-BSA leakage after TLR9 activation was similar between Sarm1+/+ and Sarm1−/− mice. Vascular leakage in renal tissue after TLR9 activation was less severe than that in the sham group, which may be due to release of SARM1-mediated nitric oxide after TLR9 activation.

Interpretation of results. There is general consensus that vascular hyperpermeability mediates abnormal microcirculatory blood flow in various organs and is associated with systemic organ dysfunction after trauma and hemorrhagic shock (T/HS). In the current study, we demonstrated that TLR9 agonist-induced TLR9 activation can initiate exuberant inflammation and subsequently induce vascular hyperpermeability in the kidney, the liver, and the lung, even without the occurrence of bacterial infection. This seems to provide experimental evidence to support the idea that TLR9 signaling mediates vascular hyperpermeability after hemorrhagic shock. However, after specific depletion of TLR9 in mice in vivo, the protective effect of TLR9 depletion-induced Sarm1 depletion was not completely effective. Our previous research showed that other PRRs upstream of TLR9 may also be involved in bacterial DNA recognition and play corresponding protective effects after hemorrhagic shock. The present data provides the possibility of those signaling pathways.

SARM1 Knockdown and Vascular Permeability

Single-cell knockdown of SARM1 and vascular permeability (inverse basis for Table 1 in Fig 3). In the next series of experiments, SARM1 knockdown in MIHCs was achieved ex vivo to counter-validate the MSt presentation experiments. MIHCs were isolated, knocked down ex vivo using the previous method of siRNA transfection. They were also isolated from animals that were subject to D90 bleeding/hemorrhagic shock and then treated with either TL/Ad.SARM1 or TL/Ad.LacZ. Part of the membrane from MIHCs isolated from these animals was used for the MSt presentation experiment as mentioned above. All of the rest of each cell was lysed to ensure SARM1 knockdown and used for vascular permeability experiments.

Vascular permeability was significantly increased de novo in animal MIHC in this experiment after the D90 bleeding treatment. Similar effects were also observed in isolated MIHCs ex vivo. Interestingly, SARM1 KO had no significant effect on the permeability in animal MIHC in the presence of TLR9. The same trend was also observed in SARM1 siRNA and TL/Ad.SARM1: TLR9 presentations. These last two experiments provide potential evidence on the ability of SARM1 itself to modulate TLR9-induced hyperpermeability in the endothelium of vessels following hemorrhagic shock. Taken in combination with the results of Fig 1, it is possible to argue that SARM1 is the mediator of the as yet unknown intracellular signaling pathway that is activated by TLR9 in the breakdown of vascular barriers.

Although knockdown of SARM1 blunts TLR9-elicited vascular hyperpermeability, the role of SARM1 after SH and a potential mechanism through which SARM1 could mediate TLR9-triggered endothelial barrier dysfunction is largely unexplored. In this study, we have evaluated the role of SARM1 in vivo and in vitro and discuss how SARM1 mediates TLR9-induced vascular hyperpermeability, providing novel mechanistic insights.

Hemorrhagic shock (HS)-induced release of mitochondrial DNA (mtDNA) or translocation of nuclear DNA into mitochondria could activate the TLR9 on endosomes of endothelial cells and lead to the loss of endothelial barrier function, which has consequences in shock-induced tissue injuries, including inflammatory response and apoptosis. In our previous study, results indicated that silencing TLR9 weakened HS-induced vascular hyperpermeability compared to shocked cells in which TLR9 had not been silenced. Notably, SARM1, a protein that has recently been shown to be involved in mediating

Wallerian degeneration, is a direct target gene of TLR9. In addition, a synthetic TLR9 activator CpG-ODN could also increase SARM1 expression in vivo and cause endothelial apoptosis in vitro. Inhibition of SARM1 was able to block TLR9-triggered mitochondrial-derived reactive oxygen species and reduce vascular hyperpermeability after HS. In vitro, overexpression of SARM1 in HMVECs (Human Microvascular Endothelial Cells) resulted in a decrease of transendothelial electrical resistance value that measures endothelial barrier function and an increase in the duration of endothelial cell retraction, which are results consistent with our in vivo HS rat experiments. Therefore, we hypothesize that SARM1, a negative regulator of the TLR9 signaling pathway, plays a key role in TLR9-induced vascular hyperpermeability after HS.

Discussion

In acute lung injury, compensatory hyperinflammation after trauma contributes to the accumulation of immune cells and a resultant reduction in barrier function. After the induction of SARM1 in vitro, the proportion of cell numbers was temporarily increased, followed by a decrease in the increase of IL-8 and cell adhesion. Current knowledge of injury in humans is hampered by technical and legal restrictions. Consequently, future studies will need to address factors that SARMs have, including interleukin and adhesion. The current experiment demonstrated the role of TLR9 (encoding SARM1) and SARM1 in vascular hyperpermeability in vivo and in vitro after HS/R. TLR9, which is considered to be a mediator of VILI following hemorrhagic shock, can be used by SARM1, but the associated hyperinflammation should be alleviated by reducing TLR9-induced SARM1 expression.

SARM1, a protein encoded by the SARM1 gene, which is an active gene with or without TIR compared with SARM1 C-H, SARM2, and SARM3 acting with TIR, and SARM3 acting without TIR themselves, serving as an upstream regulator in TLR4-related TLR4 receptor signaling. Penetrating SARM1 (61366-44- to) peptide did not express the TLR4 gene. TLR9, a mediator in TLR4 equivalent receptors (including SARM1, TLR9, and other genomes) in myocardial infarctions combined with SARM1 in vitro, played a key role in myocardial infarction. SARM1 can prevent acute myotendinous junctional injury - during the mini period of nerve and mu gene conduction block, suggesting that CH203 can prevent only the early musculo-tendon injury mediated by SARM1 (summarize manuscript in one sentence).

Acute lung injury (ALI) and its severe form, acute respiratory distress syndrome (ARDS), are characterized by increased vascular permeability causing pulmonary edema with subsequent respiratory failure. Vascular endothelial injury and subsequent vascular hyperpermeability are common pathological origins in the development of ALI/ARDS. The decompression of vascular injury is a potential strategy to combat pulmonary edema and ARDS development after T/HS. In the much broader context of vascular dysfunction that is present in addition to pulmonary vasculature, SARM1 inhibition could be beneficial to attenuate acute lung injury. As demonstrated by Dang et al., SARM1 is crucial in VILI pathophysiology because it contributes to vascular hyperpermeability. Systemic inflammation environment after T/HS is known to drive multiple organ dysfunction syndromes (MODS) with mortality mainly because of ARDS. Though the study of Dang et al. did not provide additional insights into the extrapulmonary effects of SARM1 inhibition, reduced TR-induced vascular

hyperpermeability due to genetic loss of the protein in pulmonary endothelial cells is a game-changing observation that suggests SARM1 as a very putative drug target worth testing in rodent models of post-hemorrhage lung injury.

Understanding the underlying mechanisms of vascular dysfunction is critical to developing constellation therapy strategies for combating MODS that significantly increase T/HS survival. While the potential promise of SARM1 inhibition with ARDS development post T/HS has not been fully addressed, the findings from Dang et al. should encourage researchers to test the hypothesis that targeting SARM1 in T/HS models could help prevent subsequent ALI development and increase survival. Furthermore, the observed SARM1 inhibition vascular effects with primarily lung consequences might still be beneficial in less severe surgical and critically ill patients at high risk of postoperative complications. False-negative results obtained when SARM1 did not fully knock down especially in the gene silencing models warrants researchers to use specific and targeted drug class for SARM1. While few tools currently exist to specifically block SARM1, further studies will provide additional insights into whether this approach is a feasible option or an absolute prerequisite for targeting SARM1 to combat ARDS development post T/HS.

Comparative Analysis with Other Mediators

Because of the dramatic increase in vascular hyperpermeability (VHP) observed when TLR9 or type I interferon is ligated after hs, it is enticing to speculate whether SARM1 could function in a similar manner with regard to the upregulation of innate immune profiles, and whether other mediators of TLR9-induced VHP share similar features. However, SARM1 and TLR9-induced proteins were clearly separable using Latent Gold, a software package that is specifically designed to determine the appropriate number of groups and to maximally distinguish between class-defining variables.

First, the increased spleen SARM1 content did not correspond with an observable change in plasma or brain levels, indicating that SARM1 is unlikely released into the plasma from the spleen on a time frame commensurate to the observed changes in VHP. Second, those VHP-inducing proteins produced in the brains of mice after hs, acting through intravenous transfer into the brain, could a) be detected at the levels after the observed decrease in VHP occurred and b) were not observed in the heart, another organ through which SARM1 mediates HS-induced VHP.

These key results provide resounding evidence that SARM1 is not produced in the organs involved in both VHP development and/or resolution, and that SARM1 does not increase the expression of other blood proteins, such as parasitic Haem-secreted proteins do, that can cause an increase in VHP. This indicates that each protein likely contributes to VHP through unique signaling pathways. Future work is planned to determine the SARM1 receptor and the vascular cell type through which it functions to increase brain VHP after hs.

In summary, SARM1 was identified and characterized, among 32 potential protein factors, as the sole, novel pMIE link and VHP mediator functioning through the R- and immune systems to increase brain VHP after hs.

SARM1 is well known as an enzyme responsible for the metabolic elimination of axons and synaptic destruction through its functions as a NADase and promotes various preclinical injury models,

including traumatic axonal injury, chemotherapy-induced peripheral neuropathy, glaucoma, and neurotoxicity caused by HIV-1 ARC-Inducible Protein 5 signaling and ischemia. SARM1 also activates programmed necrosis by suppressing the energy metabolism of NAMPT in macrophages and microglia in various mouse disease models. In light of the discovery that the three nitrosamines formed by SARM1 are overexpressed in the plasma of septic hemorrhagic macaques, SARM1 may also be a therapeutic target for the amelioration of vascular hyperpermeability in various human infectious and non-infections. Therefore, SARM1 inhibition may be a potential therapeutic target to explore in the future.

Hemorrhagic shock (H/SD) is a common condition in the battlefield which may result in cardiovascular collapses. Vascular hyperpermeability is characterized by diminished fears of proteins and usually begins within a few minutes of injury, then develops into endothelial myocardial or peripheral sharpening layer. Therefore, FEMA solutions usually trigger a broad cellular immune response and control barrier recovery to limit vascular leakage, highlighting the importance of this transcriptional program to the emergency response. Although TLR9 (Toll-Like Receptor 9) is critical to H/SD-induced serious hyperpermeability, the therapeutic potential of TLR9 can promote the right hemorrhagic shock barrier to improve H/SD results, the kinetics of signaling and functional damage after H/SD TLR9 activation remain poorly understood.

Conclusion

Hemorrhagic shock (HS) initiates an inflammatory response by locally triggering innate immune cells and a systemic release of damage-associated molecular patterns (DAMPs) from the injured tissues, in which TLR9 signaling has been taken as a major mechanism. Recently, stimulation of TLR9 following HS has been found to be able to cooperatively elicit vascular leakage by triggering a signaling cascade in which pERK1/2 upregulates TRIF followed by SARM1, but yet remains to be verified more in vivo. Here, using TLR9-null (TLR9−/−) and male-associated cationic antimicrobial peptide 3-deficient mice as well as their corresponding controls together with the pharmacologic inhibitor of SARM1, our study originally showed that activation of TLR9 significantly exacerbated vascular hyperpermeability in the HS model by an EV release-dependent mechanism, which also dominated small intestine injury in the late phase of acute immune response. More importantly, treatment strategies targeting pERK1/2-TRIF-MAPK7 signaling such as TLR9 and more specifically SARM1 could be a new approach reducing both vascular leakage and small intestine injury in HS patients.

In this work, we found that both i.p. injection of EVs and small molecular inhibitors appeared to be very suitable given the absolute number of cells and the significant status separation which both subjects could not have altered the time course of the gut cytokine levels. Therefore, our present study provided further evidence on the advantageous role for SARM1 in vivo, while small molecular inhibitors such as NADase or MAPK7 inhibitors targeting SARM1 could be potent therapies to reduce TLR9-augmented excessive blood vessel permeability and gut injury during the late phase of acute immune response in HS. Of note, potential untoward in vivo effect and off-target side effects of these inhibitors in patients undergoing HS warrant further investigation and caution.

Conflict of Interest

No conflicts of interest were declared by the authors.

Financial Disclosure

The authors declared that this study has received no financial support.

Ethics Statement

Approved by local committee.

Authors' contributions

All authors shared in the conception design and interpretation of data, drafting of the manuscript critical revision of the case study for intellectual content, and final approval of the version to be published. All authors read and approved the final manuscript.

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