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SASH1 Is a Scaffold Molecule in Endothelial TLR4 Signaling

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Recognition of microbial products by TLRs is critical for mediating innate immune responses to invading pathogens. In this study, we identify a novel scaffold protein in TLR4 signaling called SAM and SH3 domain containing protein 1 (SASH1). Sash1 is expressed across all microvascular beds and functions as a scaffold molecule to independently bind TRAF6, TAK1, IκB kinase α, and IκB kinase β. This interaction fosters ubiquitination of TRAF6 and TAK1 and promotes LPS-induced NF-κB, JNK, and p38 activation, culminating in increased production of proinflammatory cytokines and increased LPS-induced endothelial migration. Our findings suggest that SASH1 acts to assemble a signaling complex downstream of TLR4 to activate early endothelial responses to receptor activation. The Journal of Immunology, 2013, 191: 892–901.

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Abbreviations used in this article: FADD, Fas-associated death domain protein; HA, hemagglutinin; HMEC, human microvascular endothelial cell; IKK, IκB kinase; IP-10, IFN-γ-inducible protein 10; IRAK, IL-1R-associated kinase; ISRE, IFN-stimulated responsive element; KO, knockout; MEF, mouse embryonic fibroblast; MS, mass spectrometry; SASH1, SAM and SH3 domain containing protein 1; sh, short hairpin; SLY1, SH3 domain expressed in lymphocytes; SLY2, hematopoietic adaptor containing SH3 and SAM domains 1; WT, wild-type.

The online version of this article contains supplemental material.

Recognition of bacterial and viral products by the mammalian innate immune system is mediated, in part, by a class of receptors called TLRs (1). The intracellular signaling cascades that are initiated by ligation of TLRs leads to activation of transcription factors such as NF-κB and IFN regulatory factor (IRF), resulting in production of proinflammatory cytokines (2, 3). Signaling through TLR4 follows recognition of LPS and the subsequent recruitment of intracellular adaptor molecules TIRAP, MyD88, and TRIF. Downstream of MyD88, IL-1R–associated kinases (IRAKs) are recruited to activate the E3 ubiquitin ligase TRAF6. This results in TRAF6 autoubiquitination of lysine 63 (K63)–linked ubiquitin chains culminating in activation of NF-κB and MAPKs (4). Signaling downstream of TRIF can also result in recruitment of TRAF6 to the receptor complex and subsequent activation of NF-κB and MAPK. Alternatively, recruitment of TRAF3 leads to induction of IFN-related genes (5).

Fax-associated death domain protein (FADD) is a negative regulator of TLR4 signaling in endothelial cells and FADD-null cells show hyperactivation of NF-κB and JNK in response to LPS in a TRAF6-dependent manner (6, 7). Because LPS signaling components have been shown to localize to lipid-rich microdomains in the plasma membrane (8), we hypothesized that the hyperactivation of the LPS signal in FADD-null cells is due, in part, to an increase in LPS signaling molecules in lipid-rich microdomains. Thus, we used proteomic analysis to identify proteins that are differentially expressed in the lipid-rich microdomains of FADD wild-type (WT) versus FADD knockout (KO) cells. The hyperactivation of the signaling pathway in FADD KO cells would facilitate detection of proteins present in the cell in limiting amounts. This approach resulted in the identification of SAM and SH3 domain containing protein 1 (SASH1) as a putative candidate for positive regulation of TLR4 signaling.

SASH1 is a large protein with a predicted molecular mass of 137 kDa, and it was originally identified by expression profiling and in silico analysis as a gene that is downregulated in breast and other solid cancers, such as lung and thyroid (9). Subsequently, SASH1 mRNA has been shown to be reduced in colon cancer and post-operative metastases in the liver (10). However, these studies are correlative and no direct evidence for SASH1 as a tumor suppressor has been described. SASH1 belongs to a family of SAM and SH3 adaptor proteins, consisting of SH3 domain expressed in lymphocytes (SLY1) and hematopoietic adaptor containing SH3 and SAM domains 1 (HACS1, also called SLY2) (11, 12). The SLY family has been implicated in regulation of the adaptive immune response, suggesting that SASH1 may also function in the immune system. Indeed, SASH1 was recently identified in a global phosphoproteome analysis as a protein that is phosphorylated at multiple sites in response to LPS (13). However, a molecular function for SASH1 in the immune system has not been reported, and thus we aimed to determine whether SASH1 functions in innate immune signaling.

In this study, we show that Sash1 is preferentially expressed in microvascular endothelial cells of various organs, as well as parenchymal cells, but not in lymphocytes. SASH1 positively regulates signaling through TLR4 in human endothelial cells to increase activation of NF-κB and MAPKs, culminating in increased production of proinflammatory cytokines. Although SASH1 does not bind to MyD88 or the IRAKs, it interacts with TRAF6...
through a conserved TRAF6 binding domain in SASH1 and regulates TRAF6 ubiquitination. SASH1 also binds to TAK1 and the Isk kinase (IKK) complex, independent of TRAF6, to assemble a signaling hub that permits downstream activation of NF-κB and MAPKs. Collectively, these results demonstrate that SASH1 functions as a novel scaffold protein to regulate innate immune signaling downstream of TLR4.

**Materials and Methods**

**Recombinant plasmids, Abs, and reagents**

Hemagglutinin (HA)-SASH1, Flag-SASH1, and Flag-TAK1 constructs were generated by PCR and cloned into pcDNA3 by restriction digest. Myc-SASH1 and Myc-SASH1D was obtained by inverse PCR to generate a deletion of amino acids 852-860. Flag-TRAF6ΔN, Flag-TRAF6ΔC, and Flag-TRAF6ΔCC were cloned from Flag-TRAF6 into pcDNA3 by restriction digestion. Other plasmids were obtained as follows: Flag-TRAF6 (Tularik, San Francisco, CA); Flag-ITK, Flag-IKKγ, and Flag-IFNκ (T.D. Gilmore, Boston University, Boston, MA); Flag-UBC13 (Adegge, Cambridge, MA); Myc-Uev1A (W. Xiao, University of Saskatchewan, Saskatoon, SK, Canada); HA-Ubi-WT, HA-Ubi-K48, and HA-Ubi-K63 (Z. Chen, University of Texas, Dallas, TX, and M. Servant, McGill University, Montreal, QC, Canada); IFN-stimulated responsive element (ISRE)-Luc (Stratagene, Santa Clara, CA); and NF-κB-Luc (FR. Jirk, University of Calgary, Calgary, AB, Canada). A rabbit polyclonal Ab to human SASH1 was raised against a synthetic peptide conjugated to keyhole limpet hemocyanin to recognize amino acids 8–20 at the N terminus of the deduced SASH1 protein sequence. Human microvascular endothelial cells (HMECs), 293T cells, and FADD-deficient mouse embryonic fibroblasts (MEFs) were obtained and cultured as previously described (7). TRAF6-deficient MEFs were a gift of T. Mak (University of British Columbia, Vancouver, BC, Canada). The SASH1 Ab was purified by protein A-Sepharose chromatography. Human microvascular endothelial cells (HMECs), 293T cells, and FADD-deficient mouse embryonic fibroblasts (MEFs) were obtained and cultured as previously described (7). TRAF6-deficient MEFs were a gift of T. Mak (University of British Columbia, Vancouver, BC, Canada). A rabbit polyclonal Ab to human SASH1 was raised against a synthetic peptide conjugated to keyhole limpet hemocyanin to recognize amino acids 8–20 at the N terminus of the deduced SASH1 protein sequence.

**Lipid raft isolation**

FADD WT or FADD KO MEFs were treated with LPS (100 ng/ml, 5 min), harvested for sucrose density gradient separation, and prepared for mass spectrometry as previously described (14).

**Mass spectrometry analysis**

Lipid raft protein pellets (∼30 μg each) were solubilized in 0.2 mL CH3OH (60% [v/v]) buffered with NH4HCO3 (pH 7.9). Samples were digested overnight using a 1:20 (w/w) trypsin/protein ratio. Trypsin-catalyzed 18O (FADD WT)/16O (FADD KO) exchange/labeling was carried out as previously described (15). Peptide pools were combined and lyophilized prior to strong cation exchange fractionation. Strong cation exchange fractionation was performed using an HP 1090 LC system (Agilent Technologies, Santa Clara, CA) as previously described (16). Sixteen fractions were collected, lyophilized, and reconstituted in 0.1% trifluoroacetic acid prior to nano-flow reversed-phase liquid chromatography/Fourier transform ion cyclotron resonance mass spectrometry (MS) (LTQ-FT; Thermo Fisher Scientific, Waltham, MA), as previously described (17). MS/MS spectra were analyzed using the SEQUEST algorithm against the UniProt mouse proteome database from the European Bioinformatics Institute (http://www.ebi.ac.uk). For a peptide to be acceptably identified, it had to achieve charge state and proteolytic cleavage-dependent cross correlation (Xcorr) scores of 2.0 for [M + H]+, 2.3 for [M + 2H]2+, 3.3 for [M + 3H]3+, and a minimum delta correlation score (ΔCn) of ≥0.08. Relative abundances for differentially labeled protein-specific peptides (i.e., peptide matched to only one protein) were calculated using PepQuan (Thermo Fisher Scientific) and in-house developed software (18). Results were reported as 18O/16O (heavy/light) ratios for a particular protein identified by two or more peptides.

**Mice**

The embryonic stem cell line CC0006 from the Sanger Institute Gene Trap Resource was used to generate Sash1 gene-trap mice (C57BL/6 × 129/Ola background). A comparison of the expressed sequence tag from the Sanger database and the Gene Trap sequence suggested that the insertion site for the gene-trap construct is located within introns 14–15 of Sash1. PCR of genomic DNA, followed by DNA sequencing, was used to map the exact location of the insertion into the Sash1 gene. Genomic DNA was isolated from littermates by tail digestion with proteinase K followed by PCR using primers specific to the insertion site of the gene-trap construct to distinguish homozygous mice. All animal studies have been reviewed and approved by the University of British Columbia.

**β-galactosidase staining**

Adult mouse tissues from Sash1+/- mice and their WT littermate controls were dissected and immediately fixed in 2% paraformaldehyde at 4°C for 4 h. Tissues were cryopreserved and placed into OCT compound at −80°C until sectioning. Sections (10 μm) were fixed with 0.2% glutaraldehyde solution and stained with buffer containing 100 mM sodium phosphate (pH 7.3), 2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside, 5 mM ferrocyanide, and 5 mM ferricyanide.

**Isolation of mouse endothelial cells**

Adult lung tissue was harvested from C57BL/6 mice, digested with 2 mg/ml collagenase IV, and incubated with 0.2 mg/ml DNase. Tissue homogenate was passed through a cell strainer and RBC lysis was carried out. Cells were incubated with rat anti-mouse CD105 hybridoma (gift of E.C. Butcher, Stanford University, Stanford, CA) or rat IgG2a isotype control. Cells were sorted for CD105+ population (FACS 440; Becton Dickson, Mississauga, ON, Canada) and reuspended in TRIZol to be processed for RNA and RT-PCR.

**FIGURE 1.** Identification of SASH1 in FADD KO MEFs by mass spectrometry analysis. FADD WT and FADD KO MEFs were stimulated with LPS (100 ng/ml, 5 min), and detergent-resistant microdomains (DRM) were isolated. (A) Protein fractions isolated from sucrose density-gradient separation were immunoblotted with caveolin-1 to validate the separation procedure. (B) SASH1 peptides identified in FADD KO MEF lipid rafts. (C) MS/MS spectrum assigned to one of the SASH1 peptides predicting the amino acid sequence of FYVDVLNEEEEK. The y and b ions are labeled.
**Immunoblot analysis and immunoprecipitation**

Cells were cotransfected with 5 μg each expression plasmid, and cell lysates were collected using modified RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate plus protease inhibitors). Subsequently, protein lysate was immunoprecipitated with anti–Flag M2-agarose beads or control IgG-agarose beads (Sigma-Aldrich, St. Louis, MO). Immunoprecipitation was also performed with anti-HA, anti-Myc, or anti-TRAF6 and incubation with protein A or G agarose beads (Cell Signaling Technologies, Danvers, MA). To examine polyvalent ubiquitination, cells were lysed in buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 20 mM N-ethylmaleimide plus protease inhibitors, and endogenous TRAF6 was immunoprecipitated with anti-TRAF6 or anti-HA followed by incubation with TrueBlot anti-rabbit Ig immunoprecipitation beads (eBioscience, San Diego, CA). Flag-TAK1 was immunoprecipitated with anti–Flag M2-agarose beads.

**Luciferase reporter assay**

HMECs were cotransfected with expression plasmids or stably transduced with lentivirus, as indicated, plus an NF-κB luciferase reporter or an IFN-stimulated response element luciferase reporter (pISRE-Luc; Stratagene). The Renilla luciferase plasmid pRL-CMV (Promega, Fitchburg, WI; for pNF-κB-Luc) or pRL-TK (Promega; for pISRE-Luc) was used as a transfection normalization control. Cells were stimulated with ligands, followed by passive lysis and measurement of luciferase activity by a dual luciferase assay (Promega).

**RNA interference**

Small interfering RNAs targeting human SASH1 mRNA (NM_015278), short hairpin (sh)SASH1 5’-GCTAATGATGATGGTCAAAGA-3’, and shRandom GTTGCTTGCCACGTCCTAGAT were cloned into pLenti-lox3.7. pLKO.1 (Open Biosystems) and pLKO.1-shTRAF6 were previously described (19). Lentiviral particles were produced from HEK293T cells by cotransfection of the shRNA vector, pVSVG, pMDL g/p RRE, and RSV-REV. Viral supernatants were used to transduce target cells, and GFP+ cells were selected by flow sorting (FACS 440; Becton Dickson).

**In silico analysis**

Prediction of TRAF6 as a SASH1 interacting partner was found using the Eukaryotic Linear Motif resource (http://elm.eu.org/links.html) (20).

**ELISA**

HMECs were stimulated with LPS (100 ng/ml, 6 h), and cell culture supernatants were assayed for IL-6 (eBioscience) or IFN-γ–inducible protein 10 (IP-10; R&D Systems, Burlington, ON, Canada) by ELISA following the manufacturers’ directions.

**Transwell migration assay**

HMECs were stably transduced with lentivirus, as indicated, and GFP+ cells were used to evaluate the role of SASH1 in endothelial cell migration in response to LPS. Cellular migration was measured using an 8-μm-pore polyethylene terephthalate membrane of a transwell migration insert (BD Biosciences, Mississauga, ON, Canada). Briefly, cells were seeded into the upper chamber of the transwell migration system and allowed to adhere for 1 h with serum-free medium in the lower chamber. Following 1 h, the media in the lower chamber was replaced with MCDB 131 medium containing 10% FBS, and cells in the upper chamber were stimulated with LPS (100 ng/ml, 6 h). Following stimulation, insert membranes were washed with PBS and fixed with 4% paraformaldehyde. Cells on the upper side of the membrane were removed using a sterile cotton swab, and the membranes were stained with 0.5% crystal violet (in 20% methanol) to visualize cells that had passed through to the lower side of the membrane. The number of migrated cells was counted using an inverted microscope. Four randomly selected fields were counted for each sample.

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**FIGURE 2.** Generation of gene-targeted Sash1 mice. (A) RT-PCR of tissue RNA isolated from C57BL/6 mice using primers specific to mouse Sash1. (B) Mapping of the genomic insertion of the gene-trap vector to introns 14–15 by PCR. (C–E) Sash1 expression in the endothelium of the spleen (C), thymus (D), and lung (E) as determined by β-galactosidase activity. (F) RT-PCR using RNA of lung endothelial cells purified by flow-sorting using rat anti-mouse CD105 hybridoma. Scale bar, 50 μm; inset image scale bar, 25 μm.
Statistical analysis

Results are expressed as means ± SD. Data were analyzed using a two-tailed Student t test using the GraphPad Prism statistical program. A p value < 0.05 was considered significant. Error bars depict SD.

Results

Proteomic analysis identifies SASH1 as a putative player in the TLR4 signaling pathway

To examine the protein content of lipid microdomains from LPS-stimulated MEFs, caveolin-containing detergent-resistant membrane fractions were isolated from FADD KO and WT MEFs (Fig. 1A) and differential proteomic analysis was performed. Two peptides uniquely identified SASH1 in the detergent-resistant membrane fraction of FADD KO, but not FADD WT, cells (Fig. 1B, 1C), suggesting that SASH1 may function as a positive regulator of TLR4 signaling.

Generation of Sash1 gene-trap mice

Sash1 mRNA is ubiquitously expressed in human tissues, with the highest levels of expression found in the lung, spleen, thymus, and placenta (9). To investigate the in vivo expression pattern of murine Sash1, tissues were harvested from C57BL/6 mice and analyzed for mRNA expression by RT-PCR. Sash1 mRNA was found to be expressed in all mouse tissues examined (Fig. 2A). To examine the in vivo expression of Sash1 in murine tissues, we generated Sash1−/− mice using a gene-trap that results in a Sash1-LacZ fusion protein comprising the first 14 exons of Sash1, followed by the β-geo construct under control of the endogenous Sash1 promoter (Fig. 2B). At the cellular level, Sash1 was expressed in the parenchyma of various organs that show dysfunction in sepsis, including the liver, kidney, and brain. Notably, Sash1 was strongly expressed in the microvascular endothelium of...
all organs examined, explaining the ubiquitous organ expression of Sash1 mRNA (Fig. 2C, 2D, and Supplemental Fig. 1). Of interest, Sash1 was not expressed in lymphoid cells of the spleen and thymus (Fig. 2C–E). To confirm endothelial Sash1 expression in the lung, we examined flow-sorted, CD105+ endothelial cells by RT-PCR. Sash1 mRNA was found predominantly in the CD105+ fraction of adult lung tissue, along with several endothelial markers. In contrast, SLY1 and SLY2 were found predominantly in the nonendothelial (CD105−) population (Fig. 2F). The presence of SASH1 in the endothelium, but not in lymphoid cells of the spleen and thymus, suggests that SASH1 may function in non-immune cells to propagate the TLR4 signal.

**SASH1 positively regulates LPS signal transduction**

Given the ubiquitous microvascular endothelial expression of Sash1, we examined SASH1 function in HMECs. Lentiviral-mediated knockdown using shRNA targeting SASH1 in HMEC resulted in ∼70% reduction of SASH1 protein relative to a random shRNA (Fig. 3A). Knockdown of SASH1 was sufficient to decrease NF-κB luciferase reporter activity in response to LPS (Fig. 3B), but not in response to activation of TLR2, TLR3, TLR5, or other receptors that signal through TRAF6, such as IL-1R, IL-17R, and TGF-βR (21, 22) (Fig. 3C–H). In contrast, SASH1 knockdown did not affect activation of an ISRE coupled to luciferase, suggesting that SASH1 is not required for LPS-induced activation of IFN-regulated genes (Fig. 3K).

We were unable to detect NF-κB activation downstream of TLR9 in HMECs using several different CpG oligonucleotides, and previous reports have shown that TLR7 and TLR8 are not activate NF-κB downstream of these receptors was not assessed (23).

We next determined whether NF-κB–dependent cytokines are decreased in response to LPS when endogenous SASH1 levels are reduced. Indeed, knockdown of SASH1 resulted in decreased production of IL-6 and IP-10, in response to LPS, compared with cells transduced with a random control shRNA construct (Fig. 3H, 3I). Conversely, enforced overexpression of SASH1 in HMECs resulted in an increase in LPS-induced NF-κB activation (Fig. 4A) and increased production of IL-6 and IP-10 (Fig. 4B, 4C), but it had no effect on an ISRE-luciferase reporter (Fig. 4D). Taken together, these data suggest that SASH1 plays a positive role in the activation of NF-κB, but not IRF-regulated, signaling pathways downstream of TLR4 activation.

**SASH1 interacts with the C-terminal domain of TRAF6 through a conserved TRAF6 binding domain**

TRAF6 binding proteins CD40 and IRAK possess a conserved TRAF6 interaction motif (24, 25), and in silico analysis revealed that SASH1 contains a consensus TRAF6 binding sequence at amino acids 852–860. Interaction of SASH1 and TRAF6 was demonstrated by reciprocal coimmunoprecipitation of Flag-tagged TRAF6 and HA-tagged SASH1 in HEK293T cells cotransfected with each of these expression constructs (Fig. 5A).

To determine whether SASH1 binds to TRAF6 through the putative TRAF6 binding domain identified by in silico analysis, a SASH1 mutant lacking this domain was constructed. Deletion of this domain abolished interaction between TRAF6 and SASH1 (Fig. 5B), suggesting that the interaction between these two proteins occurs through the conserved TRAF6 binding motif. Other TRAF6 binding partners that possess a conserved TRAF6 interaction motif interact with TRAF6 at the C-terminal TRAF domain (26). To investigate which region of TRAF6 binds to SASH1, we used mutants of the TRAF6 protein. The TRAF6 mutant that lacks the N terminus coimmunoprecipitated with SASH1. However, the mutant lacking the C-terminal region of TRAF6 did not coimmunoprecipitate with SASH1, suggesting that SASH1 interacts with the C terminus of TRAF6 (Fig. 5C). Additionally, a TRAF6 deletion construct lacking the coiled-coil domain interacted poorly with SASH1, consistent with a role for SASH1 in the oligomerization and activation of TRAF6.

To determine whether the TRAF6–SASH1 interaction was LPS-dependent, interaction between endogenous TRAF6 and SASH1 was examined in LPS-stimulated HEK293-TLR4-CD14-MD2 cells. Immunoprecipitation of TRAF6 resulted in coprecipitation of SASH1 and increased production of IL-6 and IP-10 (Fig. 4B, 4C), but it had no effect on an ISRE-luciferase reporter (Fig. 4D). Taken together, these data suggest that SASH1 plays a positive role in the activation of NF-κB, but not IRF-regulated, signaling pathways downstream of TLR4 activation.

**FIGURE 4.** Enforced expression of SASH1 increases TLR4 signaling. (A–D) HMECs were transfected with Flag-SASH1 or vector control and (A) cotransfected with pNF-κB plus pRL-CMV and NF-κB activity was measured by dual luciferase assays following stimulation with LPS (100 ng/ml, 8 h) or (B and C) stimulated with LPS (100 ng/ml, 6 h) and supernatants were assayed by ELISA for (B or (C) IP-10 or (D) cotransfected with pISRE-Luc plus pRL-TK to quantify IFN activity by dual luciferase assays. Data are presented as the fold change relative to normal. *p = 0.0004, **p < 0.0001 as determined by Student t test. Error bars indicate SD; n = 3 independent experiments.
endogenous SASH1 after 5 min of LPS treatment, and this interaction was sustained for up to 60 min after stimulation (Fig. 5D). This finding suggests that the interaction between SASH1 and TRAF6 is LPS-dependent and physiologically relevant. In contrast, SASH1 does not interact with MyD88 or IRAKs (Fig. 5E–G).

TRAF6 belongs to a family of TRAF molecules that mediate signaling downstream of TNFR superfamily members (27). In silico analysis also identified four putative TRAF2 binding sites within the human SASH1 protein sequence. TRAF2 is critical for the activation of NF-κB and JNK downstream of numerous receptors, including TNFR1 and CD40 (28). The presence of multiple putative TRAF2 binding sites within SASH1 could be indicative of a broader role for SASH1 in regulating TRAF family members. However, coimmunoprecipitation analysis did not show SASH1–TRAF2 interaction (Supplemental Fig. 2A). TRAF3 functions downstream of TLR4 in MyD88-independent pathways, primarily contributing to the activation of IFN-dependent genes (5), but again coimmunoprecipitation did not reveal an interaction between SASH1 and TRAF3 (Supplemental Fig. 2B). Taken together, these results suggest that SASH1 may interact specifically with TRAF6 and not with other TRAF molecules.

SASH1 regulates TRAF6 ubiquitination

K63-linked autoubiquitination of TRAF6 is critical for formation of the signaling complex comprised of TAK1 and the adaptor proteins TAB2 and TAB3 (29). To determine whether the interaction between SASH1 and TRAF6 regulates TRAF6 activation, TRAF6 ubiquitination was examined in the presence of SASH1. Indeed, transient overexpression of SASH1 in HEK293-TLR4-CD14-MD2 cells was sufficient to induce autoubiquitination of TRAF6 in the absence of LPS stimulation (Fig. 6A). Because TRAF6 is modified by K48-linked and K63-linked ubiquitin, we wanted to determine the precise modification mediated by SASH1. We transfected HEK293-TLR4-CD14-MD2 cells with total ubiquitin, K63-linked ubiquitin, or K48-linked ubiquitin and examined TRAF6 ubiquitination by immunoprecipitation. Expression of SASH1, independent of LPS stimulation, resulted in greater K63-linked ubiquitination, compared with vector control, and no significant difference in K48-linked ubiquitin, suggesting that SASH1 is important for activating K63-linked ubiquitination but not the degradative K48-linked pathway (Fig. 6B). To determine whether the interaction between SASH1 and TRAF6 was
critical for the increase in TRAF6 ubiquitination, we transiently transfected HEK293-TLR4-CD14-MD2 cells with the TRAF6 binding domain mutant of SASH1 and assessed TRAF6 ubiquitination. Expression of this mutant did not induce autoubiquitination of TRAF6 in the absence of LPS stimulation (Fig. 6C) or increase the downstream activation of an NF-κB luciferase reporter (Fig. 6D). These results imply that binding of SASH1 to TRAF6 is important for ubiquitin-mediated activation of TRAF6 and the subsequent downstream signaling to NF-κB.

TRAF6 ubiquitination requires interaction with a heterodimeric ubiquitin conjugating enzyme composed of Ubc13 and the Ubc variant Uev1A (30). TRAF6 has been shown to bind directly to Ubc13, but not to Uev1A (31). However, we did not detect an interaction between SASH1 and either Ubc13 or Uev1A (Supplemental Fig. 3A and 3B, respectively), although we were able to detect interaction between TRAF6 and Ubc13, as well as between Ubc13 and Uev1A (Supplemental Fig. 3C and 3D, respectively). These findings suggest that the E2 ligases do not directly bind SASH1, but become incorporated into a complex by binding TRAF6 and each other.

SASH1 acts as a scaffold molecule by binding TAK1 and the IKK complex

Given that SASH1 is a large protein with multiple protein interaction domains, suggestive of a scaffolding function, coimmunoprecipitation was used to determine whether SASH1 provides the framework for constructing a molecular signaling complex around TRAF6. TAK1 has been reported to bind TRAF6 through the adaptor molecules TAB2 and TAB3, thereby facilitating activation of TRAF6 (32). Reciprocal coimmunoprecipitation experiments demonstrated that SASH1 interacts with TAK1 (Fig. 7A). TAK1 phosphorylates and activates the downstream target IKKβ, leading to NF-κB activation (33), and reciprocal coimmunoprecipitation revealed that SASH1 also binds to IKKβ (Fig. 7B). IKKβ is part of a complex consisting of an additional catalytic subunit, IKKa, and a regulatory subunit, IKKγ (34). Reciprocal coimmunoprecipitation revealed that SASH1 also interacted with IKKα, but not the regulatory subunit IKKγ, although we were able to detect interaction between IKKγ and IKKβ as a control (Fig. 7C, 7D). This suggests that SASH1 acts as a scaffold to assemble an NF-κB activation module through TRAF6, downstream of TLR4 stimulation.

To confirm that the interaction between SASH1 and TAK1 is independent of the SASH1 interaction with TRAF6, we examined the interaction between SASH1 and TAK1 in MEFs lacking expression of TRAF6. Interaction of SASH1 and TAK1 was maintained in TRAF6−/− MEFs, suggesting that TRAF6 is indispensable for the interaction between SASH1 and TAK1 (Fig. 7E). Moreover, when TRAF6 was knocked down in HEK293T cells, interaction of SASH1 with TAK1 and IKKβ was sustained (Fig. 7F, 7G). Similarly, interaction of SASH1 with either TAK1 or IKKβ was not abolished by deletion of the TRAF6 binding domain (Fig. 7H, 7I), although the SASH1–TAK1 interaction was significantly reduced, suggesting that TRAF6–SASH1 interaction may facilitate TAK1 binding to SASH1, or that the SASH1 deletion mutant through conformational or other changes binds TAK1 less efficiently. Collectively, these results suggest that SASH1 acts as a scaffold molecule to independently bind TRAF6, TAK1, and IKKβ to facilitate signaling to NF-κB.
SASH1 regulates TAK1 ubiquitination and activation of downstream MAPK

TAK1 polyubiquitination has been shown to be essential for TAK1-mediated activation of IKK/NF-κB downstream of receptors for TNF and IL-1 (35). We have shown that SASH1 interacts with TAK1, and thus we wanted to determine whether SASH1 could regulate ubiquitination of TAK1. Transient transfection of HEK293T-TLR4-MD2-CD14 cells with SASH1 resulted in an increase in TAK1 ubiquitination, even in the absence of LPS stimulation (Supplemental Fig. 4A), similar to what was seen with TRAF6. However, as with TRAF6, the SASH1–TRAF6 binding domain mutant failed to result in increased TAK1 ubiquitination in the absence of LPS stimulation, suggesting a requirement of SASH1–TRAF6 interaction for TAK1 activation. TAK1 is essential for activation of NF-κB, JNK, and p38 in response to LPS stimulation (36). Because SASH1 interacts with TAK1 and regulates its polyubiquitination, we investigated whether SASH1 is important for activation of JNK and p38 in endothelial cells. Knockdown of SASH1 in HMECs resulted in a decrease in LPS-induced JNK and p38 activation (Supplemental Fig. 4B). Taken together, these findings suggest that SASH1 is important for regulating multiple signaling cascades activated downstream of TRAF6 and TAK1.

SASH1 regulates LPS-induced endothelial cell migration

We have previously shown that LPS stimulates angiogenesis through TRAF6-dependent activation of NF-κB and JNK (37). To determine whether SASH1 might affect LPS-induced angiogenesis, we used a transwell assay to assess endothelial migration. Knockdown of SASH1 significantly reduced endothelial migration in response to LPS stimulation (Fig. 8), suggesting that SASH1 is important for endothelial cell function in innate immunity.

Discussion

The data presented in this study describe the function of a previously uncharacterized protein, SASH1. We show that SASH1, a member of the SLY family of proteins, acts as a novel TLR4...
signaling molecule that is expressed in microvascular endothelial cells. Our results support a function for SASH1 as a scaffolding molecule to assemble a molecular complex that includes TRAF6, TAK1, IKKα, and IKKβ, thereby facilitating activation of NF-κB, JNK, and p38 (Supplemental Fig. 4C). Thus, SASH1 provides the first example of a noncatalytic scaffolding molecule that functions in TLR4 signaling.

Scaffold proteins are defined as molecules that bind to at least two other proteins in a signaling cascade to regulate signaling, either by recruiting specific regulators or mediating subcellular localization of the signaling complex (38). Although scaffold molecules have been well defined in adaptive immunity in T cells, the description of classical scaffold molecules in innate immune signaling pathways has remained elusive. The Pellino proteins are a family of E3 ubiquitin ligases characterized by their interaction with IRAK molecules downstream of TLR and IL-1R complexes (39). However, in contrast to the catalytic function described for Pellino proteins, classical signaling scaffolds typically do not possess enzyme activity and act primarily as specificity elements to control signaling between binding partners (40). In addition to binding IRAK, Pellino proteins also complex with TRAF6 and TAK1 (41). However, the interaction between Pellino proteins and either TRAF6 or TAK1 has only been demonstrated downstream of IL-1R, and direct interaction between Pellino proteins and TRAF6 or TAK1 has not been shown, as Pellino proteins do not contain a classic TRAF6 binding motif (20, 41).

In this study, we show that SASH1 binds to TRAF6 through a conserved TRAF6 binding domain and that lack of TRAF6, reduced TRAF6 expression, or deletion of the TRAF6 binding domain does not abolish binding between SASH1 and TAK1 or IKKβ, suggesting that SASH1 can associate with these molecules in the absence of a SASH1/TRAF6 complex. The Pellino proteins are small molecules (~46 kDa), therefore limiting their ability to act as assembly scaffolds for multiple signaling partners simultaneously. Thus, SASH1 provides the first example of a large scaffolding molecule that is capable of binding multiple signaling proteins downstream of TLR4.

The SLY family of SAM and SH3 adaptor molecules has previously been implicated in regulation of the immune system. SLY1, named for its predominant expression in lymphoid tissues such as the spleen and thymus, is serine phosphorylated (Ser27) following B or TCR stimulation (42). Sly1<sup>−/−</sup> mice lack Ser27 and a functional nuclear localization sequence, and as a result show impaired lymphoid organ development, defects in Ag receptor–mediated lymphocyte activation, attenuated humoral immune responses, and prolonged allograft survival, suggesting that SLY1 is critical for mediating adaptive immune responses in mice (11). SLY2 is expressed in normal and hematopoietic tissues and is upregulated in B cells stimulated with IL-4, IL-13, anti-IgM, and anti-CD40, suggesting a role in B cell activation and differentiation cascades (12). Moreover, Sly2<sup>−/−</sup> mice have increased Th1 and Th2 humoral immune responses, suggesting that Sly2 acts as an immunosuppressor (43). The results presented in this study suggest that SASH1 also functions in immune regulation, but in nonlymphoid cells, and, to our knowledge, describe for the first time a molecular function for SASH1 in innate immunity.

TRAF6 is essential for activation of IKK as well as JNK and p38 MAPKs (44, 45). Our finding that SASH1 interacts with the complex of TRAF6/TAK1/IKKα/β and promotes TRAF6 ubiquitination agrees with the finding that SASH1 increases downstream activation of NF-κB and MAPKs. However, because SASH1 regulates TRAF6 ubiquitination, but is insufficient to activate NF-κB or MAPKs in the absence of signal, our findings suggest that TRAF6 and TAK1 ubiquitination is not sufficient to activate downstream pathways, and that a second, LPS-dependent event is required for full activation of the signaling cascade.

Endothelial cells are among the first cells to come into contact with invading pathogens in the bloodstream and play a critical role in the inflammatory response through recruitment and transmigration of leukocytes into infected tissue and the regulation of vascular permeability (4). The expression of Sash1 in microvascular endothelial cells, as well as the novel scaffolding function described in this study, may provide insight into the cell type–specific responses to LPS and the molecular events that lead to endothelial dysfunction and vascular collapse in sepsis. Endothelial cells, through the upregulation of surface adhesion molecules, such as E-selectin and VCAM-1, serve as important mediators of leukocyte recruitment following LPS challenge (46). However, it remains unclear whether the endothelial events initiated during inflammation are an indirect result of LPS activation of immune cells or a direct effect on the endothelium. In this study, we show that knockdown of SASH1 significantly reduces LPS-induced endothelial migration in vitro, suggesting that SASH1 may play a role in the angiogenic response during infection in vivo. In this study, we were unable to confirm SASH1 function in TLR4 signaling in vivo because the homozygous-null mice showed perinatal lethality (data not shown). Additional studies using endothelial-specific knockout mice with targeted deletions of genes important in innate immune signaling, such as Sash1, will shed light on the importance of the endothelium following LPS challenge in vivo.

**FIGURE 8.** SASH1 regulates LPS-induced endothelial migration. HMECs were transduced with lentiviral vectors encoding shRNA, as indicated, and (A) immunoblotting was used to confirm knockdown. (B and C) HMECs were seeded into the transwell migration system, stimulated with LPS (100 ng/ml, 6 h), and cells that migrated through the membrane were stained (shown in purple) and counted (B). Migrating cells were visualized by microscopy and (C) the number of migrating cells was quantified. Original magnification ×100. *p < 0.001 as determined by Student t test. Error bars indicate SD; n = 3 independent experiments.
In conclusion, in this study we have shown that SASH1 is a novel regulator of TLR4 signaling through formation of a molecular complex around TRAF6. Indeed, the importance of endothelial NF-κB signaling in the vascular sequelae of sepsis strengthens a model in which SASH1 functions as an endothelial scaffold molecule that is required for the assembly of TRAF6/TAK1/IKK to activate downstream signaling.

Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Figure 1. Sash1 is expressed in the microvasculature of the liver, kidney and brain. Sash1 expression in the (A) hepatic sinusoids, (B) renal glomeruli and (C) brain vasculature. Scale bar = 50 μM
Supplemental Figure 2. SASH1 does not interact with TRAF2 or TRAF3. A-B, 293T cells were transfected with HA-SASH1 and either Flag-TRAF2 (A) or Flag-TRAF3 (B) and lysates were immunoprecipitated (IP) and immunoblotted using antibodies to HA or Flag.
Supplemental Figure 3. SASH1 does not interact with E2 ligases, Ubc13 and Uev1A. (A-D) HEK293T cells cotransfected with (A) HA-SASH1 and Flag-Ubc13, (B) Flag-SASH1 and Myc-Uev1A, (C) HA-TRAF6 and Flag-Ubc13 or (D) Flag Ubc13 and Myc-Uev1A and lysates IPed and immunoblotted with anti-HA and anti-Flag.
Supplemental Figure 4. SASH1 regulates TAK1 ubiquitination and activation of JNK and p38. (A) HEK293-TLR4-MD2-CD14 cells cotransfected with Flag-TAK1 and HA-SASH1, Myc-SASH1ΔT6BD or vector control, stimulated with LPS (100 ng/mL, 30 min) and IPed for Flag-TAK1 and immunoblotted with antibodies to ubiquitin, Flag and SASH1. (B) LPS-induced (100 ng/mL) activation of JNK or p38 in HMEC transduced with lentiviral vectors encoding shRNA as indicated. (C) Model for the role of SASH1 in endothelial TLR4 signaling.