

**INVESTIGATION OF THE ROLE OF SASH1 IN THE REGULATION OF
TRAF6- β -ARRESTIN COMPLEX FORMATION**

by

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ABSTRACT

Proper activation and regulation of innate immune signaling pathways and inflammatory responses are essential to efficiently fight infection. Dysregulation of these responses, however, can result in the development of a severe hyper-inflammatory state, ultimately leading to sepsis. The endothelium has been shown to play a critical role in innate immune responses and the pathogenesis of sepsis. In the presence of Gram-negative infection, circulating LPS is recognized by TLR4 expressed on the surface of endothelial cells, mediating downstream activation of pro-inflammatory signal cascades and immune responses. SASH1 has been recently characterized as a novel scaffold protein in the regulation of endothelial TLR4 signaling. The work presented in this thesis further characterizes the role of SASH1 in the regulation of TRAF6 activation within the TLR4 pathway. LPS-induced auto-ubiquitination of TRAF6 is an essential regulatory step in the downstream activation of TLR4-mediated inflammatory signaling. Co-immunoprecipitation analyses confirmed the interaction of endogenous SASH1 with TRAF6 in an LPS-dependent manner in endothelial cells. Furthermore, SASH1 was required for LPS-induced auto-ubiquitination of TRAF6. To further investigate SASH1 function, a yeast two-hybrid approach was employed to identify novel SASH1-interacting proteins. β -arrestin 1 was identified as the top potential interactor of SASH1. Both β -arrestin 1 and β -arrestin 2 have been shown to act as negative regulators of the TLR4 pathway by impairing the oligomerization and auto-ubiquitination of TRAF6. It was speculated that SASH1 may coordinate the interaction of TRAF6 with the β -arrestins to mediate regulation of the TLR4 pathway. Reciprocal co-immunoprecipitation experiments confirmed the interaction of SASH1 with both β -arrestin 1 and β -arrestin 2. SASH1 was also found to interact with β -

arrestin 1 in a complex with TRAF6. Further studies aimed to characterize the role of SASH1 in coordinating the interaction of TRAF6 with the β -arrestins. Although knockdown of SASH1 did not modulate TRAF6- β -arrestin binding, the enforced expression of SASH1 was found to specifically impair TRAF6 binding with β -arrestin 1, but not β -arrestin 2. The significance of this isoform-specific regulation remains to be determined. Overall, these studies further investigate the role of SASH1 as a critical scaffold protein in the regulation of the TLR4 pathway.

PREFACE

The following is a publication that I co-authored during my graduate school career, originally published in *The Journal of Immunology*.

Dauphinee SM, **Clayton A**, Hussainkhel A, Yang C, Park Y-J, Fuller ME, Blonder J, Veenstra TD and Karsan A. (2013) SASH1 is a scaffold molecule in endothelial TLR4 signaling. *J Immunol.* 191(2):892-901.

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The work presented in this manuscript identifies and characterizes SASH1 as a novel scaffold protein in endothelial TLR4 signaling, and is therefore relevant to this thesis. I was involved in generating the following data for this manuscript:

Figure 6B. SASH1 mediates K63-linked poly-ubiquitination of TRAF6.

Figure 7E, 7F and 7G. SASH1 interacts with TAK1 and IKK β independent of TRAF6.

Figure 8. SASH1 is required for LPS-induced endothelial cell migration.

Supplemental Figure 3C and 3D. TRAF6 interacts with UBC13.

The findings of this study are discussed in detail in Chapter 1 of this thesis and were the starting point for the work presented in Chapter 3. The remainder of this thesis is original, unpublished work conceptualized by the author, Ashley Clayton, and Dr. Aly Karsan. Unless otherwise described in Chapter 2, all plasmid constructs used in this study were previously generated by Shauna Dauphinee, a former graduate student in the Karsan lab. The yeast two-hybrid analysis described in Chapter 3 identifying β -arrestin 1 as a potential interacting protein of SASH1 was a service provided by Hybrigenics. All remaining experiments in this chapter were independently carried out by Ashley Clayton and are currently unpublished.

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LIST OF ABBREVIATIONS

7TMR, seven-transmembrane receptor

AMP, anti-microbial peptide

AP1, activator protein 1

AP2, β 2-adaptin

Arrb1, β -arrestin 1

Arrb2, β -arrestin 2

BMDM, bone marrow-derived macrophages

BTK, Bruton's tyrosine kinase

CAM, cell-adhesion molecule

DD, death domain

DIC, disseminated intravascular coagulation

dsRNA, double-stranded RNA

eNOS, endothelial nitric oxide synthase

FADD, Fas-associated death domain protein

HA, Hemagglutinin

HEK293T, human embryonic kidney 293T cell

HSP, heat shock protein

HMEC, human microvascular endothelial cell

HUVEC, human umbilical vein endothelial cell

IFN, interferon

IFN β , interferon β

IFN γ , interferon γ

I κ B, inhibitor of NF- κ B

IKK, I κ B kinase
IL-1 β , Interleukin-1 β
IL-1R, Interleukin-1 receptor
IL-6, Interleukin-6
IL-8, Interleukin-8
iNOS, inducible nitric oxide synthase
IP, immunoprecipitation
IP-10, IFN γ -inducible protein 10
IRAK, IL-1R-associated kinase
IRF, interferon regulatory factor
IRF3, interferon regulatory factor 3
JNK, c-jun NH₂-terminal kinase
K48, lysine 48
K63, lysine 63
LBP, lipopolysaccharide-binding protein
LPS, lipopolysaccharide
LRR, leucine-rich repeat
MAPK, mitogen-activated protein kinase
mCD14, membrane-anchored CD14
MCP-1, monocyte chemoattractant protein-1
MEF, mouse embryonic fibroblast
miR-146a, microRNA-146a
MKK, MAPK kinase
MyD88, myeloid differentiation factor 88

NAP1, NF- κ B-activating kinase-associated protein 1
NES, nuclear export signal
NF- κ B, nuclear transcription factor- κ β
NO, nitric oxide
PAMP, pathogen-associated molecular pattern
PRR, pattern recognition receptor
RING, really interesting new gene
RIP, receptor interacting protein 1
SAM, sterile- α -motif
SASH1, SAM and SH3 domain-containing protein 1
sCD14, soluble CD14
SH3, Src homology 3
SLY1, SH3 domain expressed in lymphocytes
SLY2, hematopoietic adaptor containing SH3 and SAM domains 1
ssRNA, single-stranded RNA
T6BD, TRAF6 binding domain
sTLR4, soluble Toll-like receptor 4
TAK1, TGF- β -activated kinase 1
TANK, TNF receptor-associated factor family member-associated NF- κ B activator
TBK1, TANK-binding kinase 1
TGF- β , transforming growth factor- β
TIR, Toll/Interleukin-1 receptor
TIRAP, TIR domain-containing adapter protein
TNF, tumour necrosis factor

TNF α , tumour necrosis factor α

TLR, Toll-like receptor

TLR4, Toll-like receptor 4

TRAF, TNF receptor-associated factor

TRAF6, TNF receptor-associated factor 6

TRAM, TIR domain-containing adapter molecule 2

TRIF, TIR domain-containing adapter molecule 1

UBC13, ubiquitin-conjugating enzyme 13

UEV1A, ubiquitin-conjugating enzyme E2 variant

VE-cadherin, vascular endothelial cadherin

VEGF, vascular endothelial growth factor

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CHAPTER 1: INTRODUCTION

1.1 The Immune System

The primary role of the immune system is to recognize and eliminate invading pathogens and foreign molecules from the body [1, 2]. Under normal conditions, the immune system acts as a highly complex and interactive network of cells, organs and soluble factors to recognize foreign antigens from self, rapidly fight infection and provide long-lasting immunity [1-3]. The speed and specificity of these reactions separates the immune system into two classes: the innate immune system and the adaptive immune system [1-3]. The innate immune response provides rapid, non-specific host defense against invading pathogens, but does not provide long-lasting antigen immunity. This is in contrast to the delayed adaptive immune response, which provides antigen-specificity and immunological memory [1, 2]. These features of the adaptive immune system provide long-lasting antigen immunity, permitting enhanced immune responses upon subsequent immunogen exposure [1, 2]. Despite their functional differences, it is important to note that the innate and adaptive immune systems work together to efficiently eliminate pathogens and maintain overall health [1-3]. Moving forward, this introduction will focus on the innate immune system.

1.2 The Innate Immune System

The innate immune system rapidly acts as the first line of defense against invading pathogens [3]. The highly evolutionarily conserved nature of innate responses is indicative of the importance of this system in host survival [2]. The innate immune system can be described as providing four main barriers to infection: anatomical, physiological, cellular and inflammatory [3, 4].

Anatomical or physical barriers to infection include the skin and mucous membranes [5, 6]. The skin acts as a mechanical barrier against the invasion of microorganisms, and provides an acidic environment to inhibit bacterial growth [5]. Furthermore, the expression of anti-microbial peptides (AMPs), such as cathelicidins and defensins, from the skin and gut tissues can act with broad-spectrum activity against bacteria, fungi and viruses [3, 7]. In addition to these mechanisms, passages of the body exposed to the external environment are lined with mucous membranes to provide a physical mucous barrier [6]. This secreted viscous mucous barrier acts in conjunction with cilia to trap and sweep away invading microorganisms [6]. Mucosal surfaces are also home to the commensal microbiota [8]. These commensals play an important immuno-protective role by either directly combating and out-competing invading microbes, or by further mediating host anti-microbial defenses [8].

Physiological barriers of innate immunity include pH, body temperature and the production of circulating antimicrobial substances, such as complement. pH plays an important role in the gut, where many microbes cannot survive the acidic environment[1-3]. Furthermore, host body temperature is not always conducive to the growth of certain pathogens [1-3]. In addition to these physiological barriers, the complement system plays an important anti-microbial role [1, 9]. This group of serum proteins activates a microbicidal enzymatic cascade in the presence of microbial products, mediating pathogen destruction and clearance of infected cells [1, 9].

Cellular barriers to infection are a third mechanism of the innate immune system [2, 3]. These barriers include a host of specialized cells, such as macrophages and neutrophils, that are capable of rapidly recognizing pathogens via cell surface receptors, and internalizing pathogenic material through the process of phagocytosis [10, 11]. Internalization of these

materials ultimately leads to lysosomal degradation and destruction of the pathogen [10, 11]. Moreover, this phagocytic process can lead to antigen presentation and subsequent activation of cells of the adaptive immune system, such as lymphocytes [2, 10, 11].

The fourth and ultimate innate immune barrier to infection is inflammation. This process is characterized by *rubor* (redness), *tumor* (swelling), *calor* (heat) and *dolor* (pain) [12]. In the presence of an immunogen, pro-inflammatory mediators, called cytokines, promote the recruitment and extravasation of leukocytes from the blood to the site of infection. The phagocytic activity of these immune cells in tissues can result in nearby tissue damage, promoting the inflammatory process. As previously described, this innate immune response can ultimately lead to the subsequent recruitment and activation of adaptive immune cells, indicative of how these two systems can work together to mount a host immune response and clear infection [1, 2, 4, 11].

1.3 The Endothelium in Innate Immunity and Sepsis

1.3.1 The Endothelium

The vasculature is a complex network of blood vessels that circulates blood throughout the body to deliver oxygen, nutrients and immune factors [13]. The endothelium, comprised of endothelial cells lining the inner surface of blood vessels, provides a selectively permeable barrier between circulating blood and extravascular space [13]. Under normal conditions, the endothelium functions to maintain organ homeostasis, modulate and balance leukocyte recruitment and immune function, as well as regulate vascular tone, permeability, blood flow and coagulation [13-15]. Proper functioning of the endothelium is critical for the structural and functional integrity of the vasculature and circulation [13].

1.3.2 Sepsis

Sepsis is described as a systemic inflammatory response to severe infection, and is the number one cause of death in critically ill patients [14, 16, 17]. In the presence of severe infection and a systemic hyper-inflammatory state, loss or malfunction of host regulatory mechanisms can result in the uncontrolled hyper-activation of the inflammatory response and the subsequent tissue damage observed during sepsis [18]. Clinical manifestations of sepsis include this initial hyper-inflammatory response, followed by a prolonged immunosuppressive state due to extensive immune cell death and dysfunction [18]. It has become increasingly evident that the endothelium plays a key role in the pathophysiology of sepsis [17, 19, 20]. In the presence of excess systemic inflammatory mediators or severe infection, the balance and normal physiological functioning of the endothelium is completely disrupted [14, 17, 19]. The resulting endothelial dysfunction can lead to the excessive production of inflammatory mediators, severe hypotension, excess leukocyte recruitment, vascular leakage and hyper-coagulation, followed by tissue damage, multiple organ failure and often death [14, 19-21].

1.3.3 Endothelial Dysfunction in Sepsis

Endothelial dysfunction in the pathogenesis of sepsis may be a consequence of the direct interaction of immunogens with endothelial cells, or possibly a secondary effect of increased circulating inflammatory molecules produced by immune cells [17, 21, 22]. Receptors found on immune cells and endothelial cells can recognize pro-inflammatory molecules as well as specific pathogen-associated molecular patterns (PAMPs) in the circulation to further elicit inflammatory responses [17, 21]. In cases of Gram-negative bacterial sepsis, or endotoxemia, the bacterial cell wall component lipopolysaccharide (LPS) can bind to endothelial cell

surface receptors, stimulating downstream activation of several pro-inflammatory signal cascades [17, 21]. These cascades include activation of the transcription factors nuclear transcription factor- κ B (NF- κ B) and interferon regulatory factor (IRF), ultimately leading to the production of several pro-inflammatory cytokines [21, 23]. Furthermore, LPS-induced NF- κ B activation can mediate activation of both pro-survival and apoptotic responses in endothelial cells [21]. Proper balance of these signals is critical in the pathogenesis of sepsis to avoid complete vascular collapse [21]. Interestingly, specific inhibition of endothelial NF- κ B reduced organ injury and increased overall survival in a mouse model of sepsis [24]. Furthermore, recognition of bacterial components, such as LPS, by endothelial cells can lead to excess production of endothelial nitric oxide synthase (eNOS), mediating the production of the potent vasodilator nitric oxide (NO) [25]. Inducible nitric oxide synthase (iNOS) is also subsequently produced, potentiating NO production and contributing to the severe hypotension and loss of vasoregulation observed in sepsis [25-27]. Indeed, in mouse models of endotoxemia, mice deficient in eNOS production were found to be resistant to LPS-induced hypotension [25, 27].

As previously described, in the presence of infection, leukocytes are recruited to the site of inflammation and can extravasate across the endothelial barrier into affected tissues [2, 28, 29]. Excessive leukocyte recruitment and phagocytic activity, however, can contribute to the tissue and organ damage observed in sepsis [2, 28]. Leukocytes are attracted to the site of inflammation following the production of pro-inflammatory mediators, such as cytokines and chemokines [2, 29]. These circulating leukocytes are then recruited to the endothelial surface through an association of selectin molecules found on the surface of both the rolling leukocytes (L-selectins) and the endothelium (E- and P-selectins) [2, 29]. Leukocyte

adhesion is then further mediated by the tight association between integrins found on the leukocytes, and cell-adhesion molecules (CAMs) expressed on the surface of cytokine-stimulated endothelial cells [29, 30]. LPS stimulation and activation of NF-κB in endothelial cells has been shown to directly increase the expression of E-selectin and CAMs, permitting excessive leukocyte recruitment to the endothelium and promoting a hyper-inflammatory state [28, 30]. Indeed, elevated levels of E-selectins are reflective of poor prognoses in sepsis patients [31, 32].

Under normal conditions, the endothelium acts as a continuous, selectively permeable barrier, permitting the exchange of fluid and macromolecules between the circulating blood and extravascular tissues [13, 15-17]. *In vitro*, LPS stimulation of endothelial cells was found to induce actin reorganization, increase paracellular permeability and mediate detachment of endothelial cells from an underlying extracellular matrix [33]. Furthermore, LPS stimulation has been shown to downregulate vascular endothelial (VE)-cadherin and promote caspase-mediated cleavage of other proteins responsible for the maintenance of intercellular adherens junctions [33, 34]. These junctions are involved in regulating the transport of fluid and materials across the endothelial barrier, therefore disruption of this barrier function in endotoxemia can contribute to severe vascular leakage and edema [15, 33].

The endothelium is also maintained in a non-thrombogenic state under normal physiological conditions [17, 21]. This non-thrombogenic endothelial state is maintained by the activity of several endogenous anti-coagulant systems on the surfaces of endothelial cells. The pro-coagulant enzyme thrombin binds to endothelial cell thrombomodulin and amplifies the cleavage of protein C into its active form. Activated protein C negatively regulates thrombin generation, and thus prevents excess coagulation [16, 35, 36]. In the presence of

severe infection and sepsis, however, the endothelium shifts from an anti-coagulant surface to a pro-coagulant surface, thereby limiting bleeding and isolating the infection [17, 21]. In these cases, reduced expression of anti-coagulant mediators, such as thrombomodulin, shifts the activity of tissue factor and thrombin towards the cleavage of fibrinogen, and the subsequent generation of intravascular fibrin clots [16-18, 35]. Indeed, inhibition of tissue factor reduces clot formation and inflammation in both mouse models of endotoxemia and in septic patients [37]. If left untreated, the pro-coagulant activity of the vasculature can lead to disseminated intravascular coagulation (DIC) in sepsis, which can ultimately cause organ failure and patient death [18, 35, 38].

1.4 Toll-like Receptors

As previously mentioned, numerous cell types, including endothelial cells and immune cells, are capable of encountering and recognizing conserved PAMPs via pattern recognition receptors (PRRs) [21, 23, 39, 40]. These PRRs can be expressed on cell surfaces, intracellular compartments, or even secreted into the blood and extravascular fluids [39, 41]. Recognition of pathogens and immunogenic materials by PRRs plays a critical role in the innate immune response by mediating phagocytosis, activation of the complement system, regulation of coagulation cascades, as well as the stimulation of pro-inflammatory signaling pathways [39, 41].

One of most extensively studied groups of PRRs includes the Toll-like receptor (TLR) family [39, 41, 42]. *Toll* was initially identified in *Drosophila melanogaster* as a maternal-effect gene required for dorso-ventral axis formation during fruitfly embryogenesis [43]. Furthermore, *Toll* mutant flies were found to be rapidly susceptible to fungal infections, implicating a role for Toll-mediated signaling in immunity [43]. Mammalian homologues

were subsequently identified and classified as Toll-like receptors [41, 44]. Currently, there are 10 TLRs that have been identified in humans and 13 TLRs identified in mice [41, 45]. The TLRs comprise a highly conserved family of transmembrane receptors that have been identified to play a key role in the recognition of bacterial, fungal, viral and protozoal immunogenic ligands (Table 1.1) [42, 46]. These receptors are characterized by an extracellular leucine-rich repeat (LRR) domain involved in ligand recognition, and a cytoplasmic Toll/Interleukin-1 receptor (TIR) domain critical for signal transduction [39, 42, 44, 47]. This TIR domain is a highly conserved protein-protein interaction motif found in several transmembrane and cytoplasmic proteins, involved in both animal and plant immunity [39, 48]. The exact subcellular localization of the individual TLRs can vary depending on the specific ligand identified by each receptor (Figure 1.1) [42, 47]. Receptors localized to the endosomal compartment include TLR3, TLR7, TLR8 and TLR9, and have been characterized in the identification of bacterial and viral nucleic acids [42, 47]. The endosomal localization of these receptors may be important in the ability to distinguish self from foreign nucleic acids [49]. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are localized to the plasma membrane, whereas the subcellular localization of TLR11, TLR12 and TLR13 has not yet been described [41, 42, 47]. Exogenous ligands identified for these cell surface TLRs primarily include bacterial and fungal structural components [41, 42]. Several endogenous ligands, including heat shock proteins (HSPs), heparan sulphate and fibrinogen, have also been identified [47, 50]. Furthermore, cooperation through homo- and heterodimerization of these cell surface TLRs is critical for specific ligand recognition and downstream inflammatory pathway activation [39, 41, 47].

Table 1.1 Human and mouse TLRs and their associated ligands. Ligands of TLR1-13 found in humans (h) or mice (m) are shown. TLR1-10 are conserved between humans and mice, however TLR10 is non-functional in mice. TLR11-13 are not found in humans, but are present in mice. Although most of the receptors are conserved in mammals, additional species and their respective TLRs have been excluded as they are not relevant to this thesis. Characterized exogenous and endogenous (host) ligands are shown.

Receptor	Ligand	Origin of Ligand	Species (h or m)
TLR1	Triacyl lipopeptides	Bacteria	h/m
TLR2	Peptidoglycan Glycolipids Lipoteichoic acid Lipoprotein/lipopeptide Zymosan HSP70	Bacteria Bacteria Bacteria Bacteria, Fungi Fungi Host	h/m
TLR3	Double-stranded RNA (dsRNA)	Viruses	h/m
TLR4	LPS HSPs Fibrinogen Heparan sulphate Hylauronic acid	Bacteria Bacteria, Host Host Host Host	h/m
TLR5	Flagellin	Bacteria	h/m
TLR6	Diacyl lipopeptides Lipoteichoic acid Zymosan	Bacteria Bacteria Fungi	h/m
TLR7	Single-stranded RNA (ssRNA)	(RNA) viruses	h/m
TLR8	ssRNA	(RNA) viruses	h/m
TLR9	Unmethylated CpG DNA Hemozoin	Bacteria Protozoa	h/m
TLR10	Unknown	Unknown	h
TLR11	Profilin	Protozoa	m
TLR12	Unknown	Unknown	m
TLR13	Unknown	Unknown	m

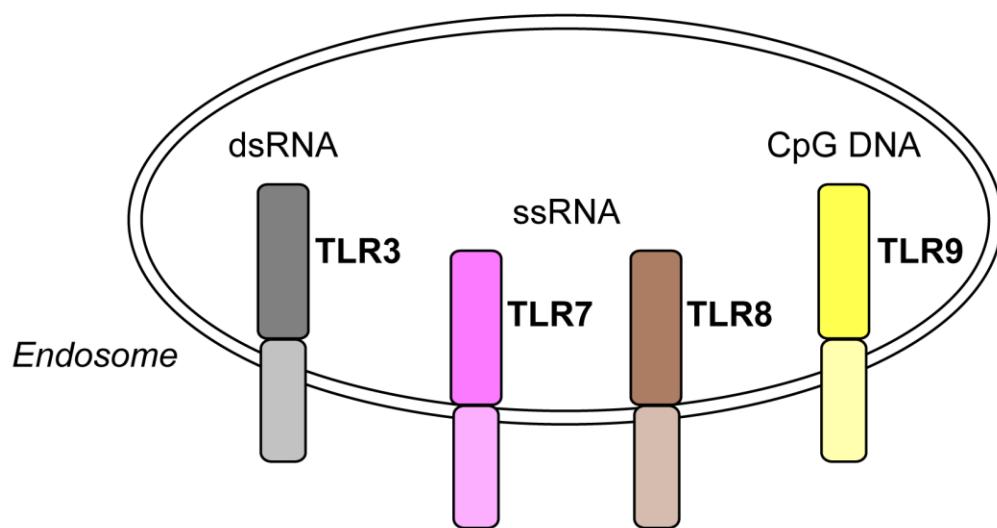
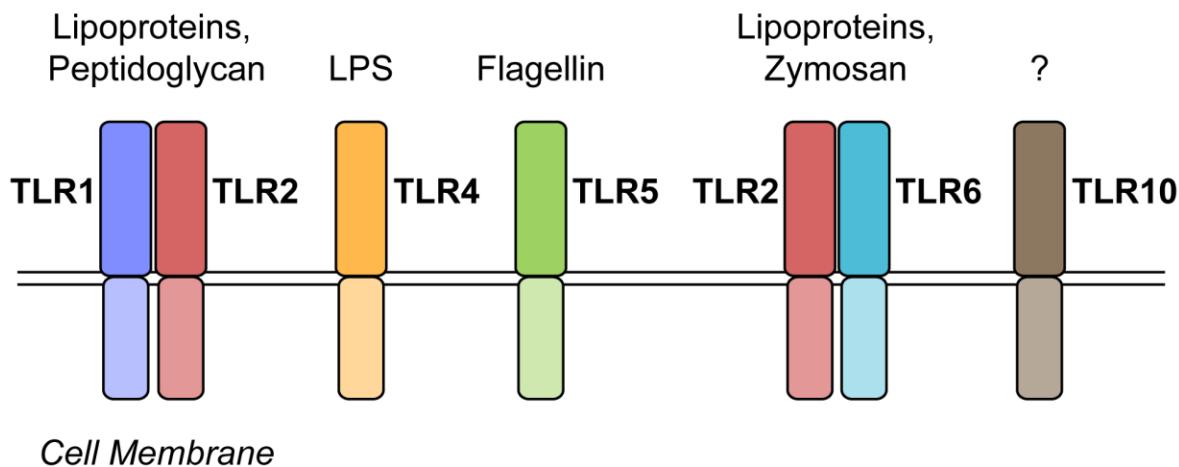


Figure 1.1 Cellular localization and ligands of TLRs. There are currently 10 TLRs that have been identified in humans and 13 TLRs identified in mice. These receptors play a critical role in pathogen recognition and activation of immune responses. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are found on the plasma membrane, whereas TLR3, TLR7, TLR8 and TLR9 are found within endosomal compartments. The localization of TLR11, TLR12 and TLR 13 is currently unknown.

1.5 The TLR4 Signaling Pathway

1.5.1 Recognition of LPS

Lipopolysaccharide (LPS), also referred to as endotoxin, is an essential component of the outer membrane of Gram-negative bacteria [51-55]. The recognition of LPS and subsequent pro-inflammatory pathway activation by immune and endothelial cells remain areas of extensive study due to their significance in the pathogenesis of endotoxemia and sepsis [51-53]. Indeed, the LPS-induced production of pro-inflammatory cytokines by endothelial cells has been shown to significantly contribute to the inflammatory process [17, 56]. LPS has been characterized as an amphiphilic molecule comprised of three key structural elements: a core oligosaccharide, a covalently linked O-specific chain and a membrane anchoring lipid A region responsible for the immunogenic properties of LPS [54, 55].

TLR4 was initially identified as the signaling receptor of LPS based on genetic analyses of the C3H/HeJ mouse strain [57, 58]. These mice were found to have a single point mutation in the TIR domain of *Tlr4*, conferring LPS-insensitivity [57]. Further studies in *Tlr4* null mice demonstrated hypo-responsiveness to LPS, confirming TLR4 as the receptor of LPS [42, 57, 58].

Recognition of LPS by various cell types, including endothelial cells, requires the coordinated interaction of several proteins at the cell surface [51, 53]. LPS-binding protein (LBP) directly binds to and mediates the transfer of LPS to the accessory molecule, CD14 [51, 53]. CD14 exists as both a membrane-anchored protein (mCD14) found on the cell surface, and as a soluble proteolytic fragment (sCD14) found in the serum [59]. Both mCD14 and sCD14 have been found to play critical roles in LPS recognition and signaling in endothelial cells, particularly in LPS-mediated E-selectin expression and leukocyte

recruitment [60, 61]. Expression of mCD14 appears to be limited to primary and early-passage endothelial cells, however, suggesting that LPS-mediated signaling in endothelial cell lines is primarily achieved through serum-borne sCD14 [21, 60-62]. Interaction of LPS with CD14 facilitates delivery, recognition and binding of the lipid A region of LPS to the receptor complex comprised of TLR4 and MD2 [21, 53, 63]. TLR4-MD2-LPS complex formation is facilitated by a characteristic horseshoe-like shape of the extracellular LRR domain of TLR4 [53, 63]. Formation of TLR4-MD2-LPS complexes directly mediates complex dimerization, resulting in a conformational change in the TLR4 intracellular TIR domain interface [42, 51, 53]. This conformational change facilitates the recruitment of several intracellular TIR domain-containing adaptor molecules to the stimulated receptor [42, 53, 63]. With the exception of TLR3, all currently characterized TLRs mediate activation of their respective pro-inflammatory signaling pathways through the activity of the adaptor molecule myeloid differentiation factor 88 (MyD88) [64]. Stimulation of TLR4, however, can mediate the activation of two distinct intracellular signaling pathways through the recruitment of different TIR-containing adaptor family molecules to the receptor [42, 53, 63, 65]. These two pathways include a rapidly-acting MyD88-dependent pathway and a delayed MyD88-independent pathway (Figure 1.2) [42, 52, 53, 65].

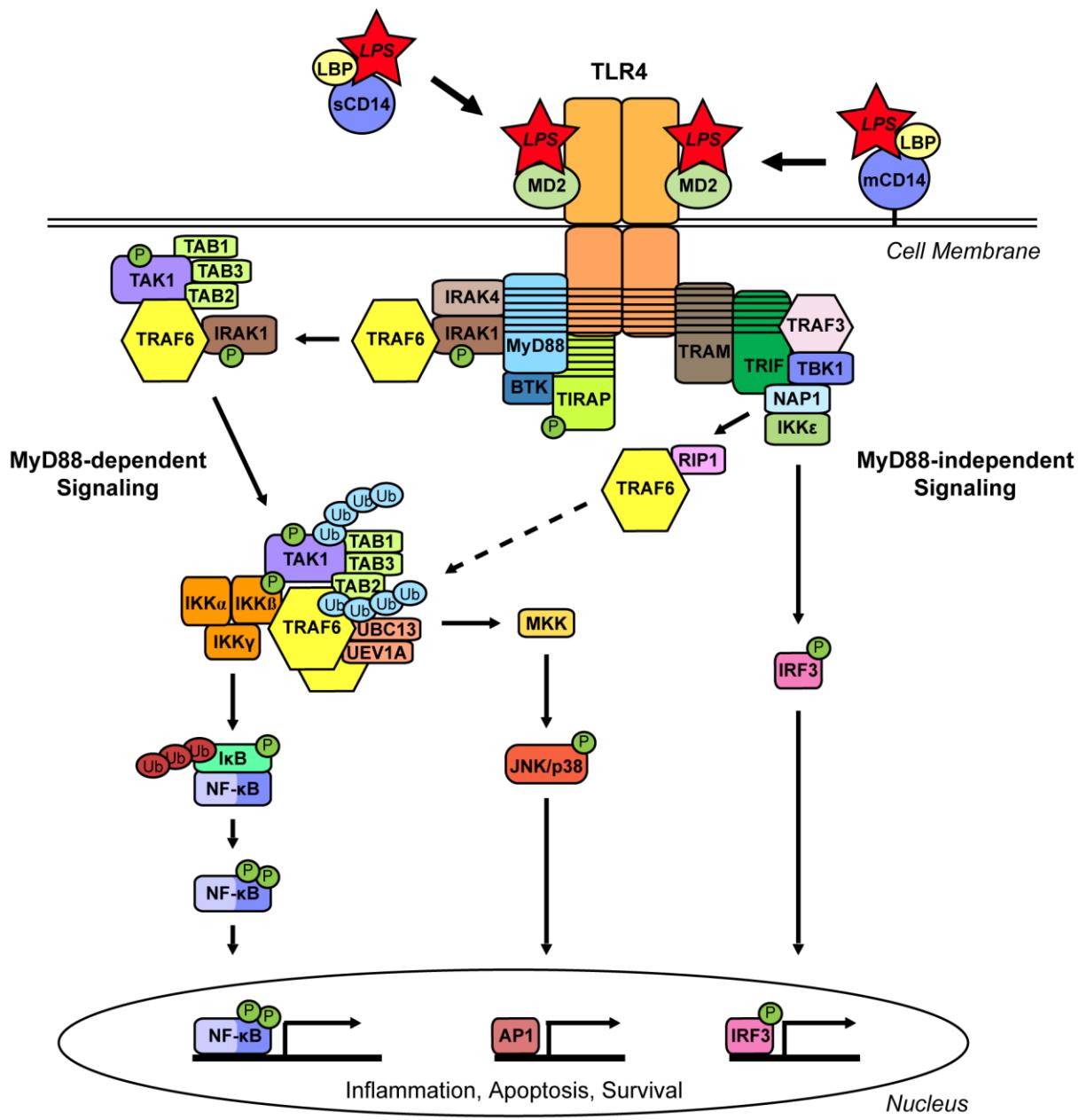


Figure 1.2 The TLR4 signaling pathway. LPS is bound by the TLR4-MD2 signaling complex. Intracellular activation of the MyD88-dependent pathway (left) involves the recruitment of the adaptor molecules MyD88, TIRAP and the IRAKs. Downstream oligomerization and auto-ubiquitination of TRAF6 mediates activation of NF- κ B and MAPKs. Activation of the MyD88-independent pathway (right) involves the recruitment of the adaptor molecules TRAM and TRIF. Association of TRAF3 or TRAF6 with TRIF mediates the downstream activation of IRF3 or NF- κ B, respectively. Activation of both pathways stimulates the production of pro-inflammatory molecules, as well as apoptotic and pro-survival responses. Horizontal lines indicate conserved TIR domains; P, phosphorylation; Ub (blue), K63-linked ubiquitin; Ub (red), K48-linked ubiquitin.

1.5.2 MyD88-Dependent TLR4 Signaling

Early studies identified MyD88 as an adaptor molecule in the Interleukin-1 receptor (IL-1R) signaling complex, possessing both an N-terminal death domain (DD) and a C-terminal TIR domain [66]. Upon LPS binding and dimerization of the TLR4-MD2 receptor complex, MyD88 is rapidly recruited to the receptor through a TIR-TIR interaction [42, 67, 68]. The recruitment of MyD88 is facilitated by another adaptor molecule, TIR-domain containing adaptor protein (TIRAP), which also interacts with the receptor via a TIR-TIR interaction [67, 68]. TIRAP is phosphorylated by TLR4-activated Bruton's tyrosine kinase (BTK), ultimately leading to downstream pro-inflammatory NF-κB and mitogen-activated protein kinase (MAPK) activation [69]. Indeed, TIRAP is required for MyD88-dependent signaling, as mice deficient in TIRAP show significantly delayed activation of TLR4-induced NF-κB and MAPK signals [70].

Assembly of TIRAP and MyD88 at the receptor mediates the recruitment of members of the IL-1R-associated kinase (IRAK) family of kinases, IRAK1 and IRAK4, via interaction with the DD of MyD88 [71, 72]. The association of IRAK4 with the receptor complex facilitates its trans-phosphorylation of IRAK1, inducing the kinase activity of IRAK1 [71, 72]. Subsequent auto-phosphorylation mediates the dissociation of IRAK1 from the receptor complex, facilitating interaction with tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6) [73, 74].

TRAF6 belongs to a family of seven TRAF proteins that have been characterized as critical signaling mediators in the TNF and Toll/Interleukin-1 receptor “superfamilies” [75]. With the exception of the TLR3, TRAF6 is essential for the downstream activation of all characterized TLR pathways [76]. Indeed, *Traf6*-deficient (*Traf6*^{-/-}) mice demonstrate

impaired LPS and Interleukin-1 β (IL-1 β) signaling [77]. TRAF6 is comprised of an N-terminal RING domain followed by five zinc fingers, as well as a C-terminal TRAF domain consisting of both a coiled-coil domain, referred to as the TRAF-N domain, and a highly conserved TRAF-C domain [73, 75]. The RING domain of TRAF6 functions as an E3 ubiquitin ligase to catalyse the synthesis of poly-ubiquitin chains, and is essential for downstream signal activation [78]. The C-terminal TRAF domain is required for TRAF6 oligomerization and protein-protein interactions [75, 78, 79].

Upon dissociation from the receptor, IRAK1 and TRAF6 associate with a complex comprised of transforming growth factor- β (TGF- β)-activated kinase 1 (TAK1) and the adaptor molecules, TAK1-binding protein 1, 2 and 3 (TAB1, TAB2 and TAB3) at the plasma membrane [74]. TAB1 regulates activation of TAK1, whereas TAB2 and TAB3 coordinate the interaction of TAK1 with TRAF6, and facilitate TRAF6 ubiquitination [80-82]. Formation of the TRAF6-TAK1-TAB1/2/3 complex results in the phosphorylation of TAK1 and translocation of the complex to the cytosol [74, 83-85]. This translocation facilitates the release and subsequent proteosomal degradation of IRAK1 [74, 83-85]. Once in the cytosol, the TRAF6-TAK1-TAB1/2/3 complex recruits an ubiquitin-conjugating (E1/E2) complex comprised of UBC13 and UEV1A, and catalyses the formation of lysine 63 (K63)-linked ubiquitin chains on TRAF6 [78, 86]. Stimulus-induced oligomerization of TRAF6 via the TRAF-N domain is essential for this RING domain-mediated auto-ubiquitination process [74, 83-85]. Indeed, deletion of the TRAF-N domain has been shown to abolish TRAF6 oligomerization and auto-ubiquitination, impairing downstream signal activation [78, 79, 86]. The synthesis of K63-linked poly-ubiquitin chains has been shown to mediate the signal transduction activity of proteins, as well as function as a scaffold in signaling complex

assembly [78, 86, 87]. This is in contrast to lysine 48 (K48)-linked poly-ubiquitin chains, which have a well-characterized role in targeting proteins for proteasomal degradation [78, 86, 87]. K63-linked poly-ubiquitination of TRAF6 is required for the subsequent ubiquitination and activation of TAK1 [78, 86, 87]. Activated TAK1 phosphorylates the inhibitor of NF-κB (IκB) kinase (IKK) complex [86, 87]. The IKK complex is comprised of two catalytic subunits, IKK α and IKK β , and a regulatory subunit, IKK γ [88]. Specific phosphorylation of IKK β by TAK1 leads to the activation of the IKK complex and subsequent IKK-mediated phosphorylation of IκB [85, 86]. We have recently identified and characterized a novel scaffold protein, SAM and SH3 domain-containing protein 1 (SASH1), as a novel scaffold molecule in endothelial TLR4 signaling that may act to assemble and facilitate TRAF6-TAK1-IKK activation [89]. Further characterization of SASH1 function will be discussed in greater detail later in this chapter. In endothelial cells, LPS-induced specific phosphorylation of the IκB α subunit of IκB is responsible for subsequent K48-linked poly-ubiquitination and targeted proteasomal degradation [90]. This degradation of IκB facilitates the release and translocation of the transcription factor NF-κB into the nucleus, mediating pro-inflammatory responses [21, 28, 42, 46, 90]. These responses include the production of pro-inflammatory cytokines, such as IL-6 and TNF α , as well as increased leukocyte recruitment through the expression of selectins and integrin adhesion molecules on the endothelial cell surface [21, 28, 30, 88, 91, 92]. Furthermore, LPS-induced NF-κB activation facilitates the expression of several anti-apoptotic factors, such as A1 and A20, in endothelial cells, mediating protection from LPS-induced apoptosis [90, 93].

Activated TAK1 also facilitates phosphorylation and activation the MAPK kinases (MKKs), leading to downstream activation of several members of the MAPK family,

including c-jun NH₂-terminal kinase (JNK) and p38 [86]. Activation of these MAPKs can mediate further activation of both NF-κB and another transcription factor complex, activator protein 1 (AP1), in endothelial cells [85, 94]. LPS-induced activation of TRAF6 and downstream activation of both NF-κB and JNK has been shown to initiate an angiogenic response in endothelial cells directly stimulated with LPS [95]. Furthermore, LPS-induced JNK activation mediates pro-apoptotic signaling in endothelial cells, also in a TRAF6-dependent manner [96]. In parallel, activation of NF-κB and p38 has been shown to up-regulate expression of the pro-inflammatory chemokines Interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) upon LPS stimulation of endothelial cells [97, 98]. Interestingly, LPS-induced activation of p38 was shown to be independent of TRAF6 in endothelial cell types [96].

In summary, TLR4 signaling via the MyD88-dependent pathway mediates the rapid downstream activation of both NF-κB and MAPK-regulated inflammatory signaling in numerous cell types, including endothelial cells [21, 42, 85, 90]. Oligomerization and K63-linked ubiquitination of TRAF6 is a critical regulatory step in the activation of these downstream pro-inflammatory pathways through the subsequent activation of TAK1 kinase activity [78, 79, 86]. Moreover, TRAF6 acts as an important bifurcation point in the regulation of LPS-induced pro- and anti-apoptotic signaling in endothelial cells [21, 93, 96, 99]. The balance of endothelial cell survival and death in the presence of infection is critical in the pathogenesis of sepsis in order to mediate effective immune responses, and to avoid the endothelial dysfunction and severe vascular collapse observed in septic patients [14, 18, 19, 21, 56].

1.5.3 MyD88-Independent TLR4 Signaling

The MyD88-independent TLR4 pathway was initially identified based on work conducted in *MyD88* null (*MyD88*^{-/-}) mice [100]. These mice retained the ability to activate LPS-induced NF-κB and MAPK pathways, albeit with delayed kinetics [100]. Further work conducted in macrophages confirmed that LPS stimulation of wild-type cells led to the production of the pro-inflammatory cytokines IL-6 and TNFα [101]. In MyD88-deficient macrophages, these cytokines were not expressed upon LPS stimulation [101]. These cells were, however, found to induce the expression of interferon (IFN)-inducible genes, such as IFNγ-inducible protein 10 (IP-10) and interferon β (IFNβ), through activation of the transcription factor interferon regulatory factor 3 (IFN3) [101]. Together, these studies led to the discovery of a functional LPS-induced MyD88-independent TLR4 pathway that acts with temporally delayed kinetics compared to the MyD88-dependent pathway [100, 101].

Activation of the MyD88-independent pathway begins following the recruitment of TIR domain-containing adaptor molecule 2 (TRAM) to the intracellular TIR domain of the stimulated TLR4 receptor complex [102-104]. TRAM acts as a bridging adaptor to mediate the recruitment of a second adapter molecule, TIR domain-containing adaptor molecule 1 (TRIF), to the receptor, permitting endocytosis of the receptor-adaptor complex into endosomes [102-104]. This receptor endocytosis is essential for TRIF-dependent signaling and downstream activation of IFNβ [103]. Association of TRIF with TRAF3 and TANK-binding kinase 1 (TBK1) at the endosomal signaling complex results in the recruitment of the non-canonical IκB kinase homolog, IKKε, and NF-κB-activating kinase-associated protein 1 (NAP1) [105, 106]. The subsequent phosphorylation and translocation of IRF3 mediates the production of IFNβ [105-107]. In parallel to IRF3 activation, receptor-bound TRIF can

recruit TRAF6 and receptor interacting protein 1 (RIP1), leading to late-phase activation of NF-κB [106, 108].

Although not extensively studied in endothelial cells, there exists some controversy as to the extent of MyD88-independent signaling in this cell type. A study by Harari *et al.* reported that TLR4 signaling may be restricted to the MyD88-dependent pathway in endothelial cells due to lack of expression of the bridging adaptor TRAM [109]. Another recent study, however, demonstrated expression of TRAM in primary and early-passage endothelial cells, and that the expression of TRAM and activation of the MyD88-independent response was dependent on the expression of endothelial mCD14 [61]. Indeed, further investigation is required to fully elucidate the functional significance of this pathway in LPS-mediated responses in endothelial cell types [61, 109]. The remainder of this thesis will thus focus on the MyD88-dependent TLR4 pathway.

1.6 Identification of SASH1 as a Novel Scaffold Protein in Endothelial TLR4 Signaling

1.6.1 Scaffold Proteins in Immune Signaling

The coordinated regulation of complex signaling cascades is essential to mediate immune signal transduction from cell surface receptors to numerous downstream effector molecules [110]. Scaffold proteins may play an important role in coordinating these complex signals [110]. The functional roles of scaffolds include coordinating the assembly and cellular localization of signaling complexes, regulating positive and negative feedback loops in signaling cascades, and protecting activated signaling molecules from subsequent inactivation or degradation [110].

Scaffold proteins have been defined as molecules that are capable of interacting with at least two other signaling proteins, mediating the regulation and cellular localization of signal

transduction pathways [110]. The ability of scaffolds to interact with multiple signaling effector proteins is often mediated by the presence of several putative or conserved protein interaction domains within these molecules [110]. As previously described for the TLR4 pathway, TLR-mediated signaling cascades can lead to numerous protein-protein interactions and signaling complex assembly [21, 42, 46, 53]. Extensive research has focused on the characterization of interactions between these TLR-mediated signaling proteins, however, the role of scaffold proteins and the mechanisms regulating these interactions remain largely unclear [110, 111].

1.6.2 SAM and SH3 Domain Containing Protein 1 (SASH1)

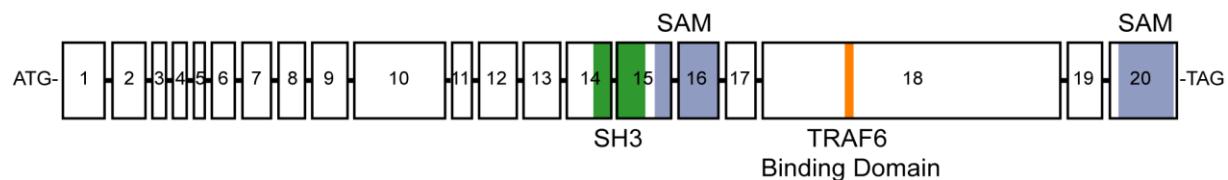
SASH1 was initially identified as a candidate tumour suppressor in several solid cancers, including breast, colon, lung and thyroid [112, 113]. Downregulation of *SASH1* in breast and colon tumours was found to correlate with increased tumour size, metastasis formation and overall poor patient survival [112, 113]. Ongoing studies aim to characterize the functional role of SASH1 in the regulation of the proliferation, apoptosis and migration/invasion capacity of several established cancer cell lines, as well as the mechanisms modulating *SASH1* expression in tumour cells [112-116].

Human *SASH1* is located on chromosome 6q24.3 and is comprised of 20 exons [112, 113]. *SASH1* encodes a large 1247 amino acid protein with a predicted molecular weight of 137 kDa, however, we and others have found that SASH1 predominantly exists with a molecular weight of 170 kDa, indicative of possible post-translational modifications [89, 112-114]. SASH1 is conserved in many organisms, with approximately 85% sequence identity between the human and mouse proteins [113]. Furthermore, SASH1 contains several putative interaction domains, including one Src homology 3 (SH3) domain and two sterile- α -

motif (SAM) domains, indicative of an adapter or scaffolding function (Figure 1.3) [113]. SH3 domains have been well-characterized to mediate protein-protein interactions through the binding of proline-rich sequences [117-119]. SAM domains have also been characterized as protein-protein interaction domains that can mediate homo- or hetero-oligomers with other SAM domain-containing or non-SAM domain-containing proteins [120].

We have recently identified and characterized SASH1 as a novel scaffold molecule in endothelial TLR4 signaling [89]. Previous studies have demonstrated that innate immune signaling components can concentrate in lipid-rich microdomains, called lipid rafts, on the plasma membrane [121]. In response to LPS, these lipid rafts have been shown to cluster TLR4 signaling components and facilitate downstream signaling [121]. Our lab has previously identified and characterized Fas-associated death domain protein (FADD) as a negative regulator of TLR4 signaling in endothelial cells by impairing the interaction of MyD88 with IRAK1 [122]. Indeed, *FADD*^{-/-} mouse embryonic fibroblasts (MEFs) demonstrate hyper-activation of NF-κB and JNK in response to LPS stimulation [122, 123]. As a result of this hyper-activation, we hypothesized that LPS signaling molecules would be enriched in the lipid rafts of *FADD*^{-/-} cells [89]. Subsequent lipid raft isolation and mass spectrometry analysis were conducted to identify innate immune signaling proteins enriched in the lipid rafts of hyper-active *FADD*^{-/-} cells that may only be detected in limiting amounts in wild-type cells [89]. This analysis resulted in the identification of SASH1 as a candidate signaling molecule in LPS-mediated pathways [89].

A



B

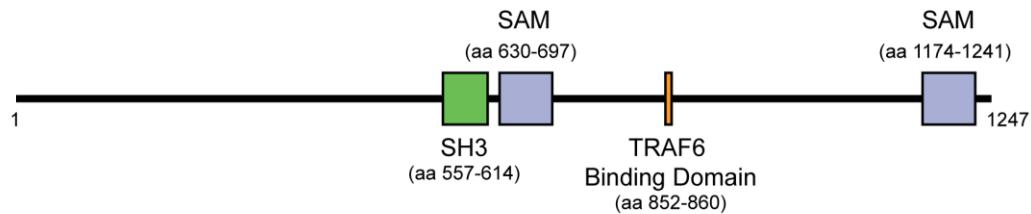


Figure 1.3 Structure of SASH1. Schematic of (A) the genomic structure of human *SASH1* and (B) the structure of the human SASH1 protein. *SASH1* is comprised of 20 exons and encodes a 1247 amino acid protein containing one SH3 domain and two SAM domains. Furthermore, a consensus TRAF6-binding motif has been identified and functionally confirmed in our lab. ATG, start codon; TAG, stop codon; aa, amino acid.

SASH1 belongs to a family of SAM and SH3 adaptor proteins comprised of SH3 domain expressed in lymphocytes (SLY1) and hematopoietic adaptor containing SH3 and SAM domains 1 (SLY2, also called HACS1 or SAMS1) [89, 112, 115]. The SLY family has been characterized in the regulation of the adaptive immune response, initially implicating a role for SASH1 in immune responses [89, 124-127]. *SASH1* mRNA is ubiquitously expressed in human tissues, with the highest levels found in the lung, placenta, spleen and thymus [112, 113]. Our lab confirmed that *Sash1* is preferentially expressed in the microvascular endothelium of numerous mouse organs, including the spleen, thymus, lungs, liver and kidneys [89]. Interestingly, *Sash1* was not expressed in the lymphoid cells of the spleen or thymus [89]. Expression of *Sash1* was confirmed in isolated mouse lung endothelial cells, whereas *Sly1* and *Sly2* were predominantly expressed in non-endothelial cell populations [89]. The preferential expression of *Sash1* in mouse endothelial cells, but not in lymphoid cells, implicated a role for SASH1 as a potential immune signaling molecule in non-immune cells [89].

Further studies conducted in our lab revealed SASH1 to function downstream of LPS to positively regulate NF-κB-mediated pro-inflammatory cytokine production in human microvascular endothelial cells (HMEC) [89]. SASH1 was also found to be required for LPS-induced phosphorylation and activation of JNK and p38 in HMEC [89]. Interestingly, SASH1 was not found to be involved in LPS-induced IRF-regulated signaling [89]. Furthermore, SASH1 expression was not found to modulate IL-1 β -induced signaling, despite the similarities in the TLR4- and IL-1R-mediated downstream pathways [89, 128]. Co-immunoprecipitation studies conducted in human embryonic kidney 293T (HEK293T) cells identified the interaction of SASH1 with TAK1 and TRAF6, but not MyD88, IRAK1 or

IRAK4 [89]. Furthermore, SASH1 was found to interact with the catalytic subunits of the IKK complex, IKK α and IKK β , but not the regulatory subunit IKK γ [89]. *In silico* analyses revealed a consensus TRAF6 binding sequence within amino acids 852-860 of human SASH1 that was functionally confirmed to be required for SASH1-TRAF6 binding (Figure 1.3) [89]. The specific interaction of SASH1 with TRAF6, but not TRAF2 or TRAF3, confirmed that SASH1 may be acting in the MyD88-dependent TLR4 signaling pathway downstream of an LPS stimulus [89].

SASH1 was further characterized to interact with the C-terminal TRAF domain of TRAF6 [89]. Interestingly, specific deletion of the coiled-coil domain within the TRAF domain of TRAF6 impaired interaction with SASH1 by co-immunoprecipitation [89]. This finding was indicative of a role for SASH1 in the oligomerization and ubiquitin-mediated activation of TRAF6 [89]. Indeed, enforced expression of SASH1 was found to mediate auto-ubiquitination of TRAF6 independent of an LPS stimulus in HEK293-TLR4-MD2-CD14 cells [89]. Further studies conducted in these cells confirmed that this observed ubiquitination was specifically activating K63-linked poly-ubiquitination on TRAF6, and not degradative K48-linked poly-ubiquitination [89]. Although SASH1 was not found to interact with the ubiquitin conjugating enzymes, UBC13 or UEV1A, interaction of SASH1 with TRAF6 may be critical for the ubiquitin-mediated activation of TRAF6 [89]. The K63-linked poly-ubiquitination of TRAF6 facilitates TRAF6-TAK1-IKK complex formation, and is required for the subsequent ubiquitination and activation of TAK1 [78, 86, 87]. Interestingly, enforced expression of SASH1 was also found to mediate the ubiquitination of TAK1 in an LPS-independent manner, possibly through the ubiquitination of TRAF6 [89]. Collectively, these experiments characterized SASH1 as a novel scaffold molecule in the MyD88-

dependent TLR4 signaling pathway in endothelial cells [89]. Upon LPS stimulation of TLR4, SASH1 may coordinate the assembly of the TRAF6-TAK1-IKK complex, facilitating downstream activation of NF-κB and MAPKs (Figure 1.4) [89]. Furthermore, SASH1 may play a critical role in the regulation of LPS-induced K63-linked poly-ubiquitination and activation of TRAF6, a critical step for downstream signal activation [89].

Attempts to evaluate the role of *Sash1* in innate immune signaling *in vivo* have remained unsuccessful thus far [89, 129, 130]. In an effort to study the essential role of *Sash1* in a mouse model of sepsis, we generated a gene-trap mouse specifically targeting *Sash1* [89, 129, 130]. Insertion of this gene trap was mapped to intron 14 of *Sash1*, disrupting the expression of the C-terminal SH3 and SAM domains, as well as the putative TRAF6 binding domain within *Sash1* [89, 129, 130]. Unfortunately, homozygous *Sash1* gene-trap mice demonstrated neonatal lethality and were unable to be used in our study [89, 129, 130]. This neonatal lethality was attributed to an apparent defect in lung development and respiration [130]. Future studies involving the conditional or inducible knockout of *Sash1* are critical in determining the role of this protein *in vivo* as an essential regulator of innate immune signaling and LPS-mediated responses in models of sepsis.

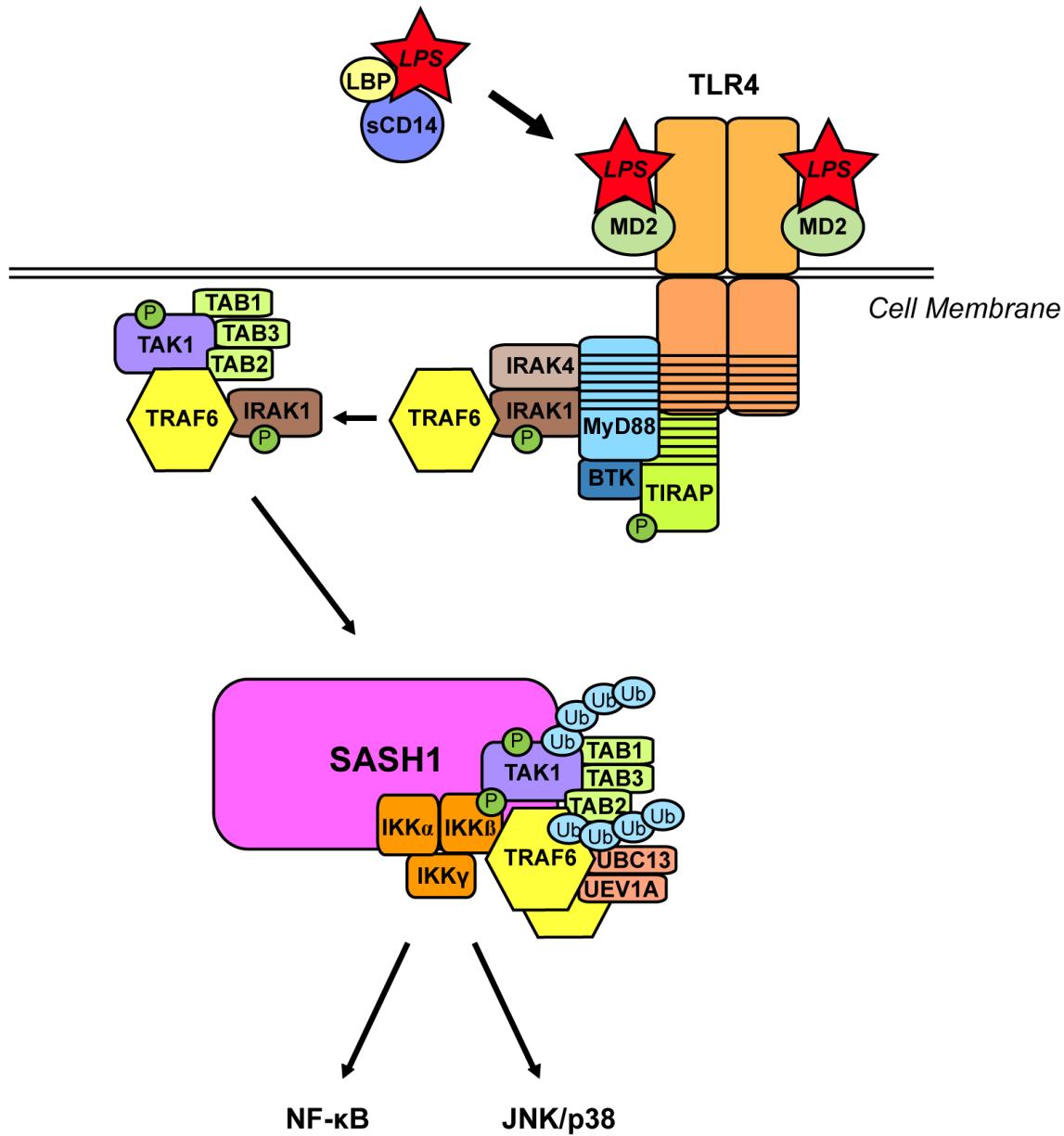


Figure 1.4 Model for SASH1 function in the MyD88-dependent TLR4 signaling pathway in endothelial cells. Upon LPS stimulation of TLR4, SASH1 acts as a scaffold to coordinate the assembly of the TRAF6-TAK1-IKK complex, facilitating downstream activation of NF-κB and MAPKs. SASH1 also plays an important role in mediating the LPS-induced K63-linked poly-ubiquitination and activation of TRAF6, facilitating downstream signal transduction. P, phosphorylation; Ub, K63-linked ubiquitin.

1.7 Negative Regulation of TLR4 Signaling

In the presence of infection, TLR-mediated responses are absolutely critical for host defense and pathogen clearance [52, 53, 131, 132]. Aberrant activation of LPS-induced TLR4 signaling, however, has been associated with the pathogenesis of Gram-negative sepsis [52, 53, 131, 132]. Hyper-activation of TLR4 signaling in these cases may be due to the direct overstimulation of TLR4, or due to the dysregulation of endogenous inhibitory processes [131]. Proper negative regulation of TLR4 signaling is therefore essential to avoid the development of such uncontrolled, hyper-inflammatory syndromes [17, 52, 131-133]. Furthermore, understanding these regulatory processes is critical for the development of therapeutic strategies against inflammatory diseases, such as sepsis [17, 52, 131-133].

Negative regulation of the MyD88-dependent TLR4 pathway is mediated by numerous endogenous mechanisms, at several points throughout the pathway [131, 132]. The expression of these endogenous negative regulators is often induced upon TLR4 stimulation, acting as a negative feedback mechanism to terminate pro-inflammatory signals [131-133]. The expression of a soluble isoform of Tlr4 (sTlr4) has been shown to impair LPS-induced NF- κ B and TNF α production in mouse macrophages *in vitro* [134]. sTLR4 may also function in humans to bind MD2 and impair TLR4-MD2-LPS complex formation and downstream pathway activation, although further investigation is required [134]. At the intracellular level, several inhibitory proteins can interact with the receptor complex to impair adapter recruitment and downstream signaling [132]. LPS-induced expression of MyD88s, a splice variant of MyD88, impairs the recruitment of IRAK4 to the receptor complex, inhibiting subsequent trans-phosphorylation and activation of IRAK1 [71, 135]. As previously

described, our lab has characterized FADD as a negative regulator of endothelial TLR4 signaling by functionally impairing the interaction of MyD88 with IRAK1 [122].

Downstream of the TLR4 receptor complex, several mechanisms act to impair LPS-induced signaling at the level of TRAF6 activation [132]. Upon LPS stimulation, oligomerization and K63-linked poly-ubiquitination of TRAF6 are required for the downstream activation of NF-κB and MAPKs [76, 78, 79, 86, 87]. The LPS-induced anti-apoptotic protein A20 has recently been characterized as a negative regulator of TRAF6 activation [136-138]. The NF-κB-dependent expression of A20 allows this protein to function in a negative feedback manner to de-ubiquitinate TRAF6 and terminate downstream signaling [136-138]. Similarly, Protein Phosphatase 4 (PP4) was shown to interact with TRAF6 in an LPS-dependent manner, impairing downstream NF-κB activation [139]. Interestingly, PP4 did not demonstrate de-ubiquitinase activity, but may instead impair TRAF6 ubiquitination through physical interaction or conformational changes [139]. At the post-transcriptional level, the LPS-induced, NF-κB-dependent expression of microRNA-146a (miR-146a) has been shown to target and decrease the expression of both TRAF6 and IRAK1 in a negative feedback manner [140, 141].

Collectively, the mechanisms briefly described above demonstrate the diverse modes of endogenous negative regulation of TLR4 signaling, particularly in the regulation of TRAF6 activation [136-141]. These diverse negative regulatory mechanisms remain an area of active research due to the pathophysiological nature of inflammatory signals upon their dysregulation [131, 132]. A complete summary of the negative regulatory mechanisms of TLR4 signaling, however, is beyond the scope of this thesis. The following section will focus

on the characterized roles of the two additional negative regulators of TLR4 signaling, β -arrestin 1 and β -arrestin 2, specifically in the regulation of LPS-induced TRAF6 activation.

1.8 Regulation of TLR4 Signaling by the β -arrestins

The arrestin family of proteins includes two visual arrestin isoforms, found exclusively in retina, as well as the ubiquitously expressed β -arrestin isoforms, β -arrestin 1 and β -arrestin 2 [142]. The β -arrestins were initially characterized as “arresting” proteins involved in the desensitization and termination of β -adrenergic receptor signaling [143, 144]. Further studies revealed that the β -arrestins could function in the internalization of numerous seven-transmembrane receptors (7TMRs), a process critical for receptor recycling and degradation [144, 145]. By acting as adaptors for β 2-adaptin (AP2) and clathrin, the β -arrestins have been shown to recruit activated 7TMRs to clathrin-coated pits, facilitating receptor endocytosis [146-148]. In addition to their classic roles in receptor desensitization and internalization, the β -arrestins have been characterized as scaffolds for MAPK signaling, nuclear trafficking molecules, transcriptional regulators and, as will be further discussed, as regulators of immune signaling [144, 145, 149, 150].

The β -arrestin 1 (*Arrb1*) and β -arrestin 2 (*Arrb2*) proteins share approximately 78% sequence identity, and are comprised of two major domains: an N-domain and a C-domain (Figure 1.5) [145, 150]. β -arrestin 1 is found in both the nucleus and cytosol, whereas β -arrestin 2 is primarily confined to the cytosol by the presence of a C-terminal nuclear export signal (NES) [145, 151]. Studies in knockout mice have shown *Arrb1*^{-/-} and *Arrb2*^{-/-} mice to be viable, however, double-null (*Arrb1*^{-/-} *Arrb2*^{-/-}) mice die during embryogenesis [152]. These findings may be indicative of functional redundancy between the β -arrestin isoforms [145, 152, 153].

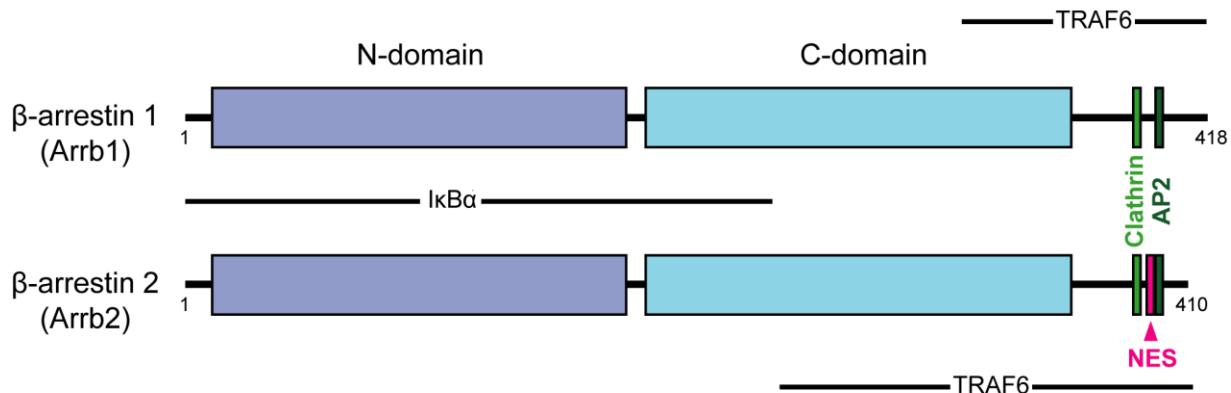


Figure 1.5 Structure of the β -arrestin proteins. β -arrestin 1 (Arrb1) is comprised of 418 amino acids and has a molecular weight of 55 kDa, whereas β -arrestin 2 (Arrb2) is comprised of 410 amino acids and has a molecular weight of 50 kDa. Structurally, both β -arrestins consist of a defined N-domain and C-domain. β -arrestin 1 is found in both the nucleus and cytosol, whereas β -arrestin 2 is primarily confined to the cytosol by the presence of a C-terminal nuclear export signal (NES). The β -arrestins have been extensively characterized in 7TMR receptor desensitization and internalization through association with clathrin and AP2 (protein interaction sites indicated). As negative regulators of immune signaling, both β -arrestins have been shown to interact with IkB α to impair TNF α -induced activation of NF- κ B. β -arrestin 1 and β -arrestin 2 have also been shown to interact with TRAF6 and negatively regulate LPS-induced activation of NF- κ B and AP1. The approximate protein interaction regions for IkB α and TRAF6 are shown.

The β -arrestins were initially characterized in innate immune signaling as negative regulators of TNF α -induced NF- κ B activation [154, 155]. These studies revealed a novel interaction of the β -arrestins with I κ B α , functionally impairing the phosphorylation and degradation of I κ B α and attenuating downstream NF- κ B activation [154, 155]. Furthermore, the β -arrestins were shown to interact with IKK α and IKK β , suggesting that these proteins can interact with multiple innate immune signaling components to facilitate regulation [155]. A later study by Wang *et al.* revealed the interaction of both β -arrestin 1 and β -arrestin 2 with TRAF6 [156]. Furthermore, TRAF6 was confirmed to interact directly with the β -arrestin 1 isoform [156]. Interestingly, endogenous interaction of TRAF6 with the β -arrestins was enhanced upon LPS stimulation of both human monocytic cell lines and mouse-derived splenocytes [156]. Co-immunoprecipitation studies in HEK293T cells revealed that the C-termini of the β -arrestins were required for interaction with TRAF6 (Figure 1.5) [156]. Furthermore, the β -arrestins were found to interact specifically with the TRAF-N domain of TRAF6 (Figure 1.6) [156]. As previously described, this TRAF-N domain consists of a coiled-coil domain required for TRAF6 oligomerization and K63-linked poly-ubiquitination [77-79, 81, 86]. Further experiments revealed that the enforced expression of both β -arrestin 1 and β -arrestin 2 was capable of impairing TRAF6 oligomerization and auto-ubiquitination in HEK293T cells [156]. This was reflective of impaired NF- κ B and AP-1 activity in these cells, as well as decreased LPS- and IL-1 β -induced pro-inflammatory cytokine production in monocytes upon overexpression of the β -arrestins [156, 157]. Conversely, knockdown of the individual β -arrestins was found to increase LPS-induced NF- κ B and AP-1 activity and pro-inflammatory cytokine production, and restored IL-1-induced TRAF6 auto-ubiquitination [156, 157]. Furthermore, LPS-induced TRAF6 auto-ubiquitination was shown to be

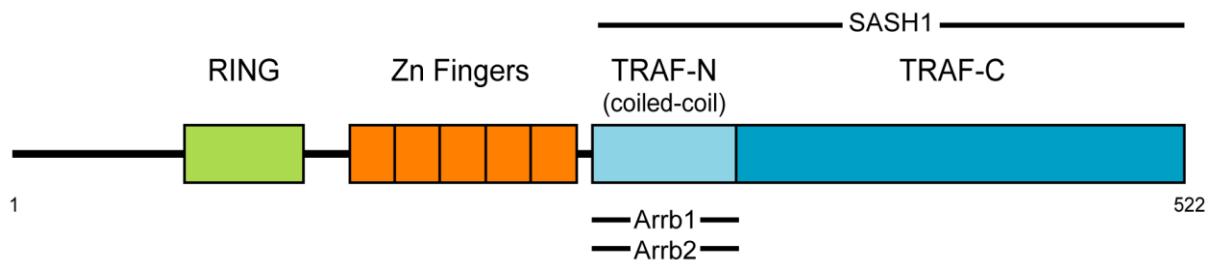


Figure 1.6 Structure of TRAF6 and characterized SASH1 and β -arrestin interaction regions. TRAF6 belongs to a family of TRAF proteins that have been characterized as critical signaling mediators in innate immune signaling. TRAF6 is essential for the downstream activation of all characterized TLR pathways, except TLR3. TRAF6 is a 522 amino acid protein (molecular weight of approximately 60 kDa) consisting of an N-terminal RING domain, followed by five zinc (Zn) fingers and a C-terminal TRAF domain. The TRAF domain is comprised of a coiled-coil domain, referred to as the TRAF-N domain, and a highly conserved TRAF-C domain. The RING domain of TRAF6 functions as an E3 ubiquitin ligase to mediate auto-ubiquitination of TRAF6 following stimulation of TLR4. The TRAF domain is required for TRAF6 oligomerization and protein-protein interactions. Oligomerization TRAF6 through the TRAF-N domain is essential for auto-ubiquitination and downstream signal activation. We have shown that the TRAF domain is required for SASH1-TRAF6 binding, and that SASH1 may play a critical role mediating auto-ubiquitination of TRAF6. β -arrestin 1 (Arrb1) and β -arrestin 2 (Arrb2) have been shown to interact with the TRAF-N domain of TRAF6 to directly impair TRAF6 oligomerization and auto-ubiquitination downstream of TLR4.

enhanced in *Arrb1*^{-/-} *Arrb2*^{-/-} MEFs [156]. Studies in mice have focused primarily on the role of the β-arrestin 2 isoform as a negative regulator of Gram-negative sepsis [156, 158-160]. Indeed, *Arrb2*^{-/-} mice have been found to be more susceptible to endotoxic shock compared to wild-type, as demonstrated by increased circulating cytokine levels, more severe lung damage, higher bacterial loads and overall poorer survival in mouse models of endotoxemia [156, 158-160]. In summary, these studies characterized a novel role for the β-arrestin proteins as negative regulators of LPS-induced pro-inflammatory signaling.

Several recent studies suggest that the β-arrestins do not always function with redundant roles in immune signaling [149, 159-162]. Furthermore, β-arrestin-mediated regulation of pro-inflammatory signaling may vary in a cell-type specific manner [149, 160]. Despite the characterization of both β-arrestin 1 and β-arrestin 2 as negative regulators of TLR4-mediated signals, studies in mouse splenocytes have demonstrated isoform-specific regulation of LPS-induced pro-inflammatory cytokine production [149, 159, 160]. LPS-induced IL-6 and TNFα production was decreased in splenocytes isolated from *Arrb1*^{-/-} mice compared to wild-type [149, 159]. In *Arrb2*^{-/-} splenocytes, however, LPS-induced IL-6 and TNFα production was increased compared to wild-type [149, 159, 160]. Similar studies in macrophages demonstrate differential roles for the β-arrestins in regulating LPS-mediated signaling [149, 159, 160, 162]. Interestingly, LPS stimulation was found to decrease β-arrestin 1, but not β-arrestin 2, expression at both the transcriptional and post-translational levels in primary mouse peritoneal macrophages [162]. Furthermore, studies have found that loss of β-arrestin 2 alone in *Arrb2*^{-/-} peritoneal macrophages and bone marrow-derived macrophages (BMDMs) had no effect on LPS-induced IL-6 or TNFα production compared to wild-type cells [159, 160]. This was in disagreement, however, with previous reports

showing significantly increased levels of LPS-induced IL-6 and TNF α in BMDMs isolated from *Arrb2*^{-/-} mice [156]. The discrepancy in these results may have been due to different sources and concentrations of LPS used in these studies [156, 159, 160]. It remains clear, however, that the β -arrestins may regulate TLR4-mediated responses in both an isoform-specific and cell type-dependent manner, and that further studies are required to fully elucidate these differential regulatory mechanisms [149, 159-162].

To date, few studies have examined the role of the β -arrestins in endothelial cells. Moreover, there are currently no studies evaluating the role of these proteins in the regulation of endothelial TLR4 signaling.

1.9 Aim of the Present Study

As previously described, we have recently identified and characterized SASH1 as a novel scaffold protein in endothelial TLR4 signaling [89]. SASH1 was found to positively regulate LPS-induced NF- κ B and MAPK activation in endothelial cells, and was shown to interact with several components of the MyD88-dependent TLR4 pathway, including TRAF6 [89]. Furthermore, enforced expression of SASH1 was found to mediate LPS-independent K63-linked poly-ubiquitination of TRAF6, suggesting a critical role for SASH1 in the regulation of TRAF6 activation [89].

The overarching aim of this study was to further characterize the critical role of SASH1 in the regulation of TRAF6 activation in the endothelial TLR4 pathway. We confirmed the interaction of endogenous SASH1 with TRAF6 in an LPS-dependent manner in endothelial cells. Furthermore, SASH1 was found to be required for LPS-induced TRAF6 auto-ubiquitination in this cell type. Using a yeast two-hybrid approach, we identified β -arrestin 1 as a novel interacting partner of SASH1. Both β -arrestin 1 and β -arrestin 2 have been shown

to act as negative regulators of the TLR4 pathway by directly impairing TRAF6 oligomerization and auto-ubiquitination [156]. Given the role of SASH1 as a scaffold protein, we hypothesized that SASH1 may coordinate the interaction of TRAF6 and the β -arrestins to mediate regulation of the TLR4 pathway. The specific aims of this study were thus to confirm and characterize the interaction of SASH1 with the β -arrestins, and to elucidate the role of SASH1 in the β -arrestin-mediated regulation of TRAF6 activation. Indeed, our findings confirmed the interaction of SASH1 with both β -arrestins, and attempted to characterize the functional role of SASH1-TRAF6- β -arrestin complex formation on the regulation of TRAF6 ubiquitination. Interestingly, we found that SASH1 may differentially regulate TRAF6- β -arrestin binding between the β -arrestin isoforms. These results may be reflective of differential, isoform-specific roles for the β -arrestins in endothelial cells through specific interaction with SASH1.

Further research into the regulation of the TLR4 pathway and LPS-mediated signaling in endothelial cells is critical to understand the underlying mechanisms contributing to the endothelial dysfunction observed in Gram-negative sepsis [14, 17, 52, 56]. Taken together, the work presented in this thesis further investigates the critical role of SASH1 as a scaffold molecule in TLR4-mediated signaling, contributing to our understanding of the molecular mechanisms involved in the activation and regulation of innate immune responses.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Culture

The human microvascular endothelial cell line (HMEC) was provided by the Centers for Disease Control and Prevention (Atlanta, GA). HMEC were cultured in MCDB 131 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT), 2 mM glutamine (Sigma-Aldrich, St. Louis, MO) and 100 U each of penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO). Human embryonic kidney 293T (HEK293T) and HEK293-TLR4-MD2-CD14 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated (FBS), 2 mM glutamine and 100 U each of penicillin and streptomycin. HEK293-TLR4-MD2-CD14 medium was further supplemented with 100 µg/mL Normocin™, 10 µg/mL Blasticidin and 50 µg/mL of HygroGold™ (InvivoGen, San Diego, CA). All cells were cultured at 37°C in 5% CO₂.

For experiments involving LPS stimulation, cells were washed once with serum-free medium and subsequently incubated overnight in medium containing 2% FBS for 12 hours. Cells were stimulated with 100 ng/mL LPS (purchased from Sigma-Aldrich, St. Louis, MO) for all indicated times.

2.2 Recombinant Plasmids and Gene Transfer

Unless otherwise described, all plasmid constructs used in this study were generated in our lab by a previous graduate student, Shauna Dauphinee. These include pcDNA3-Flag-SASH1, pcDNA3-HA-SASH1, pcDNA-HA-SASH1ΔSH3, pcDNA3-HA-SASH1ΔSAM1, and pcDNA3-Myc-SASH1ΔTRAF6BD [129]. The following rat β-arrestin 1 (Arrb1) and β-arrestin 2 (Arrb2) constructs were also generated by Shauna Dauphinee and used in this

study: pcDNA-Arrb1-HA, pcDNA-Arrb1-HA (aa 1-179), pcDNA-Arrb1-HA (aa 179-418), pcDNA-Arrb1-HA (aa 319-418), pcDNA-Arrb2-HA, pcDNA-Arrb2-HA (aa 1-185), pcDNA-Arrb2-HA (aa 185-410) and pcDNA-Arrb2-HA (aa 241-410), which were cloned from either pEGFPN3-Arrb1 or pEGFPN3-Arrb2 (gift from M. Caron, Duke University, Durham, NC). pRK5-Flag-TRAF6 was obtained from Tularik, (San Francisco, CA). Transfections of plasmid constructs were carried out using the TransIT®-LT1 Transfection Reagent (Mirus Bio Corporation, Madison, WI) according to the manufacturer's instructions.

2.3 RNA Interference

Target cells were transduced with shRNAs targeting either human SASH1 mRNA (shSASH1 573-GCTAATGATGGTCAAAGATTCAAGAGA-593) or a control random sequence (shRandom GTTGCTTGCCACGTCCTAGAT) using lentiviral-mediated gene transfer. The shSASH1 construct used in this study was one of two that has previously been used in our lab to demonstrate impaired activation of LPS signaling upon knockdown of SASH1 [89, 129, 130]. Lentiviral particles were produced from HEK293T cells co-transfected with 5 µg pLentilox-shSASH1 or pLentilox-shRandom vector, 3 µg pVSVG, 3 µg pREV and 3 µg pRRE per 100 mm dish. Transfections were carried out using TransIT®-LT1 Transfection Reagent (Mirus Bio Corporation, Madison, WI) according to the manufacturer's instructions. 48 h and 72 h post-transfection viral supernatants were filtered and used to transduce target cells. shRNA-mediated knockdown of SASH1 was confirmed by immunoblotting.

2.4 Protein Assay

Cell lysates were assayed for total protein concentration using the colorimetric BioRad DC™ Protein Assay Kit (BioRad Laboratories, Hercules, CA). Protein samples were diluted

5-fold in distilled water, and 5 µL of each diluted sample were added to a dye containing 25 µL alkaline copper tartarate solution, 0.5 µL surfactant solution and 170 µL Folin reagent. Following 15 min incubation at room temperature, absorbances of the protein-dye mixtures were measured at 650 nm. Protein concentrations were calculated against a standard curve of bovine serum albumin (BSA) at concentrations of 0, 0.125, 0.25, 0.5, 1.0 and 2.0 mg/mL.

2.5 Immunoblotting

Immunoblotting was conducted using 25-50 µg of protein, diluted in Laemmli sample buffer (60mM Tris-HCl, pH 6.8, 2% SDS, 10% Glycerol, 5% β-Mercaptoethanol, 0.01% Bromophenol Blue). Protein samples were boiled, denatured and reduced at 95°C for 8 min before being loaded onto 10% acrylamide gels. A pre-stained protein ladder (Thermo Scientific, Rockford, IL) was loaded adjacent to protein samples to allow for molecular weight determination. Immediately following electrophoresis, proteins were electro-transferred from acrylamide gels onto nitrocellulose membranes (BioRad Laboratories, Hercules, CA). Upon completion of transfer, membranes were blocked in 5% skim milk powder (w/v) in Tris-buffered saline containing Tween-20 (TBS-T; 50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.05% Tween-20) for 1 h at room temperature. Blocked membranes were then incubated overnight at 4°C with the primary antibody of interest diluted in 5% skim milk powder (w/v) in TBS-T. The primary antibody concentrations used in this study were as follows: anti-TRAF6 (1:1000), anti-Ubiquitin (1:1000), anti-β-arrestin 1 (Arrb1; 1:500), and anti-β-arrestin 2 (Arrb2;1:500) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-FlagM2 (1:5000) and anti-GAPDH (1:10000) from Sigma-Aldrich, St. Louis, MO; anti-HA (1:1000; Covance, Emeryville, CA); anti-GFP (1:1000; Roche, Mississauga, ON); and anti-SASH1 (1:1000; Novus Biologicals, Oakville, ON). Following overnight incubation, primary

antibodies were removed and membranes were washed three times with TBS-T for 15 min each. Membranes were then incubated for 1 h at room temperature with the corresponding species of secondary antibody (1:5000-1:10000, HRP-conjugated IgG; Sigma-Aldrich, St. Louis, MO) in 5% skim milk powder (w/v) in TBS-T. Following this incubation, membranes were washed again three times with TBS-T for 15 min each. Upon completion of the washes, membranes were incubated in a 1:1 mixture of enhanced chemiluminescence (ECL) reagents (PerkinElmer Life Sciences, Boston, MA) for 1 min. Excess ECL reagent was removed from the membranes with a paper towel. Membranes were then wrapped in plastic wrap for autoradiography.

2.6 Co-Immunoprecipitation

HEK293T or HEK293-TLR4-MD2-CD14 cells were co-transfected with 2-5 µg of each expression plasmid using the TransIT®-LT1 Transfection Reagent (Mirus Bio Corporation, Madison, WI). Transfections were carried out according to the manufacturer's instructions. Cell lysates were collected for immunoprecipitation 48 h post-transfection using a modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, plus protease inhibitors). Cell lysates were assayed for protein concentration and subsequently 1-3 mg of lysates were pre-cleared by incubation with Protein A agarose beads (EMD Millipore Corporation, Billerica, MA) for 1 h at 4 °C. Pre-cleared lysates were subsequently used for immunoprecipitation with anti-SASH1 (1 µg; Novus Biologicals, Oakville, ON), anti-β-arrestin 1 (Arrb1, 2 µg; Santa Cruz Biotechnology, Santa Cruz, CA) anti-HA (1 µg; Covance, Emeryville, CA) or control isotype IgG (Sigma-Aldrich, St. Louis, MO) overnight at 4°C, followed by an incubation with TrueBlot® Anti-Rabbit Ig IP Beads (eBioscience, San Diego, CA) for an additional 3 h at 4°C. Following

immunoprecipitation, the beads were washed four times with RIPA buffer and boiled in Laemmli sample buffer for subsequent immunoblot analysis.

For the immunoprecipitation of Flag-tagged proteins, cell lysates were collected 48 h post-transfection using a Flag-modified RIPA buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, plus protease inhibitors). Cell lysates were assayed for protein concentration and 1-2 mg of lysates were precleared by incubation with Protein G agarose beads (EMD Millipore Corporation, Billerica, MA) for 1 h at 4 °C. Pre-cleared lysates were added to 50 µL anti-FlagM2-agarose beads or control isotype IgG-agarose (Sigma-Aldrich, St. Louis, MO) and incubated overnight at 4°C. Following immunoprecipitation, beads were washed four times with a Flag-modified wash buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Triton X-100, plus protease inhibitors) and incubated with 0.2 mg/mL FlagM2 peptide (Sigma-Aldrich, St. Louis, MO) for an additional hour at 4°C to elute Flag-tagged proteins. Following elution, samples were boiled in Laemmli sample buffer for subsequent immunoblot analysis.

2.7 TRAF6 Immunoprecipitation and Ubiquitination

To monitor poly-ubiquitination of TRAF6 in HEK293T or HEK293-TLR4-MD2-CD14 cells, cells were transfected with 2-6 µg of each expression plasmid using the TransIT®-LT1 Transfection Reagent (Mirus Bio Corporation, Madison, WI). Transfections were carried out according to the manufacturer's instructions. Cell lysates were collected for immunoprecipitation 48 h post-transfection using a specific TRAF6 IP buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 20 mM N-ethylmaleimide and protease inhibitors). For the immunoprecipitation of endogenous TRAF6 in HEK293-TLR4-MD2-CD14 cells and HMEC, lysates were also collected with this buffer.

Cell lysates were assayed for protein concentration and 3 mg of lysates were precleared by incubation with Protein A agarose beads (EMD Millipore Corporation, Billerica, MA) for 1 h at 4 °C. Pre-cleared lysates were subsequently used for immunoprecipitation with anti-TRAF6 (2 µg; Santa Cruz Biotechnology, Santa Cruz, CA) or control isotype IgG (Sigma-Aldrich, St. Louis, MO) overnight at 4°C, followed by an incubation with TrueBlot® Anti-Rabbit Ig IP Beads (eBioscience, San Diego, CA) for an additional 3 h at 4°C. Following immunoprecipitation, the beads were washed five times with TRAF6 IP buffer and boiled in Laemmli sample buffer for subsequent immunoblot analysis.

CHAPTER 3: RESULTS

3.1 SASH1 Interacts with TRAF6 in an LPS-dependent Manner in Endothelial Cells

We have previously shown that endogenous SASH1 can interact with TRAF6 in an LPS-dependent manner in HEK293-TLR4-MD2-CD14 cells [89]. This LPS-dependent interaction was once again confirmed in these cells over a time course of 0, 5, 15, 30, 45 and 60 minutes of LPS stimulation (Figure 3.1A). Upon immunoprecipitation of endogenous TRAF6, SASH1 was found to co-immunoprecipitate following 5 minutes of LPS stimulation. This interaction peaked after 45 minutes of LPS stimulation, and was sustained until the 60 minute time point investigated. To compensate for any differences in the level of endogenous TRAF6 immunoprecipitated for each time point, densitometric scans were performed to normalize the amount of co-immunoprecipitated SASH1 to the amount of immunoprecipitated TRAF6 (Figure 3.1A). To further investigate the physiological role of SASH1 in LPS signaling in endothelial cells, we examined the interaction of endogenous SASH1 with TRAF6 in HMEC over a time course of 0, 5, 15, 30 and 60 minutes of LPS stimulation (Figure 3.1B). Upon immunoprecipitation of endogenous TRAF6, SASH1 was found to co-immunoprecipitate after 5 minutes of LPS stimulation. This interaction peaked after 15 min of LPS stimulation, and was sustained until the 30 minute time point. Once again, densitometric analyses were conducted to compensate for differences in the level of TRAF6 immunoprecipitated for each time point (Figure 3.1B). These differences were likely due to inconsistencies in the elution or gel-loading steps of the immunoprecipitation and subsequent immunoblotting procedure. Collectively, these results confirmed that the interaction of TRAF6 and SASH1 is physiologically relevant and occurs in an LPS-dependent manner in endothelial cells.

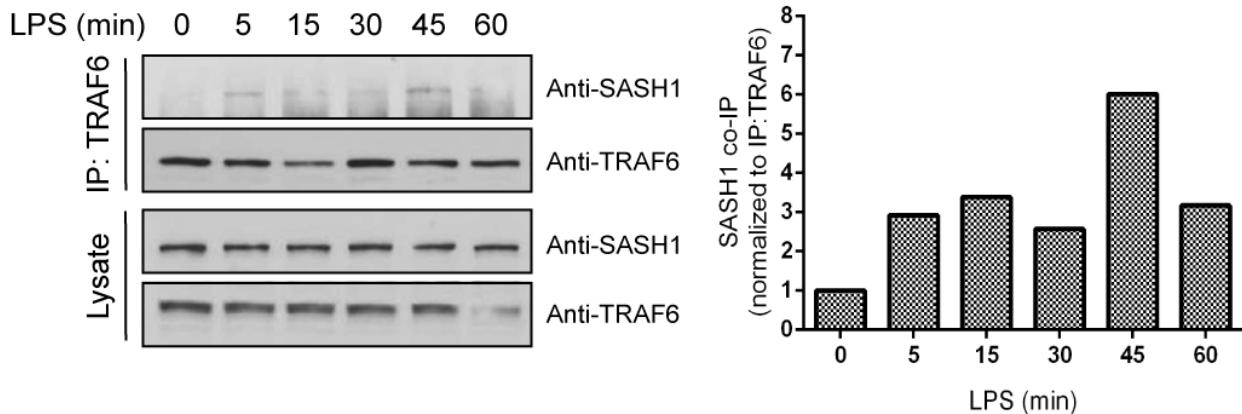
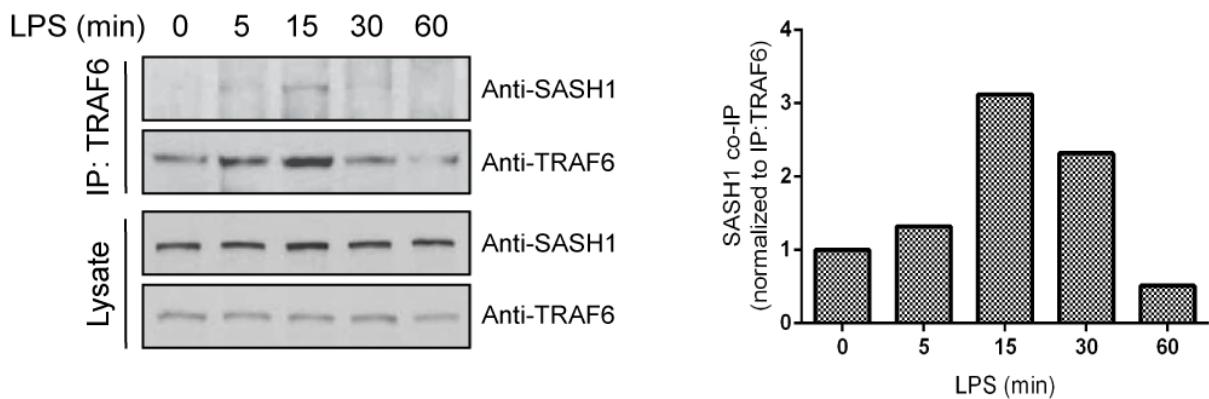
A**B**

Figure 3.1 Endogenous SASH1 interacts with TRAF6 in an LPS-dependent manner.
(A) HEK293-TLR4-MD2-CD14 cells were stimulated with LPS (100 ng/mL) for the times indicated. Lysates were immunoprecipitated (IP) with anti-TRAF6 antibodies. Interaction of SASH1 was monitored by immunoblotting with anti-SASH1. Densitometric scans were performed using ImageJ software. Co-immunoprecipitated SASH1 was normalized to the amount of immunoprecipitated TRAF6 for each time point, as indicated.
(B) HMEC were stimulated with LPS (100 ng/mL) for the times indicated. Lysates were immunoprecipitated (IP) with anti-TRAF6 antibodies. Interaction of SASH1 was monitored by immunoblotting with anti-SASH1. Densitometric scans were once again performed to normalize the amount of co-immunoprecipitated SASH1 to the amount of immunoprecipitated TRAF6 for each time point, as indicated.

3.2 SASH1 is Required for the LPS-induced Activation of TRAF6 in Endothelial Cells

Previous studies in our lab demonstrated impaired LPS-induced activation of NF-κB and MAPKs in endothelial cells upon knockdown of endogenous SASH1, suggesting a critical role for SASH1 in potentiating LPS-mediated signaling [89]. Enforced expression of SASH1 was shown to mediate K63-linked poly-ubiquitination of TRAF6, further implicating a role for SASH1 in the positive regulation of TRAF6 activation [89]. Since LPS-induced auto-ubiquitination of TRAF6 is essential for the downstream activation of NF-κB and MAPKs, we evaluated the essential role for SASH1 in LPS-induced TRAF6 auto-ubiquitination in endothelial cells. HMEC were lentivirally transduced with either shRNA specific for SASH1 (shSASH1) or a control random shRNA (shRandom), and stimulated with LPS for 0 or 30 minutes. LPS-induced poly-ubiquitination of TRAF6 was examined upon immunoprecipitation of endogenous TRAF6 in these cells (Figure 3.2). In the control shRandom cells, induction of TRAF6 poly-ubiquitination was observed following 30 minutes of LPS stimulation. In cells with suppressed SASH1 expression (shSASH1) LPS-induced poly-ubiquitination of TRAF6 was abolished. These results further confirm the critical role of SASH1 as positive regulator of endothelial TLR4 signaling, particularly at the step of mediating TRAF6 auto-ubiquitination.

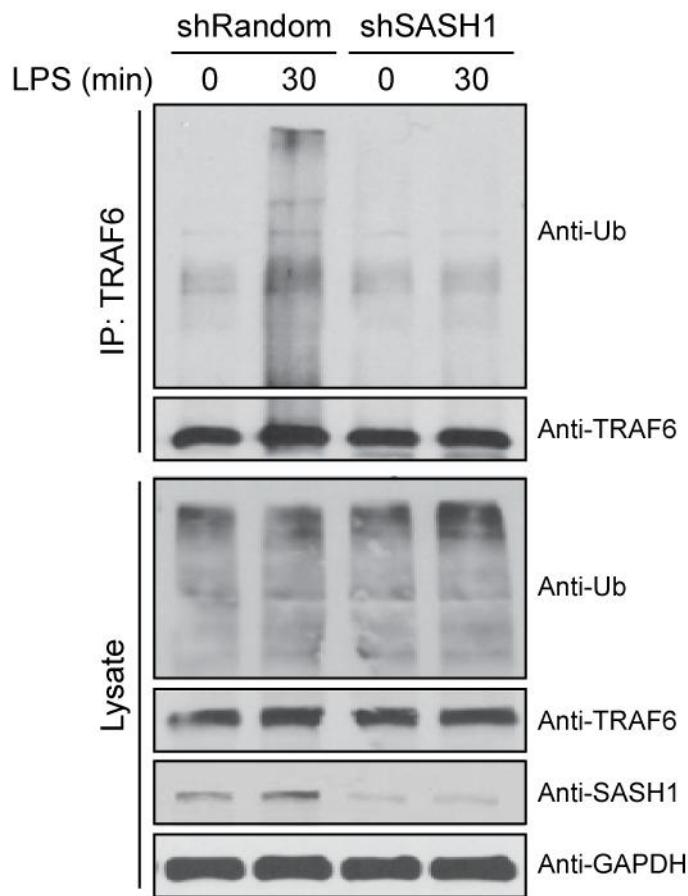


Figure 3.2 SASH1 is required for LPS-induced TRAF6 auto-ubiquitination in endothelial cells. HMEC were transduced with either shRNA specific for SASH1 (shSASH1) or a control random shRNA (shRandom). Transduced cells were subsequently stimulated with LPS (100 ng/mL) for 0 or 30 minutes. Lysates were immunoprecipitated (IP) with anti-TRAF6 antibodies. Poly-ubiquitination of immunoprecipitated TRAF6 was monitored by immunoblotting with anti-ubiquitin. Knockdown of SASH1 was confirmed by immunoblotting cell lysates with anti-SASH1.

3.3 β-arrestin 1 and β-arrestin 2 are Constitutively Expressed in Endothelial Cells

Due to the characterized role of SASH1 as a scaffold protein in the TLR4 pathway, and the presence of several putative protein-protein interaction domains within SASH1, we aimed to identify novel SASH1-interacting proteins that may also be involved in immune signaling. Following a SASH1 yeast two-hybrid analysis (conducted by Hybrigenics), β-arrestin 1 was found to be the top interacting partner of SASH1. Both β-arrestin 1 and β-arrestin 2 have been shown to act as negative regulators of TLR4 signaling in numerous cell types, however, few studies have examined the functional roles of the β-arrestins in endothelial cells [149, 156]. The expression of endogenous negative regulators is often induced upon stimulation of TLR4, acting as a negative feedback mechanism to terminate pro-inflammatory signals [131-133]. Due to the preferential expression of *Sash1* in the mouse microvascular endothelium and the characterization of SASH1 as a critical regulator of immune signaling in human endothelial cells, we initially confirmed the expression of the β-arrestin proteins in our model endothelial cell line, HMEC (Figure 3.3). Furthermore, the expression of β-arrestin 1 and β-arrestin 2 was monitored upon LPS stimulation of HMEC to evaluate constitutive or induced expression of these proteins in endothelial cells downstream of TLR4. Both β-arrestin 1 and β-arrestin 2 were found to be constitutively expressed in HMEC over 0, 5 and 10 minutes of LPS stimulation.

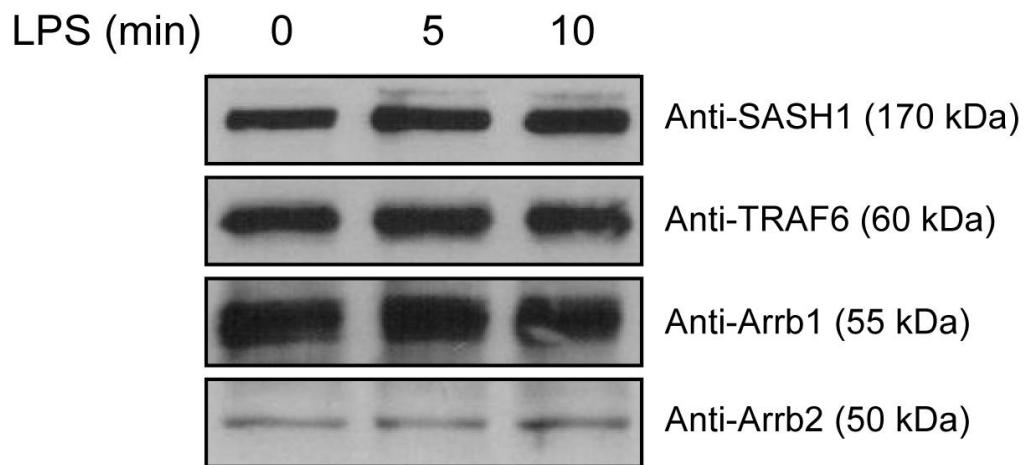


Figure 3.3 Constitutive expression of the β -arrestins upon LPS stimulation of endothelial cells. HMEC were stimulated with LPS (100 ng/mL) for the times indicated. The expression of SASH1, TRAF6, β -arrestin 1 (Arrb1), and β -arrestin 2 (Arrb2) was monitored by immunoblotting with their respective antibodies. Approximate molecular weights of the detected proteins are indicated.

3.4 SASH1 Interacts with β-arrestin 1 and β-arrestin 2

Following the yeast two-hybrid analysis, we aimed to confirm the interaction of SASH1 with β-arrestin 1, and potentially with β-arrestin 2. SASH1 was previously shown to interact with several components of the TLR4 pathway by co-immunoprecipitation studies conducted in HEK293T cells [89]. We also used these cells to evaluate the interaction of SASH1 with the β-arrestins. Interestingly, SASH1 was found to interact with both β-arrestin 1 (Figure 3.4A) and β-arrestin 2 (Figure 3.4B) in these cells by reciprocal co-immunoprecipitation.

As previously described, SASH1 is a large protein that contains several putative protein-protein interaction domains, including two SAM domains and one SH3 domain [113]. The roles of these putative interaction domains in SASH1, however, have remained previously uncharacterized. Upon confirming the interaction of SASH1 with both β-arrestin 1 and β-arrestin 2, we investigated the localization of β-arrestin binding on SASH1. We have previously generated SASH1 deletion mutants in our lab that lack the characterized SH3 (SASH1ΔSH3), SAM1 (SASH1ΔSAM1), or SAM2 (SASH1ΔSAM2) domains (Figure 3.5A). Upon immunoprecipitation of these individual deletion constructs, we monitored for interaction with β-arrestin 1 (Figure 3.5B) and β-arrestin 2 (Figure 3.5C). Both β-arrestin isoforms efficiently co-immunoprecipitated with wild-type SASH1 and SASH1ΔSH3. In contrast, the interaction with both β-arrestin 1 and β-arrestin 2 was diminished upon immunoprecipitation of SASH1ΔSAM1. These results suggest that the SAM1 domain of SASH1 is required for SASH1-β-arrestin interaction, and may be the site of β-arrestin binding. Specific deletion of the SAM2 domain of SASH1 seemed to present problems with protein conformational stability, and attempts to evaluate the role of this domain in the interaction of SASH1 with the β-arrestins were unsuccessful.

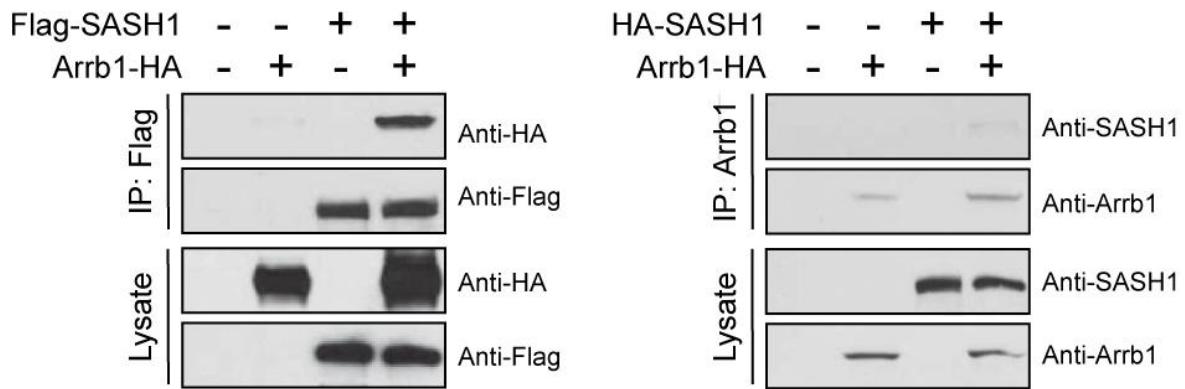
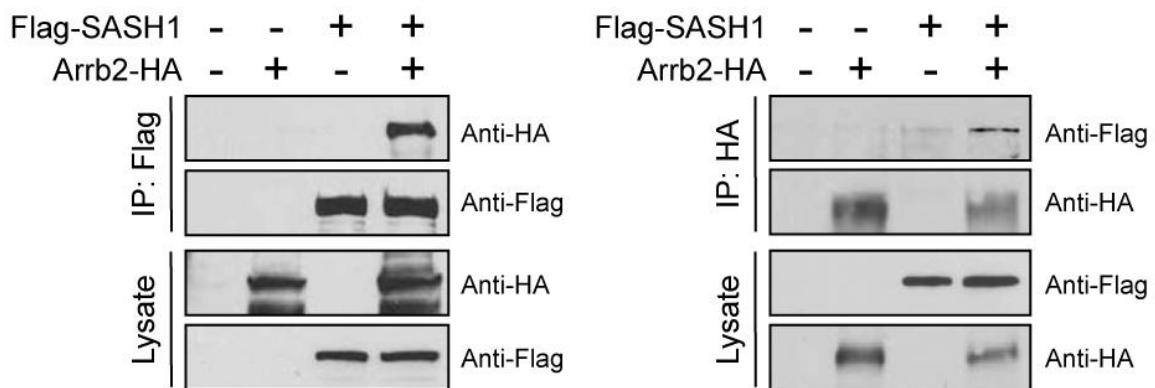
A**B**

Figure 3.4 SASH1 interacts with β -arrestin 1 and β -arrestin 2. HEK293T cells were co-transfected with Flag-SASH1 or HA-SASH1, as indicated, and either HA-tagged (A) β -arrestin-1 (Arrb1) or (B) β -arrestin-2 (Arrb2). Lanes labeled with (-) were transfected with the control empty vector. Lysates were immunoprecipitated (IP) with anti-Flag, anti-Arrb1 or anti-HA antibodies, as indicated. Interactions were monitored by immunoblotting with anti-HA, anti-SASH1 or anti-Flag.

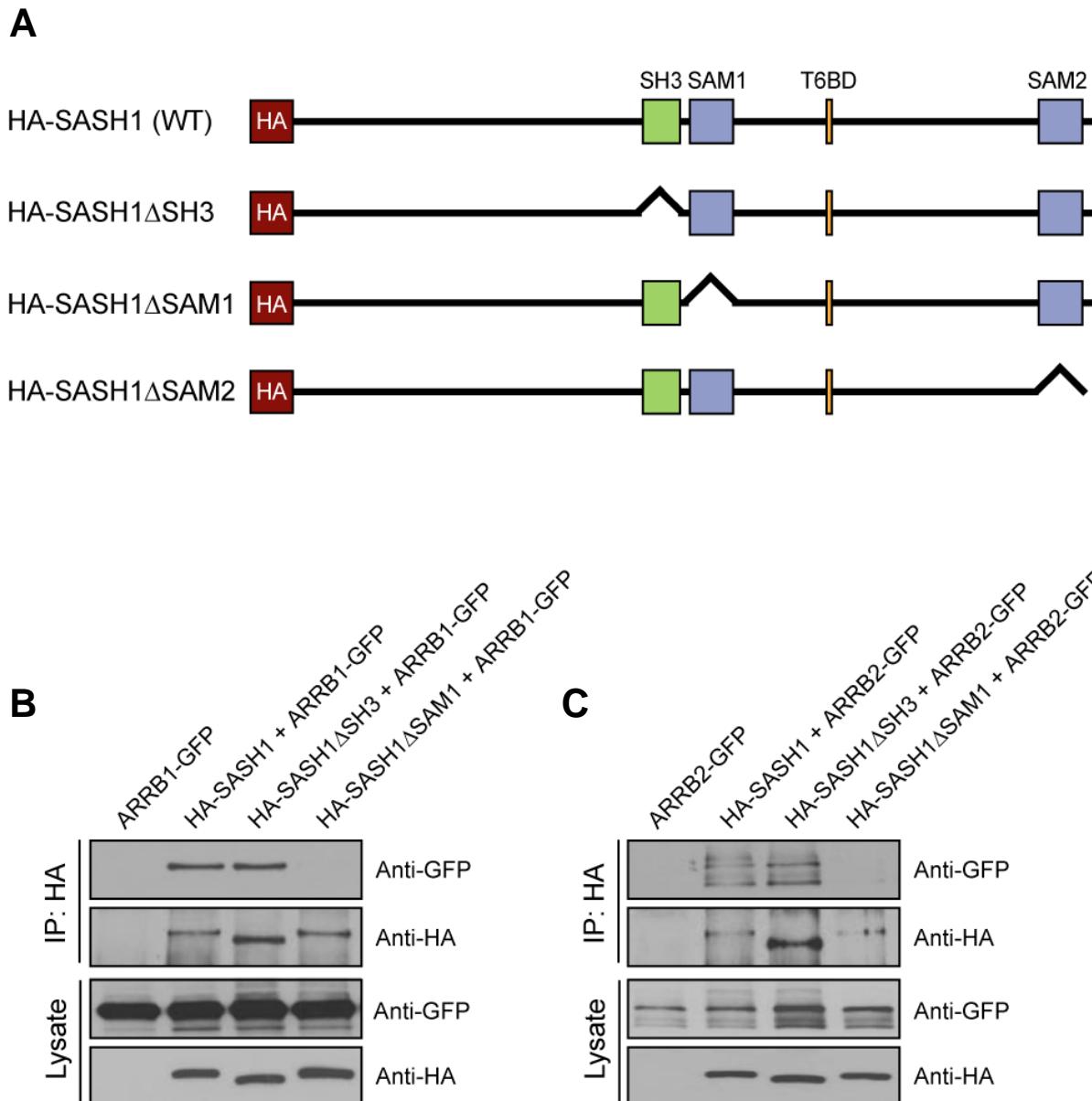


Figure 3.5 The SAM1 domain of SASH1 is required for interaction with β -arrestin 1 and β -arrestin 2. (A) Schematic of HA-tagged SASH1 deletion mutant constructs. Due to instability in protein expression, the SASH1 deletion mutant lacking the SAM2 domain (HA-SASH1 Δ SAM2) was unable to be used in this study. HEK293T cells were co-transfected with an HA-tagged wild-type (WT) or deletion mutant of SASH1, as indicated, and either GFP-tagged (B) β -arrestin 1 (Arrb1) or (C) β -arrestin 2 (Arrb2). Lysates were immunoprecipitated (IP) with anti-HA antibodies. Interactions were monitored by immunoblotting with anti-GFP.

We further investigated the localization of SASH1 binding on the β -arrestin proteins. Using β -arrestin 1 deletion mutants generated in our lab (Figure 3.6A), we monitored the interaction of these mutants with immunoprecipitated SASH1. As shown Figure 3.6B, wild-type β -arrestin 1 (amino acids 1-418) co-immunoprecipitated with SASH1, however, this interaction was abolished with a β -arrestin 1 C-domain deletion mutant (amino acids 1-179). In contrast, an N-domain deletion mutant (amino acids 179-418) and a C-terminal fragment (amino acids 319-418) of β -arrestin 1 were both capable of interacting with SASH1 in a similar manner as wild-type β -arrestin 1. These results suggest that the C-terminus of β -arrestin 1 is required for β -arrestin 1-SASH1 interaction, and may be the site of SASH1 binding. Similar findings were discovered upon investigating the interaction of SASH1 with β -arrestin 2 deletion mutants (Figure 3.7A). As shown in Figure 3.7B, wild-type β -arrestin 2 (amino acids 1-410) interacted with immunoprecipitated SASH1, but this interaction was diminished with a β -arrestin 2 C-domain deletion mutant (amino acids 1-185). Similar to the findings for β -arrestin 1, an N-domain deletion mutant (amino acids 185-410) was capable of interacting with SASH1. Furthermore, a C-terminal fragment of β -arrestin 2 (amino acids 241-410) was also found to be sufficient to maintain interaction with immunoprecipitated SASH1. Collectively, these results suggest that the C-termini of both β -arrestin 1 and β -arrestin 2 are required for interaction with SASH1, and may be the site of SASH1 binding on these proteins.

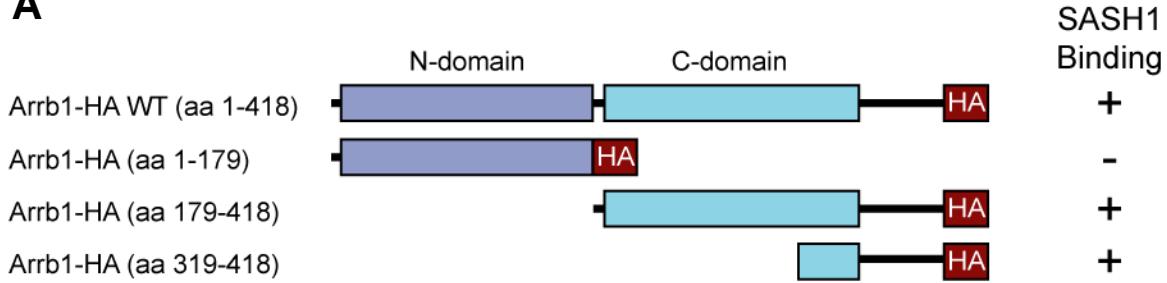
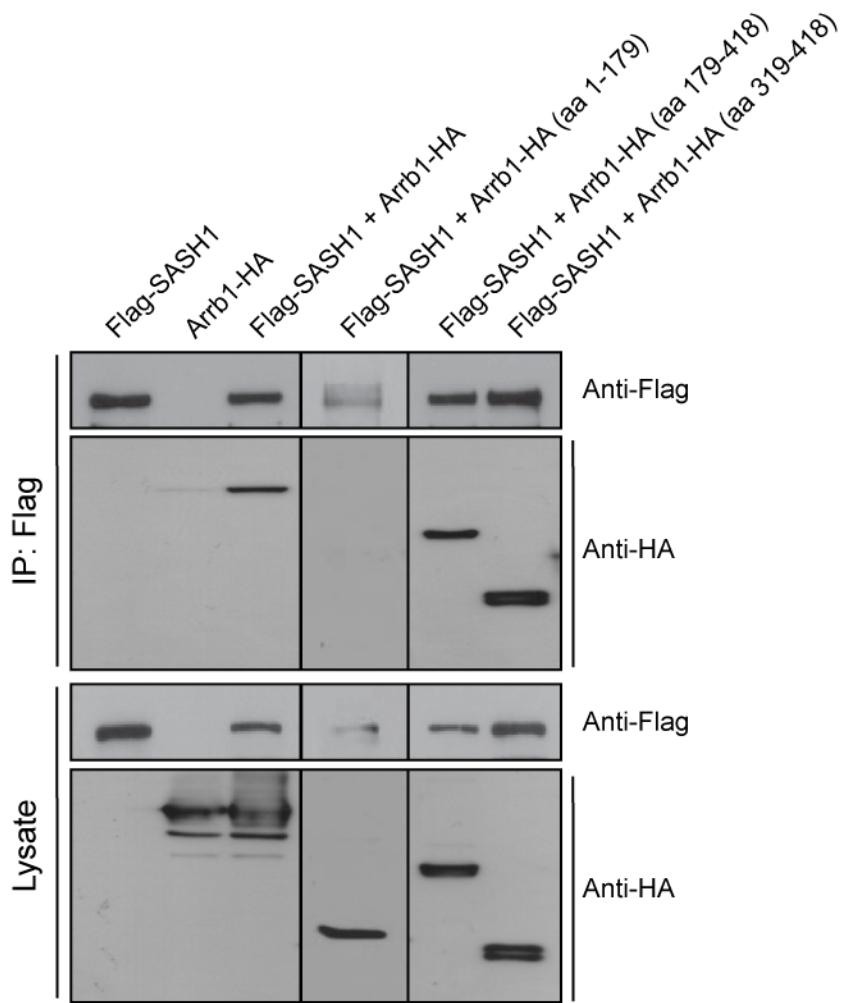
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Figure 3.6 The C-domain of β -arrestin 1 is required for interaction with SASH1. (A) Schematic of HA-tagged β -arrestin 1 (Arrb1) deletion mutant constructs. Amino acid (aa) composition of each construct is indicated. Deletion mutants that were shown to maintain an interaction with SASH1 are indicated by (+). (B) HEK293T cells were co-transfected with Flag-SASH1 and either an HA-tagged wild-type (WT) or deletion mutant of Arrb1, as indicated. Lysates were immunoprecipitated (IP) with anti-Flag antibodies. Interactions were monitored by immunoblotting with anti-HA antibodies.

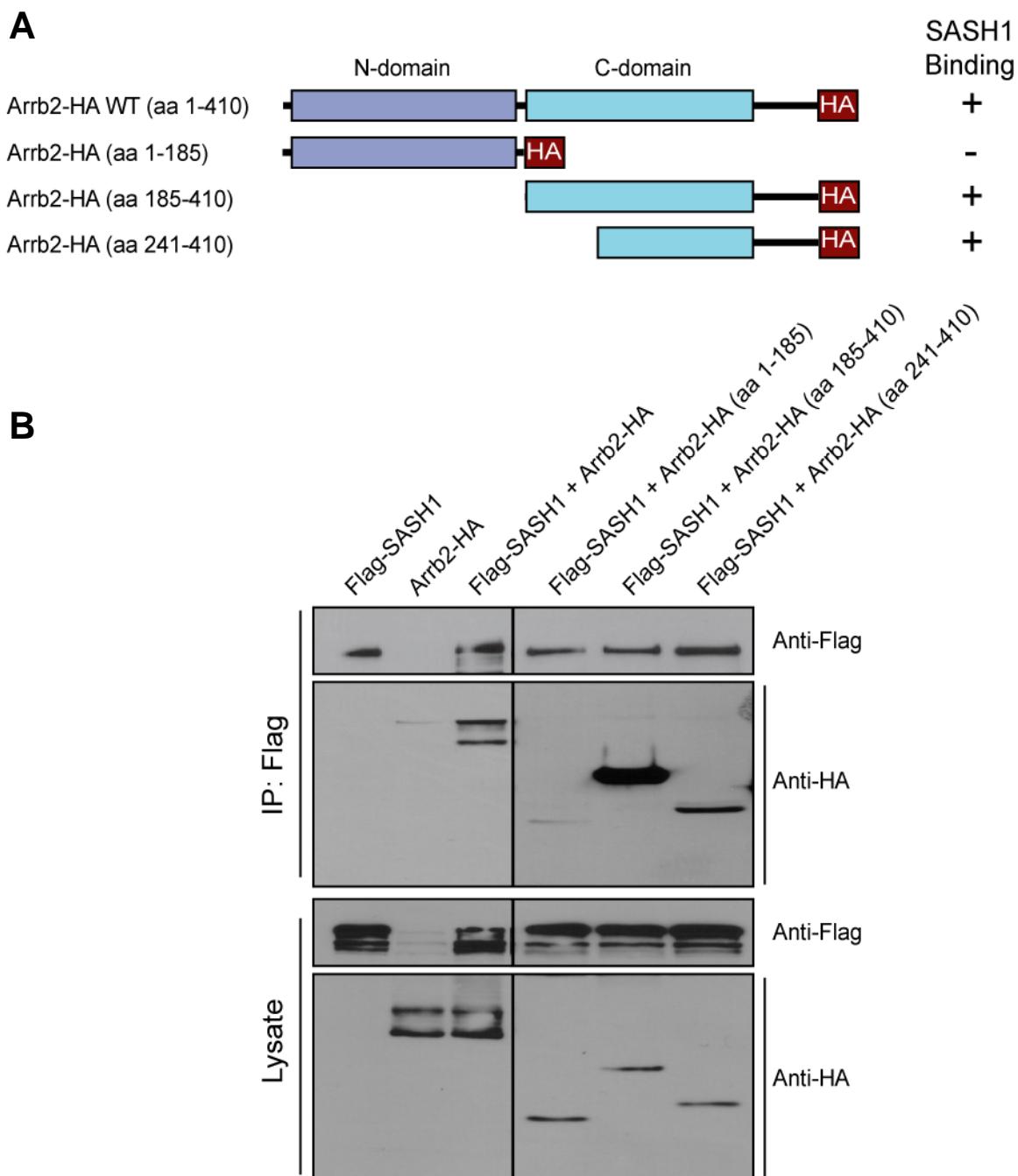


Figure 3.7 The C-domain of β-arrestin 2 is required for interaction with SASH1. (A) Schematic of HA-tagged β-arrestin 2 (Arrb2) deletion mutant constructs. Amino acid (aa) composition of each construct is indicated. Deletion mutants that were shown to maintain an interaction with SASH1 are indicated by (+). (B) HEK293T cells were co-transfected with Flag-SASH1 and either an HA-tagged wild-type (WT) or deletion mutant of Arrb2, as indicated. Lysates were immunoprecipitated (IP) with anti-Flag antibodies. Interactions were monitored by immunoblotting with anti-HA antibodies.

3.5 SASH1 Interacts in a Complex with TRAF6 and β-arrestin 1

SASH1 was found to interact with several components of the TLR4 pathway, including TRAF6, and act as positive regulator of endogenous TRAF6 auto-ubiquitination and activation [89]. The β-arrestins have also been shown to interact with TRAF6, but act to negatively regulate TRAF6 auto-ubiquitination by physically impairing TRAF6 oligomerization [156]. Only the β-arrestin 1 isoform, however, has been shown to interact directly with TRAF6 [156]. Furthermore, although both β-arrestin 1 and β-arrestin 2 were shown to interact with SASH1 by co-immunoprecipitation, only β-arrestin 1 was found in our yeast two-hybrid analysis. We thus focused our studies on evaluating the physiological significance of SASH1-β-arrestin 1 interaction on the regulation of TRAF6. Reciprocal co-immunoprecipitation experiments were conducted in HEK293T cells to monitor TRAF6-SASH1-β-arrestin 1 complex formation (Figure 3.8). As previously reported, both SASH1 and β-arrestin 1 were found to interact with TRAF6 (Figure 3.8A). The reciprocal immunoprecipitation of SASH1 confirmed interaction with both TRAF6 and β-arrestin 1 (Figure 3.8B). Interestingly, interaction of β-arrestin 1 with immunoprecipitated SASH1 was markedly enhanced in the presence of TRAF6. These results confirm that TRAF6, SASH1 and β-arrestin 1 interact together in a complex.

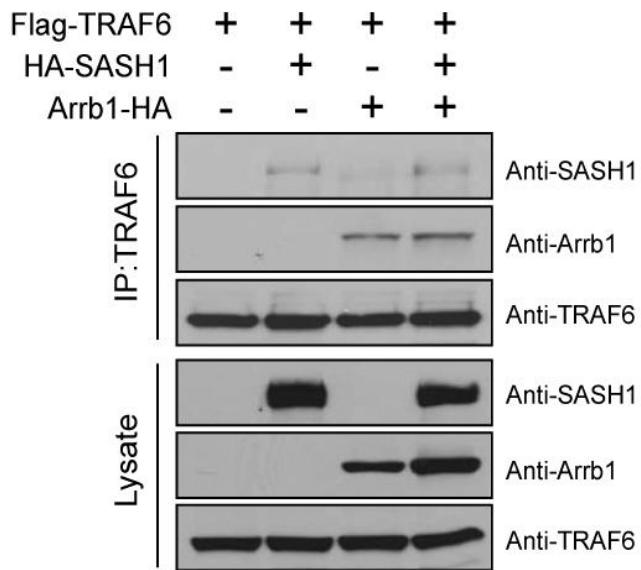
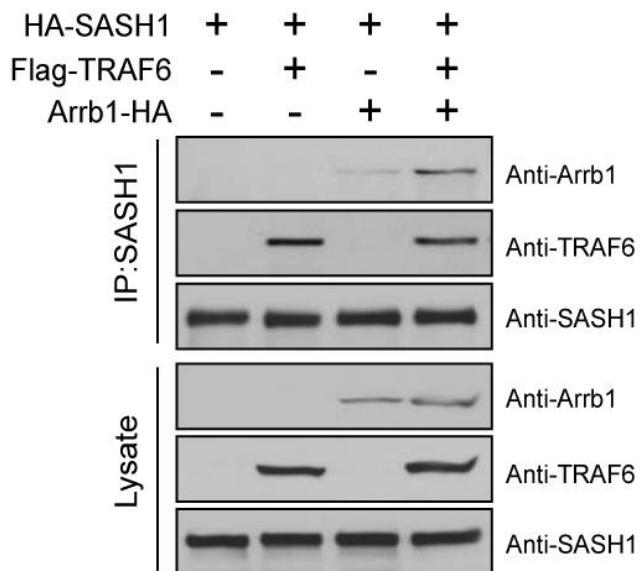
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Figure 3.8 SASH1 interacts in a complex with TRAF6 and β -arrestin 1. HEK293T cells were co-transfected with Flag-TRAF6, HA-SASH1 and HA-tagged β -arrestin 1 (Arrb1), as indicated. Lanes labeled with (-) were transfected with the control empty vector. Lysates were immunoprecipitated (IP) with either (A) anti-TRAF6 or (B) anti-SASH1 antibodies. Interactions were monitored by immunoblotting with anti-TRAF6, anti-SASH1 and anti-Arrb1 antibodies, as indicated.

3.6 Expression of β-arrestin 1 does not Impair SASH1 Oligomerization

Overexpression of TRAF6 has been shown to mediate TRAF6 oligomerization and subsequent K63-linked auto-ubiquitination, independent of an LPS stimulus [136]. We have also previously shown that overexpression of SASH1 can mediate LPS-independent K63-linked auto-ubiquitination of endogenous TRAF6, possibly by mediating TRAF6 oligomerization [89]. It was speculated that the oligomerization of SASH1 itself may be involved in this process. SASH1 oligomerization was subsequently monitored upon co-immunoprecipitation of differentially epitope-tagged SASH1 constructs (Figure 3.9). We confirmed co-immunoprecipitation of HA-SASH1 with immunoprecipitated Flag-SASH1, suggesting that SASH1 is indeed capable of forming oligomers (Figure 3.9A). The characterization of SASH1 oligomerization, however, and the essential role of this oligomerization in the activation of TRAF6 remain to be investigated. Enforced expression of β-arrestin 1 has been reported to impair TRAF6 oligomerization [156]. Upon confirming formation of a TRAF6-SASH1-β-arrestin 1 complex (Figure 3.8), we investigated whether β-arrestin 1 expression could modulate SASH1 oligomerization in a similar manner as reported for TRAF6. As shown in Figure 3.9B, we once again confirmed oligomerization of SASH1 upon co-immunoprecipitation of HA-SASH1 with immunoprecipitated Flag-SASH1. In cells overexpressing β-arrestin 1, levels of co-immunoprecipitated HA-SASH1 did not change, despite the fact that decreased Flag-SASH1 was immunoprecipitated in these cells. These results may suggest that, although overexpression of β-arrestin 1 does not impair SASH1 oligomerization in the same manner TRAF6, β-arrestin 1 expression may actually enhance SASH1 oligomerization. These findings should, however, be considered preliminary and must be confirmed.

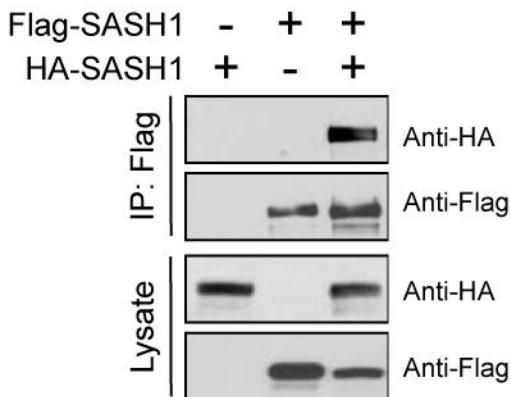
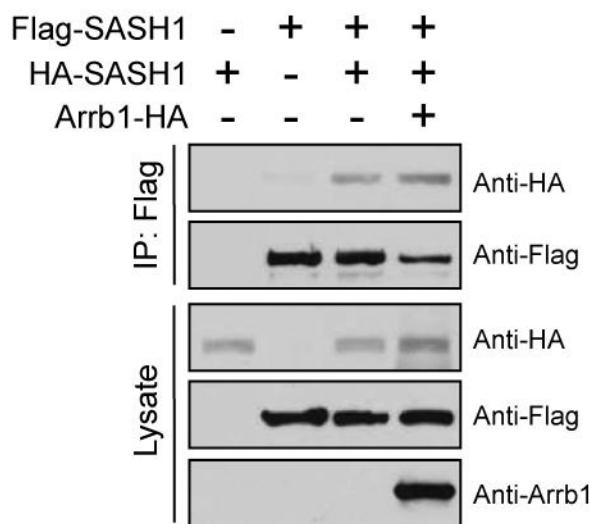
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Figure 3.9 Enforced expression of β -arrestin 1 does not impair SASH1 oligomerization.
HEK293T cells were co-transfected with (A) HA-SASH1 and Flag-SASH1, or (B) HA-SASH1, Flag-SASH1 and HA-tagged β -arrestin 1 (Arrb1). Lanes labeled with (-) were transfected with the control empty vector. Lysates were immunoprecipitated (IP) with anti-Flag antibodies. Oligomerization of SASH1 was monitored by immunoblotting with anti-HA antibodies. Overexpression of β -arrestin 1 was confirmed by immunoblotting cell lysates with anti-Arrb1.

3.7 TRAF6 Interacts with β-arrestin 1 and β-arrestin 2 Independent of SASH1

Upon confirming the interaction of SASH1, TRAF6 and β-arrestin 1 together in a complex, we evaluated whether SASH1 was required for the interaction of TRAF6 with β-arrestin 1. HEK293T cells were lentivirally transduced with either shRNA specific for SASH1 (shSASH1) or a control random shRNA (shrandom). Knockdown of endogenous SASH1 in shSASH1-transduced cells was confirmed by immunoblotting (Figure 3.10). Interaction of β-arrestin 1 with immunoprecipitated TRAF6 was subsequently monitored in both the control shrandom and shSASH1 cells (Figure 3.10A). In control cells, β-arrestin 1 co-immunoprecipitated with TRAF6. In the shSASH1 cells, co-immunoprecipitation of β-arrestin 1 with TRAF6 was sustained in a manner similar to control cells. These results suggest that SASH1 is not required for TRAF6-β-arrestin 1 binding. To monitor for any differences in SASH1-mediated TRAF6 binding between the β-arrestin isoforms, we examined whether SASH1 was required for the interaction of TRAF6 and β-arrestin 2 in shRandom and shSASH1 cells (Figure 3.10B). Interaction of β-arrestin 2 with immunoprecipitated TRAF6 was confirmed in the control (shrandom) cells. Furthermore, interaction of β-arrestin 2 with immunoprecipitated TRAF6 was sustained in shSASH1 cells. Although the amount of co-immunoprecipitated β-arrestin 2 with TRAF6 appeared slightly decreased in shSASH1 cells compared to control cells, this was likely reflective of lower amounts of immunoprecipitated TRAF6 in the shSASH1 samples. Collectively, these results suggest that SASH1 is not required for the interaction of TRAF6 with β-arrestin 1 or β-arrestin 2.

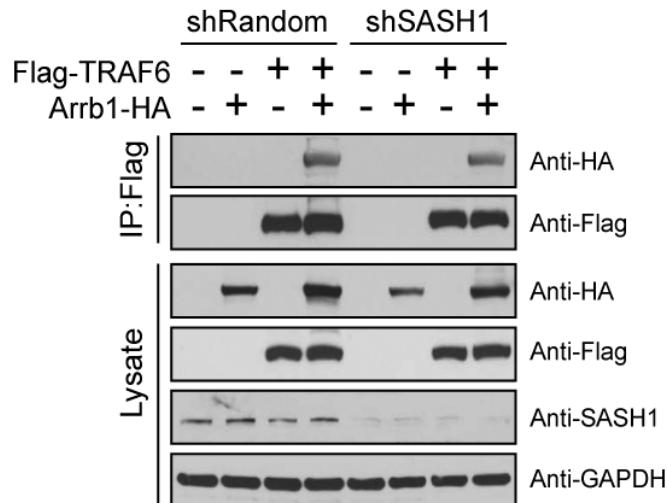
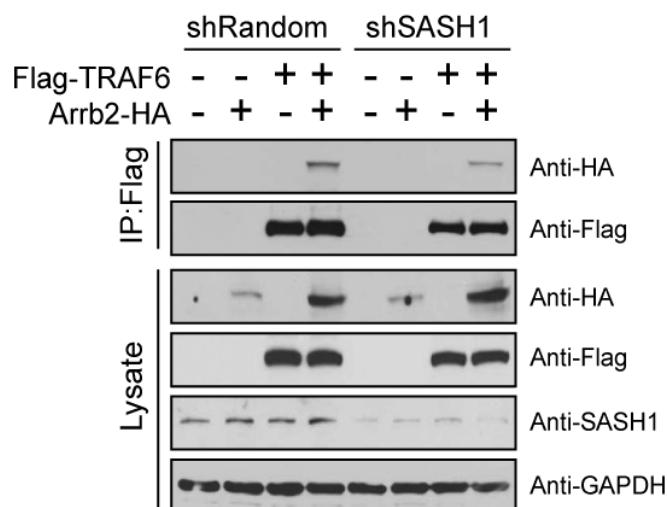
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Figure 3.10 SASH1 is not required for the interaction of TRAF6 with β -arrestin 1 and β -arrestin 2. HEK293T cells were transduced with either shRNA specific for SASH1 (shSASH1) or a control random shRNA (shRandom). Transduced cells were co-transfected with Flag-TRAF6 and either HA-tagged (A) β -arrestin-1 (Arrb1) or (B) β -arrestin-2 (Arrb2). Lanes labeled with (-) were transfected with the control empty vector. Lysates were immunoprecipitated (IP) with anti-Flag antibodies. Interactions were monitored by immunoblotting with anti-HA. Knockdown of SASH1 was confirmed by immunoblotting cell lysates with anti-SASH1.

3.8 Overexpression of SASH1 Negatively Regulates TRAF6 Binding to β-arrestin 1

To further investigate the role of SASH1 in TRAF6-SASH1-β-arrestin 1 complex formation, we examined the effects of enforced SASH1 expression on TRAF6-β-arrestin 1 binding (Figure 3.11A). In the absence of overexpressed SASH1, β-arrestin 1 interacted with immunoprecipitated TRAF6. Upon overexpression of SASH1, however, co-immunoprecipitation of β-arrestin 1 with TRAF6 was found to be impaired. These results suggest that, although SASH1 expression is not required for TRAF6-β-arrestin 1 binding, the enforced expression of SASH1 may functionally impair the ability of β-arrestin 1 to interact with TRAF6. To once again monitor for SASH1-mediated, isoform-specific regulation of β-arrestin binding with TRAF6, we monitored whether overexpression of SASH1 could also impair the interaction of TRAF6 with β-arrestin 2 (Figure 3.11B). Interestingly, the interaction of β-arrestin 2 with immunoprecipitated TRAF6 was not altered in the presence of overexpressed SASH1. Taken together, these results suggest that the overexpression of SASH1 may specifically regulate the interaction of TRAF6 with β-arrestin 1.

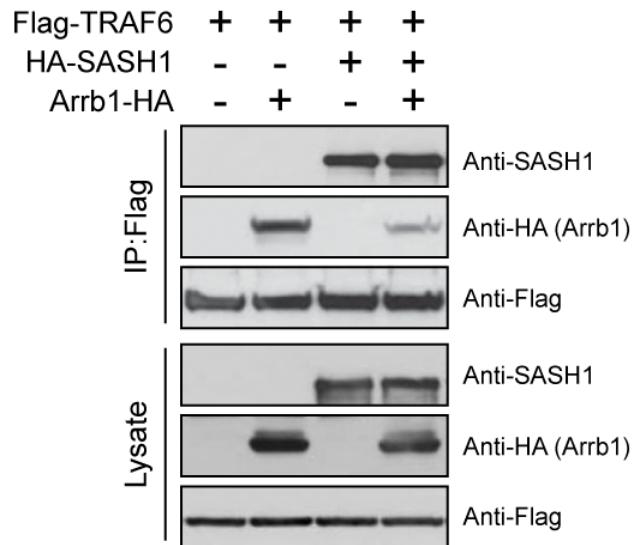
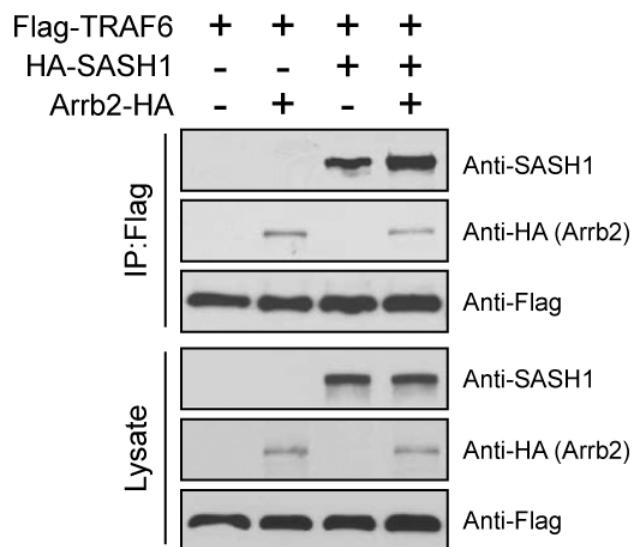
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Figure 3.11 Enforced expression of SASH1 impairs the interaction of TRAF6 with β -arrestin 1, but not β -arrestin 2. HEK293T cells were co-transfected with Flag-TRAF6 and HA-SASH1, as indicated, and either HA-tagged (**A**) β -arrestin-1 (Arrb1) or (**B**) β -arrestin-2 (Arrb2). Lanes labeled with (-) were transfected with the control empty vector. Lysates were immunoprecipitated (IP) with anti-Flag antibodies. Interactions were monitored by immunoblotting with anti-HA and anti-SASH1.

3.9 SASH1 may Functionally Regulate TRAF6-β-arrestin 1 Binding through Interaction with TRAF6

As previously demonstrated in Figure 3.8, SASH1, TRAF6 and β-arrestin 1 can interact together in a complex. Interestingly, the interaction of SASH1 with β-arrestin 1 was found to be markedly enhanced in the presence of TRAF6. We speculated that the enhanced interaction of β-arrestin 1 with SASH1 upon overexpression of SASH1 and TRAF6 may be impairing TRAF6-β-arrestin 1 interaction. Once again, we confirmed that overexpression of wild-type SASH1 impaired TRAF6-β-arrestin 1 binding (Figure 3.12A). To monitor whether binding of SASH1 to β-arrestin 1 was impairing the ability of β-arrestin 1 to interact with TRAF6, we assessed the ability of a mutant SASH1ΔSAM1 to modulate TRAF6-β-arrestin 1 interaction (Figure 3.12A). This SASH1ΔSAM1 mutant was previously found to be incapable of interacting with both β-arrestin 1 and β-arrestin 2 (demonstrated in Figure 3.5), but was confirmed to interact with TRAF6 (Figure 3.12). Interestingly, overexpression of the SASH1ΔSAM1 mutant impaired the interaction of β-arrestin 1 with immunoprecipitated TRAF6 in a similar manner as the overexpression of wild-type SASH1 (Figure 3.12A). We also monitored the effects of SASH1 or SASH1ΔSAM1 overexpression on the interaction of TRAF6 with β-arrestin 2 (Figure 3.12B). Once again, no changes were observed in the interaction of β-arrestin 2 with immunoprecipitated TRAF6. These results further indicate that SASH1 may be specifically impairing the interaction of β-arrestin 1 with TRAF6, but that SASH1-β-arrestin 1 binding is not required for this process.

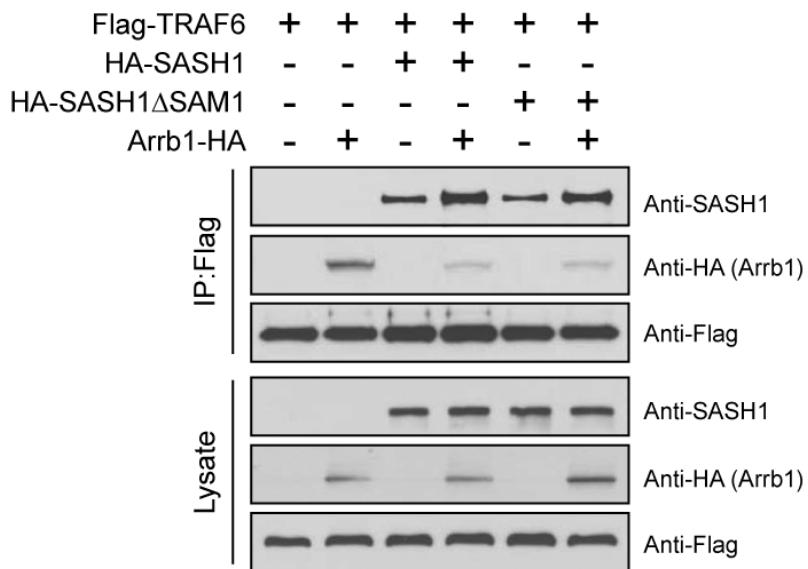
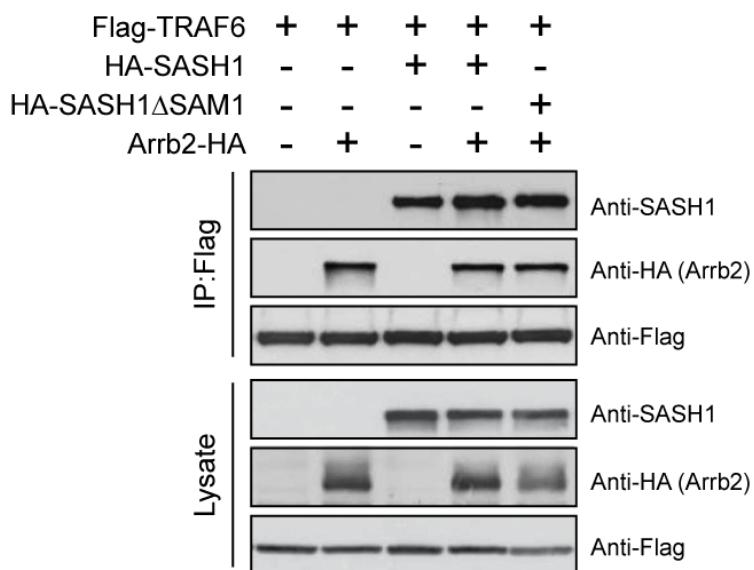
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Figure 3.12 SASH1-β-arrestin 1 binding is not required to impair the interaction of TRAF6 with β-arrestin 1 upon enforced expression of SASH1. HEK293T cells were co-transfected with Flag-TRAF6 and HA-SASH1 or HA-SASH1 Δ SAM1, as indicated, and either HA-tagged (A) β-arrestin-1 (Arrb1) or (B) β-arrestin-2 (Arrb2). Lanes labeled with (-) were transfected with the control empty vector. Lysates were immunoprecipitated (IP) with anti-Flag antibodies. Interactions were monitored by immunoblotting with anti-HA and anti-SASH1.

We further speculated that it may actually be the interaction of SASH1 with TRAF6 that is impairing TRAF6- β -arrestin 1 interaction. We have previously identified and characterized a TRAF6 binding domain (T6BD) on SASH1 that is required for SASH1-TRAF6 interaction (Figure 3.13A) [89]. To monitor whether binding of SASH1 to TRAF6 was impairing the ability of β -arrestin 1 to interact with TRAF6, we assessed the ability of a mutant SASH1 Δ T6BD to modulate TRAF6- β -arrestin 1 binding (Figure 3.13B). As previously observed, overexpression of wild-type SASH1 was capable of impairing interaction of β -arrestin 1 with immunoprecipitated TRAF6. Upon overexpression of SASH1 Δ T6BD, however, the ability of β -arrestin 1 to co-immunoprecipitate with TRAF6 may have been slightly recovered. These results are preliminary, therefore further investigation is required to confirm that the interaction of SASH1 with TRAF6 may functionally impair β -arrestin 1 binding to TRAF6. Once again, we did not observe any modulation of TRAF6- β -arrestin 2 binding upon overexpression of wild-type SASH1 or SASH1 Δ T6BD (Figure 3.13C). In summary, these results suggest that SASH1 may regulate TRAF6- β -arrestin binding in an isoform-specific manner.

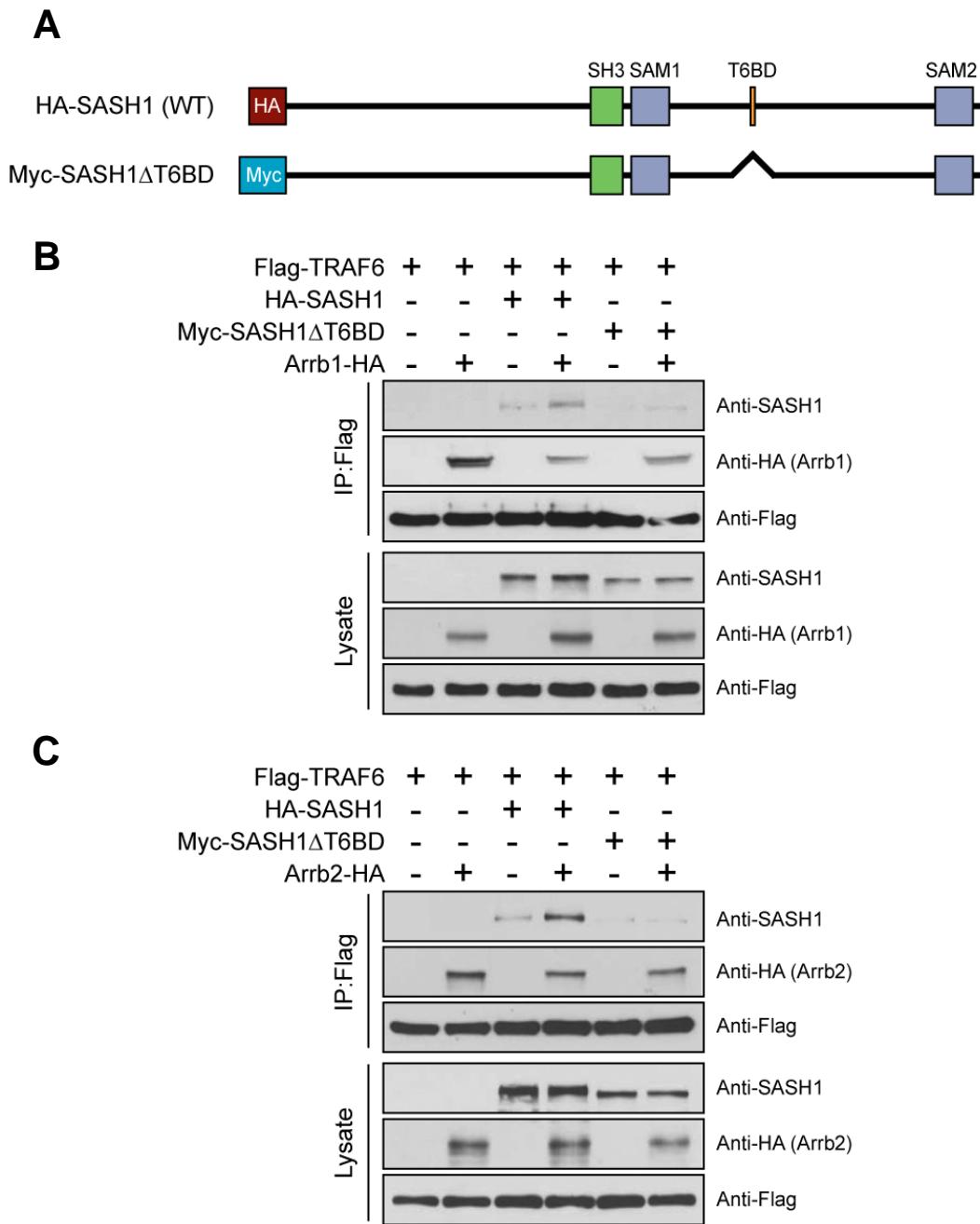


Figure 3.13 SASH1-TRAF6 binding may be responsible for the impaired interaction of TRAF6 with β -arrestin 1 upon enforced expression of SASH1. (A) Schematic of HA-tagged wild-type (WT) SASH1 and a Myc-tagged deletion mutant of SASH1 lacking the characterized TRAF6 binding domain (T6BD). HEK293T cells were co-transfected with Flag-TRAF6 and HA-SASH1 or Myc-SASH1 Δ T6BD, as indicated, and either HA-tagged (A) β -arrestin-1 (Arrb1) or (B) β -arrestin-2 (Arrb2). Lanes labeled with (-) were transfected with the control empty vector. Lysates were immunoprecipitated (IP) with anti-Flag antibodies. Interactions were monitored by immunoblotting with anti-HA and anti-SASH1.

3.10 Expression of β-arrestin 1 does not Impair TRAF6 Auto-Ubiquitination

We further investigated the role of SASH1 in the β-arrestin-mediated regulation of TRAF6 ubiquitination and activation. Both β-arrestin 1 and β-arrestin 2 have been previously characterized to bind the coiled-coil domain of TRAF6 to impair TRAF6 oligomerization and subsequent auto-ubiquitination [156]. Upon demonstrating that enforced expression of SASH1 could impair TRAF6-β-arrestin 1 binding, we evaluated whether SASH1 expression could also impair the β-arrestin 1-mediated negative regulation of LPS-induced TRAF6 poly-ubiquitination previously reported by Wang *et al.* [156]. Impaired or controlled binding of a negative regulator to TRAF6 by SASH1 would support our previous findings of SASH1 acting as a positive regulator of TRAF6 activation [89]. LPS-induced poly-ubiquitination of endogenous TRAF6 was monitored in HEK293-TLR4-MD2-CD14 cells upon 0 and 30 minutes of LPS stimulation (Figure 3.14). In control cells (absence of overexpressed SASH1 or β-arrestin 1), induction of TRAF6 poly-ubiquitination was observed following 30 minutes of LPS stimulation. In cells overexpressing SASH1, TRAF6 poly-ubiquitination was observed at 0 and 30 minutes LPS stimulation, as previously reported [89]. Overexpression of β-arrestin 1 alone also appeared to increase baseline (0 min LPS) TRAF6 poly-ubiquitination compared to control cells, but did not modulate the level of induction of TRAF6 poly-ubiquitination following 30 minutes LPS stimulation. Similar results as the β-arrestin 1 overexpressing cells were also observed in the cells overexpressing both β-arrestin 1 and SASH1. The absence of the reported β-arrestin 1-mediated negative regulation of LPS-induced TRAF6 poly-ubiquitination was unexpected. As a result, the role of SASH1 in modulating this negative regulation could not be determined.

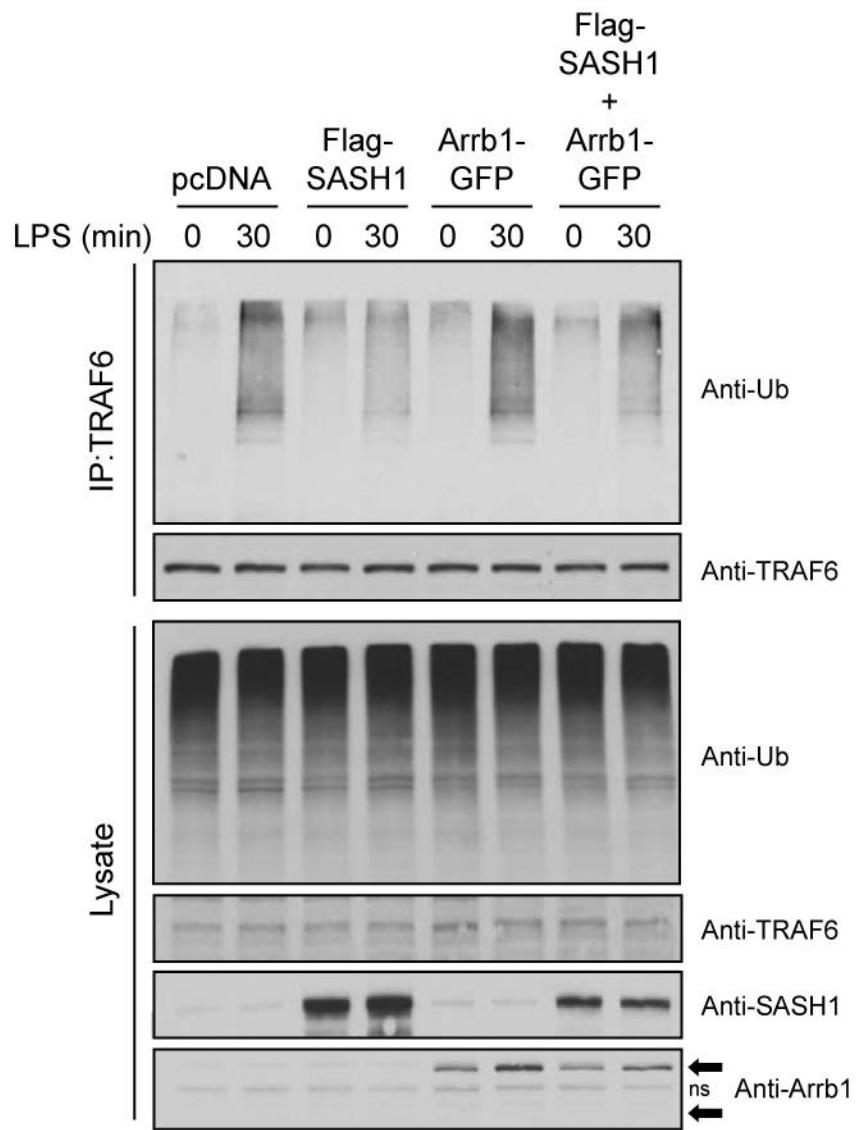


Figure 3.14 Enforced expression of β -arrestin 1 alone, or β -arrestin 1 and SASH1, does not modulate LPS-induced poly-ubiquitination of TRAF6. HEK293-TLR4-MD2-CD14 cells were transfected with Flag-SASH1 or Arrb1-GFP, or co-transfected with Flag-SASH1 and Arrb1-GFP. Transfected cells were subsequently stimulated with LPS (100 ng/mL) for 0 or 30 minutes. Lysates were immunoprecipitated (IP) for endogenous TRAF6 with anti-TRAF6 antibodies. Poly-ubiquitination of immunoprecipitated TRAF6 was monitored by immunoblotting with anti-ubiquitin. ns, non-specific.

As previously described, the enforced expression of TRAF6 has been shown to mediate TRAF6 oligomerization and auto-ubiquitination in the absence of an LPS stimulus [136]. Using HEK293T cells, Wang *et al.* demonstrated that overexpression of β-arrestin 1 could impair this auto-ubiquitination upon binding to TRAF6 [156]. We thus monitored whether SASH1 expression could modulate the β-arrestin 1-mediated negative regulation of TRAF6 auto-ubiquitination in HEK293T cells by impairing TRAF6-β-arrestin 1 binding (Figure 3.15). Increased auto-ubiquitination was confirmed upon overexpression of TRAF6 alone. Co-overexpression of SASH1 did not modulate this increased TRAF6 auto-ubiquitination, as previously observed in our lab (unpublished). Furthermore, co-overexpression of β-arrestin 1 alone, or co-overexpression of both β-arrestin 1 and SASH1 together, also demonstrated no effect on the level of TRAF6 auto-ubiquitination. Once again, despite previous reports, β-arrestin 1 was not found to have a negative effect on TRAF6 auto-ubiquitination, therefore the role of SASH1 in this process could not be evaluated.

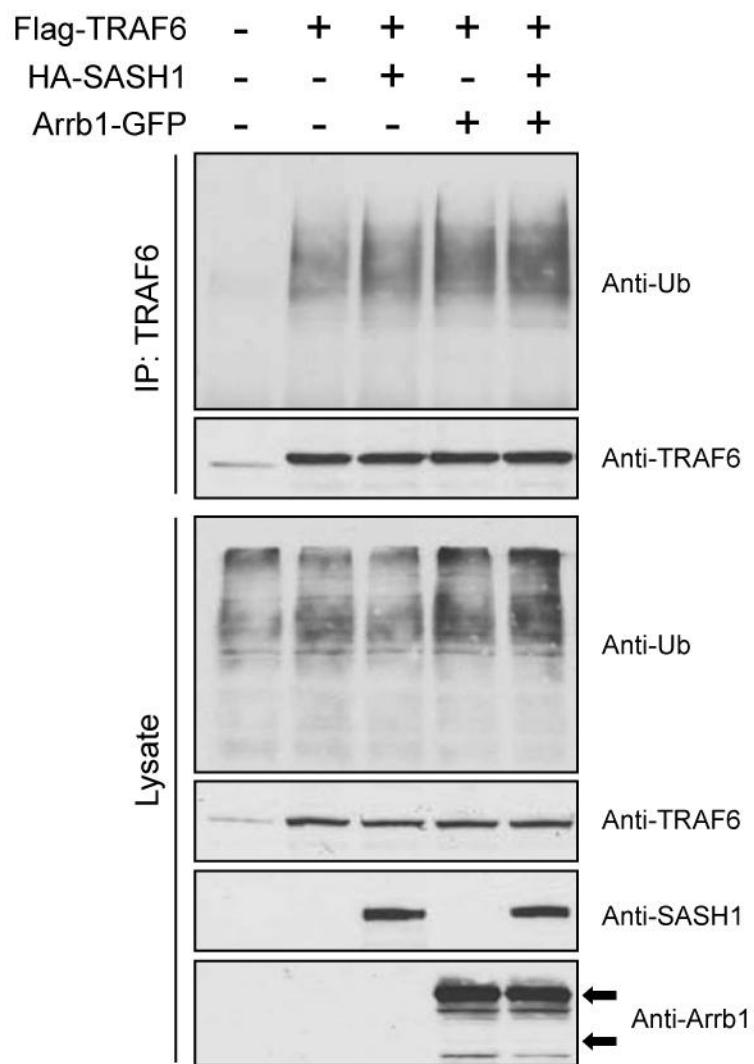


Figure 3.15 Enforced expression of β -arrestin 1 alone, or β -arrestin 1 and SASH1, does not modulate auto-ubiquitination upon overexpression of TRAF6. HEK293T cells were transfected with Flag-TRAF6 and either HA-SASH1 or GFP-tagged β -arrestin 1 (Arrb1) alone, or co-transfected with HA-SASH1 and Arrb1-GFP. Lanes labeled with (-) were transfected with the control empty vector. Lysates were immunoprecipitated (IP) for total TRAF6 with anti-TRAF6 antibodies. Poly-ubiquitination of immunoprecipitated TRAF6 was monitored by immunoblotting with anti-ubiquitin.

Wang *et al.* also demonstrated that the enforced expression of β-arrestin 2 could functionally impair TRAF6 auto-ubiquitination in HEK293T cells [156]. To confirm this β-arrestin 2-mediated regulation in our system, we monitored TRAF6 auto-ubiquitination in HEK293T cells upon co-overexpression of TRAF6 and either β-arrestin 1 or β-arrestin 2 (Figure 3.16). Furthermore, the effects of increased β-arrestin protein expression were monitored to confirm both isoform- and dosage-dependent β-arrestin-mediated regulation of TRAF6 auto-ubiquitination. HEK293T cells were co-transfected with TRAF6 and either 3 µg or 6 µg of each β-arrestin overexpression construct to represent “low” or “high” dose of β-arrestin expression, respectively. Once again, we confirmed increased TRAF6 auto-ubiquitination upon overexpression of TRAF6 alone. Low expression of β-arrestin 1 did not seem to impair this TRAF6 auto-ubiquitination, and may have actually increased levels of auto-ubiquitination. Furthermore, high β-arrestin 1 expression did not demonstrate significant impairment of TRAF6 auto-ubiquitination. Overexpression of β-arrestin 2, however, resulted in a significant decrease in TRAF6 auto-ubiquitination, independent of the expression dosage. These results suggest that, despite previous reports, β-arrestin 1 may not function to negatively regulate TRAF6 auto-ubiquitination in the same manner as β-arrestin 2.

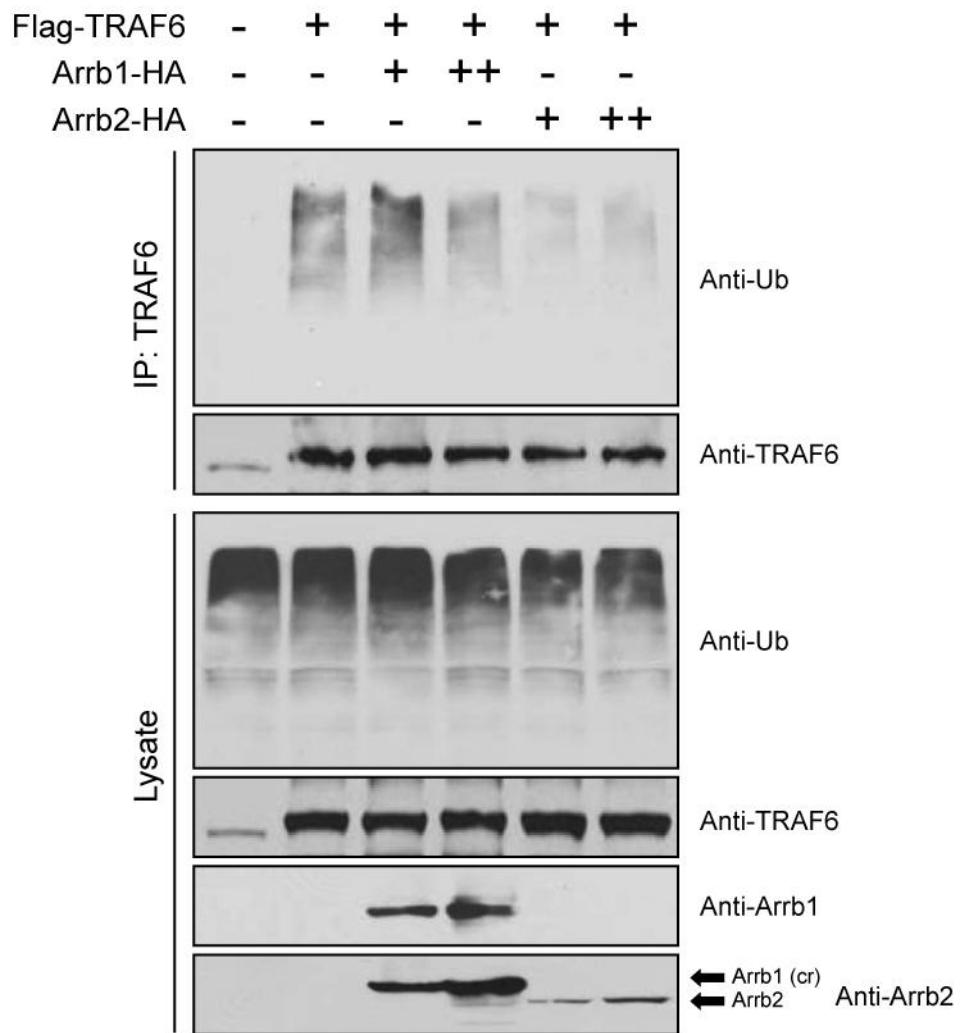


Figure 3.16 β-arrestin 1 does not function to impair TRAF6 auto-ubiquitination in the same manner as β-arrestin 2. HEK293T cells were transfected with Flag-TRAF6 and either 3 µg (+) or 6 µg (++) of HA-tagged β-arrestin 1 (Arrb1) or β-arrestin 2 (Arrb2). Lanes labeled with (-) were transfected with the control empty vector. Lysates were immunoprecipitated (IP) for total TRAF6 with anti-TRAF6 antibodies. Poly-ubiquitination of immunoprecipitated TRAF6 was monitored by immunoblotting with anti-ubiquitin. cr, cross-reacted.

3.11 Summary of Findings

The results presented in this chapter continue previous work conducted in our lab characterizing SASH1 as an important scaffold protein in the regulation of the endothelial TLR4 signaling [89]. Following up on previous findings in HEK293-TLR4-MD2-CD14 cells, we confirmed the interaction of endogenous SASH1 with TRAF6 in an LPS-dependent manner in endothelial cells. Furthermore, we found that endogenous SASH1 is required for LPS-induced TRAF6 auto-ubiquitination in these cells, an essential step in downstream pathway activation. Using a yeast two-hybrid approach, we identified β -arrestin 1, a reported negative regulator of TLR4 signaling, as a novel SASH1-binding protein. Due to the preferential expression of SASH1 in endothelial cells, we confirmed the expression of both the β -arrestin 1 and β -arrestin 2 isoforms in this cell type. Using HEK293T cells as a model interaction system, we verified the interaction of SASH1 with both β -arrestin 1 and β -arrestin 2, and characterized the domains required for these interactions. Focusing on the β -arrestin 1 isoform, we confirmed that TRAF6, SASH1 and β -arrestin 1 interact together in a complex. Interestingly, enforced expression of β -arrestin 1 was not found to impair SASH1 oligomerization, as previously reported for TRAF6. We further investigated the role of SASH1 in mediating TRAF6- β -arrestin interaction. Knockdown of SASH1 expression did not modulate the interaction of TRAF6 with both β -arrestin 1 and β -arrestin 2. Conversely, enforced expression of SASH1 was found to impair the interaction of TRAF6 with β -arrestin 1, but not β -arrestin 2. Preliminary results suggest that the interaction of SASH1 with TRAF6 may functionally impair TRAF6- β -arrestin 1 binding, possibly as a mechanism to positively regulate LPS-induced signals. We further aimed to monitor whether enforced expression of SASH1 could also impair β -arrestin 1-mediated negative regulation of TRAF6 activation.

Both the LPS-induced auto-ubiquitination of endogenous TRAF6, as well as enforced TRAF6 auto-ubiquitination were examined upon overexpression of β -arrestin 1 alone, or β -arrestin 1 and SASH1. Despite previous reports, these experiments did not reveal β -arrestin 1-mediated impairment of TRAF6 auto-ubiquitination, therefore the specific role of SASH1 in this process could not be evaluated. We were able to confirm, however, β -arrestin 2-mediated negative regulation of TRAF6 auto-ubiquitination in our system. Moving forward, further studies are required to characterize the potential isoform-specific roles for the β -arrestins in the regulation of TRAF6 activation in endothelial cells, and the significance of SASH1 in mediating this regulation.

CHAPTER 4: DISCUSSION AND FUTURE PERSPECTIVES

4.1 The Endothelium in Innate Immunity and Sepsis

The recognition of invading pathogens and the subsequent activation of immune and pro-inflammatory responses is critical in maintaining overall health and host survival [1-4]. Members of the TLR family of transmembrane receptors recognize specific PAMPs to mediate inflammatory responses [2, 17, 21, 23, 39]. TLR4 is expressed on the surface of immune and endothelial cells, and plays an essential role in the recognition and activation of immune responses to LPS, a major component of the outer membrane of Gram-negative bacteria [2, 17, 21, 23, 39]. Upon bacterial infection, circulating LPS is recognized and bound by TLR4, stimulating the downstream activation of several pro-inflammatory signal cascades and innate immune responses [2, 17, 21, 42, 85]. In the presence of severe infection and a systemic hyper-inflammatory state, the loss or malfunction of host regulatory mechanisms can result in the uncontrolled hyper-activation of inflammatory responses, contributing to the pathogenesis of sepsis [17, 52, 53, 131, 132]. In cases of Gram-negative sepsis, hyper-activation of TLR4 signaling may be due to the direct overstimulation of TLR4 by circulating LPS, or due to the dysregulation of endogenous negative regulatory mechanisms [131].

It has become increasingly evident that the endothelium plays a critical role in the pathophysiology of sepsis [14, 17, 19, 20]. The activation of endothelial cells during infection is critical for the production of pro-inflammatory mediators, leukocyte recruitment to the site of infection, as well as the regulation of vascular permeability and tone [17, 19-21]. In the presence of severe infection, however, the balance and normal physiological functioning of the endothelium is completely disrupted [14, 17, 19, 20]. The resulting

endothelial dysfunction can lead to the over-production of inflammatory mediators, severe hypotension, excess leukocyte recruitment, vascular leakage, and hyper-coagulation. These functional effects can ultimately lead to severe tissue damage, multiple organ failure and death [14, 17, 19-21]. Proper regulation of inflammatory responses is therefore essential to avoid the development of such uncontrolled, hyper-inflammatory syndromes [17, 52, 131-133]. Furthermore, understanding the mechanisms underlying this regulation is critical for the development of therapeutic strategies against such threatening inflammatory diseases as sepsis [17, 52, 131-133].

4.2 SASH1 is an Essential Scaffold Protein in Endothelial TLR4 Signaling

Although not extensively studied in TLR-mediated pathways, scaffold proteins may play a critical role in the coordination and regulation of pro-inflammatory signaling cascades and immune signal transduction [110, 111]. Our lab has recently identified and characterized SASH1 as a novel scaffold protein in endothelial TLR4 signaling [89]. SASH1 was found to positively regulate LPS-induced NF- κ B activation and pro-inflammatory cytokine production downstream of TLR4 in endothelial cells [89]. Furthermore, co-immunoprecipitation studies conducted in HEK293T cells revealed the interaction of SASH1 with several components of the MyD88-dependent TLR4 pathway, including TAK1, IKK α , IKK β and TRAF6 [89]. TRAF6 is an essential component of the TLR4 pathway, as *Traf6*-deficient mice demonstrate impaired NF- κ B and MAPK activation in response to LPS [77]. The interaction of SASH1 and TRAF6 was found to occur in an LPS-dependent manner in HEK293-TLR4-MD2-CD14 cells, suggesting physiological relevance for this interaction in this cell type [89]. Furthermore, overexpression of SASH1 in these cells was found to mediate K63-linked polyubiquitination of TRAF6 independent of an LPS stimulus, implicating a role for SASH1 in

the positive regulation of TRAF6 activation [89]. Although the HEK293-TLR4-MD2-CD14 cell line provides a model system for LPS-induced signaling, we aimed to confirm the interaction of SASH1 with TRAF6 in endothelial cells and evaluate the role for SASH1 in the LPS-induced activation of TRAF6 activation in this cell type. Although SASH1 and TRAF6 are constitutively expressed in HMEC, the interaction of these proteins was dramatically induced upon LPS stimulation of these cells. These results confirm that SASH1 and TRAF6 interact in an LPS-dependent manner in endothelial cells, further confirming physiological relevance for this interaction. We further examined the essential role for SASH1 in mediating LPS-induced auto-ubiquitination of TRAF6 in endothelial cells. Interestingly, shRNA-mediated knockdown of SASH1 in HMEC was found to abolish LPS-induced auto-ubiquitination of TRAF6. Although SASH1 was not found to interact with the ubiquitin-conjugating enzymes, UBC13 and UEV1A, these results suggest that SASH1 may play a critical role in coordinating TRAF6 oligomerization, essential for the TRAF6 auto-ubiquitination process [78, 79, 86, 89]. Oligomerization and auto-ubiquitination of TRAF6 are also essential for the activation of NF- κ B and MAPK downstream of TLR4, therefore these results complement our previous findings of impaired LPS-induced NF- κ B and MAPK activation in HMEC upon knockdown of SASH1 [78, 79, 86, 89]. Collectively, these results further characterize the role of SASH1 as a positive regulator of the endothelial TLR4 pathway, particularly at the step of TRAF6 activation. Although SASH1 has been shown to interact with other components of the TLR4 pathway, such as TAK1 and IKK β , independent of TRAF6, SASH1 may play an essential role in the assembly of these components to mediate downstream pathway activation [89].

4.3 SASH1 Interacts with β-arrestin 1 and β-arrestin 2

The role of SASH1 as a scaffold protein is supported by the presence of several putative protein-protein interaction domains within the structure of SASH1 [113]. Due to the characterized role of SASH1 as a scaffold protein in the TLR4 pathway, we aimed to identify novel SASH1-interacting proteins that may also be involved in immune signaling. Using SASH1 as bait in a yeast two-hybrid system, β-arrestin 1 was identified as a top interacting partner. Apart from their well-characterized roles in the regulation of 7TMR internalization and signal attenuation, both β-arrestin 1 and β-arrestin 2 have been identified as critical regulators of innate immune signaling [144, 145, 149]. In the context of TLR4 signaling, the β-arrestins have been characterized as endogenous negative regulators of LPS-induced NF-κB and AP1 activation *in vitro* upon impairment of TRAF6 oligomerization and auto-ubiquitination [149, 156]. The expression of such endogenous negative regulators is often induced upon TLR4 stimulation, acting as a negative feedback mechanism to terminate pro-inflammatory signaling [131-133]. Indeed, the expression of A20, another negative regulator of TRAF6 ubiquitination, has been shown to be induced in an NF-κB-dependent manner following LPS stimulation of endothelial cells [90, 93, 136-138]. Due to the preferential expression of *Sash1* in the mouse microvascular endothelium and the characterization of SASH1 as a critical regulator of immune signaling in human endothelial cells, we initially confirmed the expression of the β-arrestin proteins in our model endothelial cell line, HMEC. Interestingly, both β-arrestin 1 and β-arrestin 2 were found to be constitutively expressed in this cell line, independent of an LPS stimulus.

As well as coordinating the assembly of signaling complexes, scaffold proteins can play a critical role in the regulation of negative feedback mechanisms in signaling cascades [110].

The identification of β -arrestin 1 as a potential interacting partner of SASH1 suggested that SASH1 may also act as a scaffold for negative regulators of the TRAF6 signaling complex, thereby also coordinating negative regulation of the TLR4 pathway. We have previously confirmed the interaction of SASH1 with several components of the TLR4 pathway, including TRAF6, TAK1, IKK α and IKK β [89]. These interactions were modeled by reciprocal co-immunoprecipitation experiments using overexpression constructs in HEK293T cells [89]. We also confirmed the interaction of SASH1 with both β -arrestin 1 and β -arrestin 2 by reciprocal co-immunoprecipitation in this cell type. Although these interactions were also characterized in an overexpression system, we justified modeling our experiments in a consistent approach to further elucidate SASH1 functions within the TLR4 pathway. Similar to the interaction of SASH1 with TRAF6, the interaction of endogenous SASH1 with the β -arrestins must be confirmed in endothelial cells. Although we confirmed the constitutive expression of both β -arrestin 1 and β -arrestin 2 in HMEC, attempts to monitor the co-immunoprecipitation of these proteins with SASH1 were unsuccessful. Furthermore, although the yeast two-hybrid analysis may imply the direct interaction of SASH1 with β -arrestin 1, confirmation of the direct binding of SASH1 with the β -arrestin proteins remains to be determined.

4.4 Characterization of SASH1- β -arrestin Binding

Despite showing tremendous sequence diversity, structural SAM domains can mediate protein homo-oligomerization, as well as interactions with non-SAM domain-containing proteins [120]. The roles of the two SAM domains within SASH1, however, have remained previously uncharacterized. Further characterization of the interaction of SASH1 with the β -arrestins revealed a novel role for the SAM1 domain of SASH1. Interestingly, we found that

the SAM1 domain was required for the interaction of SASH1 with both β -arrestin 1 and β -arrestin 2, providing insight into the localization of β -arrestin binding. Due to the technical limitations of co-immunoprecipitation, however, we cannot conclude the direct binding of the β -arrestins to the SAM1 domain of SASH1. Further *in vitro* interaction experiments are therefore required to confirm the localization of β -arrestin 1 and β -arrestin 2 binding on SASH1.

Several studies have used β -arrestin deletion mutants to investigate the localization of protein binding on each β -arrestin isoform [154, 156, 163]. In a similar manner, we monitored the interaction of SASH1 with N-domain and C-domain β -arrestin deletion mutants. We found that the C-domains of both β -arrestin 1 and β -arrestin 2 were required for the interaction of these proteins with SASH1. Once again, these experiments provide insight into the localization of SASH1 binding on the β -arrestins, however, the direct interaction of SASH1 with the C-domain of either β -arrestin 1 or β -arrestin 2 remains to be determined. Interestingly, TRAF6 has been shown to interact with the C-domains of the β -arrestins in a similar manner [156]. TRAF6 was also shown to interact directly with β -arrestin 1 [156]. Indeed, TRAF6 may interact directly with the C-domain of both β -arrestin 1 and β -arrestin 2, however, the specific binding site has not yet been determined [156]. Similarly, SASH1 may also interact directly with the C-domain of the β -arrestins, or this interaction may be indirectly mediated by TRAF6.

4.5 Identification of a Novel TRAF6-SASH1- β -arrestin 1 Complex

Although both β -arrestin 1 and β -arrestin 2 were shown to interact with SASH1 by reciprocal co-immunoprecipitation, only β -arrestin 1 was revealed as a SASH1-binding protein in our yeast two-hybrid analysis. Since β -arrestin 1 has also been shown to interact

directly with TRAF6, we focused our studies on the identification and characterization of a novel TRAF6-SASH1- β -arrestin 1 complex [156]. Indeed, formation of this complex was confirmed by reciprocal co-immunoprecipitation. Once again, although these experiments were conducted in an overexpression system in HEK293T cells, they serve as a model to evaluate TRAF6-SASH1- β -arrestin complex formation. Interestingly, the interaction of SASH1 and β -arrestin 1 appeared to be enhanced in the presence of TRAF6. As previously speculated, the interaction of SASH1 with the C-domain of β -arrestin 1 may be stabilized or mediated indirectly by TRAF6. Alternatively, β -arrestin 1 may preferentially interact with SASH1 upon formation of a SASH1-TRAF6 complex, as the direct binding of β -arrestin 1 to TRAF6 may stabilize β -arrestin 1 binding to the SAM1 domain of SASH1. Further studies upon knockdown of TRAF6, or in *Traf6*^{-/-} cells, would ideally confirm the requirement of TRAF6 in mediating the interaction of SASH1 with β -arrestin 1.

4.6 Investigation of SASH1 Oligomerization

As previously discussed, SASH1 may play a role in mediating TRAF6 oligomerization to facilitate auto-ubiquitination of TRAF6. Indeed, the enforced expression of SASH1 has been shown to mediate LPS-independent K63-linked auto-ubiquitination of endogenous TRAF6, possibly by mediating TRAF6 oligomerization [89]. We speculated that the oligomerization of SASH1 itself may be involved in this process. Unfortunately, attempts to evaluate the role of SASH1 in TRAF6 oligomerization were inconclusive in this study. We were able to confirm the oligomerization of SASH1, however, the essential role of this oligomerization in the activation of LPS-mediated signaling remains to be determined. Furthermore, characterization of the domains required for SASH1 oligomerization is an area of future research. The presence of proline-rich sequences within the N- and C-terminus of

SASH1 may play a role in SASH1 oligomerization through interaction with the SH3 domain on another SASH1 molecule [117-119]. SAM domains have also been characterized to play a role in protein oligomerization, however, the role of the two SAM domains within SASH1 remain to be characterized in this process [120]. As demonstrated by several components of the TLR4 pathway, homo- and hetero-oligomerization of signaling proteins can be a critical regulatory step in pathway activation [42, 53, 63, 78]. Indeed, both endogenous and synthetic inhibitors of TLR4- and IL-1R-mediated signaling act to impair the interaction and oligomerization of several proteins throughout the pathway [71, 122, 135, 156, 164]. As previously described, β -arrestin 1 has been shown to directly interact with TRAF6 to impair TRAF6 oligomerization and subsequent auto-ubiquitination [156]. We speculated that the interaction of β -arrestin 1 in a complex with TRAF6 and SASH1 may also impair SASH1 oligomerization. The potential interaction of β -arrestin 1 with the SAM1 domain of SASH1, a possible site of SASH1 oligomerization, further supported this investigation. Upon overexpression of β -arrestin 1, however, no impairment in SASH1 oligomerization was observed. These results should be considered preliminary, however, as we were unable to confirm the reported β -arrestin 1-mediated impairment of TRAF6 oligomerization as a positive control in our system. Interestingly, our findings suggest that overexpression of β -arrestin 1 may actually enhance SASH1 oligomerization, although this must be confirmed. Moving forward, the role of β -arrestin 1 expression on the modulation of SASH1 oligomerization is an area of future investigation. The role of SASH1 oligomerization in the regulation of TRAF6- β -arrestin binding must also be evaluated. The formation of SASH1 oligomers may act as a regulatory mechanism to facilitate the oligomerization and positive

regulation of TRAF6 activation. Indeed, SASH1 oligomerization may modulate binding of negative regulators, such as the β -arrestins, to the TRAF6 signaling complex.

4.7 SASH1 Differentially Regulates TRAF6- β -arrestin Binding

To further model TRAF6-SASH1- β -arrestin 1 complex formation, we evaluated the essential role of SASH1 in mediating TRAF6- β -arrestin 1 binding in HEK293T cells. Interestingly, shRNA-mediated knockdown of SASH1 did not affect the co-immunoprecipitation of β -arrestin 1 with TRAF6 in these cells, suggesting that TRAF6- β -arrestin 1 binding can occur independent of SASH1. Similarly, knockdown of SASH1 did not affect the relative co-immunoprecipitation of β -arrestin 2 with TRAF6, confirming that SASH1 is not required for isoform-specific binding of the β -arrestins to TRAF6. Due to the limitations in shRNA-mediated knockdown efficiency, however, future studies in *Sash1*^{-/-} cells are required to confirm that complete loss of SASH1 expression does not modulate TRAF6- β -arrestin binding.

Although knockdown of SASH1 did not affect TRAF6- β -arrestin binding, we did not rule out a role for SASH1 in the regulation of TRAF6- β -arrestin complex formation. Corresponding to a role as a scaffold protein, SASH1 may interact with TRAF6 and the β -arrestins to physically coordinate and stabilize TRAF6- β -arrestin binding. Indeed, the potential binding of the β -arrestins to the SAM1 domain of SASH1 would bring the β -arrestins in close proximity to SASH1-bound TRAF6. The interaction of TRAF6 with both β -arrestin 1 and β -arrestin 2 was subsequently investigated upon enforced expression of SASH1 to monitor whether SASH1 could enhance TRAF6- β -arrestin binding. Interestingly, overexpression of SASH1 was found to impair binding of β -arrestin 1, but not β -arrestin 2, to TRAF6. We further confirmed impaired binding of TRAF6 to β -arrestin 1, but not β -arrestin

2, upon overexpression of SASH1 Δ SAM1. This SASH1 Δ SAM1 deletion mutant can interact with TRAF6, but was previously shown in this study to be incapable of interacting with the β -arrestins. These studies suggested that the impaired TRAF6- β -arrestin 1 binding observed upon the overexpression of SASH1 was not due to enhanced SASH1 binding and sequestration of β -arrestin 1 from TRAF6.

Moving forward, we speculated that the enforced interaction of SASH1 with TRAF6 may actually be impairing TRAF6- β -arrestin 1 binding. Preliminary studies monitored the interaction of TRAF6 with β -arrestin 1 upon overexpression of SASH1 Δ T6BD, which cannot bind to TRAF6. Interestingly, the interaction of TRAF6 and β -arrestin 1 may have been very slightly recovered in the absence of enforced SASH1-TRAF6 binding. These results are preliminary, however, and must be confirmed. Once again, there was no modulation of TRAF6- β -arrestin 2 binding in these studies, confirming a regulatory role for SASH1 that is specific for the β -arrestin 1 isoform. β -arrestin 1 has been characterized to interact directly with the coiled-coil domain within the TRAF domain of TRAF6 to physically impair TRAF6 oligomerization [156]. This TRAF domain is also required for the interaction of SASH1 with TRAF6 [89]. Furthermore, we have previously shown the impaired interaction of SASH1 with a TRAF6 deletion mutant specifically lacking the coiled-coil domain [89]. These findings may implicate a role for SASH1 in the regulation of TRAF6 oligomerization and subsequent auto-ubiquitination through interaction with the TRAF and coiled-coil domains [89]. Furthermore, the interaction of SASH1 with these domains on TRAF6 may modulate the binding of regulatory proteins that target TRAF6 oligomerization and auto-ubiquitination, such as the β -arrestins. Upon the enforced expression and interaction of SASH1 with TRAF6, however, SASH1 may physically occupy the TRAF domain and β -arrestin binding

site on TRAF6. The impaired or controlled binding of a negative regulator to TRAF6 by SASH1 would support our previous findings of SASH1 as a positive regulator of TRAF6 activation. It remains to be determined, however, why this SASH1-mediated negative regulation of TRAF6-β-arrestin binding appears to be specific for the β-arrestin 1 isoform. Furthermore, the specific mechanisms underlying this SASH1-mediated regulation are subject to future investigation.

4.8 β-arrestin 2, but not β-arrestin 1, Impairs Auto-Ubiquitination of TRAF6

As previously described, the enforced expression of SASH1 is capable of mediating K63-linked poly-ubiquitination of TRAF6, independent of an LPS stimulus [89]. Indeed, this SASH1-mediated positive regulation of TRAF6 activation may be due to the physically impaired binding of TRAF6 with β-arrestin 1. We thus attempted to evaluate the effects of SASH1 expression on the β-arrestin 1-mediated negative regulation of LPS-induced TRAF6 auto-ubiquitination using the HEK293-TLR4-MD2-CD14 cell line. We have previously used this cell line to model the role of SASH1 expression on LPS-induced TRAF6 auto-ubiquitination [89]. We confirmed LPS-independent poly-ubiquitination of TRAF6 upon overexpression of SASH1, as previously described [89]. Overexpression of β-arrestin 1, however, did not result in impaired LPS-induced TRAF6 poly-ubiquitination as reported by Wang *et al.* [156]. In fact, overexpression of β-arrestin 1 may have actually increased baseline levels of TRAF6 poly-ubiquitination in a similar manner as SASH1 expression. The discrepancy in our findings with the study by Wang *et al.* may have been due to different sources and concentrations of LPS used in our studies, as well as the use of a different model cell line in our system [156]. Moving forward, we monitored the effects of SASH1 and β-arrestin 1 expression on TRAF6 auto-ubiquitination in HEK293T cells upon overexpression

of TRAF6. TRAF6 overexpression has been shown to mediate the oligomerization and auto-ubiquitination of TRAF6 in the absence of an LPS stimulus [136]. Wang *et al.* demonstrated that overexpression of both β-arrestin 1 and β-arrestin 2 could impair this increased TRAF6 auto-ubiquitination in HEK293T cells [156]. We thus designed our experiments accordingly to recapitulate this β-arrestin 1-mediated negative regulation in HEK293T cells, and monitor the role of SASH1 expression in this process. We have previously observed that the overexpression of SASH1 alone does not modulate or enhance TRAF6 auto-ubiquitination upon overexpression of TRAF6 (unpublished). Unexpectedly, we also did not observe significant modulation of TRAF6 auto-ubiquitination upon the overexpression of β-arrestin 1. Due to these results, the role of SASH1 in the β-arrestin 1-mediated regulation of TRAF6 activation could not be interpreted in this study. As a separate control, we were able to confirm the impaired auto-ubiquitination of TRAF6 in our system upon overexpression of β-arrestin 2. The critical role of SASH1 in the β-arrestin 2-mediated regulation of TRAF6 auto-ubiquitination, however, remains to be determined. Collectively, these results suggested that, unlike β-arrestin 2, β-arrestin 1 may not function as a negative regulator of TRAF6 auto-ubiquitination as previously reported.

Moving forward, the functional significance of the interaction of TRAF6 with β-arrestin 1 should be reconsidered. Indeed, the study by Wang *et al.* remains as the only report of β-arrestin-mediated negative regulation of TRAF6 auto-ubiquitination [156]. We were capable of recapitulating β-arrestin 2-mediated negative regulation of TRAF6 auto-ubiquitination in our system, confirming numerous reports of this isoform functioning as a critical negative regulator of TLR4-mediated signaling [156, 158-160]. Although we did not observe negative regulation of TRAF6 activation through β-arrestin 1, our findings may support recent studies

confirming the non-redundant, isoform-specific roles for the β-arrestin proteins, particularly in response to LPS-induced inflammation [149, 159, 160, 162]. For example, studies conducted in mouse splenocytes have demonstrated impaired LPS-induced TNFα and IL-6 production in cells isolated from *Arrb1*^{-/-} mice compared to wild-type, indicative of a role for β-arrestin 1 as a positive regulator of pro-inflammatory cytokine production [149, 159]. Conversely, LPS-induced TNFα and IL-6 production was markedly increased in splenocytes isolated from *Arrb2*^{-/-} mice compared to wild-type, suggesting negative regulation by this isoform [149, 159]. Furthermore, *Arrb2*^{-/-} mice are significantly more susceptible to endotoxic shock compared to wild-type mice, suggesting that β-arrestin 1 does not function in a redundant manner to negatively regulate hyper-inflammatory responses [149, 156, 158-160]. The β-arrestin proteins have also been shown to function with cell-type specificity, demonstrating differential roles in the regulation of pro-inflammatory signaling in multiple cell types, including macrophages and splenocytes [149, 156, 159, 160].

4.9 Roles for the β-arrestins in Endothelial Cells

To date, no studies have characterized the role of the β-arrestin proteins in the regulation of LPS-mediated signaling in endothelial cells. Furthermore, the potential isoform-specific roles for the β-arrestin proteins in this regulation remain to be determined. Due to the characterized role of SASH1 as a critical scaffold protein in endothelial TLR4 signaling, the significance of the interaction with both β-arrestin 1 and β-arrestin 2 is of great interest in this cell type. Although our studies were conducted in HEK293T cells, they serve as a model to characterize TRAF6-SASH1-β-arrestin complex formation. Indeed, the differential regulation of TRAF6-β-arrestin binding by SASH1 may be reflective of isoform-specific roles for the β-arrestins in inflammatory signaling in endothelial cells. Moving forward, the

individual roles for β -arrestin 1 and β -arrestin 2 on the regulation of TRAF6 activation remain to be investigated in endothelial cell lines. Furthermore, due to the essential role for SASH1 in LPS-induced TRAF6 auto-ubiquitination and downstream pathway activation, future studies will focus on the critical role of SASH1 in coordinating β -arrestin-mediated regulation of pro-inflammatory signaling in endothelial cells.

Interestingly, the β -arrestins have been implicated in the vascular endothelial growth factor (VEGF)-mediated endocytosis of VE-cadherin and the regulation of endothelial cell permeability [163, 165]. In human umbilical vein endothelial cells (HUVEC), β -arrestin 1 was found to negatively regulate both VE-cadherin promoter expression and cell surface expression upon stimulation with VEGF [163]. As previously described, LPS stimulation has been shown to downregulate VE-cadherin expression in endothelial cells, contributing to the loss of endothelial barrier integrity, vascular leakage and edema presented in sepsis [15, 17, 33, 34]. Although the specific roles of SASH1 and the β -arrestins in the LPS-mediated regulation of VE-cadherin expression have not yet been examined, future studies will examine the interplay of these proteins in the regulation of endothelial function and barrier integrity in response to LPS.

4.10 Characterization of *Sash1* Function *in vivo*

Attempts to evaluate the role of *Sash1* in innate immune signaling *in vivo* have remained unsuccessful. In an effort to study the essential role of *Sash1* in a mouse model of sepsis, we generated a gene-trap mouse specifically targeting *Sash1* [89, 129, 130]. It was speculated that TLR4-mediated responses would be impaired in these mice due to the impaired activation of LPS-induced signaling observed *in vitro* in human cell lines upon knockdown of SASH1 [89]. Unfortunately, homozygous *Sash1* gene-trap mice demonstrate

neonatal lethality due to an apparent defect in lung development and respiration [89, 129, 130]. These mice succumb to severe respiratory distress syndrome, secondary to defects in surfactant production and alveolar maturity, suggesting a critical role for Sash1 in lung development (unpublished). Interestingly, β -arrestin double-null mice ($Arrb1^{-/-}Arrb2^{-/-}$) also demonstrate neonatal lethality due to respiratory distress [152]. These mice present with impaired surfactant production and impaired differentiation of alveolar epithelial cells, suggesting a potential convergence of function with Sash1 [152]. The endothelium has been shown to play a critical role in signaling the differentiation and maturation of the lung epithelium, particularly within the alveoli [166, 167]. Due to the highly vascularized nature of the lungs and alveolar compartments, and the preferential expression of *Sash1* in the endothelium, we speculate that Sash1 may play an important role in mediating the endothelial signaling required for maturation of the lung epithelium. Furthermore, we speculate that the β -arrestins, through interaction with Sash1, may also be involved in this process. Future work in our lab will aim to generate a conditional knockout mouse, specifically targeting *Sash1* expression in the endothelium. Generation of this mouse line will facilitate our examination of the role of endothelial Sash1 in organogenesis and development, particularly in the lungs. Furthermore, if these mice are viable, we can begin to evaluate the specific role of endothelial Sash1 in the regulation of innate immune signaling and the pathogenesis of sepsis.

4.11 Summary and Concluding Remarks

Sepsis is described as a systemic inflammatory response to severe infection, and is the leading cause of death in critically ill patients [14, 16, 17]. In the presence of severe infection, the loss or malfunction of host regulatory mechanisms can result in the

uncontrolled hyper-activation of inflammatory responses, contributing to the pathogenesis of this disease [18]. In cases of Gram-negative sepsis, hyper-activation of TLR4 signaling may be a consequence of the direct overstimulation of TLR4 by LPS, or the dysregulation of endogenous negative regulatory mechanisms [131]. The endothelium plays a critical role in the pathophysiology of sepsis [14, 17, 19, 20]. Activation of the endothelium during infection is critical for the production of pro-inflammatory mediators, leukocyte recruitment, and vasoregulation [17, 19-21]. Upon severe infection, however, the balance and normal physiological functioning of the endothelium is completely disrupted [14, 17, 19, 20]. This endothelial dysfunction can ultimately lead to severe tissue damage, multiple organ failure and potentially death [14, 19-21]. Proper regulation of inflammatory signaling is therefore essential to avoid the development of such uncontrolled, hyper-inflammatory syndromes [17, 52, 131-133].

Scaffold proteins may play a critical role in the coordination and regulation of pro-inflammatory signaling cascades and immune signal transduction [110]. We have recently identified and characterized SASH1 as a novel scaffold protein in endothelial TLR4 signaling [89]. The work presented in this thesis extends these findings to examine the role for SASH1 in the regulation TRAF6 activation, a critical step in downstream TLR4 pathway activation. Indeed, SASH1 was confirmed to interact with TRAF6 in an LPS-dependent manner in endothelial cells, and was required for LPS-induced auto-ubiquitination of TRAF6. Furthermore, we characterized the novel interaction of SASH1 with two previously identified negative regulators of TRAF6 activation, β -arrestin 1 and β -arrestin 2. Although these interaction experiments were conducted in HEK293T cells, they serve to model SASH1 function in a consistent manner with previous findings in our lab [89]. Indeed, we confirmed

the formation of a TRAF6-SASH1- β -arrestin 1 complex in these cells. Although endogenous SASH1 expression was not required for the interaction of either of the β -arrestins with TRAF6, enforced expression of SASH1 was found to specifically impair TRAF6- β -arrestin 1 binding. The functional significance of this differential TRAF6- β -arrestin regulation remains to be determined, but may be reflective of isoform-specific roles for the β -arrestin proteins in endothelial cells through interaction with SASH1. We speculated that SASH1 may functionally regulate TRAF6- β -arrestin 1 binding within the TRAF6-SASH1- β -arrestin 1 complex to mediate TRAF6 auto-ubiquitination and activation. Attempts to evaluate the role of SASH1 in coordinating β -arrestin 1-mediated negative regulation of TRAF6 activation, however, were unsuccessful. Unlike β -arrestin 2, we were unable to demonstrate β -arrestin 1-mediated negative regulation of TRAF6 auto-ubiquitination, as previously reported [156]. Future studies are required to confirm the functional significance of TRAF6- β -arrestin 1 binding in the regulation of TLR4 signaling in endothelial cells, and the role of SASH1 in this regulation.

Taken together, the work presented in this thesis further investigates the role of SASH1 as a scaffold protein in the regulation of TLR4-mediated signaling. Furthermore, these studies contribute to our understanding of the molecular mechanisms involved in the activation and regulation of inflammatory signaling and innate immune responses.

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