

**A novel pathway to sequester ASC at mitochondria-associated membranes dampens  
inflammasome activation during early *Salmonella* infection**

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B.Sc., The University of British Columbia, 2008

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF  
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Genome Science and Technology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2012

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## Abstract

*Salmonella enterica* is an intracellular bacterial pathogen that injects effector proteins into host cells and induces rapid cell death. Mitochondria associated membranes (MAMs) are important contact sites between mitochondria and ER and selectively facilitate  $\text{Ca}^{2+}$  uptake from ER lumen into mitochondria.

In the context of early *Salmonella* infection of THP-1 cells, a discontinuous sucrose gradient was used in combination with Stable Isotope Labeling by Amino acids in Cell culture (SILAC) to profile organelle proteomes. Protein profiles were generated for >800 mitochondria and ER proteins. I observed unique protein recruitments to MAMs during early *Salmonella* infection. The inflammasome adaptor protein, ASC is co-purified with MAM markers. SILAC immunoprecipitation (IP) experiments showed that ASC interacts directly with the VDAC/Stress-70 complex at MAMs, suggesting that ASC is specifically recruited to MAMs during *Salmonella* infection.

SILAC IP experiments identified an interaction between Flightless-I and ASC. Flightless-I is an actin binding protein and also interacts with *Salmonella* flagellin (FliC). siRNA knockdown of Flightless-I and mitofusin 2 (a structural protein of MAMs) both leads to significant increase of IL-1 $\beta$  during *Salmonella* infection. Simultaneous knockdown of Flightless-I and mitofusin 2 does not have additive effect, suggesting that they are in the same pathway to dampen inflammasome activation. Actin isolation experiments showed that ASC is enriched at actin filaments during *Salmonella* infection. Therefore, I proposed a model that during early *Salmonella* infection, FliC interacts with Flightless-I that then interacts with and transports ASC to MAMs via actin filaments. This process specifically sequesters ASC at MAMs to dampen inflammasome activation in the cytosol. Experimental

data from  $\Delta fliC\Delta fljB$  *Salmonella* infection of THP-1 cells and inflammasome reconstitution in 293T cells supported this model.

This study was the first characterization of MAM protein composition during bacterial infection. Furthermore, enrichment of ASC at MAMs was identified during early *Salmonella* infection, with Flightless-I interacting with and transporting ASC to MAMs via actin filaments at the presence of *Salmonella* flagellin. This novel pathway dampened the inflammasome activation during early *Salmonella* infection. This is the first report of the negative regulation of pyroptosis by *Salmonella* flagellin.

## **Preface**

Work involving *Salmonella* has been approved with Biosafety Certificate # B12-0088.

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## List of Abbreviations

ATP	adenosine-5'-triphosphate
ASC	apoptosis-associated speck-like protein containing a CARD
BCA	bicinchoninic acid assay
BES	bis-(2-hydroxyethyl)-2-amino-ethansulfonic acid
CARD	caspase activation and recruitment domains
DAMPs	danger-associated molecular patterns
DMEM	Dulbecco's Modified Eagle Medium
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERP	endoplasmic reticulum protein
FBS	fetal bovine serum
FLII	flightless-I
GLD	gelsolin-like domain
GRP	glucose-regulated protein
HRP	horseradish peroxidase
IAA	iodoacetamide
ICAT	isotope-coded affinity tags
IFN	interferons
IL-1 $\beta$	interleukin-1 beta
IL-18	interlukin-18
IL-33	interlukin-33
IP	immunoprecipitation
IPAF	ice protease-activating factor
iTRAQ	isobaric tags for relative and absolute quantitation
LB	Luria's Broth
LPS	lipopolysaccharides
LRRFIP1	leucine-rich repeat (in Flightless I) interacting protein-1
LRRFIP2	leucine-rich repeat (in Flightless I) interacting protein-2
LRR	leucine-rich repeats
MAKP	mitogen-activated protein kinase
MAMs	mitochondria-associated membranes
MAVS	mitochondrial antiviral-signaling protein
NLR	nucleotide-binding domain, leucine-rich repeat proteins
NLRC	NLR family CARD domain-containing protein
NF-kB	nuclear factor-kappaB
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PBST	phosphate buffered saline containing 0.1% Tween
pcp	protein correlation profiling
PVDF	polyvinylidene fluoride

RIG-I	retinoic acid inducible–gene I
ROS	reactive oxygen species
SCV	<i>Salmonella</i> -containing vacuoles
SILAC	stable isotope labeling by amino acids in cell culture
Sif	<i>Salmonella</i> -induced filament
shRNA	small hairpin RNA
siRNA	small intefering RNA
SPI	<i>Salmonella</i> pathogenicity island
STAGE tip	stop-and-go extraction tip
T3SS	type-III secretion system
T4SS	type-IV secretion system
TMB	tetramethyl benzidine
TMT	tandem mass tages
TLR	toll-like receptor
VDAC	voltage-dependent anion-selective channel protein

## Acknowledgements

I'd like to express my most sincere appreciation for my supervisor, Dr. Leonard Foster, for giving me this opportunity to explore a project of my own interest. Thank you for your guidance, patience and encouragement for teaching me to conduct true scientific research in the past five years. And thank you for your caring heart for students.

I'd also like to thank my supervisory committee, Dr. R.E.W. Hancock and Dr. Jürgen Kast for your valuable advice and support through the past two years.

I'd like to thank all Foster lab members who have always shared their time and experience with me. Special thanks to Dr. Queenie Chan who introduced me into the proteomics field, Dr. Lindsay Rogers who introduced me into the *Salmonella* world, Dr. Nikolay Stoyanov who provided all technical help for mass spectrometry, Dr. Nat Brown who provided valuable guidance, and Dr. Hongbing Yu who gave me on-going input on my ideas and data in this project. I'd like to also thank Dr. Queenie Chan, Dr. Hongbing Yu and Louise Lund for proof-reading my thesis.

I'd like to thank my husband, Ning Tu (Tony). You have loved, supported and took care of me through the past two years. You are my best friend and companion. I'd also like to thank my coming baby. Thank you for accompanying mom in the final stages to wrap up the project and to write out the thesis. I'd like to thank my mom and dad. Thank you for your hard work to support my undergraduate degree in Canada and for being such good role models as diligent and responsible people. I love you all.

I'd like to thank the GSAT program for generously providing a three-year scholarship.

## **Dedication**

To my parents, my husband, and my baby in the womb.

# **1. Introduction**

## **1.1 *Salmonella* and *Salmonella* infection**

*Salmonella enterica* is a Gram-negative, facultative intracellular pathogen. There are over 2,500 serovars that are categorized into six subspecies (Ochman, Groisman 1994). This enteric pathogen is acquired orally and causes four types of symptoms: typhoid fever, diarrhea, bacteremia and asymptomatic carriage (Coburn, Grassl et al. 2007). It causes 200 million to 1.3 billion cases of enterocolitis and around 3 million deaths worldwide every year (WHO website 2005).

*Salmonella* infection is studied mainly in two models: 1) a typhoid fever model, and 2) an intestinal inflammation model. In the typhoid model, *Salmonella* survives gastric acidity and invades across the intestinal epithelium via phagocytic epithelial M cells at Peyer's patches or via intestinal dendritic cells (Jones, Ghorri et al. 1994, Rescigno, Urbano et al. 2001). *Salmonella* translocates to the spleen and liver by hijacking lymphocytes and eventually resides inside macrophages, dendritic cells, neutrophils, splenocytes, and hepatocytes (Vazquez-Torres, Jones-Carson et al. 1999, Richter-Dahlfors, Buchan et al. 1997, Nakoneczna, Hsu 1980). In this model, survival and replication inside phagocytes are crucial for the bacterial invasion (Fields, Swanson et al. 1986). In the intestinal inflammation model, *Salmonella* causes acute inflammatory responses and diarrhea in the intestine. The local inflammation is marked by recruitment of neutrophils to the site of infection and accompanied by rapid fluid flux (Fields, Swanson et al. 1986, McGovern, Slavutin 1979).

*Salmonella* encodes two type III secretion systems (T3SS) that inject a cascade of over thirty effector proteins directly into the host cytosol to manipulate host cell functions. The

genes for the two T3SS apparatuses and some of their effectors are encoded on two separate *Salmonella* pathogenicity islands (SPI) that are likely to be obtained by horizontal transfer (Brown, Finlay 2011). The SPI-1 T3SS and its effectors are mainly responsible for bacterial invasion (Galan, Curtiss 1989, Zhang, Santos et al. 2002), while SPI-2 T3SS and its effectors are responsible for the intracellular survival and the systematic infection (Shea, Hensel et al. 1996, Coburn, Li et al. 2005).

*Salmonella* is capable of rapidly invading phagocytes and non-phagocytic epithelial cells because SPI-1 effectors can potently induce phagocytosis by membrane deformation and actin rearrangement. This can effectively facilitate bacterial internalization upon contact with the host cell. SPI-1 effectors (SipA, SipC, SopB, SopD and SopE/E2) are involved in this process (Srikanth, Mercado-Lubo et al. 2011). Another SPI-1 effector, SptP, antagonizes this process and returns the host cytoskeleton to its resting state after bacterial internalization (Srikanth, Mercado-Lubo et al. 2011). Once inside the host cell, most *Salmonella* are located in a special membrane-bound vacuole called the *Salmonella* Containing Vacuole (SCV). SCVs avoid fusing with lysosomes and traffics towards Golgi apparatus where the bacteria have access to cellular nutrients. SCVs eventually position themselves in the perinuclear region. SPI-1 effectors (SopE, SopB, SipA) and SPI-2 effectors (SseJ, SteC, SseI and SspH2) are involved in the proper maturation and trafficking of SCVs (Srikanth, Mercado-Lubo et al. 2011). Once at the perinuclear area, *Salmonella* starts to replicate rapidly inside the SCV. Meanwhile, membrane protrusions called *Salmonella* induced filaments (Sif) start to form. SPI-1 effectors (SipA, SopB) and SPI-2 effectors (SseG, SseF, SifA, PipB2) are involved in SCV positioning and Sif formation (Srikanth, Mercado-Lubo et al. 2011). The SPI-2 effectors, SseJ and SpvB antagonize Sif formation (Srikanth, Mercado-Lubo et al. 2011). The

interplays among effectors and between effectors and host signaling proteins ensure the proper timing for *Salmonella* invasion and intracellular survival (Cain, Hayward et al. 2008, McGhie, Brawn et al. 2009). In addition to the SPI-1 and SPI-2 T3SS systems, the *sci*-encoded T6SS and the ZirTS secretion system contribute to *Salmonella* virulence and modulate the biological processes during *Salmonella* infection (Filloux, Hachani et al. 2008, Gal-Mor, Gibson et al. 2008).

## **1.2 Pathogen-induced cell death**

There are four principal types of cell death induced by pathogenic infection: apoptosis, oncosis/necrosis, pyroptosis and autophagic cell death (Labbe, Saleh 2008). Apoptosis and autophagic cell death are non-inflammatory and do not involve membrane rupture or release of intracellular contents (Labbe, Saleh 2008). Oncosis and pyroptosis are pro-inflammatory with membrane swelling and rupture, and with release of intracellular contents into the extracellular space (Labbe, Saleh 2008). Apoptosis and pyroptosis are associated with DNA fragmentation and nuclear condensation, while oncosis and autophagic cell death are not (Labbe, Saleh 2008). Apoptosis and pyroptosis are mediated by cysteinyl aspartate-specific proteases (caspases), while the other two forms of cell death are not (Labbe, Saleh 2008). The caspases are expressed as inactive precursors (pro-caspases) and they are typically activated by catalytic cleavage by other already activated caspases or by autoproteolysis induced by close proximity (Siegel 2006).

Apoptosis is a well-studied form of programmed cell death and is mediated by the apoptotic caspases: caspase-2, 8, 9, 10 as the initiator caspases and caspase-3, 6, 7 as the executioner caspases (Siegel 2006). Apoptosis is initiated by two pathways. The first one



involves mitochondria outer membrane permeabilization and release of cytochrome c from mitochondria into cytosol (Labbe, Saleh 2008). Cytosolic cytochrome c can activate caspase-9 in a multi-protein complex called the apoptosome and caspase-9 then cleaves and activates caspase-3, 6, 7 (Labbe, Saleh 2008). The executioner caspases then cleave cellular proteins and cause cell death (Labbe, Saleh 2008). The second pathway involves TNF receptor and Fas on cell membrane that can activate caspases-8 and caspases-10 (Labbe, Saleh 2008). Similarly, caspase-3, 6, 7 are then cleaved and activated with further protein cleavage and cell death (Labbe, Saleh 2008). During apoptosis, the following cell morphology is typically observed: DNA fragmentation, nuclear condensation, membrane shrinking, formation of small, membrane-bound vacuoles called apoptotic bodies (Labbe, Saleh 2008).

Pyroptosis is mediated by one proinflammatory caspase, caspase-1, and is observed in macrophages and dendritic cells (Miao, Rajan et al. 2011). This type of rapid cell death is accompanied by secretion of proinflammatory cytokines: IL-1 $\beta$ , IL-18 and IL-33 (Miao, Rajan et al. 2011). Pyroptosis is activated by formation of multi-protein complexes called inflammasomes involving NLR proteins (discussed in detail in Section 1.3). Upon activation by ligands or stimuli, NLR proteins oligomerize via their NATCH domains and recruit pro-caspase-1 via the CARD domain with the help of an adaptor protein, ASC (Miao, Rajan et al. 2011). The close proximity of multiple pro-caspase-1 molecules initiates its autoproteolytic activity to produce p10 and p20 peptides that then form the active caspase-1 enzyme (Miao, Rajan et al. 2011). Activated caspase-1 is responsible for the rapid cell death, pore formation on membranes and the cleavage of proinflammatory cytokines (Miao, Rajan et al. 2011). During pyroptosis, the following cellular morphology is typically observed: loss of mitochondrial membrane potential, DNA fragmentation, nuclear condensation, plasma

membrane swelling and rupture, and release of cytoplasmic contents into the extracellular matrix (Labbe, Saleh 2008). Both the cell lysis and the secretion of proinflammatory cytokines make pyroptosis a highly proinflammatory process (Labbe, Saleh 2008).

Oncosis or necrosis is a rapid form of cell death independent of caspases. It is induced by extracellular stress or by high loads of intracellular bacteria (Labbe, Saleh 2008). It is believed to be accidental and less controlled, although there are some specific pathways that have been reported recently (Labbe, Saleh 2008). The cellular morphology changes include membrane swelling, increased permeability and rupture. It is also very proinflammatory due to release of intracellular contents (Labbe, Saleh 2008).

Autophagy is a highly regulated cellular process important for the removal and degradation of damaged organelles and proteins (Klionsky, Emr 2000). It involves lysosomal degradation of intracellular components inside double-membrane vacuoles (autophagosomes) (Klionsky, Emr 2000). Autophagic cell death is observed with excessive autophagy during some microbial infections (Labbe, Saleh 2008, Levine, Deretic 2007). The cellular morphological characteristics include excessive vacuolization and internalization by neighboring cells (Labbe, Saleh 2008). This process is non-inflammatory and intracellular contents are not released (Labbe, Saleh 2008).

*Salmonella* can typically stimulate apoptosis in epithelial cells and pyroptosis in macrophages. Autophagy and autophagic cell death are also observed. More details are discussed in section 1.5.

### **1.3 Inflammasomes**

The innate immune system includes two families of germline-receptors for recognition of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) (Schroder, Tschopp 2010). Proteins in the Toll-like receptor (TLR) family can recognize pathogen-associated ligands on cell surface and in intracellular compartments (Akira, Takeda 2004). Proteins in the NOD-like receptor (NLR) family can recognize pathogen-associated ligands and host danger-associated molecules in the cytosol (Schroder, Tschopp 2010). Crosstalk between TLR and NLR pathways regulates the activation of both the innate and adaptive immune systems. During NLR signaling, TLR pathways first activate the NF- $\kappa$ B pathway and up-regulate the expression of NLR proteins and proinflammatory cytokines (Hiscott, Marois et al. 1993). NLR signaling activates the proinflammatory caspase-1 that leads to pyroptosis and maturation of proinflammatory cytokines (Martinon, Burns et al. 2002). Therefore, the TLR and NLR pathways work stepwise to activate the innate immunity at different locations within the host cell.

The NLR protein family in humans encodes 22 genes in three distinct families: NODs, NLRPs and IPAFs (Schroder, Tschopp 2010). All the proteins in this family contain a central NOD domain that allows homotypic or heterotypic oligomerization with itself or with other members of the NLR family (Schroder, Tschopp 2010). Typically, NLR proteins contain C-terminal leucine-rich repeats (LRR) that are originally proposed to participate in ligand sensing, although the specific ligands remain unknown (Schroder, Tschopp 2010). The LRR domain in NLRC4 is responsible for auto-inhibition of its activation (Poyet, Srinivasula et al. 2001). Similarly, NLR proteins contain an N-terminal effector domain (CARD domain or PYRIN domain) that is responsible for the recruitment of pro-caspase-1 (Schroder, Tschopp

2010). The CARD domain can recruit pro-caspase-1 directly and the PYRIN domain typically interacts with an adaptor protein ASC to indirectly recruit pro-caspase1 (Schroder, Tschopp 2010). Some NLR proteins contain the FIIND domain for further protein-protein interactions (Schroder, Tschopp 2010) and HIN-200 family proteins contain the HIN domain that can directly detect microbial dsDNA (Roberts, Idris et al. 2009).

Multiple copies of NLR proteins and pro-caspase-1 can form high molecular weight complexes called inflammasomes that lead to caspase-1 activation and pyroptosis in macrophages and dendritic cells (Petrilli, Dostert et al. 2007). The apoptosis-associated speck-like protein containing a CARD (ASC) is an important adaptor for inflammasome formation (Agostini, Martinon et al. 2004). It is composed of an N-terminal PYRIN domain and C-terminal CARD domain (Liepinsh, Barbals et al. 2003). It bridges between pro-caspase-1 and NLR proteins lacking the CARD domain and stabilizes the interaction between pro-caspase1 and NLR proteins containing the CARD domain (Agostini, Martinon et al. 2004). Inflammasomes are formed and activated in the cytosol (Agostini, Martinon et al. 2004). Upon ligand activation, NLR proteins self-oligomerize and subsequently recruit ASC and pro-caspase-1 (Petrilli, Dostert et al. 2007). The resulting close proximity of pro-caspase-1 induces the auto-proteolytic activity of pro-caspase-1 to release the p10 and p20 peptides (Petrilli, Dostert et al. 2007). These two peptides form the catalytic caspase-1 that then cleaves cellular proteins and proinflammatory cytokines. Proinflammatory cytokines, IL-1 $\alpha$ , IL-1 $\beta$ , IL-18 and IL-33 have been reported to be cleaved by caspase-1 and secreted during inflammasome activation (Petrilli, Dostert et al. 2007).

So far, there are four well-studied inflammasomes that respond to various pathogens or danger signals (Schroder, Tschopp 2010). The NLRP1 inflammasome responds to the lethal

toxin from *Bacillus anthracis* and bacterial muramyl dipeptides (Boyden, Dietrich 2006, Faustin, Lartigue et al. 2007). The PYHIN inflammasomes (mediated by AIM2 and FIF16) are activated by dsDNA during viral and intracellular bacterial infections (Roberts, Idris et al. 2009, Fernandes-Alnemri, Yu et al. 2009, Burckstummer, Baumann et al. 2009). The NLRP3 inflammasome responds to a variety of cellular danger signals: potassium efflux, generation of reactive oxygen species (ROS) and phagosomal membrane destabilization (Cruz, Rinna et al. 2007, Hornung, Bauernfeind et al. 2008). Some single molecules can potently activate the NLRP3 inflammasome: alum, high concentration of extracellular ATP, uric acid crystal etc. (Hornung, Bauernfeind et al. 2008, Mariathasan, Weiss et al. 2006, Martinon, Petrilli et al. 2006). Notably, ATP, P2X<sub>7</sub> ion channel opening and phagolysosomal membrane destabilization can activate both the NLRP1 and NLRP3 inflammasomes, suggesting a partial functional overlap between these two pathways (Ali, Timmer et al. 2011, Newman, Leppla et al. 2009). In addition, the NLRP3 inflammasome is activated by several bacterial pathogens possibly by pore formation on cell membranes or destabilization of phagosomes (Rathinam, Vanaja et al. 2012a). The NLRC4 inflammasome mainly responds to bacterial flagellin and T3SS with the help of other NLR proteins (Schroder, Tschopp 2010). Bacterial flagellin and proteins in some T3SS and T4SS apparatuses can activate the NLRC4 inflammasome via NAIP5 and NAIP2 in mouse cells (Zhao, Yang et al. 2011, Kofoed, Vance 2011). In human cells, the NAIP protein was proposed to play a similar role in recognizing the T3SS apparatus in *Chromobacterium violaceum* (Zhao, Yang et al. 2011). The specific adaptors to recognize the other Gram-negative organisms (*Salmonella*, *Legionella* etc.) remain to be identified for the NLRC4 inflammasome in human cells (Zhao, Yang et al. 2011).

## 1.4 ASC regulation of innate and adaptive immune systems

In addition to its function as the adaptor protein for inflammasomes, ASC is an important regulator of the innate and adaptive immune systems. During *Legionella pneumophila* infection of macrophages, ASC negatively regulates the NF- $\kappa$ B pathway and limits intracellular bacterial growth (Abdelaziz, Gavrilin et al. 2011b, Abdelaziz, Gavrilin et al. 2011a). During chronic *Mycobacterium tuberculosis* infection, ASC is essential for proper granuloma formation and controlling intracellular bacteria replication (McElvania Tekippe, Allen et al. 2010). ASC suppresses tumor proliferation by regulating apoptosis in a similar mechanism (Siraj, Hussain et al. 2011). Deficiency in ASC causes defects in phagocytosis in dendritic cells (Ippagunta, Malireddi et al. 2011a). Consequently, these dendritic cells fail to induce the proper T cell response (Ippagunta, Malireddi et al. 2011a). In addition, deficiency in ASC causes defects in normal cell migration of B and T lymphocytes (Ippagunta, Malireddi et al. 2011a). These studies suggest that ASC is involved in essential biological processes to boost effective adaptive immunity.

Several recent studies revealed that ASC finely tunes NLRC4 inflammasome activation. Earlier data suggested that although ASC is not required for the NLRC4 inflammasome activation, it effectively enhances the NLRC4 signaling pathway by stabilizing the inflammasome complex (Franchi, Stoolman et al. 2007, Mariathasan, Newton et al. 2004, Suzuki, Franchi et al. 2007). Recent data from two separate groups suggested that there are two types of inflammasome protein complexes formed during *Salmonella* and *Legionella* infection: one includes ASC and the other one does not (Broz, von Moltke et al. 2010, Case, Roy 2011). The association of ASC with pro-caspase-1 effectively leads to activation of caspase-1 and cytokines (IL-1 $\beta$  and IL-18) while the NLRC4/pro-caspase-1 complex is

responsible for rapid cell death (Broz, von Moltke et al. 2010, Case, Roy 2011). However, these two groups reported different readouts for these two types of complexes. One group showed that the NLRC4-pro-caspase-1 complex cannot induce any IL-1 $\beta$  processing or secretion, and both complexes contribute to cell death (Broz, von Moltke et al. 2010). The other group showed that both complexes contribute to the processing of cytokines (IL-1 $\beta$  and IL-18), and the ASC-pro-caspase-1 complex cannot not induce cell death (Case, Roy 2011). The second group has further shown that the ASC-pro-caspase-1 complex can recruit NLRC4 to modulate cell death. Nevertheless, both reports support the idea that ASC can tune the downstream signaling of the NLRC4 inflammasome.

### **1.5 Activation of innate immunity by *Salmonella***

*Salmonella* can potently activate the innate immune system during the invasion process. *Salmonella* LPS is detected by TLR4 and flagellum is detected by TLR5 on the host cell membrane (Akira, Takeda 2004). The signaling pathways of TLR4 and TLR5 activate the NF- $\kappa$ B pathway and up-regulate the expression of cytokines and NLR proteins (Franchi 2011). Specifically, the proinflammatory cytokine IL-8 is responsible for recruitment of neutrophils to the site of infection (Franchi 2011). SPI-1 effectors can activate the mitogen-associated protein kinase (MAPK) pathway to further activate the NF- $\kappa$ B pathway through Cdc42 (Chen, Hobbie et al. 1996, Hobbie, Chen et al. 1997, Bruno, Hannemann et al. 2009). Specifically, SipA is important for neutrophil recruitment (Lee, Silva et al. 2000) and SopE is critical for the MAPK pathway activation (Hardt, Chen et al. 1998). In addition, the SPI-1 effector SipB activates caspase-1 through unidentified mechanisms (Hersh, Monack et al. 1999). Activation of caspase-1 leads to pyroptosis, the rapid proinflammatory macrophage

death. Under the SPI-2 condition, pyroptosis can also occur, and the SPI-2 effectors SpvB and SseL are the ligands (Browne, Hasegawa et al. 2008, Rytönen, Poh et al. 2007).

There are some effector proteins that act to suppress the host innate immune response. The SPI-1 effector SptP is a GAP and tyrosine phosphatase, and it inhibits MAPK pathway activation (Murli, Watson et al. 2001, Lin, Le et al. 2003). AvrA, another SPI-1 effector, suppresses Jnk activation (Jones, Wu et al. 2008). The SPI-2 effectors SpvC and SseL inhibit the Erk/Jnk/MAKP and NF- $\kappa$ B pathways (Mazurkiewicz, Thomas et al. 2008, Le Negrate, Faustin et al. 2008).

*Salmonella* invasion also strongly activates the NLR signaling pathways. Nod1 and Nod2 proteins sense the peptidoglycan-related ligands and contribute to the activation of MAKP and NF- $\kappa$ B pathways (Inohara, Nunez 2003). The NLRP3 inflammasome is responsible for SPI-2-dependent pyroptosis through unknown mechanisms (Broz, Newton et al. 2010). The NLRC4 inflammasome is significantly activated by *Salmonella* flagellin and SPI-1 T3SS (Miao, Alpuche-Aranda et al. 2006, Miao, Mao et al. 2010b). Monomeric flagellin is secreted into the host cytosol by the flagella T3SS and the SPI-1 T3SS, and this process is mediated by bacterial sensing of lysophospholipids (Subramanian, Qadri 2006, Sun, Rolan et al. 2007). Cytosolic flagellin can activate the NLRC4 inflammasome and lead to caspase-1 activation, pyroptosis, and activation of proinflammatory cytokines (Miao, Alpuche-Aranda et al. 2006). In mouse cells, NAIP5 interacts with *Salmonella* flagellin directly and bridges flagellin and NLRC4, while in human cells, the corresponding receptor is not yet identified (Zhao, Yang et al. 2011, Kofoed, Vance 2011). PrgJ, a rod protein for SPI-1 T3SS, can also activate the NLRC4 inflammasome via the NAIP2 protein in mouse cells (Zhao, Yang et al. 2011, Kofoed, Vance 2011). The downstream signaling and consequence of the NLRC4



inflammasome activation is identical to the flagellin-NAIP5-NLRC4 pathway (Zhao, Yang et al. 2011, Kofoed, Vance 2011). Still, the specific receptor for PrgJ is not identified in human cells (Zhao, Yang et al. 2011, Kofoed, Vance 2011).

Upon invasion, *Salmonella* significantly down-regulates flagellin and SPI-1 T3SS, likely to evade detection by the NLRC4 inflammasome. A recent paper showed that expression of flagellin under a SPI-2 promoter can significantly attenuate the virulence during the systemic infection, suggesting that the NLRC4 inflammasome is critical to detect and clear *Salmonella*, and intracellular *Salmonella* down regulates flagellin to evade host recognition (Miao, Leaf et al. 2010). Notably, the SPI-2 T3SS is not detected by the NLRC4 inflammasome (Miao, Mao et al. 2010b). However, the NLRC3 inflammasome is activated during the SPI-2 conditions by unknown mechanisms (Broz, Newton et al. 2010) and pyroptosis is an effective mechanism for bacterial clearance (Miao, Leaf et al. 2010).

Taken together, there is a close interplay between *Salmonella* and the host innate immune system. Pyroptosis and inflammation via inflammasome activation are important mechanisms to restrict infection and clear bacteria although they cause tissue damage to the host. *Salmonella* has evolved mechanisms to reduce the innate immunity activation by antagonizing effector proteins and by down-regulating ligands for the NLRC4 inflammasome.

## 1.6 MAMs

The mitochondria-associated endoplasmic reticulum membrane (MAM) is a specialized subdomain of the smooth endoplasmic reticulum (ER) membrane where ER tubules come in close contact with mitochondria outer membranes (Fujimoto, Hayashi 2011). The ER membranes are highly dynamic; they originate from the nuclear envelope and extend throughout the cytosol (PALADE 1956). They form various close membrane contacts with other organelles and cellular structures, such as the Golgi apparatus, endosomes, lysosomes, peroxisomes, plasma membranes (Voeltz, Rolls et al. 2002). Similar to MAMs, a subdomain of smooth ER also associates with plasma membranes (Rapoport 2007). This structure is called plasma membrane-associated membranes (PAMs). They are involved with the lipid transport to the plasma membrane (Rapoport 2007) and facilitate  $\text{Ca}^{2+}$  entry from the extracellular space (Lebiedzinska, Szabadkai et al. 2009).

The MAM structure was first reported by microscopic observation of fish gill cells in 1959 (COPELAND, DALTON 1959) and since then, experiments involving detergent-free homogenization have repeatedly shown the co-sedimentation of ER membranes and mitochondria (Fujimoto, Hayashi 2011). Like the ER, the MAM structure is highly heterogeneous: ER tubules cover mitochondria from 10% to 80% in different types of cells and different structures (Csordas, Renken et al. 2006a, Dai, Kuo et al. 2005, de Meis, Ketzer et al. 2010). There are unique protein and lipid components at MAMs to differentiate them from the bulk ER membranes (Fujimoto, Hayashi 2011). MAMs function in various cellular processes: lipid biogenesis and transportation, regulation of mitochondrial  $\text{Ca}^{2+}$  uptake, mitochondrial morphology, protein degradation, apoptosis, and innate immunity activation during viral infection (Fujimoto, Hayashi 2011).

A group of proteins are specifically enriched at MAMs and they characterize functions of MAMs. The IP3 receptors are more concentrated at MAMs than other subdomains of ER (Hayashi, Su 2007a). Together with the cytoplasmic chaperone Stress-70 protein, mitochondrial VDAC and cytochrome c, the IP3 receptors regulate  $\text{Ca}^{2+}$  uptake by mitochondria and facilitate apoptosis (Szabadkai, Bianchi et al. 2006). Meanwhile, the Bcl-2 family proteins associate with IP3 receptors at MAMs to negatively regulate apoptosis (Decuypere, Monaco et al. 2011), highlighting the idea that MAMs are hubs that regulate apoptosis. Besides the cytosolic chaperone, ER chaperones are also enriched at MAMs: GRP-78, calnexin, calreticulin and Sigma-1 receptors (Hayashi, Su 2007b, Mironov, Symonchuk 2006, Myhill, Lynes et al. 2008a). Proteins involved in the proteasome protein degradation pathway are also present at MAMs, suggesting the linkage between MAMs and ER-stress related protein degradation (Goetz, Nabi 2006, Gilady, Bui et al. 2010). Structurally, proteins involved in mitochondria fusion and fission are important for MAM structure (Yoon, Pitts et al. 1998, de Brito, Scorrano 2008). Mitofusin 2 and PACS-2 stabilize the structure of MAMs (de Brito, Scorrano 2008, Myhill, Lynes et al. 2008b). Knockdown of these two proteins caused ER-mitochondria dissociation at MAMs and mitochondria fragmentation, suggesting that MAMs and mitochondria morphology are tightly co-regulated (de Brito, Scorrano 2008, Myhill, Lynes et al. 2008b). During viral infection, the stimulator of interferon genes (STING) associates with the MAVS/IPS-1 protein complex at MAMs (Ishikawa, Barber 2008). Knockdown of mitofusin 2 increased IFN- $\beta$  synthesis while overexpression inhibited the NF- $\kappa$ B pathway (Yasukawa, Oshiumi et al. 2009). Cytomegalovirus (HCMV) utilizes MAMs for processing and trafficking virally

produced proteins (Zhang, Williamson et al. 2011). Therefore, MAMs are very important sites that are closely regulated by the host innate immune system and viral pathogens.

MAMs are also characterized by a very specialized lipid composition. They have higher levels of cholesterol and ceramides than the other regions of ER (Hayashi, Fujimoto 2010), possibly due to on-site lipid synthesis (Vance 1990, Bionda, Portoukalian et al. 2004) and its high capacity to accumulate these two lipids from their free states (Hayashi, Fujimoto 2010, Hayashi, Su 2003). Cholesterol and ceramides are important for retaining signaling proteins (Sigma-1 receptors and type-3 IP3 receptors) at MAMs (Hayashi, Fujimoto 2010). Ceramides may also contribute to the regulation of apoptosis by MAMs as they are highly pro-apoptotic for mitochondria (Fujimoto, Hayashi 2011).

Taken together, MAMs are unique structural and functional hubs for various signaling pathways and cellular functions. Recent studies have revealed their roles in regulating apoptosis via mitochondrial  $\text{Ca}^{2+}$  homeostasis, ER stress, anti-apoptotic proteins, and one pro-apoptotic lipid (Fujimoto, Hayashi 2011). MAMs are regulated by both the host cells and by viral pathogens during viral infection (Fujimoto, Hayashi 2011). However, the roles of MAMs in pyroptosis and in bacterial infection remain to be revealed.

## **1.7 Flightless-I**

Flightless-I was first identified in *Drosophila melanogaster* and the phenotypes of flightless-I mutants include irregular actin organization and defective flight muscles (Campbell, Schimansky et al. 1993). Flightless-I homologues were identified in both mouse and human genomes (Campbell, Fountain et al. 2002, Campbell, Fountain et al. 1997). In mice, defects in this gene are embryonic lethal (Campbell, Fountain et al. 2002), and in

humans, this gene maps to a region linked to developmental and behavioral abnormalities (Chen, Gunaratne et al. 1995). Flightless-I negatively regulates the wound healing process and positively regulates the hair follicle regeneration (Kopecki, Cowin 2008, Waters, Lindo et al. 2011).

Flightless-I is composed of a C-terminal gasoline-like domain (GLD) that is involved in actin remodeling, and N-terminal leucine-rich repeats (LRR) that are implicated in ligand sensing. In particular, two Flightless-I interacting proteins, LRRFIP1 and LRRFIP2 (Liu, Yin 1998, Fong, de Couet 1999) are involved in TLR signaling pathways. LRRFIP1 interacts directly with TLR3 (Bagashev, Fitzgerald et al. 2010); it is recruited to RNA-containing endosomes, and positively regulates type I interferon response during viral infection (Bagashev, Fitzgerald et al. 2010). LRRFIP2 interacts with MyD88 and positively regulates the NF- $\kappa$ B pathway and cytokine production in macrophages (Dai, Jeong et al. 2009). Flightless-I interacts with MyD88 and TRIF directly via their TIR domains and negatively regulates the TLR4-MyD88 pathway (Wang, Chuang et al. 2006). A recent study reported that Flightless-I is a negative regulator of two proinflammatory caspases in mouse macrophages: caspase-1 and caspase-11 (Li, Yin et al. 2008). Specifically, it interacts with and transports caspase-11 to the cell peripheral via actin filaments and inhibits caspase-1 activation directly (Li, Yin et al. 2008). In conclusion, current literature suggests that Flightless-I is involved in various signaling pathways in innate immunity. It is a negative regulator of the TLR4 and caspase-1, and its two interacting proteins are positive regulators of TLR signal transduction.

## 1.8 Quantitative proteomics and protein correlation profiling

Mass spectrometry is a powerful technology for analyzing complex biological samples. With recent advances in sensitivity and accuracy of mass spectrometers, and the development of various reagents to incorporate mass tags into proteins or peptides, quantitative proteomics has emerged as an important tool to compare the differences in protein composition between biological conditions (Bantscheff, Schirle et al. 2007). For relative quantitation, mass tags are introduced into proteins or peptides in two major ways: metabolic or chemical/enzymatic reactions (Bantscheff, Schirle et al. 2007). For absolute quantitation, internal standards of known concentration are spiked into samples (Bantscheff, Schirle et al. 2007). Although less accurate, label-free quantitation methods also exist and are continually being improved by rigorous computational algorithms (Bantscheff, Schirle et al. 2007).

Metabolic labeling is by far the most widely used method to differentiate biological samples (Bantscheff, Schirle et al. 2007). Stable isotope signatures are introduced into proteins during the normal cell growth and division.  $^{15}\text{N}$  labeling or Stable Isotope Labeling of Amino acids in Cell culture (SILAC) are the two major methods (Oda, Huang et al. 1999, Ong, Blagoev et al. 2002).  $^{15}\text{N}$  labeling has been applied to *Caenorhabditis elegans*, *Drosophila melanogaster* and rats (Krijgsveld, Ketting et al. 2003, Wu, MacCoss et al. 2004), but it is quite impractical to apply in heterotrophic eukaryotic systems. Instead, SILAC is widely applied to immortalized eukaryotic cell lines, as well as to bacteria, yeast cells, and even mice (Wu, MacCoss et al. 2004) (Ong 2012).

SILAC is usually applied by using arginine and lysine isotopologues so that all tryptic peptides (except sometimes the C-terminal peptide) carry one labeled amino acid with the expected mass increments. As with all metabolic approaches, complete labeling is critical for

SILAC experiments, so cells are grown for at least six doublings in SILAC medium. The SILAC medium is a basic medium using dialyzed FBS, so certain cell lines that are sensitive to changes in media composition may not grow normally in this condition. In other cases, some cells can partially synthesize arginine, so optimization experiments are required to achieve complete labeling. To label bacteria and yeast cells, auxotrophic strains are often required.

Currently, there are three sets of reagents for SILAC labeling: L-lysine/L-arginine (“light”), D<sub>4</sub>-lysine/<sup>13</sup>C<sub>6</sub>-arginine (“medium”) and <sup>13</sup>C<sub>6</sub><sup>15</sup>N<sub>2</sub>-lysine/<sup>13</sup>C<sub>6</sub><sup>15</sup>N<sub>4</sub>-arginine (“heavy”) (Ong, Blagoev et al. 2002). Experimental conditions are often applied to two cell populations while the third cell population serves as the control. Upon treatment, the three cell populations are mixed as intact cells, thereby eliminating inaccuracies in quantitation resulting from sample handling. Peptide identification is based on the fragment spectra of at least one of the co-eluting peptides and relative quantitation is based on the ion intensity of the isotope clusters of intact peptides. In addition to monitoring changes in protein expression across the whole proteome, SILAC is also widely applied to many types of studies: quantitation of post-translational modifications, confident identification of protein-protein interactions by affinity purification, calculation of protein turnover rates, generation of analytical reagents, to name a few. (Ong 2012). Overall, SILAC is a very convenient and sensitive technique to compare proteomes of different biological conditions.

There are a variety of chemical reagents available to label and differentiate between protein samples. The most widely used ones include <sup>18</sup>O labeling during protease digestion (Yao, Freas et al. 2001), isotope-coded affinity tag (ICAT) (Gygi, Rist et al. 1999), isotope tags for relative and absolute quantitation (iTRAQ) (Ross, Huang et al. 2004), tandem mass

tags (TMT) (Thompson, Schafer et al. 2003), and derivatization of specific sites (e.g. dimethylation of peptide N-termini and lysine side chains) (Hsu, Huang et al. 2003). ICAT is mainly applied to cysteine-containing peptides (Gygi, Rist et al. 1999). The iTRAQ protocol allows the comparison up to eight conditions (Ross, Huang et al. 2004). Dimethylation using formaldehyde is a very fast and cheap method allowing comparisons among three conditions (Boersema, Raijmakers et al. 2009). Overall, chemical labeling methods are important alternatives to metabolic labeling and can be directly applied to clinical tissue samples and primary cells. However, these methods suffer from inaccuracy in quantitation and are not as sensitive as SILAC (Bantscheff, Schirle et al. 2007). Moreover, chemical labeling typically occurs at the peptide level, so sample handling steps prior to the labeling step are likely to introduce errors into quantitation.

Protein correlation profiling (pcp) is a technique to identify proteins for cellular organelles and protein complexes on a global scale (Dengjel, Jakobsen et al. 2010). In combination with density gradients or liquid chromatography, this method can generate protein profiles based on their relative abundance across fractions. Typically, proteins can be grouped into different organelles or complexes if they share the same profiles as the established markers. The key steps are the reproducibility of separation and the accuracy of protein profiling. For organelle profiling using density gradients, discontinuous or continuous gradients are commonly used. Continuous gradients will generate more accurate data through superior resolution but they involve more optimization to establish the gradients, whereas discontinuous gradients are relatively easier to establish. The combination of protein correlation profiling and SILAC can efficiently identify novel proteins in specific organelles. In a typical pcp-SILAC experiment, the L-lysine/L-arginine cells are used as the internal



control. This sample is often fractionated, re-mixed and spiked into each fraction of the D<sub>4</sub>-lysine/<sup>13</sup>C<sub>6</sub>-arginine sample. The SILAC ratios of proteins will reflect the relative abundance over the spike-in base level. Since the spike-in concentration is uniform across all the fractions, the protein profiles generated from SILAC ratios are highly accurate. Often, additional comparison between different biological treatments can be included with a third cell population with the <sup>13</sup>C<sub>6</sub><sup>15</sup>N<sub>2</sub>-lysine/<sup>13</sup>C<sub>6</sub><sup>15</sup>N<sub>4</sub>-argine labeling. For large-scale studies to generate unbiased organelle proteomes or protein complexes, rigorous algorithms are required. This method has been successfully applied to global organelle maps (Foster, de Hoog et al. 2006) and proteome composition of specific cellular organelles (Andersen, Wilkinson et al. 2003, Wiese, Gronemeyer et al. 2007).

## **1.9 Summary of research goals and data**

In this study, the initial aim was to investigate the cellular proteome changes in response to *Salmonella* infection, specifically under conditions that activate inflammasomes. The pc-SILAC method was applied to resolve organelle proteome changes in THP-1 monocytes during early *Salmonella* infection. The mitochondrial and ER membrane fractions (35%-60% sucrose) were analyzed in depth. Novel protein recruitments were observed at MAMs, including the inflammasome adaptor protein ASC. With mitofusin 2 knockdown to partially disrupt MAM structure, enhanced inflammasome activation was observed, suggesting that MAMs can negatively regulate inflammasome activation during early *Salmonella* infection.

The second phase was to further explore the mechanism behind the enrichment of ASC at MAMs. The SILAC-IP method was applied to identify interacting proteins of ASC during early *Salmonella* infection. One actin-binding protein, Flightless-I was identified to interact

with ASC, and enrichment of ASC at actin filaments was observed during *Salmonella* infection. A novel pathway was proposed to involve *Salmonella* flagellin, Flightless-I, ASC and MAMs. This pathway could partially dampen inflammasome activation by sequestering ASC away from cytosol, the major site for inflammasome activation.

## **2. Method**

### **2.1 Tissue culture and SILAC**

THP-1 cells were grown in RPMI-1640 medium (Fisher/Hyclone) supplemented with 10% heat-inactivated fetal bovine serum (Gibco/Invitrogen), 2 mM L-glutamine (Invitrogen) and 1 mM sodium pyruvate (Invitrogen). 293T cells were grown in DMEM medium (Fisher) supplemented with 10% fetal bovine serum and 2 mM L-glutamine.

Stable Isotope Labeling of Amino acids in Cell culture (SILAC) technique was used to label the arginine and lysine residues in THP-1 cells through six doublings. The SILAC medium consists of 1 L of RPMI-1640 without L-arginine and L-lysine (Cassion Labs), 100 mL of heat-inactivated dialyzed FBS (Gibco/Invitrogen), 2 mM L-glutamine, 1 mM sodium pyruvate and in-house lysine and arginine supplements. In the light condition, 1 mL of L-lysine (146 mg/mL) and L-arginine (84 mg/mL) were added; in the medium condition, 1 mL of D<sub>4</sub>-lysine (150 mg/mL) and <sup>13</sup>C<sub>6</sub>-arginine (87 mg/mL) were added; in the heavy condition, 1 mL of <sup>13</sup>C<sub>6</sub><sup>15</sup>N<sub>2</sub>-lysine (154 mg/mL) and <sup>13</sup>C<sub>6</sub><sup>15</sup>N<sub>4</sub>-argine (89 mg/mL) were added.

### **2.2 *Salmonella* infection of THP-1 cells**

*Salmonella enterica* serovar Typhimurium wild-type strain SL1344 was used to infect THP-1 cells. Bacteria were grown overnight in Luria-Bertani (LB) broth supplemented with 50 µg/mL streptomycin and then sub-cultured in antibiotics-free LB at 1:33. After 3 h of exponential growth (mid-log phase), bacteria were harvested and used for infecting THP-1 cells with LPS priming (100ng/mL, 3h). THP-1 cells were harvested at 30 min post-infection.

Cells were pelleted by centrifuging at 600 rcf for 5 min, washed twice with cold PBS and stored at -80 °C until further analysis.

## **2.3 ELISA**

Enzyme-linked immunosorbent assays (ELISA) were used to determine IL-1 $\beta$  secretion during *Salmonella* infection. All ELISA assays were performed with the IL-1 $\beta$  ELISA kit (Thermo). Cell supernatant were harvested and centrifuged at 800 relative centrifugal force (rcf) and 16,000 rcf to pellet intact cells and bacteria. 50  $\mu$ l of sample was added to IL-1 $\beta$  antibody pre-coated wells and then mixed with 50  $\mu$ l of biotin-linked IL-1 $\beta$  antibody solution. The mixture was incubated at room temperature for 3 h and then the wells were washed 10 times with ELISA wash buffer. One hundred microlitres of HRP-linked Streptavidin reagent was added to each well. The plate was incubated at room temperature for 30 min then washed 10 times with ELISA wash buffer. 100  $\mu$ L TMB development reagent was added to each well and the plate was incubated at room temperature for 30 min. One hundred microliters of stop reagent was added and absorbance at 450 nm was measure with a background reference at 550 nm. IL-1 $\beta$  concentration was calculated according to the standard curve in each assay.

## **2.4 Western blot**

Protein samples were resolved in pre-cast gradient gels (4%-12% Bis-acetate, Invitrogen). Proteins were transferred onto PVDF membrane (0.45  $\mu\text{m}$ , Millipore) with the semi-dry transfer system (BioRad) at 50 mA per gel for 70-90 min. Membranes were blocked in 5% skimmed milk in PBST (0.1% Tween) overnight. After a brief wash, membranes were incubated with primary antibodies (1:2500-1:1000 dilution) for 2 h at room temperature and then washed with PBST for 30 min. Membranes were then incubated with horseradish peroxidase (HRP)-coupled secondary antibodies (1: 2500) for 1 h at room temperature and then washed with PBST for 30 min. HRP signal was amplified using the Chemiluminescence Reagent Plus Kit (PerkinElmer). The membranes were then transferred to a dark chamber for film development.

Primary antibody source: caspase-1 p10 (rabbit, Santa Cruze); ASC (rabbit, Enzo Life Science); Flightless1 (mouse, Covance); HA (mouse, Covance); VDAC (rabbit, Abcam); MFN2 (mouse, Abcam); Flag (rabbit, Thermo); Calreticulin (rabbit, Abcam); ERP57 (rabbit, Abcam). HRP-linked secondary antibodies were purchased from Jackson Labs.

## **2.5 Immunoprecipitation**

If the IP samples were for mass spectrometry analysis, antibodies were covalently linked to NHS-activated sepharose 4 fast flow beads (amersham/GE). Sepharose beads were pre-washed with 1 mM HCl by inverting for 5 min and then pelleted at 1000 rcf for 1 min. After the supernatant was removed, specific antibody solution (diluted in PBS at the ratio of 0.5 mg of antibody per 1mL of sepharose) was added to the beads and the mixture was rotated at 4 °C overnight. After antibody conjugation, Tris buffer (50 mM Tris, 0.5 M NaCl, pH=8.5)

was added to the beads to block any unreacted sepharose and the mixture was rotated at 4 °C for 4 h. The beads were then washed alternatively in high (the same Tris buffer, pH=8.5) and low pH (100mM NaAc, 0.5 mM NaCl, pH=4) buffers for five times. The antibody conjugated sepharose beads were then ready for use and store in PBS at 4 °C.

For the SILAC-IP samples, medium and heavy cell populations were infected with *Salmonella* while the light cells were untreated. Cells were lysed in 20 mM Tris-HCl, 150 mM NaCl, 1% NP-40 buffer supplemented with protease and phosphatase inhibitors (Halt/Thermo) on ice. Lysates were then centrifuged at 800 rcf and 16000 rcf for 5 min to pellet nuclei and cell debris, respectively. Cell lysates were then pre-cleared with empty sepharose beads for 2 h at 4 °C and then incubated with antibody-conjugated sepharose beads by rotating at 4 °C overnight. HA antibody-conjugated beads were added to the light and medium cell lysate and mouse IgG conjugated beads were added to the heavy cell lysate. Following IP, beads were washed once with lysis buffer and then beads from three cell populations were mixed together and then washed with lysis buffer for three times. Proteins were extracted by boiling beads in non-reductive SDS loading buffer for 5 min and were subject to in-gel digestion and mass spectrometry analysis.

If the IP samples were for western blot analysis, there was no covalent coupling process. 100 uL of empty sepharose 4 fast flow protein G beads (Amersham/GE) were added to cell lysate and the mixture was rotated for 2 h at 4 °C for pre-clearing. Primary antibody was added to cell lysate and the mixture was rotated for 1 h at 4 °C for antibody-protein binding. 50 uL of sepharose 4 fast flow protein G beads were then added to the sample and the mixture was rotated at 4 °C overnight. Beads were then washed five times with lysis buffer.

Proteins were eluted by boiling beads in non-reductive SDS loading buffer for 5 min and kept for further SDS-PAGE and western blot analysis.

## **2.6 Dis-continuous sucrose gradient**

A discontinuous sucrose gradient was used to fractionate organelles from treated THP-1 cells. Cells were lysed by syringing through a 22G needle for 30 times in PBS supplemented with protease and phosphatase inhibitors (Halt/Thermo). The cell lysate was centrifuged at 800 rcf for 5 min to pellet any unlysed cells and intact nuclei. The supernatant was loaded onto a discontinuous sucrose gradient (10%-80%, at 5% steps, 1mL per fraction). Samples were then centrifuged at 100,000 rcf for 18 h at 4 °C. Fractions were collected by inserting a 22G needle at the bottom of the tube and siphoning out 1 mL fractions using clean syringes. To analyze the protein contents of each fraction, 100 µL samples were diluted with 100 µL distilled water to prevent sucrose from precipitating out in ethanol. Proteins were precipitated by adding 1 mL of 100% ethanol at pH=5 for 1.5 h at room temperature. Precipitated proteins were pelleted at 16,000 rcf and pellets were solubilized by boiling in SDS loading buffer for 5 min.

For the SILAC protein-correlation profiling experiment (pcp-SILAC), the light and medium cell populations were infected with *Salmonella* and the heavy cell population was untreated. Following cell treatment, medium and heavy cells were mixed at 1:1 ratio, and lysed together. The lysate was resolved across a 10%-80% sucrose gradient. The light cell population were lysed and resolved across an identical gradient by itself. After collecting sucrose fractions, all 15 fractions from the light sample were re-mixed and manually aliquoted into 15 equal samples and spiked into each fraction of the medium and heavy

mixture to serve as an internal control. This way, the protein profiles can be determined according to the medium/light and heavy/light ratios to determine the difference between infected (medium) and resting (heavy) THP-1 cells.

## **2.7 In-gel digestion, mass spectrometry analysis and data processing**

In-gel digestion was performed as previously described (Shevchenko, Wilm et al. 1996). Protein samples were resolved on 12% acrylamide gels and stained with Coomassie Blue (Thermo) for 30 min. Gels were de-stained with distilled water and protein bands were cut Out for in-gel digestion. Gel pieces were cut into 1 mm<sup>3</sup> cubes and de-stained in 50% ethanol in 25mM ammonium bicarbonate. They were reduced in 10 mM DTT for 45 min at 56 °C, then alkylated in 55 mM IAA for 30 min at 37 °C. Proteins trapped in the gel matrix were digested with 12.5 ng/μL trypsin (Promega, sequencing grade) in 50 mM ammonium bicarbonate, pH=8.5. This mixture was incubated for 20 h at 37 °C. After digestion, the solution was acidified until pH<2, and peptides were extracted from the gel matrix using 30% acetonitrile with 0.1% acetic acid and 100% acetonitrile to dehydrate the gel. Acetonitrile in the peptide extracts was evaporated in a vacuum concentrator (SpeedVac, Eppendorf). Peptides were resuspended in 1% TFA, 3% acetonitrile and concentrated on a C18 STop And Go Extraction (STAGE) tip (Rappsilber, Ishihama et al. 2003). Peptides were eluted with 0.1% TFA 80% acetonitrile, dried by vacuum centrifugation, re-suspended in 1% TFA with 3% acetonitrile and 0.1% acetic acid, then analyzed in an LTQ-Orbitrap Velos. For the pcpi-SILAC samples, five gel fractions were analyzed for each sucrose fraction. For the SILAC-IP samples, two gel fractions were analyzed for each IP sample. LC-MS/MS data were analyzed by MaxQuant v1.0.1.13. Fixed modifications include cysteine



carbamidomethylation, and variable modifications include oxidation at methionine, N-terminal acetylation, and triple SILAC. Proteins were identified by searching the fragment spectra against the human IPI database (v3.47, 144,389 sequences), allowing a 1% false discovery rate at the protein level. Protein profiles were generated by SILAC ratios across the sucrose fractions.

## **2.8 Isolation of crude mitochondria**

To isolate crude mitochondria, THP-1 cells were lysed by syringing through a 22G needle for 30 times in phosphate-buffered saline (PBS) supplemented with protease and phosphatase inhibitors (Halt/Thermo). Lysates were centrifuged at 800 rcf for 5 min to pellet nuclei and intact cells. The supernatant was centrifuged at 7,000 rcf for 5 min to pellet crude mitochondria, which contains both mitochondria and mitochondria-associated membranes (Wieckowski, Giorgi et al. 2009). The crude mitochondria pellet was washed twice in PBS and re-pelleted at 7,000 rcf. Mitochondria pellets were solubilized by boiling in 1% sodium deoxycholate, 50 mM ammonium bicarbonate for 5 min, and protein concentrations were determined by the BCA assay.

## **2.9 Actin fractionation**

Actin fractionation protocol was optimized according to published papers (Yamamoto, Hilgemann et al. 2001). THP-1 cells were lysed in 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, pH=7.5 buffer supplemented with protease and phosphatase inhibitors (Halt/Thermo). The cell lysate was centrifuged at 800 rcf to pellet nuclei and intact cells and

further centrifuged at 7,000 rcf to pellet intact mitochondria or intracellular bacteria (in infected cells). The supernatant was centrifuged at 16,000 rcf to pellet the cross-linked actin filament (low-speed pellet) and at 200,000 rcf (high-speed pellet) to pellet the shorter actin oligomers (Li, Yin et al. 2008). The low- and high-speed pellets were washed once with the same lysis buffer and re-pelleted. The final actin pellets were solubilized by boiling in SDS loading buffer for 5 min.

## **2.10 Cloning and calcium phosphate DNA transfection**

PCR reactions were performed using Pfu turbo DNA polymerase (Invitrogen) with the standard procedure with annealing temperature at 50°C and extension temperature at 68 °C. All cloned cDNA were sequenced to ensure 100% correctness.

ASC cDNA was obtained from the Gumucio lab (University of Michigan). Two copies of HA tags were added to the N-terminus of Asc and the sequence was cloned into pcDNA3.1 vector (Invitrogen). HA-tagged ASC gene was clone into the lentivirus pLVX-puro vector (Clontech) between the XhoI and XbaI sites. The primer sequences were as following: first round of cloning, forward, 5'-GGA TGG GGC GCG CGC GCG ACG CCA T-3'; reverse, 5'-GAA ATC TCG AGT CAG CTC CGC TCC AG-3'; second round of cloning, forward, 5'-GAA ATC TCG AGG CCA CCA TGT ACC CA-3'; reverse, 5'-GCA CGT CTA GAG ATC AGC TCC GCT CC-3'.

NLRC4 cDNA was obtained from the Finlay Lab (University of British Columbia). Flag tag sequence was added to the N-terminus of Nlrc4 and the gene was cloned directly into the lentivirus pLVX-puro vector and the pcDNA3.1 vector between the XhoI and XbaI sites. The

primer sequences were as follows: forward, 5'-GAA CTC TCG AGT TGC CAT GGA CTA CA-3', reverse, 5'-GCA CGT CTA GAT TAA GCA GTT ACT AG-3'.

Pro-caspase-1 cDNA on the pcDNA3.1 vector was obtained from the Roy lab (Yale University). Pro-IL1 $\beta$  cDNA was obtained from the Ting lab (University of North Carolina) and was cloned into the pcDNA 3.1 vector between the XhoI and XbaI sites. The primer sequences were as follows: forward, 5'-ATG CTC GAG ACC ATG GCA GAA GTA CC-3', reverse, 5'-ATG TCT AGA TTA GGA AGA CAC AAA TT-3'.

HA-tagged Flightless-I and FliC cDNA on the pcDNA 3.1 vectors were obtained from Finlay lab (University of British Columbia).

The calcium-phosphate method was used to transfect DNA plasmids into 293T cells. For the inflammasome reconstitution experiment using a 24-well plate, plasmids (input DNA ) including pcDNA3.1-derived-ASC (5 ng), -pro-caspase1 (5 ng), -pro-IL1 $\beta$  (5 ng), -FliC (10ng), -Flightless-I (10 ng) and empty pcDNA3.1 plasmid (to normalize total DNA input to the same amount) were transfected into each well in a 24-well plate. For each well, 2.25  $\mu$ L of 2 M calcium chloride were mixed with input DNA and PCR-grade water to a total volume of 18  $\mu$ L, followed by the addition of 2x BES buffer (275 mM sodium chloride, 2 mM disodium phosphate, 50 mM BES, pH=7.0) dropwise to the calcium chloride/DNA mixture. The whole transfection mixture was incubated at room temperature for 60 min before it was added to 293T cells (20% confluency, exponential growth stage, with 300  $\mu$ L DMEM medium per well). After 24 h of transfection, supernatants from each well were harvested for IL-1 $\beta$  ELISA analysis.

## 2.11 Lentivirus packaging and infection

Lentivirus plasmids pLVX-puro (Clontech)-containing genes of interest were transfected into 293T cells using Fugene transfection reagent (Promega). Briefly,  $1.5 \times 10^6$  293 T cells were seeded into 5 mL media the day before lentiviral transduction. After 24 h, 94  $\mu$ L of opti-MEM, 1  $\mu$ g of lentiviral plasmid and 1  $\mu$ g of packaging plasmid and 6  $\mu$ L of Fugene transfection reagent were mixed sequentially in a polypropylene tube. After an incubation period of 10 min at room temperature, the mixture was added to 293 T cells dropwise and incubated overnight. After 48 h, media from the transfected 293 T cells were collected and filtered through a 0.45  $\mu$ m membrane, and the flow-through containing the virus was added to THP-1 cells. Polybrene (Millipore) was added to the medium at a final concentration of 8-10  $\mu$ g/mL. THP-1 cells and the virus were incubated for 4 h and the media was replaced with fresh RPMI. After 24 h, the viral infection procedure was repeated. Infected THP-1 cells were recovered and amplified in RPMI medium. A GFP control experiment showed that close to 100% of THP-1 cells expressed a strong GFP signal after this procedure, indicating a very high efficiency in transfection and protein expression. Therefore, puromycin selection was not necessary.

## 2.12 Mutagenesis of FliC and FljB

Flagellin genes, *fliC* and *fljB*, were deleted in *Salmonella enterica* serovar Typhimurium wild-type strain SL1344 using two rounds of homologous recombinations. The first homologous recombination allows plasmids to integrate into *Salmonella* genome either upstream or downstream of the gene of interest. The second homologous recombination

allows the gene of interest to be removed from the genome. The  $\Delta fliC$  strain was first made , and  $\Delta fliC \Delta fljB$  was made in the  $\Delta fliC$  background by deletion of *fljB*.

One kilobase (kb) of sequence upstream and downstream of each gene were PCR-amplified using Platinum pfx DNA polymerase (Invitrogen). The upstream and downstream sequences were digested by SalI restriction enzyme, ligated into a 2 kb fragment, PCR amplified and cloned into the pRE112 vector between SphI and XbaI sites. The pRE112 vector is a suicidal vector that requires a *pir* gene to replicate, and it contains a chloramphenicol resistant gene and a *sacB* gene that confers the plasmid sucrose sensitivity. So the presence of the plasmid can be selected by chloramphenicol, while the absence of the plasmid can be selected by sucrose (5%). The primer sequences were as follows: FliC-upstream forward, 5'- ATG TCT AGA GTA TTG CTC TGA CGC TCA ATG -3'; FliC-upstream reverse, 5'- ATG GTC GAC GTT TGT ATT AAT GAC TTG TGC C-3'; FliC-downstream forward, 5'- ATG GTC GAC CTC TCT TTA CTG CGT TAA TCC-3'; FliC-downstream reverse, 5'- ATG GCA TGC CAG TTC TTC TGC TGC TCA TTC -3'. FljB-upstream forward, 5'- ATG TCT AGA GTC ATC CAG GTT ATC GAC AC -3'; FljB-upstream reverse, 5'- ATG GTC GAC GTT AGT GTT GAT TAC TTG TGC -3'; FljB-downstream forward, 5'- ATG GTC GAC AAC GTG CTG TCT CTG TTA CGT -3'; FljB-downstream reverse, 5'- ATG GCA TGC TAC AGT ATC ACA GAA GGC GTT -3'.

The pRE112 plasmid containing the flanking sequences of *fliC* or *fljB* was amplified in a DH5 $\alpha$  *E. coli* strain carrying *pir* that is required for the plasmid replication. It was then transformed into an *E. coli* donor strain MFD (*pir*<sup>+</sup>) and horizontally transferred into the SL1344 strain via a bacterial conjugation method. As pRE112-derived plasmid cannot replicate in *Salmonella* that does not have the *pir* gene, chloramphenicol resistance can only

be obtained by integration of the plasmid into *Salmonella* genome by homologous recombination. *Salmonella* was selected twice on streptomycin/chloramphenicol (50 µg/mL) plates and single colony was cultured in LB containing streptomycin but no salt for 4 h, which allows the deletion of genes of interest via the second homologous recombination. The resulting *Salmonella* strains were further selected twice on streptomycin/sucrose plates to screen for proper *Salmonella* mutants. The deletion of *fliC* was confirmed by PCR amplification of the 200 bp upstream and downstream of the *fliC* gene (double homologous recombination generated a 400 bp band, while single homologous recombination generated a 400 bp band and a 2000 bp band). The deletion of *fljB* was confirmed by PCR amplification of the 200 bp upstream and the first 200 bp of the *fljB* gene (double homologous recombination failed to generate a PCR product while single homologous recombination generated a 400 bp band). The PCR primers used to confirm mutants are as follows: FliC-mutation-forward, 5'-GCG TTA TCG GCA ATC TGG AG-3'; FliC-mutation-reverse, 5'-CGA TGG TAC GAA TCG TCG TG-3'. FljB-mutation-forward, 5'-GGT TTG CAA TCT TGC CAC TGA-3'; FljB-mutation-reverse, 5'-CTT CAG TGG TCT GCG CAA TG.

$\Delta fljF$  mutant strain was obtained from Finlay Lab (University of British Columbia).

### **2.13 siRNA transfection**

siRNA for human Flightless-I and mitofusin 2, and non-target siRNA were purchased from Dharmacon/Thermo. Smartpool of each siRNA set is a mixture of four on-target siRNA sequences. siRNA duplexes were resuspended in 1x siRNA buffer (Invitrogen) at a final concentration of 10 µM. In 24-well plates, THP-1 cells were suspended in 400 µL serum-free RPMI medium at  $3 \times 10^5$  cell/mL. 2.5 µL siRNA was diluted in 50 serum-free RPMI medium

and incubated for 5 min at room temperature. Three hundred thirty nanolitres of Dharmafect1 reagent was diluted in 50  $\mu$ L of serum-free RPMI medium and incubated for 5 min at room temperature. The siRNA and Dharmafect1 were mixed and incubated for 30 min at room temperature and 400  $\mu$ L of this mixture was added to cells in each well. After 6 h, the medium was replaced with fresh RPMI media. After 72 h, cells were harvested or treated with corresponding stimuli.

## **2.14 Assays for protein concentration**

In this study, protein concentrations were determined by Bradford or BCA assays. In both assays, BSA standards were used to plot standard curves. The Bradford assay is faster but is significantly interfered by some detergent such as sodium deoxycholate and SDS while BCA assay is compatible with most detergents at low concentrations. For the Bradford assay, the Bradford reagent (Thermo/Pierce) was first diluted 1:1 in water and then protein sample was diluted 1:100-1:1000 in the Bradford reagent and incubated for 5 min at room temperature. Absorbance at 595 nm was determined with reference absorbance at 590 nm in a Bio-Rad spectrometer. For BCA assay, BCA reagents A and B (Thermo/Pierce) were mixed immediately prior to the assay at a ratio of 50:1. Protein sample was diluted 1:20 in the BCA reagent and the mixture was incubated at 37 °C for 30 min. Absorbance at 562 nm was determined in the Nanodrop spectrometer.

### 3. Results

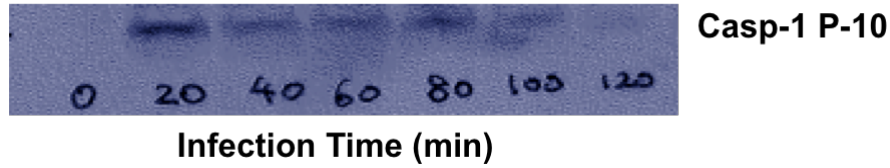
#### 3.1 *Salmonella* infection of THP-1 cells

*Salmonella* can induce inflammasome activation in primary macrophages and monocytic cell lines (Miao, Alpuche-Aranda et al. 2006, Miao, Mao et al. 2010b). To monitor inflammasome activation, THP-1 cells were infected with *Salmonella enterica* serovar Typhimurium (wild-type strain SL1344) at a multiplicity of infection (MOI) of 50 for 2 h. Samples (cells pellets and supernatant) were harvested every 20 min to determine IL-1 $\beta$  secretion and caspase-1 activation. To quantify IL-1 $\beta$  secretion during the early *Salmonella* infection, THP-1 cells were treated with LPS (100 ng/mL, 3 h) to stimulate the expression of the pro-IL-1 $\beta$ . Caspase-1 was activated before 20 min and IL-1 $\beta$  was secreted into the supernatant during the initial 40 min (Figure 1a, 1b). Therefore, 30 min was chosen to be the optimal infection time to track inflammasome activation. For all the wildtype *Salmonella* infections in this study, THP-1 cells were primed with LPS and then infected for 30 min. To ensure biological observations were not due to LPS priming, validation experiments were performed to determine the difference between LPS primed cells and *Salmonella* infected cells.

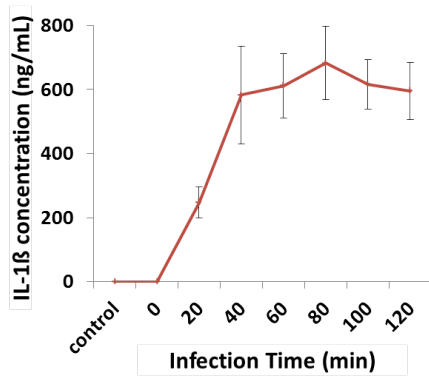
For the infection with aflagellated *Salmonella* ( $\Delta fliF$  and  $\Delta fliC\Delta fliB$  strains), MOI was increased to 200 to improve cell-bacteria contacts. Immediately after immobile *Salmonella* were added to cells on 6-well plates, the whole plate was centrifuged at 1500 rcf for 2 min to force cell-bacteria contacts. Similarly, IL-1 $\beta$  secretion was monitored for 2 h with 20 min intervals for  $\Delta fliF$  strain. A lower concentration but a similar pattern for IL-1 $\beta$  secretion was observed: an initial linear increase and then plateauing (Figure 1c) IL-1 $\beta$  secretion time was delayed to 40 to 80 min, while during wild type *Salmonella* infection, the secretion time was



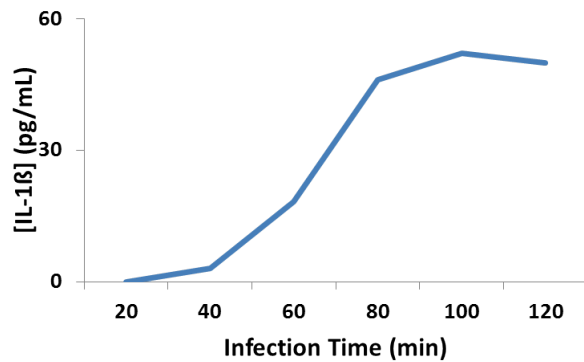
0 to 40 min. Therefore, for experiments performed with aflagellated *Salmonella*, THP-1 cells were primed with LPS and then infected for 70 min.



(a)



(b)



(c)

**Figure 1: Inflammasome activation during early *Salmonella* infection.**

(a) Western blot for caspase-1 p10 peptides in THP-1 cells during 2 h of wildtype *Salmonella Typhimurium* infection with 20 min intervals. (b) ELISA assay of IL-1 $\beta$  secretion by THP-1 cells during 2 h of wildtype *Salmonella Typhimurium* infection with 20 min intervals. (c)

ELISA assay of IL-1 $\beta$  secretion by THP-1 cells during 2 h of  $\Delta$ *fliF* *Salmonella Typhimurium* infection with 20 min intervals.

### **3.2 MAM proteome during *Salmonella* infection**

Protein correlation profiling (pcp) in combination with Stable Isotope Labeling of Amino acids in Cell culture (SILAC) was used to profile organelle proteins during *Salmonella* infection. There were three THP-1 cell populations, light, medium and heavy, each with labeled arginine and lysine residues. The light cell population was LPS primed and infected with *Salmonella* to serve as the internal standard for protein profiling. The medium cell population was primed with LPS and infected with *Salmonella*. The heavy cell population was left untreated and was mixed with the medium cells immediately after they were treated. The mixture of medium and heavy cells was lysed and the resulting cytosol was resolved across a discontinuous sucrose gradient (10%-80%, with 5% steps) to ensure an accurate comparison between untreated cells and *Salmonella* infected cells. The light cells were lysed and also resolved across a separate discontinuous sucrose gradient. For the light cells, fractions collected were re-mixed and manually aliquoted into 15 identical fractions that were spiked into each fraction obtained from the medium and heavy mixture. Through this method, the medium to light ratios were used for organelle profiling and the medium to heavy ratios reflect the difference between infected and untreated cells (Figure 2a).

Proteins from six sucrose fractions (35%, 40%, 45%, 50%, 55%, 60%) were analyzed in depth by a mass spectrometer. Over 2300 proteins were identified and protein profiles for over 800 mitochondrial and ER proteins were confidently generated using the MaxQuant software package. Profiles for major markers of mitochondria and ER were generated. Beyond these, some MAM marker proteins were enriched in *Salmonella* infected THP-1

cells in comparison to resting cells. These include the previously reported MAM proteins which can be classified into the following categories: lipid metabolism enzymes ACAT, FACL4, PSS-1; ion channel or transporters IP3R-1, calcium pump; ER chaperones: GRP-78, calnexin, calreticulin, ERP-44, ERP-57; lipid rafts associated protein: Erlin-2; and Ras-related G protein: Rab-32 (Table 1a). Novel protein recruitments to MAMs were further identified during *Salmonella* infection (Table 1b). Some MAM makers were not regulated by *Salmonella* infection (Table 1c) and some others were not identified in this experiment (Table 1d). Rab-32 was previously reported to regulate apoptosis onset by modulating calcium handling, PKA signaling and calnexin enrichment at MAMs (Bui et al. 2010). Enrichment of Rab-32 at MAMs was observed during early *Salmonella* infection. Additionally, protein profiles for twenty-five Ras-related proteins were also generated. Among these Rab-7, 14, 21 were also enriched at MAMs during *Salmonella* infection.

Erlin-1 and Erlin-2 are two lipid raft-associated proteins at MAMs (Fujimoto, Hayashi 2011). During *Salmonella* infection, Erlin-1 was not regulated and Erlin-2 was up regulated at MAMs. Cytochrome c is released from mitochondria to MAMs (Szabadkai, Bianchi et al. 2006). During *Salmonella* infection, it was not regulated but cytochrome b and cytochrome 450 enzymes were more enriched at MAMs. In conclusion, MAM protein composition was described in resting and in *Salmonella* infected THP-1 cells. To my knowledge, this was the first characterization of the MAM proteome during bacterial infection.

**Table 1: MAM proteome during early *Salmonella* infection.**

(a) MAM marker proteins that were enriched in *Salmonella* infected cells. (b) Novel MAM proteins that were co-purified and enriched with MAM markers in *Salmonella* infected THP-1 cells. (c) MAM marker proteins that were not regulated by *Salmonella* infection. (d) MAM marker proteins that were not identified in this project.

<b>MAM proteins regulated during <i>Salmonella</i> infection</b>	<b>Functions</b>
<b>Acyl-CoA cholesterol acyltransferase</b>	<b>Lipid metabolism</b>
<b>CDP-diacylglycerol acyltransferase</b>	
<b>Phosphatidylserine synthase-1</b>	
<b>Inositol 1,4,5-triphosphate receptor-1</b>	<b>Ion channels or transporter</b>
<b>Calcium transportation ATPase</b>	
<b>78 kDa glucose-regulated protein</b>	<b>Protein chaperone</b>
<b>Calnexin</b>	
<b>Calreticulin</b>	
<b>Endoplasmic reticulum resident protein 44</b>	<b>Protein disulfide isomerase</b>
<b>Endoplasmic reticulum resident protein 57</b>	
<b>Erlin-2</b>	<b>Lipid raft-associated protein</b>
<b>Rab-32</b>	<b>Ras-related G protein</b>

(a)

<b>Novel MAM proteins during <i>Salmonella</i> infection</b>	<b>Functions</b>
<b>ASC</b>	<b>Pyroptosis, inflammasome activation</b>
<b>Cytochrome b reductase</b>	<b>Cytochrome enzymes</b>
<b>Cytochrome p450 reductase</b>	
<b>S100-A8</b>	<b>Calcium binding</b>
<b>Hippocalcin-like protein 1</b>	
<b>Heat Shock 70 kDa protein</b>	<b>Protein chaperone</b>
<b>GRP-170</b>	
<b>ERP-70</b>	<b>Protein disulfide isomerase</b>
<b>ERP-60</b>	
<b>ERP-18</b>	<b>Protein thiol disulfide oxidase</b>
<b>Rab-7</b>	<b>Ras-related G protein</b>
<b>Rab-14</b>	
<b>Rab-21</b>	
<b>GRP-94/endoplasmic</b>	<b>Protein processing and secretion</b>
<b>ERP-28</b>	
<b>Endopeptidase SP18</b>	<b>Protein processing</b>
<b>Microsomal signal peptidase</b>	

(b)

<b>MAM proteins not regulated during <i>Salmonella</i> infection</b>	<b>Functions</b>
<b>Sigma-1 receptor</b>	<b>Molecular chaperone and calcium signaling</b>
<b>GRP-75</b>	<b>Molecular chaperone</b>
<b>Cytochrome c</b>	<b>Electron transport chain protein</b>
<b>Dynamin-related protein</b>	<b>Mitochondria fission</b>
<b>Bax</b>	<b>Apoptosis</b>
<b>Erlin-1</b>	<b>Lipid raft-associated protein</b>

(c)

<b>MAM proteins NOT identified</b>	<b>Functions</b>
<b>Diacylglycerol acyltransferase 2</b>	<b>Lipid metabolism</b>
<b>Phosphatidylserine synthase-2</b>	
<b>Ryanodine receptors</b>	<b>Ion channel</b>
<b>Bak</b>	<b>Regulation of apoptosis</b>
<b>Bcl-2</b>	
<b>Bcl-XL</b>	
<b>S-100B</b>	<b>MAM structure</b>
<b>Mitofusin 2</b>	
<b>Phosphoacidic cluster sorting protein 2</b>	
<b>MET30 E3 ubiquitin ligase</b>	<b>Ubiquitin ligase</b>
<b>Autocrine motility factor receptor</b>	

(d)

### 3.3 Association of ASC with mitochondria-associated membranes during *Salmonella* infection

Notably, the Apoptosis-associated Speck-like protein containing a CARD (ASC) was highly enriched near 50% sucrose in *Salmonella* infected cells while in untreated cells this protein was evenly distributed across these sucrose fractions (Figure 2b). The protein profile for ASC showed correlation with other MAM marker proteins in *Salmonella* infected cells, suggesting that ASC was co-purified with MAMs.

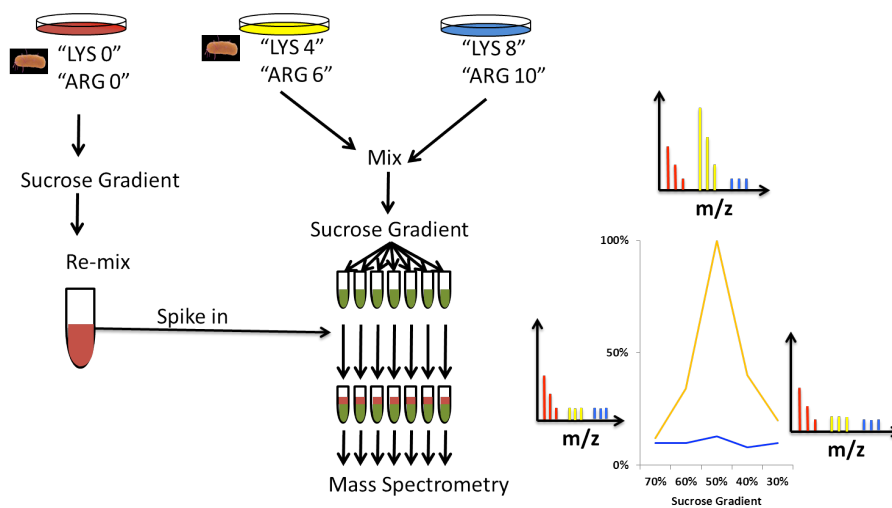


Figure 2 (a)

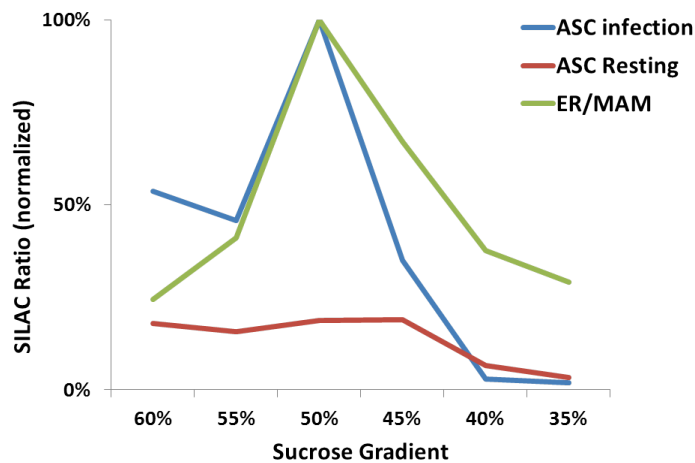


Figure 2 (b)

**Figure 2: Co-purification of ASC with ER/MAM proteins on a discontinuous sucrose gradient.**

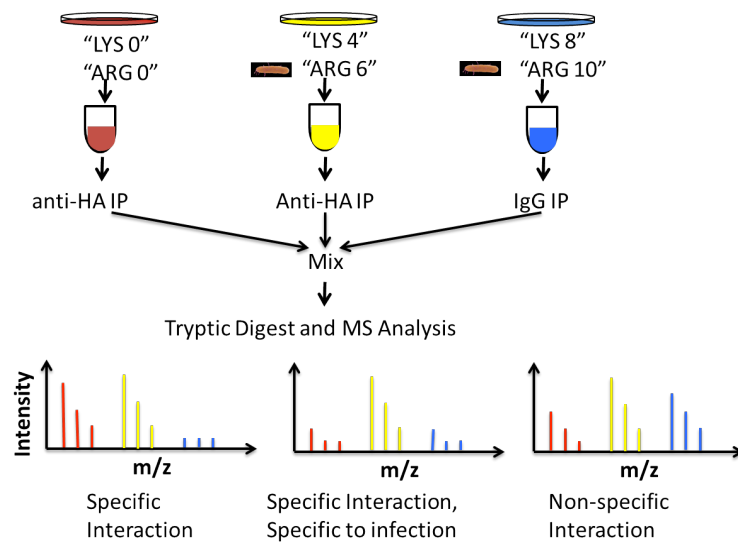
(a) Experimental scheme for the pcip-SILAC experiment and one example of protein profile specific for infected cells. (b) SILAC protein profile for the ASC protein in resting or infected cells and the profile for ER/MAM proteins. SILAC ratios were normalized to the highest ratio for each protein and the ER/MAM profile was the mean value of ten MAM marker proteins. The ten MAM proteins exhibited very similar protein profiles.

Since ASC is an important adaptor protein for inflammasomes and the pcip-SILAC data revealed its co-enrichment with MAM proteins during *Salmonella* infection, I explored the potential mechanisms through which this might occur using quantitative immunoprecipitation (SILAC-IP). A THP-1 cell line that stably expresses HA-tagged ASC was generated by a lentivirus transduction method. Three THP-1 cell populations were labeled with SILAC reagents. The light cells were untreated, and the medium and heavy cells were primed with LPS and infected with *Salmonella*. Standard immuno-precipitation procedures with covalently linked anti-HA antibody were performed. The light and medium cell lysates were immuno-precipitated with anti-HA antibody, and the heavy cell lysate was

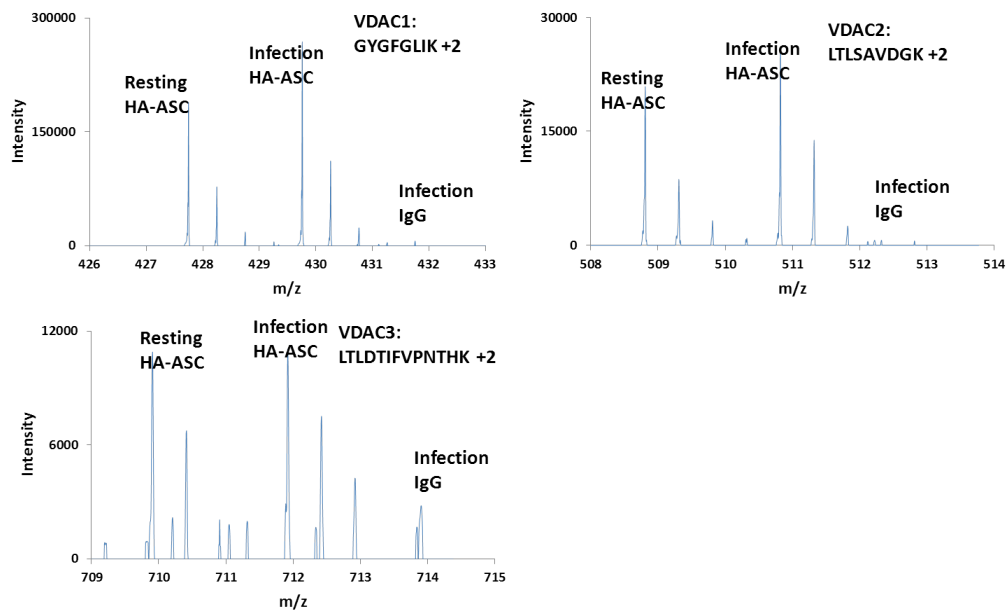


immuno-precipitated with the IgG antibody. The medium to light ratios reflected the difference between untreated cells and infected cells. The medium to heavy ratios identified specific interactions. (Figure 3a)

SILAC-IP results showed that ASC specifically interacted with all three isoforms of voltage-gated anion channels (VDAC) on mitochondria membranes (Figure-3b). There was also a weak interaction between ASC and Stress-70 protein (Table 2). VDAC and Stress-70 protein form a complex linking mitochondria and ER membranes at MAMs (Szabadkai, Bianchi et al. 2006). Therefore, ASC is likely to interact with the VDAC/Stress-70 protein complex at the MAMs.



(a)



(b)

**Figure 3: Interaction between ASC and VDAC 1, 2, 3.**

(a) Experimental Scheme and data interpretation for SILAC-IP. (b) Mass Spectra for three peptides from VDAC1, 2, 3. ASC-VDAC interaction was shown by high HA/IgG ratios for all three isoforms of VDAC. N=2.

**Table 2: Interaction between ASC and Stress-70 protein.**

HA/IgG ratios for Stress-70 protein showed a weak interaction between ASC and Stress-70 protein. N=2.

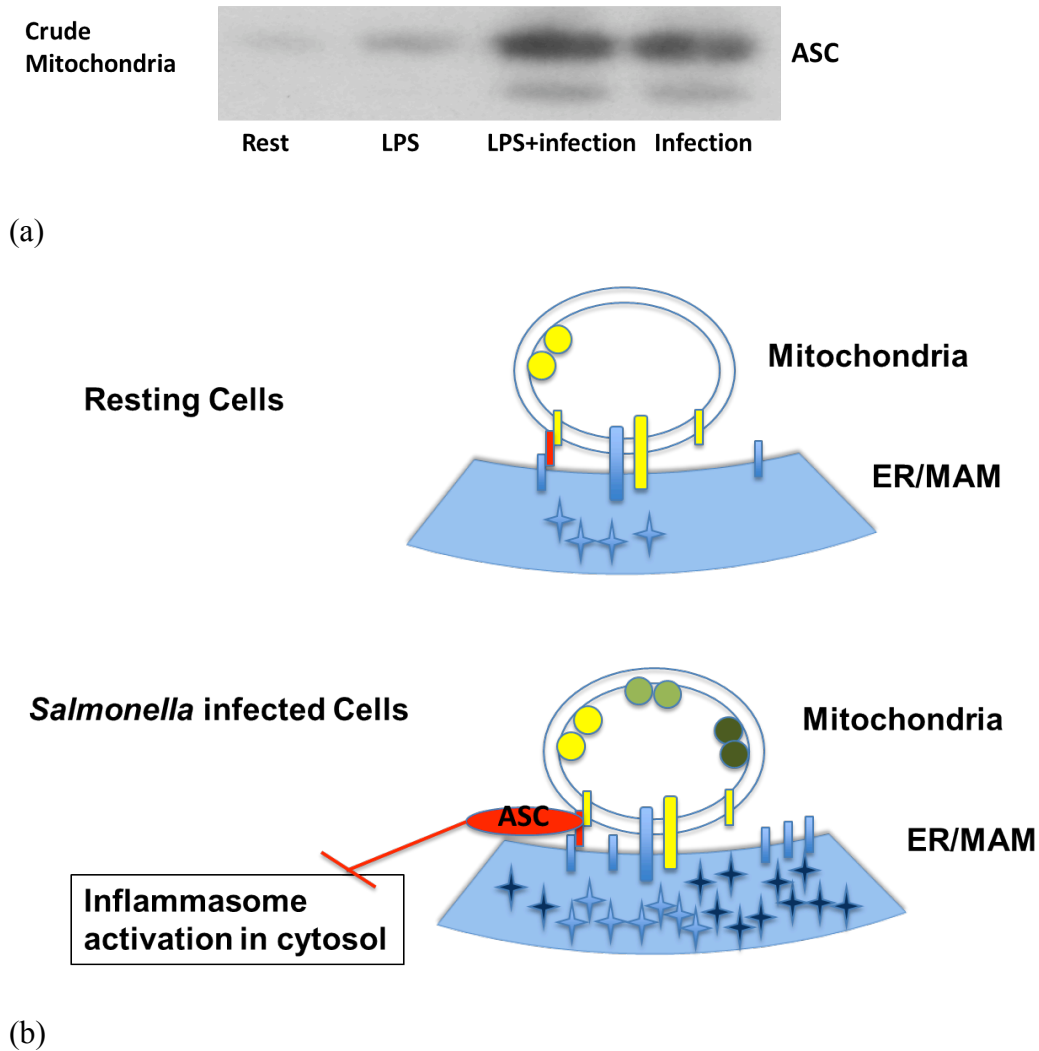
	REP1	REP2
<b>Stress-70 protein</b>	<b>2.000</b>	<b>1.74</b>

To validate the mass spectrometry data, MAMs were purified directly from the cytosol but the isolation was ultimately unsuccessful, as MAMs didn't separate from mitochondria, possibly due to low levels of starting materials. However, immune-blot analysis of the crude mitochondria pellet (containing both "free" mitochondria and MAMs) revealed that ASC is significantly enriched in the crude mitochondria in *Salmonella* infected THP-1 cells only (Figure 4a), which supports the speculation that ASC is recruited to MAMs during *Salmonella* infection.

Since ASC is an important adaptor protein for inflammasomes (Schroder, Tschopp 2010), and the NLRC4 inflammasome is typically activated during *Salmonella* infection (Miao, Alpujch-Aranda et al. 2006, Miao, Mao et al. 2010b), I tested the possibility that the NLRC4 inflammasome is recruited at MAMs during early *Salmonella* infection. Having created a stable cell line using THP-1 cells that stably expresses FLAG-tagged NLRC4, I performed immune-blot analysis using to detect pro-caspase-1 and FLAG-NLRC4 across the discontinuous sucrose gradient and in crude mitochondria samples. Pro-caspase-1 showed a different protein profile from ASC and MAM proteins and was not enriched in crude mitochondria fractions during early *Salmonella* infection (data not shown). Similarly, Flag-NLRC4 protein was not identified in the MAMs fractions and it was not enriched in crude mitochondria (data not shown). Therefore, ASC was recruited to MAM independent of the NLRC4 inflammasome complex during early *Salmonella* infection.

Taken together, ASC was co-purified with MAM proteins and it interacted with the VDAC/Stress-70 complex at MAMs. I proposed a working model that during early *Salmonella* infection, many proteins were specifically recruitment to MAMs, including housekeeping MAM marker proteins and other ER and cytosolic proteins. ASC was

specifically recruited to MAMs during early *Salmonella* infection while other members of the NLRC4 inflammasome complex were not (Figure 4b).



**Figure 4: Recruitment of ASC to MAMs during early *Salmonella* infection.**

(a) A significantly higher concentration of ASC was found in crude mitochondria (containing both mitochondria and MAMs) in *Salmonella* infected cells than in resting cells. (b) Cartoon showing protein recruitment at MAMs during early *Salmonella* infection, and the interaction between ASC and the VDAC/Stress-70 tethering complex at MAMs. Circles: cytochrome complexes in mitochondria; light blue stars: housekeeping MAM proteins; dark blue stars:

ER proteins recruited to MAMs during *Salmonella* infection; blue rectangles: ion channels and tethering proteins on ER at MAMs; yellow rectangles: ion channels and tethering proteins on mitochondrial outer membranes at MAMs; red rectangle: cytosolic chaperone.

### **3.4 Interaction between ASC and Flightless-I**

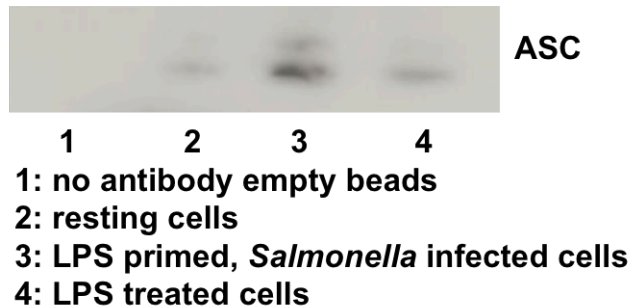
The SILAC-IP data also revealed the interaction between ASC and a gasoline-family protein, Flightless-I. Flightless-I was initially identified in *Drosophila melanogaster* and was reported to inhibit caspase-1 and caspase-11 activation in murine cells. SILAC IP data indicated that the interaction between ASC and Flightless-I is enhanced in *Salmonella* infected cells compared to resting THP-1 cells (Table 2). To confirm this observation, I performed reciprocal IPs with anti-Flightless-I antibody and analyzed the IP eluent by immune-blotting with an anti-ASC antibody. Results were consistent with the SILAC-IP data that ASC-Flightless-I interaction is enhanced in *Salmonella* infected THP-1 cells (Figure 5a).

As the Flightless-I was previously reported to transport caspase-11 to pericellular regions via actin filaments, I performed the actin purification experiment (Li, Yin et al. 2008). In *Salmonella* infected THP-1 cells, ASC was significantly enriched in both cross-linked and short oligo actin fractions compared to resting cells (Figure 5b). Notably, this enrichment was not observed for pro-caspase-1, suggesting that this is an inflammasome-independent mechanism. Since Flightless-I protein is a known actin binding protein and was previously reported to transport cellular proteins through actin filaments (Li, Yin et al. 2008), it could similarly transport ASC during *Salmonella* infection. Based on the preliminary data on enrichment of ASC at MAMs and actin filaments, and its interaction with Flightless-I, I hypothesized that Flightless-I transports ASC to MAMs through actin filaments in *Salmonella* infected cells.

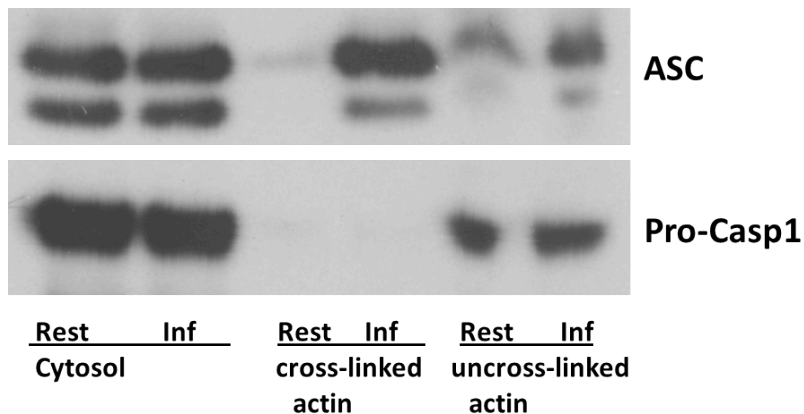
**Table 3: Interaction between Flightless-I and ASC.**

Infection/resting ratios for Flightless-I showed enhanced ASC-Flightless-I interaction in *Salmonella* infected THP-1 cells. N=2.

	REP1	REP2
<b>Protein flightless-1</b>	<b>17</b>	<b>3.2</b>



(a)



(b)

**Figure 5: Enhanced interaction between ASC and Flightless-I and enrichment of ASC in actin filaments during *Salmonella* infection.**

(a) IP with anti-Flightless-I antibody and WB with anti-ASC antibody in resting THP-1 cells, LPS primed and *Salmonella* infected THP-1 cells and LPS primed THP-1 cells, with no antibody as control. (b) Resting or *Salmonella* infected THP-1 cells were lysed and subjected to actin isolation. Cross-linked actin and uncross-linked actin samples were analyzed by WB with anti-ASC and anti-caspase-1 antibody.

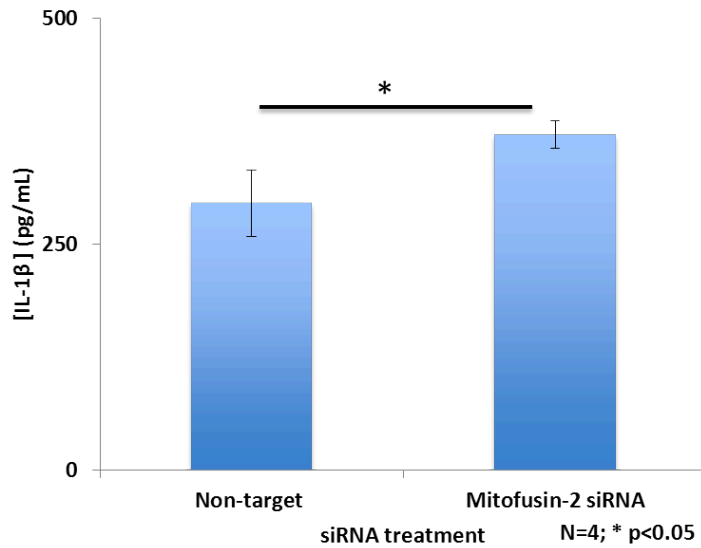
### 3.5 Biological relevance of ASC association with MAMs and with

#### Flightless-I

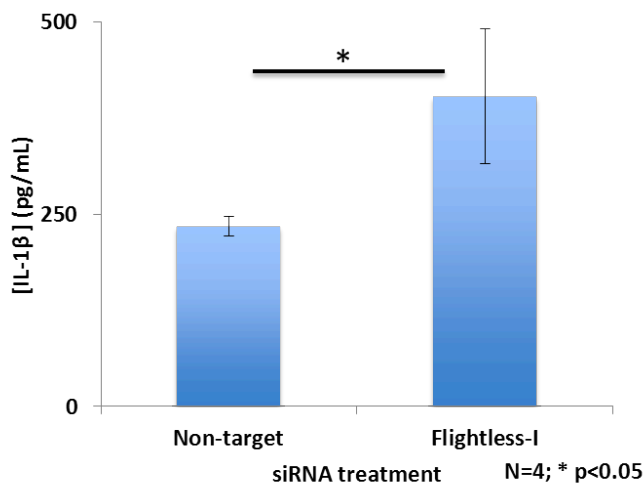
To test the above hypothesis, siRNA knockdown experiments were performed to determine the biological relevance of the association between ASC and MAMs and with Flightless-I. Mitofusin 2 is one of the most important structural proteins that form tethering complexes between mitochondrial outer membranes and ER membranes at MAMs. Previous studies showed that it is involved in ~50% of tethering at MAMs and knockdown of mitofusin 2 enhances RIG-I mediated antiviral signaling (Yasukawa, Oshiumi et al. 2009). A pool of four on-target siRNA for mitofusin 2 was transfected into THP-1 cells using Dharmafect1 transfection reagents. After 72 h, cells were harvested for assaying knockdown efficiency or infected with *Salmonella* after LPS priming. Knockdown efficiency for mitofusin 2 was ~50% and knockdown led to a significant increase in IL-1 $\beta$  secretion during *Salmonella* infection (Figure 6a) (The increase in IL-1 $\beta$  secretion was normalized to knockdown efficiency). Data suggested that MAMs negatively regulated inflammasome activation during early *Salmonella* infection.

Similarly, Flightless-I was knocked down in THP-1 cells and IL-1 $\beta$  secretion was assayed during *Salmonella* infection. Knockdown efficiency for Flightless-I was ~20% and knockdown led to a significant increase in IL-1 $\beta$  secretion during *Salmonella* infection (Figure 6b) (the increase in IL-1 $\beta$  secretion was normalized to knockdown efficiency). Since both mitofusin 2 and Flightless-I knockdown led to significant increase in IL-1 $\beta$  secretion, I performed a double knockdown to determine whether there would be additive effects. Simultaneous knockdown of mitofusin 2 and Flightless-I led to significant increase in IL-1 $\beta$  at a level similar to single knockdown and no additive effect was observed (Figure 6c). This

observation suggested that these two proteins were likely to function in the same pathway to dampen inflammasome activation, supporting the hypothesis that Flightless-I transports ASC to MAMs. This process negatively regulated inflammasome activation, possibly by decreasing the concentration of ASC in the cytosol.

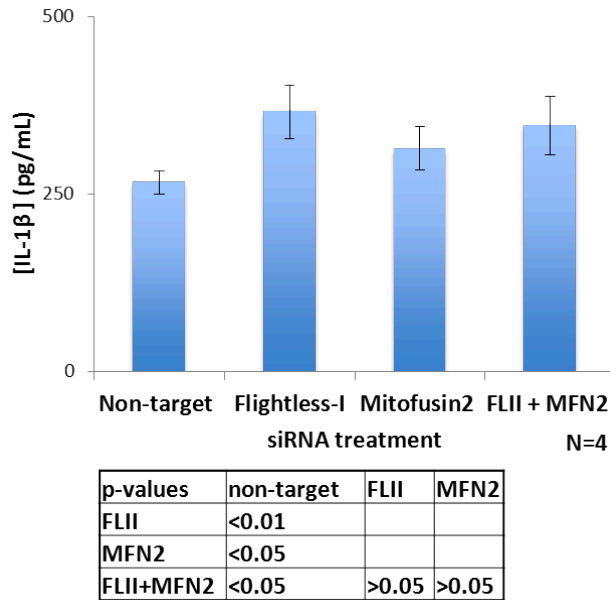


(a)



(b)





(c)

**Figure 6: IL-1 $\beta$  secretion during *Salmonella* infection with mitofusin 2, Flightless-I or double siRNA knockdown.**

(a) Mitofusin 2 siRNA knockdown. (b) Flightless-I siRNA knockdown. (c) mitofusin 2 and Flightless-I siRNA single and double knockdown.

### 3.6 Signal transduction of FliC through Flightless-I

In a previous *Salmonella* virulence factor screening project, Flightless-I was found to specifically interact with FliC, the *Salmonella* flagellin protein (Figure 7) (H.B. Yu, B.B. Finlay, L.J. Foster, unpublished data), so I hypothesize that Flightless-I is involved in the signal transduction for *Salmonella* flagellin.

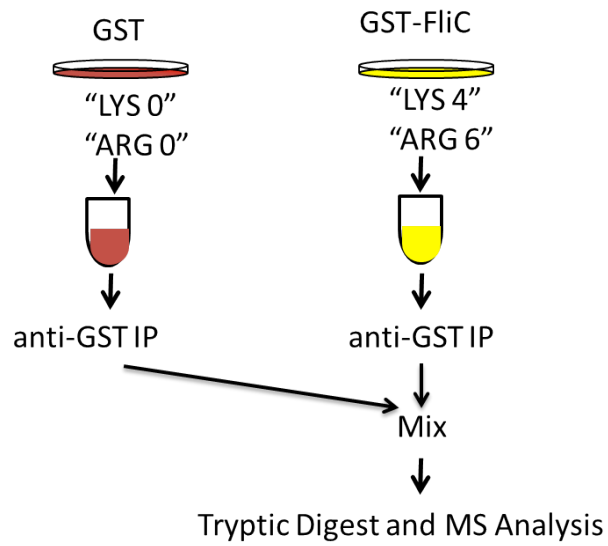
To test whether the interaction between Flightless-I and FliC is relevant for the interaction between Flightless-I and ASC, I made a *fliC/fljB* double knockout in the *Salmonella Typhimurium* wildtype strain SL1344. The *fljB* gene was also knocked out

because it is highly homologous to *fliC* and in some *Salmonella* strains it forms functional flagella during a process called phase variation (Ikeda, Schmitt et al. 2001). THP-1 cells were infected with the wild type strain, the  $\Delta fliF$  strain and the  $\Delta fliC\Delta fliB$  strain, and untreated cells were used as the control. Both the  $\Delta fliF$  and the  $\Delta fliC\Delta fliB$  strains do not have flagella and cannot invade cells. These two mutants were centrifuged onto THP-1 cells instead. After treatment, THP-1 cells were lysed and treated with standard immuno-precipitation procedures with the anti-Flightless-I antibody. IP eluents were analyzed by immuno-blotting to quantify the amount of ASC co-purified with Flightless-I. As observed before, the Flightless-I and ASC interaction was significantly stronger in wild type *Salmonella* infected cells than in resting cells. Interestingly, this interaction was stronger in  $\Delta fliF$  infected cells than in  $\Delta fliC\Delta fliB$  infected cells. Although  $\Delta fliF$  *Salmonella* does not form flagella, it expresses FliC that can be secreted into the host cell cytosol by the SPI-1 type-III secretion system (Subramanian, Qadri 2006, Sun, Rolan et al. 2007), therefore, the comparison between the  $\Delta fliF$  bacteria and the  $\Delta fliC\Delta fliB$  bacteria revealed the difference that appears to be due to the absence of FliC. The interaction between Flightless-I and ASC was stronger in  $\Delta fliF$  *Salmonella* infected cells than in  $\Delta fliC\Delta fliB$  *Salmonella* infected cells (Figure 8a), suggesting that FliC is essential for the interaction between Flightless-I and ASC.

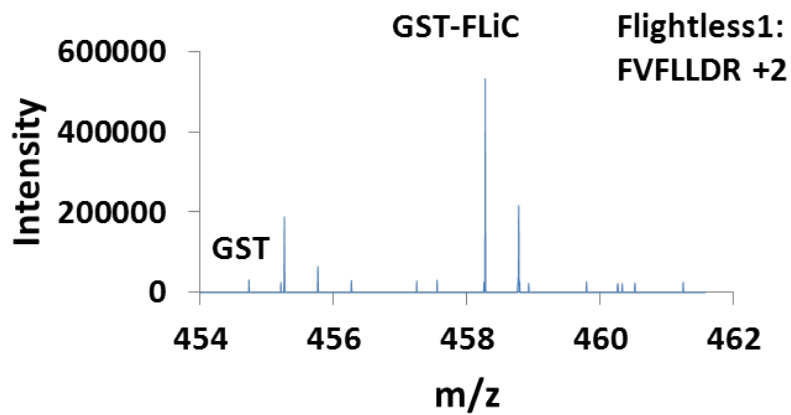
To further validate this model, I reconstituted the caspase-1 activation complex in 293T cells and expressed Flightless-I and FliC to test the effects of Flightless-I and FliC on inflammasome activation in a system isolated from other inflammasomes or TLR signaling complexes. IL-1 $\beta$  was activated and secreted when pro-caspase-1, ASC and pro-IL-1 $\beta$  cDNA are co-expressed in 293T cells (Figure 8b). FliC alone did not interfere with IL-1 $\beta$  activation (Figure 8b). This is possibly due to the lack of NAIP and NLRC4, both of which are critical

components of inflammasome complex required for the activation of caspases-1. Flightless-I alone inhibited IL-1 $\beta$  activation (Figure 8b), likely due to its interaction with ASC or with pro-caspase-1. When FliC and Flightless-I were co-expressed, IL-1 $\beta$  secretion was further inhibited (Figure 8b). The inflammasome reconstitution data supported the proposed model that FliC induces the interaction between Flightless-I and ASC, which dampens inflammasome activation.

Taken together, data suggested that during early *Salmonella* infection, FliC interacted with Flightless-I. Flightless-I then transported ASC to MAMs through actin cytoskeleton. This process was likely to sequester ASC away from cytosol to dampen inflammasome activation and IL-1 $\beta$  secretion (Figure 9).



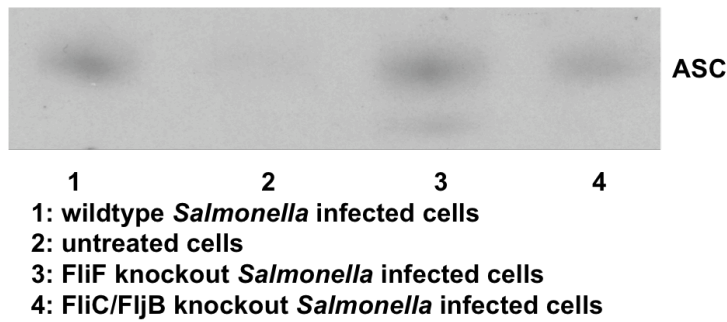
(a)



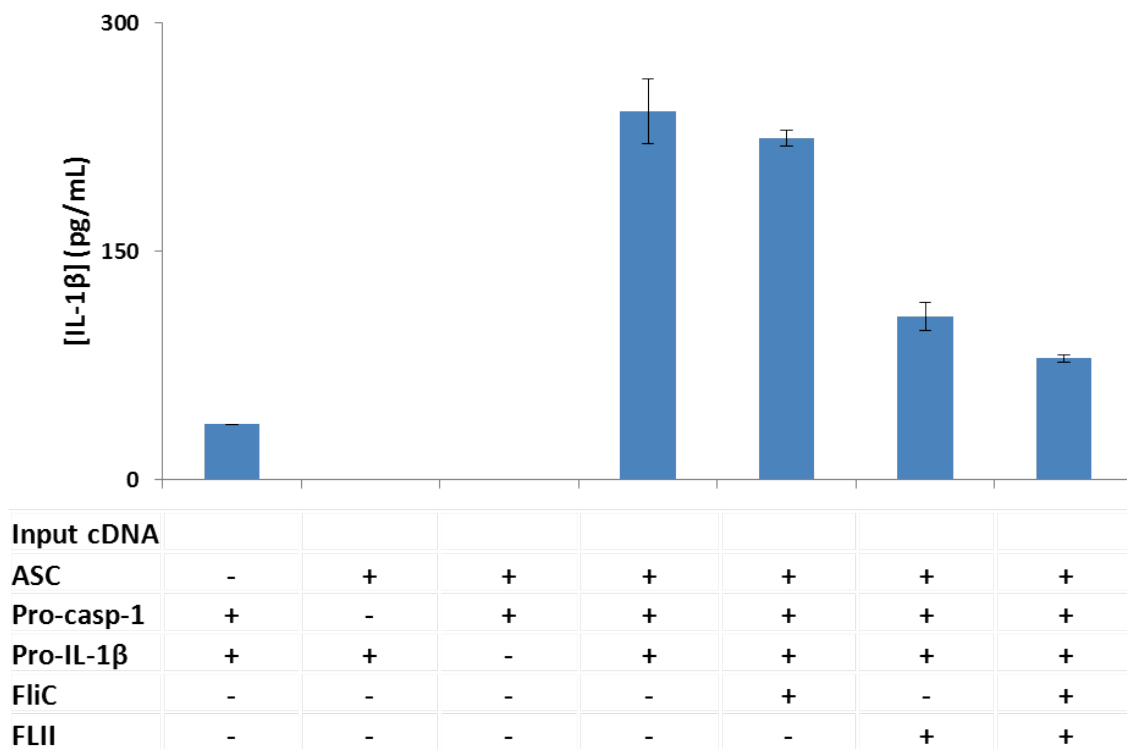
(b)

**Figure 7: Interaction between FLiC and Flightless-I.**

(a) Experimental scheme for SILAC-IP experiment to determine FLiC interacting proteins. (b) Mass Spectra for one Flightless-I peptide in the SILAC-IP analysis.



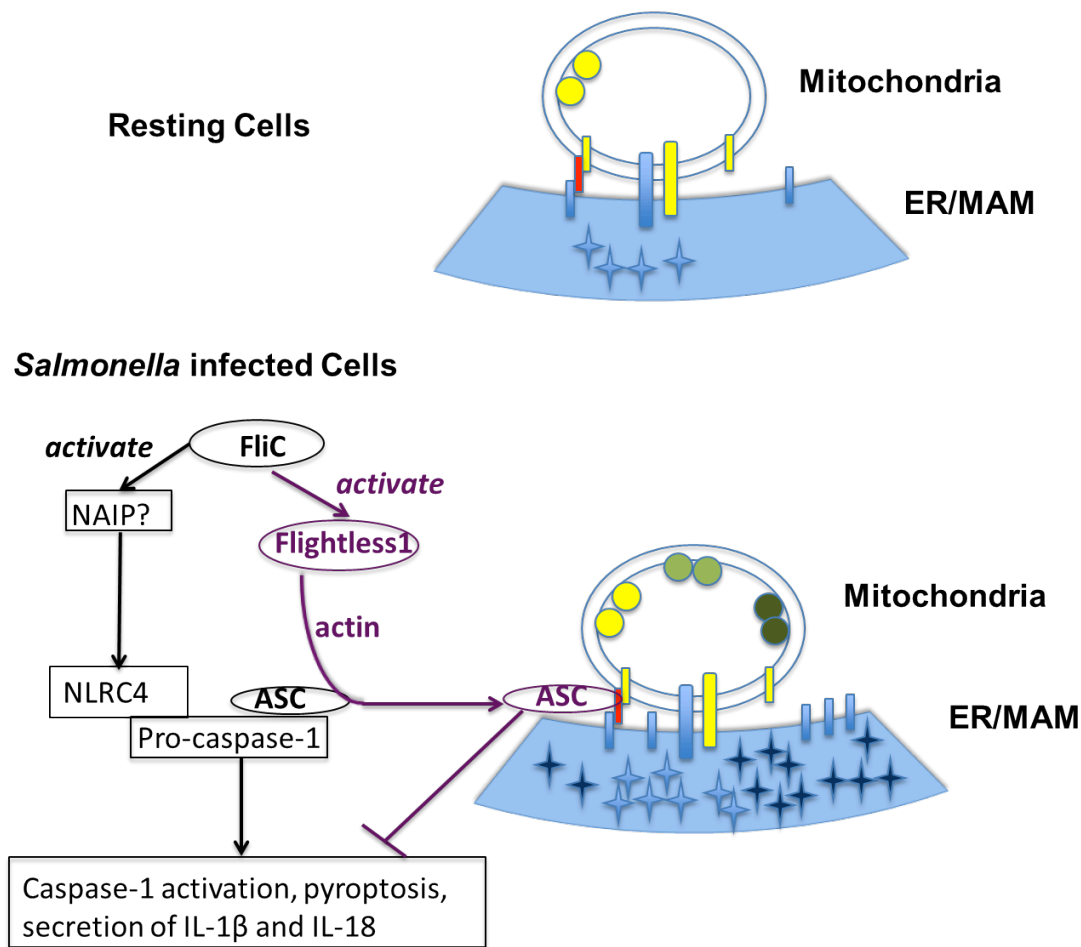
(a)



(b)

### Figure 8: FliC important for the interaction between Flightless-I and ASC.

(a) IP with anti-Flightless-I antibody and WB with anti-ASC antibody in THP-1 cells infected with wildtype,  $\Delta fliF$  and  $\Delta fliCfliB$  *Salmonella*. (b) in vitro inflammasome reconstitution. 293 T cells were seeded at 20% confluency in 24-well plates. cDNA were transfected into 293T cells with the following amount: 5 ng ASC, 5 ng proCASP1, 5 ng proIL1  $\beta$ , 10 ng FliC, 10 ng Flightless-I. Empty vector was added to normalize the total amount of cDNA for transfection.



**Figure 9: Proposed model.**

During *Salmonella* infection, flagellin activates the NLRC4 inflammasome pathway to induce caspase-1 mediated pyroptosis and secretion of proinflammatory cytokines.

Meanwhile, flagellin interacts with Flightless-I that then interacts with and transports ASC to MAMs via actin filaments. This process sequesters ASC away from cytosol and dampens inflammasome activation. Through unknown mechanisms, some other MAM proteins and cytosolic proteins are recruited to MAMs. Circles: cytochrome complexes in mitochondria; light blue stars: housekeeping MAM proteins; dark blue stars: ER proteins recruited to MAMs during *Salmonella* infection; blue rectangles: ion channels and tethering proteins on ER at MAMs; yellow rectangles: ion channels and tethering proteins on mitochondrial outer membranes at MAMs; red rectangle: cytosolic chaperone.

## 4. Discussion

### 4.1 MAM and pyroptosis during *Salmonella* infection

There is increasing evidence for a link between mitochondria-mediated cell stress and regulation of inflammation (Kepp, Galluzzi et al. 2011). Mitochondria are important for the regulation of cellular  $\text{Ca}^{2+}$  homeostasis and producing intracellular reactive oxygen species (ROS) (Kepp, Galluzzi et al. 2011); these two factors contribute to cellular stress and sterile inflammation. Mitochondrial membrane permeabilization is a potent trigger for apoptosis and necrosis (Kroemer, Galluzzi et al. 2007).

Recent papers have shown the linkage between mitochondria and NLRP3 inflammasome activation (Nakahira, Haspel et al. 2011, Zhou, Yazdi et al. 2011). ROS generated by mitochondrial respiratory chain complex I and III enhances NLRP3 inflammasome activation (Zhou, Yazdi et al. 2011). Abolishing mitochondrial ROS by overexpression Bcl-2 protein or by knocking down VDAC decreases NLRP3 inflammasome activation (Zhou, Yazdi et al. 2011). Release of mitochondrial DNA into cytosol also activates the NLRP3 inflammasome, resulting in maturation and secretion of the proinflammatory cytokine IL-1 $\beta$  (Nakahira, Haspel et al. 2011). Shimada et al. further confirmed that the  $\text{K}^{+}$  efflux and ROS result in oxidized mitochondria DNA that can potently activate the NLRP3 inflammasome (Shimada, Crother et al. 2012). Autophagy negatively regulates the NLRP3 inflammasome, possibly by preventing mitochondrial damage and ROS production (Nakahira, Haspel et al. 2011, Zhou, Yazdi et al. 2011). Of particular interest, Zhou et al. showed that NLRP3 and ASC both co-localize with mitochondria and MAMs when the NLRP3 inflammasome is activated by monosodium urate and nigerion (Zhou, Yazdi et al. 2011).

Meanwhile, recent studies have revealed the linkage between MAMs and apoptosis (Fujimoto, Hayashi 2011). MAMs are the close contact site between mitochondria and ER membranes. There is no direct membrane fusion between the two organelles (Lebiedzinska, Szabadkai et al. 2009), but several proteins and protein complexes tether the two organelles together and modulate biological functions at this unique inter-organelle interface. The previously reported tethering complexes include PACS2, VDAC/Stress70/IP3R, MFN2/MFN1, MFN2/MFN2 (Szabadkai, Bianchi et al. 2006, de Brito, Scorrano 2008, Myhill, Lynes et al. 2008b). Among these, mitofusin 2 is responsible for 40 to 50% of the mitochondria-ER linkage (de Brito, Scorrano 2008). MAMs actively regulate lipid biosynthesis,  $\text{Ca}^{2+}$  homeostasis and apoptosis (Hayashi, Rizzuto et al. 2009). Disruption of MAM structure decreases the direct uptake of  $\text{Ca}^{2+}$  from ER into mitochondria, and this process dampens apoptosis in most cases (Giorgi, Wieckowski et al. 2011). In some cases, however, sustained retention of  $\text{Ca}^{2+}$  within the ER lumen eventually leads to apoptosis via ER stress mediated pathways (Csordas, Renken et al. 2006b, Simmen, Aslan et al. 2005). Taken together, MAMs can modulate apoptosis in both ways (Fujimoto, Hayashi 2011) and the majority of data shows its pro-apoptotic function by rapidly increasing the mitochondrial  $\text{Ca}^{2+}$  concentration upon stimuli.

MAMs are an important hub for antiviral signaling pathways and, as a result, some viruses have evolved specific mechanisms to target this site. During RNA virus infection, RIG-I is recruited to MAMs to bind to MAVS (Horner, Liu et al. 2011). HepC NS3/4A protease cleaves MAVS at MAM (not at “the free mitochondria”) to potentiate viral infection (Horner, Liu et al. 2011). However, another paper showed that disrupting MAMs by silencing mitofusin 2 enhances RIG-I mediated antiviral signaling, suggesting that MAMs



can negatively regulate the RIG-I antiviral pathway (Yasukawa, Oshiumi et al. 2009). A recent proteomic study reported the up-regulation of major MAM proteins, as well as pro-apoptotic regulator proteins, at MAM sites during human cytomegalovirus infection, suggesting that MAM is highly regulated by viral infection (Zhang, Williamson et al. 2011).

So far, there is no report of MAM function during bacterial infection. My data provided novel insights into MAM composition and functions during *Salmonella* infection. Unbiased protein correlation profiling data showed unique protein recruitments to MAMs during early *Salmonella* infection. Some housekeeping MAM proteins were more enriched at MAMs in the infected cells than in resting cells, while some other MAM markers were not regulated (Table-1). An interesting example of this was seen with some of the lipid raft-associated proteins: Erlin-2 was up regulated at MAMs in *Salmonella* infected cells while Erlin-1 showed no difference. I identified novel MAM proteins that have similar functions to previously reported MAM markers. I identified three novel Ras-related proteins, Rabs-7, 14, 21, that are recruited to MAMs during *Salmonella* infection, where only Rab-32 has been reported before (Bui et al. 2010). Cytochrome c is released from mitochondria to MAMs (Szabadkai, Bianchi et al. 2006). During *Salmonella* infection, it was not regulated but cytochrome b and cytochrome 450 enzymes were more enriched at MAMs. Type-3 IP<sub>3</sub>R is the most reported IP<sub>3</sub> receptor at MAMs (Fujimoto, Hayashi 2011), and I identified the type-1 IP<sub>3</sub>R to be enriched during *Salmonella* infection. I have also identified novel proteins with previously unknown functions at MAMs. S100-A8 protein was specifically recruited to MAMs in *Salmonella* infected cells. It is a calcium binding protein and mediates proinflammatory responses (Viemann, Strey et al. 2005, Ahmad, Bayley et al. 2003). To my knowledge, this is the first report of the MAM proteome during bacterial infection and my

data showed that MAM proteins were dynamically regulated during early *Salmonella* infection with recruitment of some housekeeping MAM markers and many other cytosolic proteins.

Interestingly, the inflammasome adaptor protein ASC was co-purified with MAMs. Immuno-precipitation of ASC identified its interaction with the VDAC/Stress70 protein complex at MAMs. My data suggested that ASC was specifically recruited to MAMs during early *Salmonella* infection. Since previous papers reported the co-localization of NLRP3 and ASC at MAMs during the NLRP3 inflammasome activation (Zhou, Yazdi et al. 2011), I explored this possibility for the NLRP4 inflammasome that is typically activated during *Salmonella* invasion (Schroder, Tschopp 2010). Neither NLRP4 nor pro-caspase-1 was co-purified with the MAM fraction on the sucrose gradient, suggesting that the ASC was recruited to MAMs independent of the NLRP4 inflammasome protein complex. Disrupting MAMs partially by silencing mitofusin 2 caused a significant increase of IL-1 $\beta$  secretion during *Salmonella* infection, suggesting that MAMs negatively regulated inflammasome activation possibly by sequestering ASC away from cytosol. Although ASC is not essential for the NLRP4 inflammasome complex formation, it enhances the stability of NLRP4 complex (Schroder, Tschopp 2010). There are other inflammasomes activated during *Salmonella* infection, and ASC can be important for them. A recent paper reported a novel NLRP7 inflammasome activated by microbial acetylated lipopeptides (Khare, Dorfleutner et al. 2012). NLRP3 inflammasome and pyroptosis are important for clearing *Salmonella* during the system infection stage (Broz, Newton et al. 2010, Miao, Leaf et al. 2010). Both NLRP3 and NLRP7 require ASC to form functional inflammasomes (Martinon, Burns et al.

2002, Khare, Dorfleutner et al. 2012), and they can be significantly disrupted if ASC is sequestered away from cytosol.

The intracellular localization of ASC is important for its function. During *E. coli* infection, endogenous ASC re-localizes to cytosol from the nucleus (Bryan, Dorfleutner et al. 2009). Inhibition of the nuclear export of ASC prevents IL-1 $\beta$  secretion (Bryan, Dorfleutner et al. 2009). During the activation of the NLRP7 inflammasome by *Mycoplasma*, intracellular redistribution of ASC from nucleus to cytosol is also observed (Khare, Dorfleutner et al. 2012). My data further proposed another mechanism to sequester ASC at the membranes (MAM) to modulate inflammasome activation in the cytosol.

Since VDAC is involved in the ER-mitochondria tethering (Szabadkai, Bianchi et al. 2006) and I identified its interaction with ASC, I silenced VDAC1,2,3 to determine the impact on IL-1 $\beta$  secretion during *Salmonella* infection. With the siRNA transfection of VDAC1,2,3 individually or all together, I observed a slight decrease in IL-1 $\beta$  secretion following *Salmonella* infection, but the difference between scramble siRNA treated cells and the VDAC siRNA treated cells was not significant (data not shown). This observation was consistent with the previous report that VDAC knockdown can negatively regulate IL-1 $\beta$  secretion by dampening mitochondrial ROS (Zhou, Yazdi et al. 2011). As VDACs are involved with various mitochondrial functions, silencing of mitofusin 2 is a more direct assay to study the impact of MAMs on pyroptosis. To conclude, my data reported the novel recruitment of ASC at MAMs and this process negatively regulated inflammasome activation during early *Salmonella* infection.

## 4.2 The interaction between Flightless-I and ASC during *Salmonella*

### infection

Flightless-I is a member of the gasline protein family that binds to and manipulates the actin cytoskeleton (Silacci, Mazzolai et al. 2004). Several studies proposed its involvement with signal transduction in the innate immune system. Flightless-I interacts with MyD88 and TRIF directly via the TIR domain and can negatively regulate the TLR4-MyD88 pathway (Wang, Chuang et al. 2006). It also negatively regulates caspase-1 and caspase-11 (Li, Yin et al. 2008). With its actin binding function, Flightless-I transports caspase-11 to cell peripheries via the actin filaments, away from the soluble fraction of the cytosol (Li, Yin et al. 2008). Current literature suggests that Flightless-I is mainly a negative regulator of inflammation. On the other hand, two Flightless-I interaction proteins positively regulate the TLR pathways (Liu, Yin 1998, Fong, de Couet 1999). LRP1P1 interacts with TLR3 and up-regulate Type I Interferon during viral infection (Bagashev, Fitzgerald et al. 2010). LRP1P2 interacts with MyD88 and up-regulates the NF- $\kappa$ B pathway (Dai, Jeong et al. 2009).

The SILAC-IP data reported here revealed a novel pathway for Flightless-I during early *Salmonella* infection. It interacted with ASC in *Salmonella* infected THP-1 cells but not in resting cells, and this interaction dampened inflammasome activation during early *Salmonella* infection. Enrichment of ASC in actin fractions in infected cells suggested Flightless-1 might transport ASC via actin filaments in a mechanism similar to the previously reported transportation of caspase-11 to change the cellular localization of target proteins. Enrichment of ASC at MAMs suggested that MAMs could be the destination. siRNA knockdown of Flightless-I and mitofusin 2 both led to significant increase in IL-1 $\beta$  secretion during *Salmonella* infection. No additive effects were observed by the knock down of both

proteins, which suggested that Flightless-I and MAMs were in the same pathway for dampening inflammasome activation. To further test this hypothesis, I knocked down Flightless-I and then determined the enrichment of ASC in the actin fractions and in the crude mitochondria. I could not see an obvious response (data not shown), possibly due to the low knockdown efficiency of Flightless-I in THP-1 cells (20% knockdown). Further knockdown experiments using Flightless-I shRNA is likely to address this question.

### **4.3 The FliC-Flightless1-MAM pathway to dampen inflammasome activation**

NLRP3 and NLRC4 inflammasomes are involved with caspase-1 activation and pyroptosis during *Salmonella* infection (Broz, Newton et al. 2010) but the mechanisms for the NLRP3 inflammasome activation by *Salmonella* have not been resolved (Broz, Newton et al. 2010). NLRP3/NLRC4 double knockout mice have a phenotype similar to caspase-1 knockout mice, while single knockout (NLRP3 or NLRC4) mice do not (Broz, Newton et al. 2010). Recent studies revealed the ligands and receptors for NLRC4 inflammasomes in murine cells. *Salmonella* flagellin directly binds to NAIP5 that then activates NLRC4 (Zhao, Yang et al. 2011, Kofoed, Vance 2011). PrgJ, an inner rod protein from the SPI-1 type-3 secretion apparatus, binds to NAIP2 that then activates NLRC4 (Zhao, Yang et al. 2011, Kofoed, Vance 2011). In human cells, NAIP protein may play a similar role, but this has not been confirmed during *Salmonella* infection (Zhao, Yang et al. 2011). ASC is the adaptor protein required for the formation of NLRP1 and NLRP3 inflammasome complexes (Schroder, Tschopp 2010). ASC is not required for the NLRC4 inflammasome but it enhances NLRC4 inflammasome activation, possibly by stabilizing the protein complex

(Schroder, Tschopp 2010). A recent paper reported that ASC<sup>-/-</sup> BMMs have reduced activation of caspase-1 and IL-1 $\beta$ , and partially affected pyroptosis during NLRC4 inflammasome activation, suggesting that ASC is important for the NLRC4 signaling (Broz, von Moltke et al. 2010).

*Salmonella* flagellin is a potent activator for innate immunity. It is recognized by TLR5 on the cell surface and NLRC4 in the cytosol (Akira, Takeda 2004, Miao, Alpuche-Aranda et al. 2006). Recognition by TLR5 leads to the NF- $\kappa$ B pathway activation, expression and secretion of proinflammatory cytokines, chemokines and activation of other antimicrobial defenses (Franchi 2011). Flagellin is the major pro-inflammatory activator in the gut, mainly via the cytokine IL-1 $\beta$  (Carvalho, Nalbantoglu et al. 2012). Meanwhile, *Salmonella* has evolved mechanisms to inhibit its recognition by the host. During the intracellular replication and systemic infection stages, flagellin expression is repressed (Miao, Mao et al. 2010a). A recent study made one special *Salmonella* strain, FliC<sup>ON</sup> that expresses flagellin under SPI-2 conditions (Miao, Leaf et al. 2010). This strain has attenuated virulence, is efficiently cleared by mice and causes a 50% lower mortality rate than the wild-type *Salmonella Typhimurium* strain (Miao, Leaf et al. 2010). Taking together, flagellin is a potent innate immunity activator during the initial invasion into gut epithelial cells and macrophages, and is down-regulated by *Salmonella* to evade host recognition when it is internalized into host cells.

My research data has proposed a novel model that during early *Salmonella* infection, flagellin could partially dampen the host innate immune activation. The SILAC IP experiment identified the interaction between flagellin (FliC) and Flightless-I, and Flightless-I interacted with ASC specifically in *Salmonella* infected cells. I hypothesize that flagellin

binds to and activates Flightless-I, which is required for the Flightless-I/ASC/MAM pathway to dampen inflammasome activation.

My data from *Salmonella* infection with two aflagellated strains supported this model. Since  $\Delta fliC\Delta fliB$  bacteria are immobile and cannot invade host cells as efficiently as wild type *Salmonella*, I used the  $\Delta fliF$  *Salmonella* strain as the control. Both strains do not have flagella and the only difference is whether flagellin is expressed within the bacterial cytosol. Flagellin is normally secreted by the flagellum type-III secretion apparatus that shares high homology with the SPI-1 and SPI-2 type-III secretion systems (Hueck 1998, Pallen, Beatson et al. 2005). Flagellin can be translocated into host cytosol via both SPI-1 and SPI-2 type-3 secretion apparatus (Sun, Rolan et al. 2007, Miao, Leaf et al. 2010). And the sensing of lysophospholipids triggers secretion of flagellin monomers by *Salmonella* (Subramanian, Qadri 2006). During the infection with  $\Delta fliF$  *Salmonella*, flagellin is likely to be translocated into the host cytosol by the SPI-1 T3SS. The interaction between Flightless-I and ASC in  $\Delta fliC\Delta fliB$  infected cells was weaker than that in  $\Delta fliF$  infected cells, suggesting that flagellin was essential for the interaction between Flightless-I and ASC.

Previous studies reported that the C-terminal region of FliC is sufficient to activate the NLRC4 inflammasome, while the N-terminal region cannot by itself but can enhance the activation (Lightfield, Persson et al. 2011). It is also possible that certain domains of FliC are responsible for interacting with Flightless-I. A co-IP experiment expressing different domains of FliC and Flightless-I is likely to address this question.

My data from the inflammasome *in vitro* reconstitution experiments also supported the hypothesis that FliC interacts with and activates Flightless-I that then inhibits inflammasome activation. Expression of ASC, pro-caspase-1 and pro-IL-1 $\beta$  led to IL-1 $\beta$  maturation and

secretion by 293T cells. Expression of pro-caspase-1 and pro-IL-1 $\beta$  led to a very low level of IL-1 $\beta$  secretion, probably due to the background level of caspases-1 auto-proteolysis activity. Expression of ASC greatly enhanced IL-1 $\beta$  secretion. Therefore, in this study, the co-expression of ASC, pro-caspase-1 and pro-IL-1 $\beta$  reconstituted the caspase-1 activation complex for IL-1 $\beta$  processing. Co-expression of Flightless-I decreased IL-1 $\beta$  activation, possibly by its interaction with pro-caspase-1 and/or ASC. Co-expression of FliC had no effect on IL-1 $\beta$  activation, probably due to the lack of ligand receptors. Co-expression of Flightless-I and FliC led to a further and significant decrease in IL-1 $\beta$  activation. This observation confirmed the speculation that FliC enhances the negative regulation of inflammasome by Flightless-I.

#### **4.4 Inflammasome regulation and proposed model**

Recent studies indicate that inflammasomes plays a very important role in innate immunity against microbial pathogens and host damages. Different inflammasome complexes are activated during various bacterial, viral and fungal infections and during cell damage conditions (Schroder, Tschopp 2010). Inflammasome activation also bridges the innate and adaptive immune systems (Ghiringhelli, Apetoh et al. 2009), and facilitates clearance of pathogens (Schroder, Tschopp 2010). On the other hand, dys-regulations in NLR proteins (often due to genetic mutations) are tightly linked with auto-immune or auto-inflammatory disorders (Agostini, Martinon et al. 2004, Ting, Kastner et al. 2006). The pro-inflammatory cytokines produced during pyroptosis can cause significant tissue damages. Therefore, inflammasomes activation is tightly regulated by the host immune system at several checkpoints to prevent uncontrolled damages. Many pathogens have also evolved



effective mechanisms to control inflammasome activation to enhance their survival and replication in the host (Rathinam, Vanaja et al. 2012b).

On the host side, interferons negatively regulate inflammasome activation by inducing anti-inflammatory cytokines (e.g. IL-10) to inhibit the expression of pro-IL-1 $\beta$  (Guarda, Braun et al. 2011). IFN- $\beta$  dampens NLRP3 inflammasome activation in monocytes (Guarda, Braun et al. 2011). IFN- $\gamma$  suppresses pro-IL-1 $\beta$  expression triggered by LPS (Masters, Mielke et al. 2010). Effector and memory T cells attenuate the proteolytic activation of caspase-1 and IL-1 $\beta$  in macrophages and dendritic cells during NLRP1 and NLRP3 inflammasome activation (Guarda, Dostert et al. 2009). Autophagy is another general mechanism to negatively regulate inflammasome activation by recycling of cellular proteins and damaged organelles (Harris, Hartman et al. 2011, Zhou, Yazdi et al. 2010). Autophagy also modulates the transcription and secretion of IL-1 $\beta$  in human cells (Crisan, Plantinga et al. 2011, Dupont, Jiang et al. 2011). Additionally, proteins in the TRIM family, Pyrin-only proteins, CARD-only proteins and an antimicrobial peptide (LL-37) have all been reported to negatively regulate inflammasome activation (Rathinam, Vanaja et al. 2012b).

On the microbial side, several viruses express proteins that can directly inhibit inflammasome activation. The mechanisms include binding to and inhibiting ASC, inhibiting the transcription of IL-1 $\beta$  and inhibiting caspase-1 activity (Rathinam, Vanaja et al. 2012b). A viral homolog of NLRP1, Orf-63 inhibits NLRP1 and NLRP3 oligomerization (Gregory, Davis et al. 2011). Bacterial pathogens evolved similar mechanisms to inhibit inflammasome activation. *Pseudomonas* expresses two effector proteins (ExoU and ExoS) that can negatively regulate caspase-1 and IL-1 $\beta$  activation (Galle, Schotte et al. 2008). *Mycobacterium* encodes a zinc metalloprotease to inhibit caspase-1 activation (Master,

Rampini et al. 2008). *Legionella* interferes with ASC protein expression (Abdelaziz, Gavrillin et al. 2011a). *Yersinia* effector protein YopK interacts with its own T3SS to prevent recognition by host cells (Schotte, Denecker et al. 2004). *Salmonella* down-regulates flagellin and SPI-1 T3SS during the systemic infection stage (Miao, Mao et al. 2010a). While flagellin and PrgJ (an inner rod protein in the SPI-1 apparatus) are potent activators of the NLRC4 inflammasome, SPI-2 T3SS is not recognized (Miao, Mao et al. 2010a). Expression of flagellin under SPI-2 conditions facilitates the clearance of *Salmonella* by the host immune system (Miao, Mao et al. 2010a). Therefore, microbes have evolved mechanisms to down-regulate or evade the inflammasome pathways to enhance their survival inside the host.

My data proposed a novel pathway to regulate inflammasome activation during early *Salmonella* infection. During the early stage of infection (invasion stage), *Salmonella* expresses high levels of flagellin and SPI-1 apparatus, both of which strongly activate the host innate pro-inflammatory pathways, mainly via TLR4, TLR5 and the NLRC4 inflammasome. *Salmonella* also potently induces pyroptosis at this stage of infection (Miao, Alpuche-Aranda et al. 2006). At later stages of infection, *Salmonella* down-regulates both flagellin and SPI-1 T3SS to enhance its survival inside host cells (Miao, Mao et al. 2010a). My data, for the first time, proposed that *Salmonella* has also evolved a mechanism to dampen inflammasome activation during the invasion stage. Flagellin is a potent activator of TLR5 and NLRC4 pathways. My data suggested that it could also interact with and activate Flightless-I that is a host inhibitor of inflammasome activation. This dual function or negative feedback loop is not uncommon for bacterial virulence factors. One example is the pneumolysin in *Streptococcus pneumoniae* where it activates the IL-1 $\beta$  response via the NLRP3 inflammasome and caspase-1 (McNeela, Burke et al. 2010, Witzenrath, Pache et al.

2011) and inhibits inflammasome activation in human dendritic cells (Littmann, Albiger et al. 2009) Flagellin may play a similar role during *Salmonella* invasion. One previous paper reported that Flightless-I inhibits caspase-1 activation in mouse cells; my data proposed an additional mechanism whereby Flightless-I interacted with and transported ASC to MAMs to sequester it away from the cytosol. The protein re-localization process was also via actin filaments and was only observed at the presence of FliC.

Two research groups have recently proposed two types of inflammasome complexes with different effects on pyroptosis and secretion of proinflammatory cytokines: an ASC-dependent complex and an ASC-independent complex (Broz, von Moltke et al. 2010, Case, Roy 2011). Although the two groups reported different read-outs for the two types of protein complexes, it is clear that ASC can modulate the downstream signaling of the NLRC4 inflammasomes (Broz, von Moltke et al. 2010, Case, Roy 2011). Specifically, Broz et al. reported that the NLRC4 inflammasome lacking ASC cannot process or secrete proinflammatory cytokines during *Salmonella* infection (Broz, von Moltke et al. 2010). The model I proposed here may be a mechanism for *Salmonella* to modulate proinflammatory cytokine activation by specifically targeting ASC.

This novel inflammasome inhibition pathway involved ASC but not other members of the NLRC4 inflammasome complex. This is not an uncommon observation: *Legionella* specifically interferes with ASC protein expression (Abdelaziz, Gavrilin et al. 2011a) ASC-deficient mice have a very distinct phenotype with defect in antigen presentation (by dendritic cells) and lymphocyte migration and this is not observed in NLRC4-deficient or caspase-1-deficient mice (Ippagunta, Malireddi et al. 2011a). Lack of ASC is linked to impaired actin polymerization via Dock2 that can mediate Rac-dependent signaling

processes, and this mechanism is also independent of the NLRC4 inflammasome (Ippagunta, Malireddi et al. 2011b). In the model I proposed that sequestering ASC away from cytosol dampened inflammasomes in general. ASC stabilizes the NLRP4 protein complex to enhance its activation (Schroder, Tschopp 2010) and can modulate its downstream signaling pathways (Broz, von Moltke et al. 2010, Case, Roy 2011). There are other inflammasomes activated during *Salmonella* infection (Broz, Newton et al. 2010), and lack of ASC in the cytosol can potentially interrupt other NLR signaling pathways.

## 5. Summary and Conclusion

This study was the first characterization of MAM protein composition during bacterial infection. Furthermore, I identified the enrichment of ASC at MAMs during early *Salmonella* infection, with Flightless-I interacting with and transporting ASC to MAMs via actin filaments at the presence of *Salmonella* flagellin. This novel pathway negatively regulated the inflammasome activation during early *Salmonella* infection possibly by sequestering the inflammasome adaptor protein away from cytosol. This was the first report of the negative regulation of inflammasome activation by *Salmonella* flagellin.

The research experiments were conducted using a widely used human monocytic cell line, THP-1 cells. There might still be differences between primary monocytes and THP-1 cells. Further validation using human primary monocytes is needed. In this study, siRNA knockdown was applied to study biological relevance of certain proteins. The knockdown efficiency was not optimal in THP-1 cells. Further experiments using shRNA may add strength to the current data. Various biochemical methods were used in this study while cell biology approaches (e.g. immunofluorescence) were relatively unsuccessful due to the shape and morphology of THP-1 cells. More imaging techniques need to be tested to validate the biochemical data.

In this study, a novel pathway was proposed to negatively regulate inflammasome activation during early *Salmonella* infection. Multiple inflammasomes are activated by *Salmonella* (Miao, Alpuche-Aranda et al. 2006, Broz, Newton et al. 2010), and my data did not reveal which inflammasome complexes were specifically dampened. One future goal is to study the impact of this pathway on different inflammasome complexes. In addition, my data described a unique MAM proteome during early *Salmonella* infection. The functions

and interplay between many of the specifically recruited proteins are unknown. Another future direction is to study the biological relevance of these proteins.

My data was the first report of MAM function during bacterial infection, specifically during early *Salmonella* infection. Methods and assays used in this study can be applied to infections with other bacterial pathogens to further study the functions of MAMs in innate immunity. Although the pathway discovered here was induced by *Salmonella*, the interaction between Flightless-I and ASC can potentially be applied to control undesired inflammation induced by inflammasome activation that is observed in sterile inflammatory and autoimmune diseases.

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