CHARACTERIZATION OF PORCINE NLRP3 INFLAMMASOME-MEDIATED INTERLEUKIN-1 BETA PRODUCTION IN RESPONSE TO INFLUENZA A VIRUSES

A Thesis
Submitted to the College of Graduate and Postdoctoral Studies
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy
in the
Department of Veterinary Microbiology,
Western College of Veterinary Medicine,
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ABSTRACT

Influenza A virus (IAV) causes respiratory infections in humans and animals. Pigs are infected not only by swine influenza virus (SIV), but also by human and avian influenza viruses. Pigs serve as the mixing vessel for the generation of novel reassortant viruses with pandemic potential as evidenced by the emergence of 2009 human pandemic IAV (pdm09). Therefore, IAV infection in pigs poses both an economic concern and a threat to public health. Virus-host interplay dictates the outcome of virus infection. The host innate immune system can sense the virus infection and produce multiple cytokines and chemokines to control inflammation as well as to mount antiviral responses. On the other hand, the host innate immune response can be antagonized by IAV-encoded proteins, one being the non-structural protein 1 (NS1). Thus, a better understanding of the porcine innate immunity to IAV infection and the innate immune evasion strategies by IAV is required for a rational design to combat IAV infections in pigs.

Innate immune cells in lungs, particularly the alveolar macrophages, are indispensable for host protection against IAV infection. They produce pro-inflammatory cytokines including interleukin-1 beta (IL-1β) that recruits other immune cells and promotes phagocytic activities. IL-1β production is tightly regulated by a cytosolic multiprotein complex called the nucleotide-binding domain and leucine-rich repeat-containing protein 3 (NLRP3) inflammasome. The NLRP3 inflammasome is comprised of NLRP3, apoptosis-associated speck-like protein containing caspase activation and recruitment domain (ASC), and pro-caspase-1. The molecular mechanism underlying the NLRP3 inflammasome-mediated IL-1β production upon IAV infection in the swine host, and the mechanism by which IAV NS1 protein counteracts the porcine IL-1β response remain largely unknown. This PhD project was centered on the characterization of the NLRP3 inflammasome-mediated IL-1β production in response to IAV infection in the swine host; specifically, I first characterized the IL-1β production in primary porcine alveolar macrophages (PAMs) in response to different isolates of IAV. Next, I investigated the molecular mechanisms by which viral NS1 protein modulates the porcine NLRP3 inflammasome activity, and finally I examined the molecular pathways that are involved in inflammasome activation upon IAV infection in PAMs.

This study showed that while various SIV strains could induce NLRP3 inflammasome-mediated IL-1β production in primary PAMs, the human pdm09 virus induced much less IL-1β than did SIVs. Subsequent analyses revealed that the NS1 C-terminus of pdm09, but not that of

SIV, was responsible for the significant inhibition of the NLRP3 inflammasome-mediated IL-1β production. Mechanistically, the NS1 C-terminus of pdm09 disrupted the interaction between NLRP3 and ASC, a prerequisite for the NLRP3 inflammasome assembly; this led to an impaired formation of ASC specks, a hallmark of NLRP3 inflammasome activation. NS1 C-terminus of pdm09 also suppressed the ubiquitination of porcine ASC. Furthermore, two lysine (K) residues, K110 and K140, on porcine ASC were identified as the ubiquitination acceptor sites; mutation of these two lysine residues diminished the ubiquitination of ASC and significantly reduced the IL-1β production in response to the pdm09 virus with the NS1 C-terminal deletion. These results revealed that the NS1 C-terminus of pdm09 suppresses ASC ubiquitination to support the immune evasion by the virus.

Further attempts were made to dissect the upstream mechanism of the NLRP3 inflammasome-mediated IL-1β production upon SIV infection, focusing on the mitochondrial dynamics regulated by dynamin-related protein 1 (DRP1). This study showed that SIV infection induced not only phosphorylation of DRP1 at serine 579 (S579) that is required for mitochondrial fission activity of DRP1, but also mitochondrial fission in PAMs. The reactive oxygen species produced from mitochondrial fission was also related to the IL-1β production. Phospho-mimetic mutation at S579 on DRP1 could upreguate the NLRP3 inflammasome activity, leading to an increased IL-1β production. Furthermore, the requirement of the kinase activity of receptor-interacting protein kinase 1 (RIPK1) for the IL-1β production and the association of RIPK1 with DRP1 suggested that RIPK1 is an upstream kinase for DRP1 phosphorylation. These results indicated an integral role of the RIPK1/DRP1 signaling axis in modulating the porcine NLRP3 inflammasome-mediated IL-1β production in SIV-infected PAMs.

Taken together, this study defined the mechanism by which SIV induces porcine NLRP3 inflammasome-mediated IL-1β production and elucidated the strategies pdm09 employs to circumvent the host innate immunity. The obtained information will enhance our knowledge of the innate immunity to IAV infection in the swine host.

ACKNOWLEDGEMENTS

First of all, I would like to express the deepest gratitude to my supervisor, Dr. Yan Zhou, for her thoughtful guidance and support that enabled me to complete the study. Without her dedicated mentorship, it would have been much harder for me to get through this journey. I want to extend my appreciation to the advisory committee members, Dr. Suresh Tikoo, Dr. Joyce Wilson, and Dr. Alexander Zakhartchouk for their constructive criticism with suggestions, and the graduate chair, Dr. Janet Hill for keeping me on the right track.

I would like to acknowledge my former and current colleagues, Dr. Hyun-Mi Pyo, Dr. Li Lin, Dr. Fang Xu, Dr. Sathya N. Thulasi-Raman, Dr. Amit Gaba, Leo Guanqun Liu, Magda Langton, Fangzheng Wang, Yao Lu, and Shelby Landreth for their assistance and inspiration. Among others, I give special thanks to Leo for stimulating me not only with the way of doing science, but also through helpful discussions, and to Sathya for providing valuable resources and advice. I also feel thankful to the VIDO-InterVac scientists and staff, especially Dr. Qiang Liu, Donna Dent and Ken Lai, and to the faculty and staff in the Department of Veterinary Microbiology, Western College of Veterinary Medicine for their kind help and support.

Further, I feel indebted to the funding sources, the Natural Sciences and Engineering Research Council of Canada (NSERC) grant to Dr. Yan Zhou as well as the Western College of Veterinary Medicine, University of Saskatchewan that provided scholarship/fellowship to me.

Finally and most importantly, I thank my parents for all their love and support.

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LIST OF ABBREVIATIONS

aa	amino acid
AIM2	absent in melanoma 2
AM	alveolar macrophage
AMP	adenosine monophosphate
ANOVA	analysis of variance
AP-1	activator protein 1
ASC	apoptosis-associated speck-like protein containing caspase activation and recruitment domain
ATD	acidic transactivation domain
ATP	adenosine triphosphate
BALF	bronchoalveolar lavage fluid
BIR	baculovirus inhibitor of apoptosis protein repeat
bp	base pairs
BM18	influenza A/Brevig Mission/1/1918/H1N1
BSA	bovine serum albumin
CARD	caspase activation and recruitment domain
CDK1	cyclin-dependent kinase 1
cGAS	cyclic GMP-AMP synthase
CHX	cycloheximide
CIITA	MHC class II transactivator
CPE	cytopathic effect
CPSF30	30 kDa subunit of cleavage and polyadenylation specificity factor
cRNA	complementary RNA
DAMP	danger-associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DDX	DEAD-box helicase
DED	death effector domain
DENV	dengue virus
DHX	DEAH-box helicase

DMEM	Dulbecco’s modified Eagle’s medium
DMSO	dimethyl sulfoxide
DPBS	Dulbecco’s phosphate-buffered saline
DRP1	dynamin-related protein 1
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
EDTA	ethylenediaminetetraacetic acid
eIF2a	eukaryotic translation factor 2 subunit alpha
eIF4B	eukaryotic translation initiation factor 4B
eIF4GI	eukaryotic translation initiation factor 4 gamma 1
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FBS	fetal bovine serum
FIIND	function to find domain
GFP	green fluorescent protein
GMP	guanosine monophosphate
GTP	guanosine triphosphate
GTPase	GTP hydrolase
HA	hemagglutinin
HCV	hepatitis C virus
HEK293T	human embryonic kidney 293T
Hf09	influenza A/Halifax/210/2009/H1N1
HIN200	hematopoietic IFN-inducible nuclear antigens with 200 amino acid repeats
hpi	hours post-infection
HPRT1	hypoxanthine phosphoribosyltransferase 1
hpt	hours post-transfection
hStaufen	human homologue of *Drosophila melanogaster* Staufen
IAV	influenza A virus
IFITM3	IFN-induced transmembrane protein 3
IFN	interferon
IκB	inhibitor of NF-κB

IKK	IκB kinase
IL	interleukin
IL-1α	IL-1 alpha
IL-1β	IL-1 beta
IL-1R	IL-1 receptor
IL-1Ra	IL-1 receptor antagonist
IP	immunoprecipitation
IPS-1	IFN-β promoter stimulator protein 1
IRF	IFN regulatory factor
ISG	IFN-stimulated gene
JAK	Janus kinase
LPS	lipopolysaccharide
LRR	leucine-rich repeat
LUBAC	linear ubiquitin assembly complex
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral signaling protein
MDA5	melanoma differentiation-associated protein 5
MDCK	Madin-Darby canine kidney
MEM	minimum essential medium
Mfn	mitofusin
MHC	major histocompatibility complex
MNS	3,4-methylenedioxy-β-nitrostyrene
MOI	multiplicity of infection
MyD88	myeloid differentiation primary response protein 88
NA	neuraminidase
NAC	N-acetyl L-cysteine
NACHT	neuronal apoptosis inhibitor protein, MHC class II transactivator, plant *het* gene product involved in vegetative incompatibility, and telomerase-associated protein 1
NAIP	neuronal apoptosis inhibitor protein
Nec-1	Necrostatin-1

NEK7	NIMA-related kinase 7
NEMO	NF-κB essential modulator
NEP	nuclear export protein
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIMA	never in mitosis gene A
NLR	nucleotide-binding oligomerization domain-like receptor
NLRA	NLR family ATD-containing protein
NLRB	NLR family BIR domain-containing protein
NLRC	NLR family CARD-containing protein
NLRP	NLR family PYD-containing protein
NLRP3	NLR family PYD-containing protein 3 (or nucleotide-binding domain and leucine-rich repeat-containing protein 3)
NOD	nucleotide-binding oligomerization domain-containing protein
NP	nucleoprotein
NS1	non-structural protein 1
OAS	2'-5'-oligoadenylate synthetase
OD	optical density
ORF	open reading frame
PABPII	poly(A)-binding protein II
PACT	protein activator of the IFN-induced protein kinase
PAGE	polyacrylamide gel electrophoresis
PAM	porcine alveolar macrophage
PAMP	pathogen-associated molecular pattern
pdm09	2009 human pandemic IAV
PFU	plaque-forming unit
PBS	phosphate-buffered saline
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKR	protein kinase R
PNPP	p-nitrophenyl phosphate
PR8	influenza A/Puerto Rico/8/1934/H1N1

PRR	pattern recognition receptor
PRRSV	porcine reproductive and respiratory syndrome virus
PYD	pyrin domain
RIG-I	retinoic acid-inducible gene-I
RIPK	receptor-interacting protein kinase
RLR	RIG-I-like receptor
RNase	ribonuclease
ROS	reactive oxygen species
RSV	respiratory syncytial virus
SD	standard deviation
SDS	sodium dodecyl sulfate
siRNA	small interfering RNA
SIV	swine influenza virus
Sk02	influenza A/swine/Saskatchewan/18789/2002/H1N1
ssRNA	single-stranded RNA
STAT	signal transducer and activator of transcription
STING	stimulator of IFN genes
SUMO	small ubiquitin-like modifier
Syk	spleen tyrosine kinase
TAK1	transforming growth factor beta-activated kinase 1
TANK	TRAF family member-associated NF-κB activator
TBK1	TANK-binding kinase 1
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween 20
TIR	Toll-interleukin-1 receptor
TLR	toll-like receptor
TNF	tumor necrosis factor
TOM20	translocase of outer mitochondrial membrane 20
TRAF	TNF receptor-associated factor
TRIF	TIR-domain-containing adapter-inducing IFN-β
TRIM25	tripartite motif-containing protein 25

TSE	Tris-buffered saline with EDTA
Tx98	influenza A/swine/Texas/4199-2/1998/H1N1
TXNIP	thioredoxin-interacting protein
UTR	untranslated region
vRNA	viral RNA
vRNP	viral ribonucleoprotein
VSV	vesicular stomatitis virus
WNV	West Nile virus
WSN33	influenza A/WSN/1933/H1N1
WT	wild-type
ZBP1	Z-DNA binding protein 1

CHAPTER 1 LITERATURE REVIEW

1.1 Influenza A virus (IAV)

1.1.1 Overview

IAV is a contagious pathogen that causes airway infection in a wide range of mammalian and avian hosts. IAV is the only species in the genus *Alphainfluenzavirus*, which is one of the seven genera in the family *Orthomyxoviridae* (Ferhadian et al., 2018, Shaw and Palese, 2013). This enveloped virus contains eight segments of negative-sense, single-stranded RNAs (ssRNAs) for its genome, which enable antigenic changes by mutation and reassortment events. IAVs are divided into subtypes based on the surface antigens, hemagglutinin (HA) and neuraminidase (NA); there are currently 18 HA subtypes (H1 to H18) and 11 NA subtypes (N1 to N11) (Hutchinson, 2018). IAV has the ability to cause pandemics and is the causative agent of seasonal flu along with the genus *Betainfluenzavirus* (Teitzel, 2018), while other genera of influenza viruses, *Gammainfluenzavirus* and *Deltainfluenzavirus* do not pose as significant of a threat to public health as IAV (Zhou et al., 2018). With rapid evolution and interspecies transmission, IAV is one of the most important global health threats today. It is responsible for annual flu epidemic affecting human populations including several million severe cases and estimated up to 646,000 deaths worldwide (Girard et al., 2005, Iuliano et al., 2018). In Canada, a death toll caused by IAV infection and its complications is reported to be more than 5,000 per year (Thommes et al., 2017). The 2009 pandemic H1N1 has affected millions of people with an estimated fatality of up to 575,000 in the world (Dawood et al., 2012). The emergence of this novel swine-derived virus through the reassortment of swine influenza virus (SIV) with human and avian strains has emphasized the importance of research with SIV and immunity to SIV in pigs, since pigs are the intermediate host to generate new viruses with pandemic capacity (Nelson and Vincent, 2015). There is possibility of further reassortment among SIVs and human IAV strains in pigs leading to the production of new strains with more pathogenicity and better transmissibility among humans. Due to public health concerns, there has been a need for effective vaccines and therapeutics to cope with the potential pandemic. In parallel, a better understanding of the swine host immunity to IAV is required, since the outcome of IAV infection depends on the host immune response against infection.

1.1.2 Life cycle of IAV

The IAV genome consists of eight segments of ssRNAs: PB2, PB1 and PA segments encode the three subunits of RNA polymerase complex, PB2, PB1 and PA proteins, respectively; HA and NA segments encode the two surface glycoproteins, HA and NA, respectively; NP segment encodes the nucleoprotein (NP); M segment encodes the matrix protein M1 and envelope protein M2 that is expressed via alternative splicing; NS segment encodes the non-structural protein 1 (NS1) and nuclear export protein (NEP), which is also an alternative splicing product. Each RNA segment is associated with the RNA polymerase complex and encapsidated by viral NP (Bouvier and Palese, 2008, Shaw and Palese, 2013), forming viral ribonucleoprotein (vRNP); this is the minimum unit for IAV replication.

As shown in Figure 1.1, IAV attaches to the host cell by receptor binding activity of viral glycoprotein, HA. The globular head region of this protein binds to the cell surface receptor, sialic acid (also called *N*-acetylneuraminic acid), and then, the virus can be endocytosed to be located in the endosomal compartment (Shaw and Palese, 2013). After host cellular proteases cleave HA0, which refers to the precursor form of HA, into HA1 and HA2, viral membranes are fused with endosomal membranes at acidic pH (Hamilton et al., 2012). As a result, the fusion peptide in the HA2 is exposed and inserted into the endosome, thereby inducing the alignment of viral and endosomal membranes (Hamilton et al., 2012). Next, uncoating is initiated by the viral ion channel protein, M2 that lowers the pH inside the virion by transporting protons from the endosome into the virus (Pinto and Lamb, 2006). Consequently, the vRNP complex for each gene segment is dissociated from M1 and released into the cytoplasm (Shaw and Palese, 2013). Transcription and replication of the viral genome take place in the nucleus. Thus, nuclear entry of vRNP is required and is dependent on the nuclear localization signal on NP and three polymerase subunits (PB2, PB1 and PA) consisting the vRNP (Martín-Benito and Ortín, 2013). Viral mRNA synthesis requires 5’ cap structures from host mRNA, which is generated by host RNA polymerase II. PB2 binds to the cap structure on the 5’ end of host pre-mRNA and PB1 binds to the 5’ and 3’ ends of viral RNA (vRNA), while PA cleaves 10 to 13 nucleotides downstream of the 5’ cap. Using this cleaved fragment as the primer, viral mRNA synthesis is catalyzed by PB1 (Martín-Benito and Ortín, 2013, Shaw and Palese, 2013). Synthesized viral mRNA is transported to the cytoplasm to be translated. Incoming vRNA from the cytoplasm also serves as the template for the synthesis of complementary RNA (cRNA), which in turn, is

required for the generation of new vRNA (Shaw and Palese, 2013). After vRNA is assembled as vRNP, the vRNP is exported to cytoplasm with the help of M1 and NEP, both of which interact with a cellular export receptor (Samji, 2009, Shaw and Palese, 2013). Unlike NP, M1 and NEP that move to the nucleus to associate with newly produced vRNA, HA, NA and M2 are modified in endoplasmic reticulum (ER) and Golgi apparatus, and are transported to the cell membrane (Shaw and Palese, 2013). For virion assembly, selective packaging of vRNP is believed to occur based on the packaging signals on each vRNA (Shaw and Palese, 2013). HA and NA associate with plasma membrane domains called lipid rafts where budding takes place (Nayak et al., 2004). Finally, virion release occurs with the destruction of sialic acid by NA (Shaw and Palese, 2013).

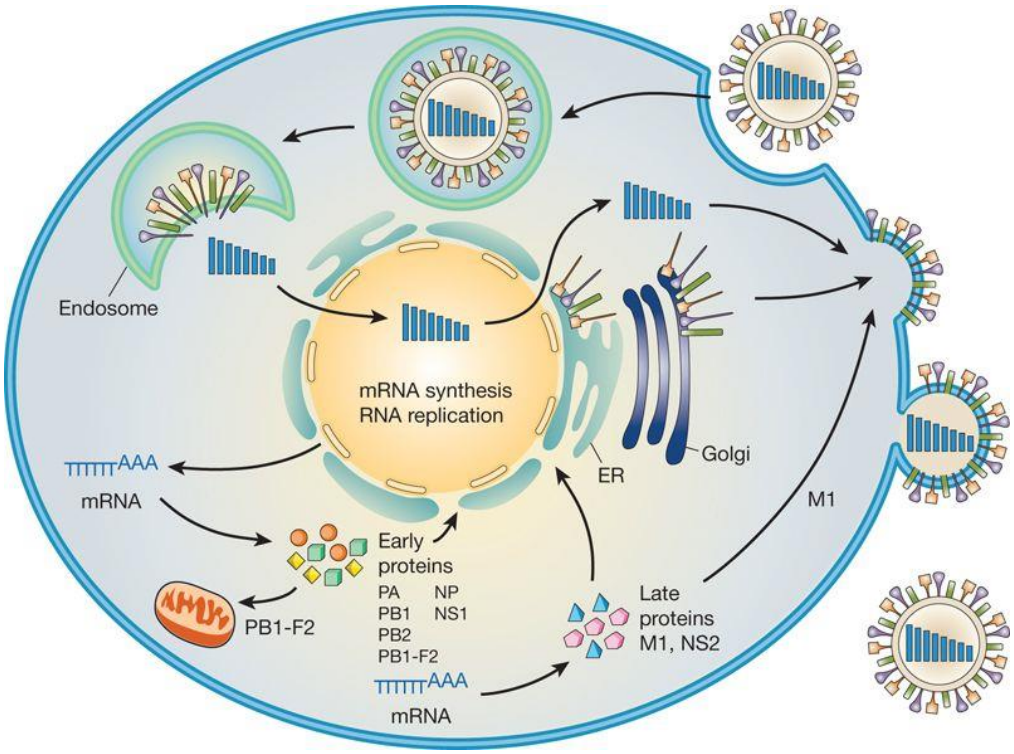

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Figure 1.1 Life cycle of IAV.
IAV attaches to the host receptor, sialic acid and is endocytosed. After cleavage of HA, the fusion between viral membranes and endosomes occurs. Uncoating allows the vRNPs to be released into cytoplasm, followed by its entry into the nucleus. With vRNAs as the template, viral mRNAs are transcribed using the 5' cap snatched from host pre-mRNAs, and cRNAs are synthesized for the vRNA replication. Viral proteins are produced and modified, and newly

assembled vRNPs are packaged for virion assembly. Upon budding, the sialic acid destruction by NA allows the virions to be released.

1.1.3 Immunology of IAV infection

In IAV-infected animals, the epithelial cells in the airway mucosa are the first to be infected (Pulendran and Maddur, 2014). IAV infection then further spreads to the lung macrophages and dendritic cells (DCs) in alveoli or within the lung epithelium, which are the pivotal cells to trigger inflammation (Iwasaki and Pillai, 2014, Short et al., 2012). The innate immunity to IAV begins with the recognition of viral genome via host immune sensors in the infected cells (Figure 1.2). The main sensors to detect IAV infection includes toll-like receptors (TLRs) and retinoic acid-inducible gene-I (RIG-I) (Ichinohe, 2010). Viral ssRNA or double-stranded RNA (dsRNA) is recognized by TLR3 or TLR7 in the endosome, and the 5'-triphosphate dsRNA is recognized by RIG-I both in the cytosol and nucleus (Iwasaki and Pillai, 2014, Liu et al., 2018). Activation of both of sensors turns on a cascade of signaling events leading to the activation of transcription factors, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), interferon (IFN) regulatory factors 3 (IRF3) and 7 (IRF7). This results in the production of pro-inflammatory cytokines, type I IFNs and a variety of IFN-stimulated genes (ISGs) that support the host antiviral status (Iwasaki and Pillai, 2014). Viral RNA of IAV can be sensed by another cytosolic sensor called the nucleotide-binding domain and leucine-rich repeat-containing protein 3 (NLRP3) (Allen et al., 2009, Thomas et al., 2009), but whether NLRP3 can sense viral RNA by a direct binding is still under debate. Recent evidence suggests that NLRP3 recognizes diverse cellular changes induced by viral infection as reviewed in the section 1.2.1.3.1. Among pro-inflammatory cytokines, interleukin (IL)-1β and IL-18 are synthesized as precursors via NF-κB pathway; then, the precursors become biologically active after processed by inflammasome complexes consisted of nucleotide-binding oligomerization domain-like receptors (NLRs) or non-NLRs along with their adaptor and effector molecules (Iwasaki and Pillai, 2014). Recently, Z-DNA binding protein 1 (ZBP1) is proposed as another important sensor; the recognition of IAV proteins by ZBP1 triggers NLRP3 inflammasome activation and cell deaths (Kuriakose et al., 2016).

Among innate immune cells, the lung macrophages are crucial in eliminating IAV particles and controlling inflammation, which largely depends on the action of pro-inflammatory

cytokines including IL-1β, IL-6 and tumor necrosis factor (TNF)-α. These cytokines can be involved in the inflammatory responses as well as the pathogenesis during the course of IAV infection (Van Reeth, 2000). IAV-induced pathological signs in the lung can be derived from the combination of direct effects of viral replication and the effects of host responses via inflammatory cytokines produced from immune cells including alveolar macrophages (Gordon and Read, 2002).

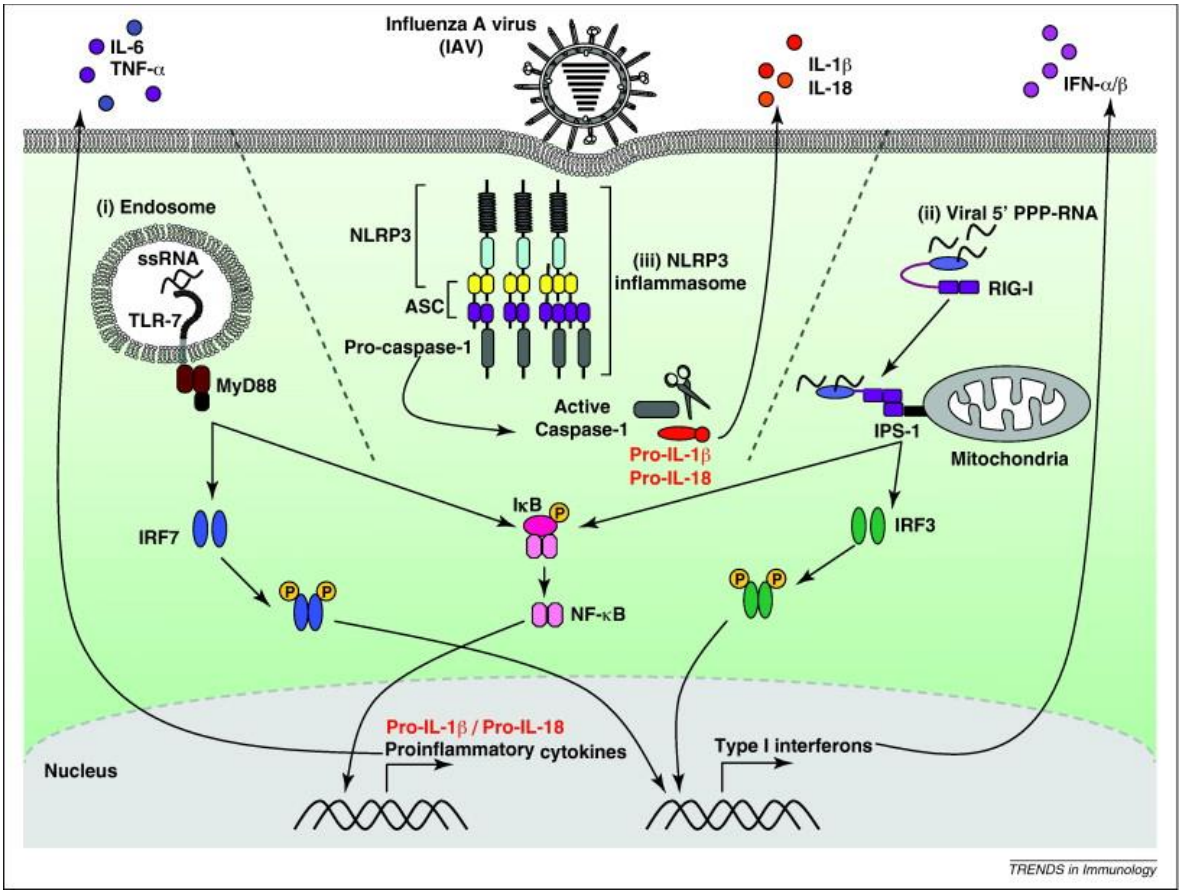

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Figure 1.2 Innate recognition of IAV infection.
Viral ssRNA is sensed by TLR7 in the endosome (i) or viral 5'-triphosphate RNA is sensed by RIG-I (ii). TLR7 signaling mediated its adaptor protein, myeloid differentiation primary response protein 88 (MyD88) activates IRF7 and NF-κB, whereas RIG-I signals mediated by its adaptor, IFN-β promoter stimulator protein 1 (IPS-1, also called mitochondrial antiviral signaling protein [MAVS]) on the mitochondria activates IRF3 and NF-κB. IRF7 and IRF3 translocate to the nucleus, thereby inducing the type I IFN, while NF-κB induces the expression of pro-inflammatory cytokines including pro-IL-1β and pro-IL-18. IAV infection also induces the

NLRP3 inflammasome activation (iii), leading to pro-caspase-1 cleavage that is critical for the conversion of pro-IL-1β and pro-IL-18 into their mature forms.

DCs are critical in linking innate immunity to adaptive immunity in response to viral infection. Upon antigen uptake in IAV-infected lungs, respiratory DCs mature and migrate to the mediastinal lymph node where they present processed antigens to naïve T cells (Ho et al., 2011, Ingulli et al., 1997). T cells are classified into two subgroups, $CD4^+$ and $CD8^+$ T cells. After antigen recognition, they possess effector functions to support antibody production and to eliminate infected cells. IAV-specific $CD4^+$ T cells can migrate to the lung. They produce cytokines like TNF-α, IL-2 and IFN-γ to induce cytotoxicity by $CD8^+$ T cells, and to activate alveolar macrophages. They also secrete IL-4 and IL-13 to activate B cells (Kreijtz et al., 2011, Zens and Farber, 2014). After the infection is resolved, some of the $CD4^+$ T cells acquire memory and can clear IAV particles more effectively in case of reinfection. Memory $CD4^+$ T cells can secrete more cytokines in a more rapid manner than naïve $CD4^+$ T cells, particularly for IFN-γ (Lai et al., 2011). Memory $CD4^+$ T cell-mediated protection against further IAV infection is executed independently of B cells or $CD8^+$ T cells; and this protection is cross-reactive to different strains (Richards et al., 2010, Zens and Farber, 2014).

With signals provided by DCs, $CD8^+$ T cells proliferate in IAV-infected lungs (Lawrence and Braciale, 2004). $CD8^+$ T cells also secrete cytokines including IFN-γ, but their main function is to eliminate IAV-infected epithelial cells through cytotoxicity (Topham et al., 1997). When recognizing antigens presented by major histocompatibility complex (MHC) class I, $CD8^+$ T cells induce apoptosis of target cells by releasing their enzymes (pore-forming proteins and granzymes), or by upregulating the TNF family members (such as Fas ligand) that bind to the target cell ligand, Fas to initiate the apoptotic signaling (Hufford et al., 2014). Following recovery from infection, some portions of $CD8^+$ T cells also remain as long-lived memory $CD8^+$ T cells in preparation for re-infection (Hufford et al., 2014, Kreijtz et al., 2011).

For humoral immunity, the surface glycoproteins HA and NA are the major targets of antibody production. Naïve B cells recognize these antigens provided by DCs in the mediastinal lymph nodes and the respiratory mucosa-associated lymphoid tissues, thereby becoming antibody-secreting cells (Chiu et al., 2014). Alternatively, some B cells can capture antigens by themselves in the lymph nodes and at the site of infection (Waffarn and Baumgarth, 2011). While antibody secretion by B cells can occur independently of T cells, it is known that $CD4^+$ T

cell-mediated B cell responses are critical for more effective antibody production against IAV infection (Mozdzanowska et al., 2005). HA-specific antibodies can neutralize IAV, and NA-binding antibodies interfere with the enzymatic activity of NA, rather than directly neutralizing (Kreijtz et al., 2011). Binding of antibodies targeting both HA and NA to infected cells can lead to antibody-dependent cell-mediated cytotoxicity executed by natural killer cells (Kreijtz et al., 2011). IgG and IgM are the predominant antibody classes produced in the lung and in particular, IgG is thought to be responsible for preventing systemic dissemination of IAV; IgA secreted from the respiratory mucosa inhibits the neutralized antigens from adhering to the epithelium (Chiu et al., 2014). After acquiring memory, B cells maintain the ability to generate both IgG and IgA, which also serves for cross-protection against other IAV strains (Waffarn and Baumgarth, 2011).

1.1.4 Swine influenza virus (SIV)

Influenza infection in pigs causes a mild or subclinical disease in the respiratory tract. Symptoms of the acute illness include fever, anorexia, lethargy, nasal discharge and coughing (Van Reeth et al., 2012). These symptoms are similarly observed upon infection by different viral subtypes, and major difference in virulence is not reported by infection with different strains (Van Reeth et al., 2012). While high morbidity is seen, mortality is generally low and affected animals can recover in 5 to 7 days. However, secondary infection with other respiratory viruses or bacteria can exacerbate the condition (Van Reeth et al., 2012). SIV transmission occurs through direct contact or by aerosols. Epithelial cells on the airway are the main sites for viral replication, while viruses preferentially target the lower respiratory tract (Van Reeth et al., 2012). The disease outcome can be affected by inflammatory cytokines such as IL-1, IL-6 and TNF-α along with IFN-α. Although they have antiviral activities, high levels of these cytokines are responsible for lung inflammation (Van Reeth et al., 2012, Van Reeth et al., 1998, Van Reeth et al., 2002). Bronchopneumonia is prominent in infected pigs and microscopic lesions include necrotizing bronchitis; bronchiolitis; airway obstruction by necrotic epithelial cells and inflammatory cells such as neutrophils (Janke, 2014, Van Reeth et al., 2012).

Although swine influenza does not often lead to fatal outcomes, it has been a concern of economic loss in the swine production industry (Schultz-Cherry et al., 2013). Among different influenza species, influenza A, C and D viruses are isolated from pigs (Su et al., 2017). However,

only IAV is considered as a critical pathogen for swine influenza (Van Reeth et al., 2012). The first case of swine influenza was reported during the pandemic in 1918, while SIV was first isolated in the United States in 1930 (Lorusso et al., 2013). As a result of infection of pigs with the 1918 pandemic strain, classical swine H1N1 was established (Zell et al., 2013). Since then, this lineage of SIV has stably circulated in North America for decades. In the late 1990s, two different genotypes of H3N2 subtype emerged, and one of them, a triple reassortant derived from avian, swine and human seasonal H3N2 strains, has spread throughout the United States. While this virus was endemic in pigs, its reassortment with the classical swine H1N1 also occurred, generating novel variant SIVs of H1N1 and H1N2 subtypes (Brockwell-Staats et al., 2009, Lorusso et al., 2013). As evidenced by the 2009 pandemic H1N1, the reassortment of IAVs from different hosts in pigs highlights the important roles played by pigs for the emergence of new viruses. The contribution of pigs to the generation of new viruses can be presented by the mixing vessel theory (Scholtissek et al., 1985) with several observations supporting this idea. Pigs are permissive to avian and human isolates (Kida et al., 1994), and reassortment among viruses of different origins has been displayed for isolates from pigs (Peiris et al., 2001). Another important factor is the receptor binding activity of HA, one of the critical determinants in the host restriction of IAVs. The binding specificity of HA depends on the types of host cell receptors differentially distributed in host animals. The molecular structures of this receptor, sialic acid linked to galactose, are divided into two types: avian-type alpha 2,3-linkage and human-type alpha 2,6-linkage that are preferably recognized by avian and human isolates, respectively (Zell et al., 2013). Swine isolates recognize both structures and pigs express both types of sialic acids (Ito et al., 1998), rendering them susceptible to infection by both avian and human strains. Later, human cells were also found to harbor both types of these receptors (Shinya et al., 2006), showing that the receptor binding activity alone may not fully support this theory. An additional model is such that the adaptation of avian strains in swine hosts, as avian-like SIVs, through repeated replication in pigs, is able to generate variants that can recognize the human-type sialic acid (Ito et al., 1998). Overall, further reassortment among IAV strains in swine hosts may lead to the generation of new strains with pandemic capacity.

1.1.5 2009 human pandemic IAV (pdm09)

The pdm09 emerged as a pandemic through the reassortment of IAVs of different origins in the intermediate hosts, pigs. The genome segments of pdm09 are derived from multiple lineages: PB2 and PA from avian strain of North American lineage; PB1 from human seasonal H3N2 strain; HA, NP and NS from classical swine H1N1 of North American lineage; NA and M from avian-like swine H1N1 of Eurasian lineage (Neumann et al., 2009). This virus is thus, antigenically close to swine H1N1, while distinct from human seasonal strains (Garten et al., 2009). The initial emergence of this H1N1 subtype virus in Mexico was quickly followed by the worldwide spread through human-to-human transmission, leading to the first IAV pandemic in the 21st century. However, it is suggested that the introduction and circulation of the original gene segments in pigs took place years before the pdm09 outbreak in humans (Girard et al., 2010, Vijaykrishna et al., 2010). In humans, the pdm09 generally induced higher rates of infection in the younger population rather than the old; this may be attributable to the possession of cross-reactive antibodies initially raised against the 1918 pandemic-like viruses in the older generation (Girard et al., 2010, Tscherne and García-Sastre, 2011). Genetic signatures for human adaptation or increased pathogenicity characteristic for highly pathogenic strains were not found in the pdm09 strains. For example, the pdm09 PB2 protein harbours glutamic acid at the 627th amino acid (aa), common for avian strains, instead of lysine that is typical for human strains. While PB1-F2 protein and C-terminal region of NS1 protein encoded by 1918 pandemic IAV strains are involved in pathogenicity, the PB1 and NS segments of pdm09 encode a truncated version of PB1-F2 and NS1 proteins, respectively, compared to those of 1918 pandemic strains (Garten et al., 2009, Tscherne and García-Sastre, 2011). Although the pdm09 infection elicited symptoms that are comparable to seasonal IAVs in humans (Tscherne and García-Sastre, 2011), it may acquire virulence by further reassortment, and this potential threat underscores the importance of ongoing surveillance in pigs.

1.1.6 Non-structural protein 1 (NS1) of IAV

NS1 protein is encoded by the NS segment of IAV and is between 202 and 237 aa in length. NS1 exerts extensive functions such as supporting viral replication and counteracting the host antiviral responses (Figure 1.3), although many of these functions are virus strain- or host-dependent (Ayllon and García-Sastre, 2014). It has two functional domains: an N-terminal RNA-

binding domain that can bind several species of RNA such as dsRNA and mRNA in a sequence-independent manner; and a C-terminal effector domain that interacts with host cellular proteins and stabilizes the RNA-binding domain (Hale et al., 2008). NS1 has one or two nuclear localization signals that are required for the nuclear import of NS1 (Hale et al., 2008). In infected cells, NS1 mainly localizes to the nucleus, while it is also found in cytoplasm indicating that NS1 functions in both compartments (Hale et al., 2008, Krug, 2015).

1.1.6.1 NS1 acting on the synthesis of viral proteins

Upon IAV infection, viral mRNAs are preferentially translated over cellular mRNAs (Hale et al., 2008). NS1 is one of many proteins that binds the 5' untranslated region (UTR) of viral mRNAs for selective translation (Garfinkel and Katze, 1993); NS1 promotes the translation of viral mRNAs, but not that of non-viral mRNAs (de la Luna et al., 1995). While NS1 does not impact viral mRNA transcription, it enhances viral mRNA translation that is dependent on the 5' UTR of mRNAs (Enami et al., 1994). NS1 can bind proteins involved in translation such as the eukaryotic translation initiation factor 4 gamma 1 (eIF4GI) (Aragón et al., 2000), and it is suggested that NS1 recruits eIF4GI to the 5' UTR of viral mRNAs (Hale et al., 2008). NS1 also binds human homologue of *Drosophila melanogaster* Staufen (hStaufen), a dsRNA-binding protein involved in the transport of viral mRNA to polyribosomes where multiple ribosomes form a complex for active translation (Falcón et al., 1999). Protein kinase R (PKR) is activated either by the recognition of viral dsRNA or via a cellular protein, PACT (which refers to 'protein activator of the IFN-induced protein kinase') (Li et al., 2006). PKR activation induces the phosphorylation of eukaryotic translation factor 2 subunit alpha (eIF2α), thereby suppressing the viral RNA and protein synthesis in infected cells (Hatada et al., 1999). However, binding of NS1 to PKR can subvert either dsRNA- or PACT-mediated PKR activation (Li et al., 2006, Min et al., 2007).

1.1.6.2 NS1-mediated restriction of the host gene expression

NS1 inhibits the transportation of polyadenylated mRNAs from the nucleus to the cytoplasm and this action is exerted by binding of NS1 to the poly(A) tails of cellular mRNAs (Hale et al., 2008, Qiu and Krug, 1994). The effector domain of NS1 binds to the 30 kDa subunit of cleavage and polyadenylation specificity factor (CPSF30) and poly(A)-binding protein II

(PABPII) (Chen et al., 1999, Twu et al., 2006). CPSF30 recognizes the AAUAAA sequence upstream of the cleavage site for polyadenylation of cellular mRNAs (Das et al., 2008). NS1 binding to CPSF30 or sequestration of CPSF30 by NS1 prevents cleavage and polyadenylation of cellular mRNAs, while viral mRNAs are polyadenylated by viral polymerases and not affected by this inhibition (Hale et al., 2008). NS1 binding to PABPII blocks the nuclear export of mature cellular mRNAs that could avoid the inhibition step at the polyadenylation level (Chen et al., 1999). NS1 also blocks the export of cellular mRNAs from the nucleus to the cytoplasm, by interacting with cellular proteins involved in nuclear export machinery (Satterly et al., 2007).

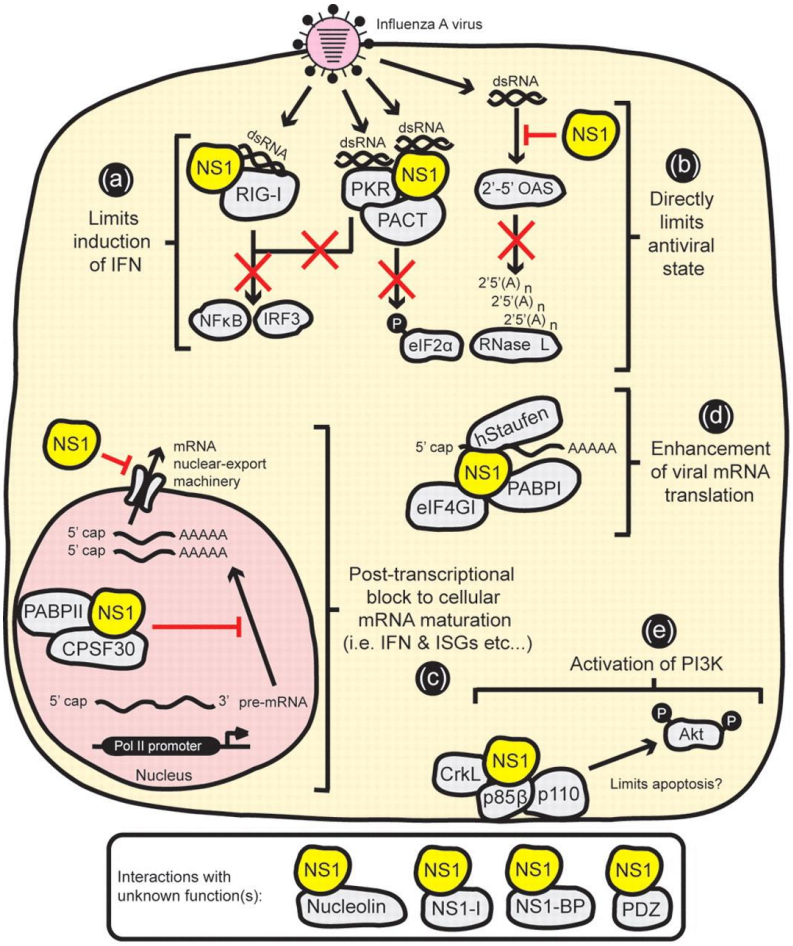

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Figure 1.3 Multiple functions of IAV NS1 protein.
(a) NS1 limits IFN induction by binding to viral RNA and RIG-I. (b) NS1 limits the antiviral status established by the PKR and 2'-5'-oligoadenylate synthetase (OAS)/ribonuclease (RNase) L pathways. (c) NS1 restricts the maturation and nuclear export of cellular mRNAs. (d) NS1

promotes the translation of viral mRNA. (e) NS1 limits apoptosis by activating the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt pathway.

1.2 Inflammasomes and interleukin-1 beta (IL-1β)

1.2.1 Inflammasomes

Inflammasomes are cytosolic multiprotein complexes that govern inflammation by regulating the pro-inflammatory cytokines, IL-1β and IL-18. Different immune sensors (either NLRs or non-NLRs) can assemble into the inflammasomes with apoptosis-associated speck-like protein containing caspase activation and recruitment domain (ASC) and inflammatory caspases in response to diverse stimuli. Upon this assembly, the inflammatory caspases are modified by proteolytic cleavage for their enzymatic activity that is required to process pro-IL-1β and pro-IL-18 (Pétrilli and Martinon, 2011). Inflammasomes consisting of caspase-1 are considered as canonical, while non-canonical inflammasomes involve caspase-4 and -5 (or caspase-11 in mouse) (Viganò et al., 2015). The key steps in inflammasome activation is represented by a two-signal model (Figure 1.4): signal 1 for the enhanced transcription of the inflammasome-forming immune sensors and substrates (pro-IL-1β or pro-IL-18) by NF-κB activation; signal 2 for the recruitment of adaptor and effector proteins for inflammasome assembly (Mariathasan and Monack, 2007). By finding that dysregulation of inflammasomes is related to auto-inflammatory disorders and metabolic diseases (Guo et al., 2015), the role of inflammasomes is considered to be the maintenance of homeostasis as well as the host defense against pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs).

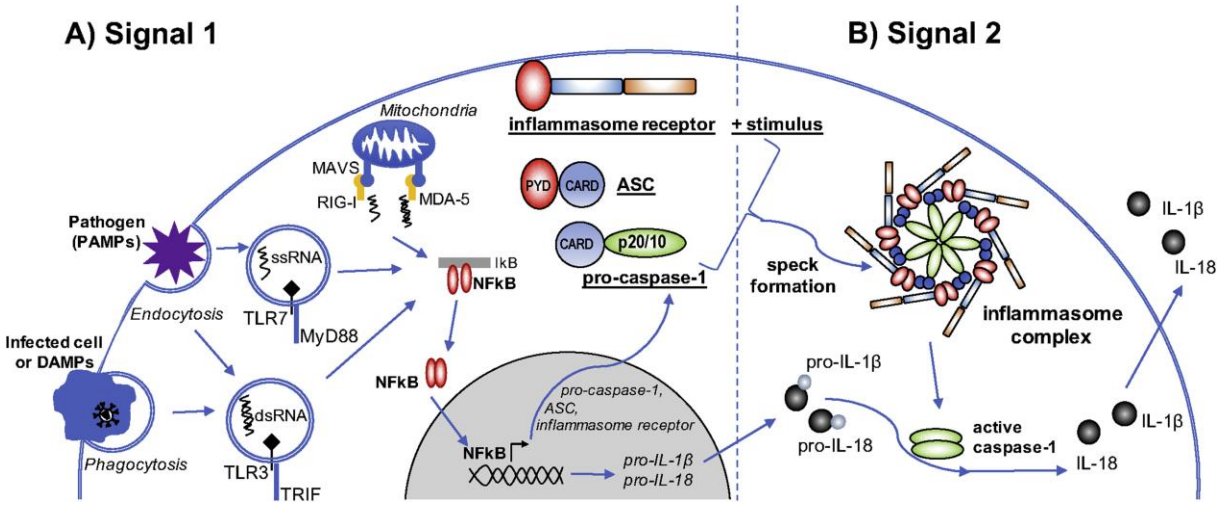

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Figure 1.4 Two-signal model for the inflammasome activation.
A) Signal 1: Upon endocytosis of PAMPs or phagocytosis of DAMPs, TLR3 and TLR7 signaling involving their adaptors are activated. Upon sensing RNA, RIG-I and melanoma differentiation-associated protein 5 (MDA5) signaling involving their adaptor is activated as well. These activate the NF-κB pathway, which upregulates the expression of inflammasome receptor (NLR or non-NLR) and pro-IL-1β or pro-IL-18. B) Signal 2: The inflammasome receptor undergoes a conformational change upon sensing stimuli, leading to the assembly of inflammasome complex with ASC and pro-caspase-1. Upon auto-proteolytic cleavage of pro-caspase-1, active caspase-1 converts pro-IL-1β or pro-IL-18 into IL-1β or IL-18.

1.2.1.1 Apoptosis-associated speck-like protein containing caspase activation and recruitment domain (ASC)

ASC is a component of inflammasomes. It was initially discovered as a pro-apoptotic protein that forms speck-like aggregates in apoptotic cells (Masumoto et al., 1999). It is constitutively expressed in many myeloid cells, and functions as a bridge between immune sensors (mostly NLRs) and the effector molecule, pro-caspase-1 (Hoss et al., 2017). ASC has two domains: N-terminal pyrin domain (PYD) that interacts with another PYD of NLRs and C-terminal caspase activation and recruitment domain (CARD) that interacts with CARD of pro-caspase-1. Upon activation, NLRs oligomerize and recruit ASC molecules, which also oligomerize and form long filaments. Diffusely distributed ASC throughout the cytoplasm and nucleus cluster to a spot where NLRs form a seed. This leads to the formation of a perinuclear speck of 1 μm in diameter per each cell (Hoss et al., 2017, Stutz et al., 2013). While ASC PYD alone can form filaments, spherical speck formation requires ASC CARD that connects between

filaments (Dick et al., 2016). Moreover, the interaction between ASC CARDs provides an additional seed for the filament formation by pro-caspase-1 CARD (Dick et al., 2016, Schmidt et al., 2016). Conclusively, ASC specks are where pro-caspase-1 recruitment and activation occur (Franklin et al., 2018), therefore, they are a hallmark of inflammasome activation. Interestingly, ASC specks can be secreted outside the cells where pro-caspase-1 recruitment and IL-1β maturation can also occur. The extracellular specks can disseminate inflammatory signals to phagocytes that then engulf them (Baroja-Mazo et al., 2014, Franklin et al., 2014).

Post-translational modifications on ASC play important roles in the regulation of inflammasome activity. Absent in melanoma 2 (AIM2) inflammasome activation upon double-stranded DNA (dsDNA) is restricted by means of the ubiquitination of ASC, which is subject to degradation via autophagy (Shi et al., 2012). NLRP3 inflammasome activity can be inhibited by an autophagy protein that upregulates the activity of an E3 ligase, TNF receptor-associated factor (TRAF) 6, which ubiquitinates ASC (Chiu et al., 2016). Linear ubiquitination of ASC is essential for the activation of NLRP3 inflammasome (Rodgers et al., 2014), whereas deubiquitination of ASC is required for the same event (Lee et al., 2017c). Ubiquitination of ASC by an E3 ligase, TRAF3 upon infection with vesicular stomatitis virus (VSV) is critical for inflammasome-mediated IL-1β production (Guan et al., 2015). Phosphorylation of ASC also exerts controls on the inflammasome activity. ASC speck formation is dependent on c-Jun N-terminal kinase and spleen tyrosine kinase (Syk), and upon inflammasome activation ASC is phosphorylated by those kinases (Hara et al., 2013). This is supported by another finding that phosphorylation of ASC by Syk is required for ASC oligomerization and NLRP3 inflammasome activity (Lin et al., 2015). ASC is also phosphorylated by another kinase, proline-rich tyrosine kinase 2 that interacts and colocalizes with ASC specks to activate the NLRP3 inflammasome (Chung et al., 2016).

Spatial regulation of ASC by inhibitor of κB kinases (IKKs) is also suggested. IKKα negatively regulates the NLRP3 inflammasome by phosphorylating serine residues on ASC, which is bound to nuclear IKKα in the resting state, whereas upon lipopolysaccharide (LPS) treatment, ASC is released from IKKα. Moreover, IKKε, by phosphorylating a different serine of ASC, promotes the translocation of ASC from the nucleus to the cytosol for NLRP3 inflammasome activation (Martin et al., 2014).

1.2.1.2 Caspase-1

Caspase-1 is a component of canonical inflammasomes. Caspases (refers to cysteine-aspartic proteases) are a family of enzymes, most of which are involved in programmed cell deaths or inflammation. Caspases are expressed as a precursor, pro-caspase, which generally consists of the N-terminal pro-domain, either CARD or death effector domain (DED), the large subunit (p20) and the small subunit (p10). The proteolytic cleavage between pro-domain and p20 as well as between p20 and p10 subunit leads to the formation of active caspase (Sollberger et al., 2014, Winkler and Rösen-Wolff, 2015). Caspases are classified into either apoptotic caspases or inflammatory caspases. Apoptotic caspase are further divided into initiator caspases (caspase-2, -8, -9, and -10) and executioner caspases (caspase-3, -6, and -7). Initiator caspases, through either CARD (for caspase-2 and -9) or DED (for caspase-8 and -10), are recruited to adaptor proteins that react to cell death signals such as cytochrome c, TNF-α or Fas ligand. Executioner caspases, which lack the pro-domain, are cleaved by macromolecular complexes containing initiator caspases, and perform apoptosis by processing their target molecules (Shalini et al., 2015, Sollberger et al., 2014). Inflammatory caspases (caspase-1, -4, -5, -11 and -12) are pivotal in the regulation of inflammatory responses, and caspase-1, the prototype in this group, is integral to the cleavage of pro-IL-1β and pro-IL-18. As with other caspases, caspase-1 is expressed as pro-caspase-1, which is enzymatically inactive. Through its N-terminal CARD, pro-caspase-1 interacts with another CARD of ASC, which is the adaptor protein of NLR or non-NLR proteins. The recruitment of pro-caspase-1 to ASC is essential for the inflammasome formation and subsequently, the auto-proteolysis of pro-caspase-1. Active caspase-1 is a tetramer composed of two molecules each of p20 and p10 (Shalini et al., 2015, Sollberger et al., 2014). In addition to the cytokine regulation, active caspase-1 may contribute to NF-κB activation, independent of its enzyme activity (Lamkanfi et al., 2004). Caspase-1 also executes an inflammatory cell death called pyroptosis by cleaving the downstream effector, gasdermin D (Shi et al., 2015).

1.2.1.3 Inflammasomes formed by nucleotide-binding oligomerization domain-like receptors (NLRs)

NLRs are cytosolic PAMP/DAMP sensors. Among them, NLRP1 is the first identified inflammasome-forming innate sensor. Its assembly with ASC, caspase-1 and caspase-5 was

found to be required for IL-1β production in LPS-stimulated human monocytes (Martinon et al., 2002). Later, a two-step mechanism of NLRP1 inflammasome activation was suggested by showing that the priming and oligomerization of NLRP1 is induced by muramyl dipeptide and adenosine triphosphate (ATP), respectively (Faustin et al., 2007). Another NLR member, NLRP3 was shown to form a complex with ASC and caspase-1 for IL-1β production, and more IL-1β secretion was observed from cells with the mutant NLRP3 (Agostini et al., 2004), indicating the involvement of inflammasome activation in abnormal inflammation. A two-signal model for NLRP3 inflammasome activation was also proposed: priming signal (signal 1) by TLR agonists and activation signal (signal 2) by ATP (Netea et al., 2009). A wide variety of PAMPs, DAMPs or cell stress have been displayed to activate the NLRP3 inflammasome. While NLRP7 can negatively regulate IL-1β production (Kinoshita et al., 2005), activation of NLRP7 inflammasome can restrict intracellular bacteria (Khare et al., 2012). NLRP12 inflammasome-mediated IL-18 production can provide host resistance against *Yersinia pestis* (Vladimer et al., 2012). Unlike other inflammasome-forming receptors, two NLRs, the NLR family CARD-containing protein 4 (NLRC4) and the neuronal apoptosis inhibitor protein (NAIP) form the inflammasome together upon sensing of bacterial ligands by NAIP (Yang et al., 2013, Zhao et al., 2011). NLRC4 inflammasome assembly, possibly with NAIP, is required for IFN-γ production from $CD8^+$ T cells in response to bacterial infection (Kupz et al., 2012).

1.2.1.3.1 NLRP3 inflammasome

Among inflammasome-forming NLRs, NLRP3 has been most widely investigated. A variety of stimuli have been known to induce the formation and activation of NLRP3 inflammasome leading to secretion of IL-1β or IL-18. Importantly, NLRP3 inflammasome-mediated IL-1β production confers protection in IAV-infected mice (Allen et al., 2009, Thomas et al., 2009).

1.2.1.3.1.1 Mechanisms of NLRP3 inflammasome activation

Upon recognition of ligands by pattern recognition receptors (PRRs), i.e. TLRs or RLRs, signal transduction is facilitated through multiple adaptor proteins of the PRRs, leading to the activation of the NF-κB pathway. As a result, transcription of NLRP3 and pro-IL-1β is initiated (Kattah et al., 2017) and this priming step is called “signal 1” (Figure 1.4). Adaptor

proteins of TLRs such as MyD88 and TIR-domain-containing adapter-inducing IFN-β (TRIF) are known to play roles on the transcriptional and post-transcriptional regulation of NLRP3 (He et al., 2016a). Priming by some stimuli may cause NLRP3 activation (He et al., 2016a), while priming alone can trigger IL-1β secretion in human monocytes (Netea et al., 2009). Diverse stimuli provide "signal 2" that is needed for NLRP3 inflammasome assembly (Figure 1.4). Unlike NLRP3, ASC is constitutively expressed in many types of myeloid cells (Hoss et al., 2017). After NLRP3 senses stimuli, it oligomerizes and its N-terminal PYD interacts with N-terminal PYD of ASC. This interaction is followed by aggregation between the two, forming a supramolecular structure termed a speck. Multiple pro-caspase-1 molecules are recruited to speck-formed ASC through CARD-CARD interactions between the ASC and pro-caspase-1 (Hoss et al., 2017). Auto-cleavage of pro-caspase-1 generates two subunits of active caspase-1, p20 and p10. Two sets of a heterodimer of p20 and p10 form a homodimer that can cleave pro-IL-1β (Walker et al., 1994). Since caspase-1 is an inflammatory caspase, NLRP3 inflammasome activation is implicated in inflammatory cell death (Fink and Cookson, 2006, Shi et al., 2015).

Different mechanisms have been suggested to elucidate the signal 2 required for the NLRP3 inflammasome activation (Figure 1.5). An early study demonstrated that IL-1β secretion is promoted by ATP or nigericin that induce the depletion of intracellular K^+ (Perregaux and Gabel, 1994). This is further supported by studies showing that K^+ efflux can be a common stimulus to induce the NLRP3 inflammasome activation (Franchi et al., 2014, Muñoz-Planillo et al., 2013, Pétrilli et al., 2007). Although K^+ efflux-mediated NLRP3 inflammasome activation can occur independent of Ca^{2+} influx (Katsnelson et al., 2015), the importance of calcium sensing mechanisms has been demonstrated. Extracellular Ca^{2+} or elevated intracellular Ca^{2+} sensed by calcium sensing receptors activates the NLRP3 inflammasome (Lee et al., 2012, Rossol et al., 2012). In addition to the Ca^{2+} influx, Ca^{2+} release from ER due to phagolysosomal rupture can augment the NLRP3 inflammasome activation (Murakami et al., 2012). In fact, the contribution of lysosomal disruption was displayed earlier. Phagocytosis of crystals induces the leakage of lysosomal contents such as cathepsin B from damaged lysosomes, thereby activating the NLRP3 inflammasome (Duewell et al., 2010, Hornung et al., 2008). Reactive oxygen species (ROS) has been proposed as another common trigger of the NLRP3 inflammasome activation. Cytosolic ROS generated after phagocytosis of particulates activates the NLRP3 inflammasome (Dostert et al., 2008). Subsequent reports revealed that NLRP3 inflammasome requires

mitochondrial ROS for its activation (Nakahira et al., 2011, Zhou et al., 2011). Furthermore, the release of mitochondrial DNA can cause NLRP3 inflammasome activation (Nakahira et al., 2011, Shimada et al., 2012).

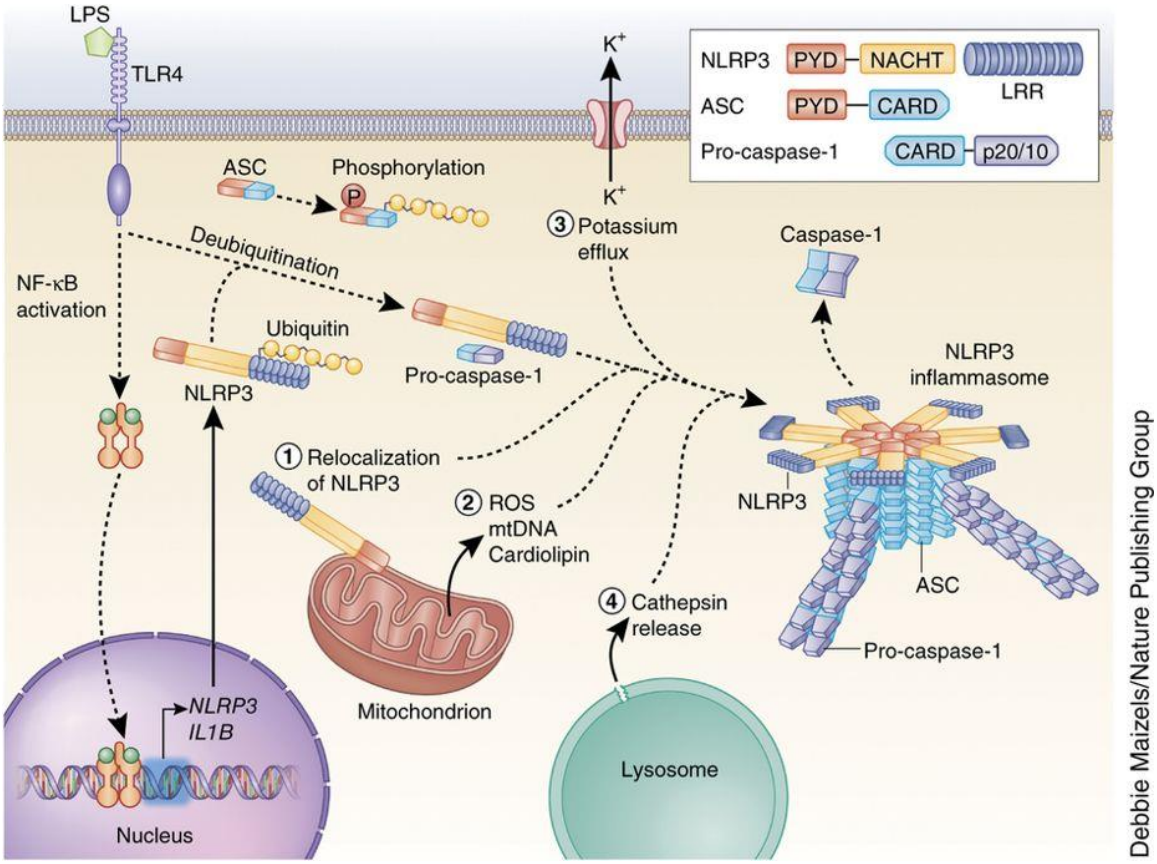

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Figure 1.5 Mechanisms of the NLRP3 inflammasome activation.
A stimulus (e.g. LPS sensed by TLR4) activates the NF-κB pathway, promoting the mRNA expression of *NLRP3* and *IL-1β*. This priming step is followed by post-translational modifications such as deubiquitination of NLRP3 and ubiquitination/phosphorylation of ASC. NLRP3 inflammasome activation requires signal 2 that include the relocalization of NLRP3 to the mitochondria, the release of mitochondrial ROS/DNA or cardiolipin, ion fluxes (e.g. K^+ efflux) and cathepsin release from disturbed lysosomes. For NLRP3 inflammasome assembly, NLRP3 nucleates ASC into filaments through PYD-PYD interaction forming the ASC speck. Pro-caspase-1 is recruited to ASC through CARD-CARD interaction, leading to the auto-proteolytic cleavage of pro-caspase-1. Active subunits of caspase-1 (p20 and p10) cleave precursor forms of IL-1β or IL-18. Domain structures of NLRP3 inflammasome components are shown in the inset.

1.2.1.3.1.2 Ubiquitination and NLRP3 inflammasome

Ubiquitination is a post-translational modification process by which ubiquitin proteins are covalently conjugated to substrate proteins. Ubiquitin is an 8.6-kDa protein, which is highly conserved among eukaryotes (Zuin et al., 2014). Its linkage to substrates was initially known to be required for proteasomal degradation of the substrate proteins; however it has been further identified to play crucial roles in a variety of cell signaling pathways. Ubiquitination is a three-step process mediated by three enzymes: E1 activating enzymes, E2 conjugating enzymes and E3 ligases. In addition, ubiquitins can be recycled after being removed from target proteins by deubiquitinases (Kattah et al., 2017). The substrate selectivity is generally determined by E3 ligases that link the C-terminal glycine of an ubiquitin molecule to the ε-amino group of a lysine (K) residue on the substrate. Monoubiquitination occurs by binding of a single ubiquitin to each lysine in the substrate; subsequent linkages of more ubiquitins to lysine residues located on the first ubiquitin leads to different types of polyubiquitination (Davis and Gack, 2015). Additional ubiquitin molecules can be linked on a lysine residue among seven lysines (K6, K11, K27, K29, K33, K48 and K63) or on the first methionine (M1) residue, eventually leading to different consequences. M1-linked ubiquitination, also called linear ubiquitination is mediated by an E3 ligase complex, a linear ubiquitin assembly complex (LUBAC), and is involved in signal transduction such as the NF-κB activation (Davis and Gack, 2015). K48-linked ubiquitination can target the substrates for proteasomal degradation, while K63-linked ubiquitination leads to signal transduction by affecting the stability or localization of target proteins; other linkages are involved in rather diverse activities including cell development, signaling regulation, autophagy or protein degradation (Davis and Gack, 2015, Kattah et al., 2017). In some cases, unanchored ubiquitin chains that are not linked to the substrate can participate in signaling events (Kattah et al., 2017).

Given that protein modifications by ubiquitins occur for a variety of cellular functions, it is of no surprise that ubiquitination and its related enzymes play pivotal roles in immune modulation. Ubiquitination and deubiquitination have been described to regulate the inflammasome activity (Bednash and Mallampalli, 2016). Deubiquitinases are required for inflammasome-dependent IL-1β production (Lopez-Castejon et al., 2013) and more specifically, deubiquitination of NLRP3 is reported to be important for the activation of the NLRP3

inflammasome (Guo et al., 2016, Han et al., 2015, Juliana et al., 2012, Kawashima et al., 2017, Py et al., 2013, Yen et al., 2015). On the contrary, autophagy-mediated or proteasomal degradation of ubiquitinated NLRP3 leads to suppression of the NLRP3 inflammasome (Song et al., 2016, Yan et al., 2015). Upon activation of several different inflammasomes, caspase-1 can be ubiquitinated, which may downregulate the inflammasome activity (Van Opdenbosch et al., 2014). A recent report shows that ubiquitination-mediated degradation of caspase-1 is prevented by Zika virus NS1 protein leading to NLRP3 inflammasome activation (Zheng et al., 2018). Pro-IL-1β also undergoes ubiquitination, which leads to either its maturation into IL-1β or its degradation (Ainscough et al., 2014, Duong et al., 2015, Harris et al., 2011). Interestingly, upon human papillomavirus infection, ubiquitination of pro-IL-1β followed by the proteasomal degradation is mediated by a viral oncoprotein that hijacks the cellular E3 ligase (Niebler et al., 2013). ASC ubiquitination was initially reported in a study of AIM2 inflammasome-related autophagy in response to dsDNA; for the inhibition of AIM2 inflammasome-mediated IL-1β, ubiquitinated ASC is delivered to an autophagic adaptor, p62 and subject to autophagy for degradation (Shi et al., 2012). Similarly, NLRP3 inflammasome activity is restricted by p62 that interacts with an E3 ligase, TRAF6, which ubiquitinates ASC (Chiu et al., 2016). Linear ubiquitination of ASC is essential for the activation of NLRP3 inflammasome (Rodgers et al., 2014), whereas a deubiquitinase, ubiquitin-specific peptidase 50 interacts with ASC and deubiquitinates the ASC for NLRP3 inflammasome activation (Lee et al., 2017c). Notably, ubiquitination of ASC by an E3 ligase, TRAF3 upon infection with VSV is critical for inflammasome-mediated IL-1β production (Guan et al., 2015).

1.2.1.3.1.3 Mitochondria acting on the NLRP3 inflammasome

Mitochondria-mediated pathophysiological changes positively or negatively regulate the NLRP3 inflammasome. Mitochondria that generate ROS are readily eliminated by mitophagy, whereas inhibition of this event causes the ROS accumulation and the loss of mitochondrial membrane potential. The correlation between autophagy and ROS-mediated NLRP3 inflammasome activation is demonstrated. While autophagy proteins can inhibit the NLRP3 inflammasome, depletion of them enhances caspase-1 activity by damaging mitochondrial homeostasis (Nakahira et al., 2011). Another study shows that upon agonist treatment, NLRP3 can localize to the ER-mitochondria that enables NLRP3 to sense ROS (Zhou

et al., 2011). ROS sensing mechanism by NLRP3 is proposed by the same group. In the resting state, thioredoxin-interacting protein (TXNIP) is bound to the antioxidant molecule, thioredoxin. Inflammasome activators let the TXNIP dissociate from thioredoxin upon ROS production; in turn, TXNIP binds to NLRP3 to activate the NLRP3 inflammasome (Zhou et al., 2010). However, NLRP3 inflammasome activation can be triggered independent of ROS, as shown by an NLRP3 agonist that induces mitochondrial dysfunction, but does not induce ROS (Iyer et al., 2013). Mitochondrial DNA released into cytosol activates the NLRP3 inflammasome as well (Nakahira et al., 2011). Another study explains this phenomenon in relation to mitochondrial membrane potential, which is a proton gradient in the inner mitochondrial membrane. NLRP3 agonists can dissipate the mitochondrial membrane potential during apoptosis; this leads to the release of oxidized mitochondrial DNA that binds to NLRP3 to activate the NLRP3 inflammasome (Shimada et al., 2012). However, it is contradicted by another finding showing that the maintenance of the intact mitochondrial membrane potential is required for the inflammasome activation upon infection with RNA viruses, including IAV. Mitochondrial uncoupling protein 2, responsible for transporting anions from the inner to the outer mitochondrial membrane, reduces the mitochondrial membrane potential, thereby suppressing the NLRP3 inflammasome activity (Ichinohe et al., 2013).

Mitochondrial components are also implicated in the regulation of the NLRP3 inflammasome. Several isoforms of voltage-dependent anion channels that are outer mitochondrial proteins are required for NLRP3 inflammasome activation (Zhou et al., 2011), whereas a mitochondrial protease, HtrA serine peptidase 2 restricts the activation (Rodrigue-Gervais et al., 2018). Cardiolipin, an inner mitochondrial membrane phospholipid has been demonstrated to interact with NLRP3 and activate the NLRP3 inflammasome (Iyer et al., 2013). Interestingly, cytochrome c released from the inner mitochondrial membrane during apoptosis inhibits the cardiolipin-NLRP3 interaction and NLRP3 inflammasome activity (Shi and Kehrl, 2016). An outer mitochondrial membrane protein, mitofusin (Mfn) 2 interacts with NLRP3 upon RNA virus infection and activates the NLRP3 inflammasome activation (Ichinohe et al., 2013). Inactive NLRP3 is cytosolic, but upon activation, NLRP3 is recruited to mitochondria by MAVS; this translocation and NLRP3 oligomerization are important for the NLRP3 inflammasome activation (Park et al., 2013b, Subramanian et al., 2013). In response to cytosolic dsRNA, MAVS can activate the NLRP3 inflammasome by inducing membrane permeabilization

(Franchi et al., 2014).

1.2.1.4 Inflammasomes formed by non-NLRs

Non-NLR proteins can also form inflammasomes. The best example is a protein called AIM2 that belongs to an IFN-inducible protein class. Unlike NLRs, it consists of the N-terminal PYD and the C-terminal domain, HIN200 (that refers to 'hematopoietic IFN-inducible nuclear antigens with 200 amino acid repeats'). Upon sensing of cytosolic dsDNA or DNA virus by HIN200 domain, the AIM2 inflammasome is activated through assembly with ASC and pro-caspase-1 (Fernandes-Alnemri et al., 2009, Hornung et al., 2009). Initially, the AIM2 inflammasome was known to be triggered by cytosolic dsDNA, but not inducers of other inflammasomes (Rathinam et al., 2010). However, further findings suggest that bacterial or fungal pathogens can activate both AIM2 and NLRP3 inflammasomes (Karki et al., 2015, Park et al., 2014, Wu et al., 2010). In spite of different structures, AIM2 and NLRP3 are commonly involved in some signaling pathways (Sagulenko et al., 2013). Recent studies have demonstrated the activation of AIM2 inflammasome upon RNA virus infection. Induction of AIM2 inflammasome upon enterovirus 71 limits viral replication (Yogarajah et al., 2017), whereas activation of AIM2 inflammasome is implicated in IAV-induced mortality (Zhang et al., 2017a). RIG-I is an inflammasome-forming RLR. Independent of NLRP3, it forms the inflammasome by binding to ASC to regulate IL-1β production upon RNA virus infection, while MDA5 only upregulates NLRP3 (Poeck et al., 2010). Its role to activate NLRP3 inflammasome via interaction with ASC and caspase-1 upon IAV infection is also described (Pothlichet et al., 2013). Pyrin is not a classical member of NLR, but it also assembles into the inflammasome that is analogous to NLRP3 inflammasome, while not affecting the NLRP3 inflammasome (Yu et al., 2006). Critical roles of pyrin inflammasome on IL-1β production upon bacterial infection have been demonstrated (Gavrilin et al., 2009, Xu et al., 2014).

1.2.2 IL-1β

The IL-1 superfamily is a group of IL-1-related proteins involved in pro- or anti-inflammatory responses. This superfamily is divided into three subfamilies, IL-1, IL-18 and IL-36 (Table 1.1). IL-1β belongs to the IL-1 subfamily along with IL-1α, IL-33 and IL-1 receptor (IL-1R) antagonist (IL-1Ra) (Dinarello, 2018). IL-1 cytokines (IL-1α and IL-1β) have specific

receptors including IL-1R1 and IL-1R2, and a co-receptor, IL-1R3. IL-1β binds either IL-1R1 to induce pro-inflammatory action or a decoy receptor, IL-1R2 to block the IL-1 signaling. For pro-inflammatory responses, IL-1β binding to IL-1R1 induces a structural change of IL-1R1 that further allows the binding of the co-receptor, IL-1R3 to IL-1R1. As a result, their adaptor protein, MyD88 is recruited, which then turns on pro-inflammatory signals via the NF-κB pathway (Dinarello, 2018).

Table 1.1 IL-1 family members with their receptors and functions.

Subfamily	Member	Receptor	Function
IL-1	IL-1α	IL-1R1	Pro-inflammatory
	IL-1β	IL-1R1	Pro-inflammatory
		IL-1R2	Anti-inflammatory
	IL-1Ra	IL-1R1	Anti-inflammatory
	IL-33	IL-1R4	Pro-inflammatory
IL-18	IL-18	IL-1R5	Pro-inflammatory
	IL-37	IL-1R5	Anti-inflammatory
IL-36	IL-36α, IL-36β, IL-36γ	IL-1R6	Pro-inflammatory
	IL-36Ra	IL-1R6	Anti-inflammatory
	IL-38	IL-1R6	Anti-inflammatory

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IL-1β is primarily produced by immune cells of the myeloid lineage such as monocytes, macrophages and DCs, and its expression is induced during disease conditions. However, IL-1α is constitutively produced from epithelial/endothelial cells in the case of sterile inflammation, which is induced by non-microbial subjects or cell injury (Dinarello, 2018, Garlanda et al., 2013). IL-1β is expressed as an inactive form and needs to be processed for biological activity; IL-1α is active either as its precursor or mature form. Transcription of IL-1β is initiated by pro-

inflammatory stimuli (including microbial ligands), inflammatory cytokines (such as TNF or IL-1 itself) or physiological changes in cells (Allan et al., 2005, Fenton, 1992). Upon activation of downstream signaling cascades, transcription factors bind the IL-1β gene promoter and the DNA regulatory region, which consists of multiple elements, for example, activator protein 1 (AP-1) and NF-κB binding sites, and the LPS response enhancer (Watkins et al., 1999). While the 5' cap and poly (A) tail prevent mRNA degradation, the stability of *IL-1β* mRNA can be enhanced by granulocyte-macrophage colony-stimulating factor, TNF or LPS (Watkins et al., 1999). Since *IL-1β* mRNA does not have a signal sequence that is required to be transported to ER-bound ribosomes, it is transported to free ribosomes for translation (Watkins et al., 1999). Post-translational processing of pro-IL-1β is necessary for its maturation into IL-1β. The proteolytic cleavage of pro-IL-1β is conducted by caspase-1, whose activation largely depends on the signal sensing by inflammasome-forming PRRs, although proteases like elastase, cathepsin G and chymase derived from neutrophils and mast cells can also cleave pro-IL-1β (Afonina et al., 2015). Different models for the IL-1β secretion pathway are proposed. While other inflammatory cytokines are secreted through a conventional pathway, i.e. transportation through the ER-Golgi route, IL-1β is not secreted in that way due to not having a signal peptide (Lopez-Castejon and Brough, 2011). The unconventional secretion pathways suggested are as follows: IL-1β can be localized to lysosomes that are exocytosed; IL-1β can be released with microvesicles that detach from plasma membranes or released with other small vesicles called exosomes that surround IL-1β; IL-1β can be released upon cell lysis (Eder, 2009, Lopez-Castejon and Brough, 2011).

IL-1β exerts a variety of functions on the immune system. Its action on vascular endothelial cells upregulates adhesion molecules and increases the vascular permeability to help leukocytes adhere to blood vessel walls (Dinarello, 2009). Macrophages stimulated with IL-1β produce cyclooxygenase-2, which is responsible for generating prostaglandins and leukotrienes that regulate the constriction and dilation of blood vessels (Tizard, 2009b). These changes are required for the recruitment and infiltration of inflammatory cells to the site of infection. Secretion of IL-1β itself along with other pro-inflammatory cytokines like TNF-α, IL-6 and IL-8 by monocytes and macrophages can be induced by IL-1β (Dinarello, 1991). In addition, IL-1β promotes phagocytosis and antigen presentation by macrophages and DCs. IL-1β also stimulates liver cells to produce acute-phase proteins that support inflammatory responses and pathogen destruction (Tizard, 2009b). Pro-inflammatory responses lead by IL-1β contribute to causing

critical signs of inflammation such as fever, redness and swelling (Tizard, 2009b). IL-1β, in synergy with other interleukins, enhances the differentiation of IL-17-producing helper T cells, which are pro-inflammatory (Acosta-Rodriguez et al., 2007, Sutton et al., 2006). IL-1β can promote B cell- and T cell-mediated antibody production as well (Dinarello, 2009, Nakae et al., 2001).

1.2.2.1 Regulation of IL-1β production by IAV proteins

While viral RNA itself has the ability to activate the NLRP3 inflammasome (Allen et al., 2009, Thomas et al., 2009), different viral proteins of IAVs have been reported to regulate the NLRP3 inflammasome-mediated IL-1β production. An early study demonstrated that the NS1 protein of the mouse-adapted strain, influenza A/Puerto Rico/08/1934/H1N1 (PR8) inhibits caspase-1 activation and IL-1β production in human macrophages (Stasakova et al., 2005). NS1 of the 1918 pandemic strain, by binding to RIG-I, can inhibit RIG-I-mediated IL-1β production, leading to increased virulence in ferrets (Pothlichet et al., 2013). Furthermore, NS1 inhibits NLRP3 inflammasome-mediated IL-1β production by downregulating pro-IL-1β transcription and by interacting with NLRP3 (Cheong et al., 2015). More recently, the residues critical for NS1 protein's RNA-binding and tripartite motif-containing protein 25 (TRIM25)-binding activities, R38/K41 and E96/E97, respectively, are found to be required for NS1 to inhibit the NLRP3 inflammasome activity (Moriyama et al., 2016). IAV M2 protein exerts an ion channel activity required for virion uncoating (Pinto et al., 1992). Perturbation of cellular ionic concentrations mediated by transmembrane region of this protein contributes to the NLRP3 inflammasome-mediated IL-1β production (Ichinohe et al., 2010). PB1-F2 is a non-structural protein, which is a virulence factor (Zamarin et al., 2006); studies have been performed since the effects of PB1-F2 on IL-1β production from different animals were evaluated (Meunier and von Messling, 2012). By challenging mice with PB1-F2 peptides, the roles of PB1-F2 to activate the NLRP3 inflammasome and recruit leukocytes were identified; this function was mapped to the C-terminus of PB1-F2 from pathogenic strains including PR8 (McAuley et al., 2013). A further study by the same group showed that PB1-F2 from PR8 and avian H7N9 strains can cause NLRP3 inflammasome-mediated IL-1β production by inducing the mitochondrial ROS (Pinar et al., 2017). In contrast, PB1-F2 that targets the mitochondria functions to inhibit the NLRP3

inflammasome by blocking the recruitment of NLRP3 to the mitochondria (Yoshizumi et al., 2014).

1.3 Innate immune cells and sensors

The innate immune system is the first line of host defense against pathogens and endogenous/exogenous danger signals. It is important for the effective clearance of pathogens and the healing process, and also for the onset of adaptive immunity. Inflammation is one of the first and the most critical responses in the innate immune system. Inflammation is intended to protect the host by eliminating the pathogens/danger signals and restoring the damaged tissues, however it has to be terminated timely to prevent prolonged inflammation as it can bring about both positive and negative consequences for the host health. Due to its double-edged sword-like roles, the tight control of inflammation is essential for the resolution of infection. Pathogens have genomes or structures that are distinguished from the normal host components. Their structural motifs or characteristic signatures of nucleic acids are called pathogen-associated molecular patterns (PAMPs). Sensing the PAMPs or uncommon physiological changes in and around the host cells by pattern recognition receptors (PRRs) triggers the innate immune signaling required for inflammation (Iwasaki and Pillai, 2014). Signals from activated PRRs are transduced to the adaptor molecules and transcription factors, so that the pro-inflammatory cytokines/chemokines, such as IFNs and ISGs are secreted and/or expressed. Subsequent events include a set of reactions to recruit other immune cells, eliminate the pathogens, process the infected or dead cells, and repair damaged tissues (Iwasaki and Pillai, 2014). Innate immune cells are specialized for these functions, although non-immune cells also express PRRs and mediate the immune signaling (Takeuchi and Akira, 2010). Successful innate immunity is accomplished through interplay between immune cells and non-immune cells. Some types of innate immune cells are responsible for antigen processing and presentation playing as a bridge between innate and adaptive immunity (Vyas et al., 2008).

1.3.1 Innate immune cells

Immune cells are leukocytes that are either from myeloid or lymphoid progenitors derived from multipotent hematopoietic stem cells in the bone marrow. Based on the shapes, leukocytes can be classified into two populations: mononuclear leukocytes and polymorphonuclear

granulocytes (neutrophils, eosinophils, basophils and mast cells) (Tizard, 2009d). Mononuclear leukocytes are further divided into two subpopulations: (phagocytic) monocytes and (non-phagocytic) lymphocytes (Tizard, 2009d). Monocytes are further differentiated into macrophages or DCs. Among others, macrophages, DCs, neutrophils and mast cells are the representative members in the innate immune system by playing crucial roles such as phagocytosis, production of immune mediators and antigen presentation (Janeway and Medzhitov, 2002). These functions lead to the protection against pathogens and prevention of injury by danger signals.

1.3.1.1 Macrophages

Phagocytosis is an important event in innate immunity, which is played by phagocytic leukocytes such as macrophages, DCs and neutrophils. While some macrophages are derived from their progenitors in yolk sac or fetal liver, most of the macrophages responding to microbial infection are derived from stem cells in the bone marrow (Gordon and Plüddemann, 2017). Monocytes developed from these stem cells circulate in the blood. The maturation of monocytes into macrophages occurs as they enter into tissues, where differentiated macrophages are distributed to exert their roles (Tizard, 2009b). At the site of infection, neutrophils are the first to show up to engulf pathogens, which is then followed by the appearance of macrophages, which can phagocytose more effectively than neutrophils. Macrophages are specialized in phagocytosis and the clearance of pathogens or dead neutrophils, but their functions are not restricted to those. They are also responsible for the immune surveillance to trigger inflammation as well as anti-inflammatory actions required for tissue repair and healing. These two-faceted functions are possible with the presence of two different populations of macrophages: M1 cells for pro-inflammatory responses and M2 cells for anti-inflammatory roles (Tizard, 2009c).

In the lungs, different types of macrophages including alveolar macrophages (AMs), interstitial macrophages and exudate macrophages reside (Nicol and Dutia, 2014). Among them, AMs are one of the first cells to respond to respiratory pathogens (Tumpey et al., 2005). These cells are located in the alveolar space formed by the epithelium consisting of type I and II pneumocytes, which are responsible for the maintenance of the space and the secretion of surfactant substances, respectively (Short et al., 2014). AMs are predominant immune cells in this space (Duan et al., 2017), and are also involved in homeostasis of the surfactant proteins (Halstead and Chroneos, 2015). Upon IAV infection, AMs produce pro-inflammatory cytokines

including IL-1β, IL-6 and TNF-α that mediate inflammation and recruit other immune cells; they produce type I IFNs that limit the spread of IAV. In addition, AMs are critical for activating the T cell response as well as antibody response upon IAV infection (He et al., 2017, Wijburg et al., 1997); more pathogenicity is observed in different animals that are deficient in AMs (Cardani et al., 2017, Kim et al., 2013, Kim et al., 2008, Purnama et al., 2014, Schneider et al., 2014). IAV infection can deplete AMs by inducing their death, which may increase the susceptibility to secondary bacterial infection (Ghoneim et al., 2013), while AM depletion upon co-infection with IAV and bacteria can be prevented by IL-1 signaling (Bansal et al., 2018). Furthermore, infection of type I pneumocytes by IAV can be inhibited by AMs (Cardani et al., 2017).

1.3.1.2 Dendritic cells (DCs)

DCs are critical for linking the innate immunity to adaptive immunity by functioning in both antigen processing and antigen presentation. Two subpopulations of DCs exist: myeloid DCs that are derived from blood monocytes, and plasmacytoid DCs that are derived from lymphoid precursors (Tizard, 2009a). Myeloid DCs are distributed in tissues and are responsible for immune surveillance; plasmacytoid DCs are located in lymphoid organs, and are responsible for type I IFN production and T cell activation. After produced from bone marrow progenitors, DCs exist as immature cells (Tizard, 2009a). Immature DCs are activated upon recognition of antigens via their PRRs or in response to pro-inflammatory cytokines; this activation enhances their recruitment to the site of infection and promotes phagocytosis (Tizard, 2009a). Their maturation by encountering antigens also leads to the migration to lymph nodes in which they interact with T cells and B cells for antigen presentation (Clark et al., 2000). Importantly, mature DCs are the only type of antigen-presenting cells that can stimulate naïve T cells (Tizard, 2009a). Critical involvement of DCs on protection against IAV infection has been well demonstrated (Bhardwaj et al., 1994, Fonteneau et al., 2003).

1.3.1.3 Neutrophils

Neutrophils are a class of polymorphonuclear granulocytes that contain multilobular nuclei and granule-filled cytoplasm. They are derived from the same progenitor in bone marrow as macrophages and constitute the major population of leukocytes (Tizard, 2009d). From the bloodstream, they are recruited to the site of infection by chemotaxis, thereby becoming the first

cells that respond to infection. Due to the lifespan of only a few days and lack of ability to repeatedly ingest, neutrophils are frequently replaced with new cells. Phagocytic activity of neutrophils is enhanced by opsonization, a process by which pathogens are coated by opsonins. By producing reactive oxygen species (ROS) or releasing antimicrobial enzymes from granules, neutrophils can destroy the ingested pathogens (Tizard, 2009d). Additionally, the cells can release their own proteins and chromatin to form extracellular networks called neutrophil extracellular traps, which are designed to kill extracellular microbes without phagocytosis (Brinkmann et al., 2004). Although neutrophils express different TLRs along with RIG-I-like receptors (RLRs), the inflammasome-forming NLRs are not defined yet (Clarke et al., 2010, Hayashi et al., 2003, Tamassia et al., 2008). In addition to microbicidal functions, they participate in the immune regulation by secreting both pro-inflammatory and anti-inflammatory cytokines (Mantovani et al., 2011). Neutrophils can attract and interplay with T cells to promote adaptive immunity, and in turn, cytokines from activated T cells can support the functions of neutrophils (Pelletier et al., 2010a, Pelletier et al., 2010b). Upon IAV infection, a number of neutrophils are recruited to the airways where they play protective roles on the host. Together with AMs, neutrophils can alleviate the IAV-induced pathology and reduce mortality (Fujisawa, 2008, Tate et al., 2008, Tumpey et al., 2005). Neutrophil-mediated IL-1β production from AMs upon IAV infection is also reported (Peiró et al., 2018).

1.3.1.4 Mast cells

Mast cells originate in the bone marrow and then migrate and mature in specific locations (Abraham and St John, 2010). Their distribution in connective tissue, the skin and respiratory/digestive mucosa allows them to encounter and respond to pathogens in the early phase of infection (St John and Abraham, 2013). The cytoplasm of mast cells is filled with granules from which regulatory molecules are released upon inflammation. Mast cells quickly initiate inflammation by secreting these molecules such as histamine and serotonin, and later, they also secrete cytokines and chemokines (Abraham and St John, 2010). Like other innate immune cells, mast cells induce inflammation upon sensing pathogens with a variety of PRRs or signals from other cells. Moreover, the cells harbor receptors for the Fc region of IgE molecules enabling mast cells to release their granule components upon recognition of antigen/allergen-bound IgE antibodies (Tizard, 2009b). Due to this behavior, mast cells can not only cause allergy

and anaphylaxis, but also play protective roles against parasites (Abraham and St John, 2010). Mast cells are activated upon IAV infection through RIG-I signaling and their roles in lung pathology are reported (Graham et al., 2013, Hu et al., 2012, Josset et al., 2012).

1.3.2 Innate immune sensors

Innate immune sensors are receptors that recognize extracellular and intracellular PAMPs or DAMPs. There are three main classes based on the domain structures: nucleotide-binding oligomerization domain-like receptors (NLRs) are cytosolic sensors to detect a wide range of molecules and cellular stress; toll-like receptors (TLRs) are membrane-bound sensors detecting both PAMPs and DAMPs in either cell surface or endosomal compartments; retinoic acid-inducible gene I-like receptors (RLRs) are cytosolic RNA sensors (Brubaker et al., 2015).

1.3.2.1 Nucleotide-binding oligomerization domain-like receptors (NLRs)

NLRs are primarily expressed in immune cells, while some NLR family proteins are expressed ubiquitously (Shaw et al., 2008). NLRs are a class of PRRs that contain three major domains: a variable N-terminal domain, a C-terminal leucine-rich repeat (LRR) domain and a central domain called NACHT, which was named after the five proteins that contain this domain (NAIP [neuronal apoptosis inhibitor protein], CIITA [MHC class II transactivator], HET- E [plant *het* gene product involved in vegetative incompatibility], and TP1 [telomerase-associated protein 1]) (Albrecht et al., 2003). The LRR domain can sense ligands or PAMPs; the N-terminal domain, which is either pyrin domain (PYD), caspase activation and recruitment domain (CARD) or baculovirus inhibitor of apoptosis protein repeat (BIR) domain, interacts with different adaptor proteins to mediate downstream signaling (Proell et al., 2008). The NACHT domain is responsible for self-oligomerization, which is further required for the signaling cascade through the N-terminal domain (Martinon and Tschopp, 2005). Based on the N-terminal domain structures, the NLRs are divided into four subfamilies: NLRs with PYD (NLRP, i.e. NLRP1 to NLRP14); NLRs with CARD (NLRC, i.e. NOD1, NOD2, NLRC3 to NLRC5); NLR with BIR (NLRB, i.e. NAIP); NLR with acidic transactivation domain (ATD) (NLRA, i.e. CIITA) (Figure 1.6). The diversity in the N-terminal domain can lead to different signaling events. The activators of NLR members and their modes of action are shown in Table 1.2.

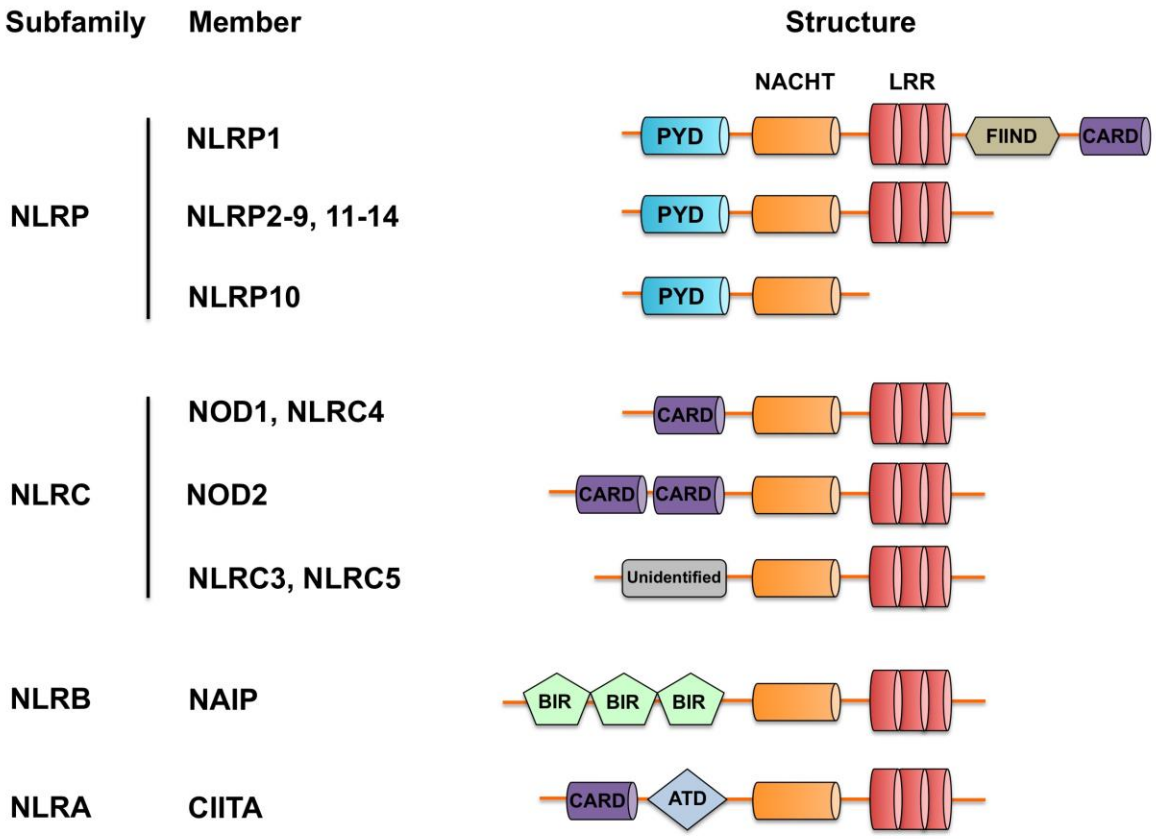


Figure 1.6 Domain architecture of the NLR family proteins.
NLRs are divided into four subfamilies (NLRP, NLRC, NLRB and NLRA) based on the N-terminal domains. PYD, pyrin domain; CARD, caspase activation and recruitment domain; BIR, baculovirus inhibitor of apoptosis protein repeat; ATD, acidic transactivation domain; NACHT, for NAIP, CIITA, HET-E and TP1; LRR, leucine-rich repeat; FIIND, function to find domain.

1.3.2.1.1 NLRs with pyrin domain (NLRP)

Among NLRs with PYD, NLRP1 and NLRP3 have been most widely investigated. These two members function by forming the protein complex, inflammasome with ASC and pro-caspase-1 to induce pro-inflammatory cytokines such as IL-1β and IL-18. NLRP1 is the first of its kind that is known to act through the assembly of the inflammasome. In LPS-stimulated human monocytes, the inflammasome consisting of NLRP1, ASC, caspase-1 and caspase-5 was discovered (Martinon et al., 2002). A structurally characteristic feature for NLRP1 is that it has

two more C-terminal domains, the ‘function to find’ domain (FIIND) and CARD besides the LRR domain. Interestingly, cleavage of the FIIND is needed for NLRP1 inflammasome activation (Finger et al., 2012, Frew et al., 2012). NLRP1-mediated caspase-1 activation upon anthrax toxin in mouse macrophages was reported (Boyden and Dietrich, 2006); a subsequent study showed the formation of NLRP1 inflammasome upon the toxin (Nour et al., 2009). Later, bacterial muramyl dipeptide was identified as a ligand for the NLRP1 inflammasome (Faustin et al., 2007). In response to both ligands, NLRP1 association with the nucleotide-binding oligomerization domain-containing protein (NOD) 2 is critical for IL-1β secretion (Hsu et al., 2008). In addition to responding to microbial products, the NLRP1 inflammasome is also activated by cellular stress such as ATP depletion (Liao and Mogridge, 2013).

Since initial discoveries have shown that mutations in the gene encoding NLRP3 are implicated in several auto-inflammatory diseases in humans (Hoffman et al., 2001), researchers have focused on the roles of NLRP3 in inflammation. Early studies displayed that NLRP3 alone can inhibit the NF-κB pathway (O'Connor et al., 2003), but by interacting with ASC, it is able to activate the pathway (Manji et al., 2002). Like NLRP1, NLRP3 forms the inflammasome with ASC and pro-caspase-1; in macrophages from an auto-inflammatory disorder patient with an NLRP3 mutation, more IL-1β secretion is observed (Agostini et al., 2004). With a growing number of studies illustrating the importance of ASC on caspase-1 activity (Mariathasan et al., 2004, Srinivasula et al., 2002, Stehlik et al., 2003), more details on the activation of the NLRP3 inflammasome started to be unveiled. Later, the specific roles of NLRP3 and ASC on NLRP3 inflammasome-mediated IL-1β production in response to bacterial RNA (Kanneganti et al., 2006b), or upon stimulation with TLR agonists followed by bacterial infection or toxin treatment (Mariathasan et al., 2006, Sutterwala et al., 2006) were demonstrated. Further, malarial hemozoin and fungal products were added to the list of agonists that can activate the NLRP3 inflammasome (Lamkanfi et al., 2009, Shio et al., 2009). Importantly, dependency of caspase-1 activity on the expression of NLRP3 and ASC upon infection with RNA viruses including IAV is reported (Kanneganti et al., 2006a). This is supported by *in vivo* studies showing that protective roles are exerted by the NLRP3 inflammasome against IAV-induced pathology (Allen et al., 2009, Thomas et al., 2009). Another study indicates that T cell responses upon IAV infection are mediated by ASC and caspase-1, while NLRP3 is not critical for the protection against IAV infection (Ichinohe et al., 2009). NLRP3 inflammasome activation by viral DNA is

demonstrated using adenovirus; this complex is activated by mammalian genomic DNA or bacterial DNA as well (Muruve et al., 2008). Afterwards, a variety of different pathogens have been reported to activate the NLRP3 inflammasome (Ito et al., 2012, Lawrence et al., 2013, Rajan et al., 2011, Segovia et al., 2012, Wang et al., 2018, Wang et al., 2017). Stimuli of NLRP3 are not restricted to microbial products, since various danger-associated particulates (Cassel et al., 2008, Dostert et al., 2008, Eisenbarth et al., 2008, Hornung et al., 2008) or even endogenous danger molecules can induce the NLRP3 inflammasome activation (Duewell et al., 2010, Martinon et al., 2006, Yamasaki et al., 2009). Because the aforementioned NLRP3 inflammasome activators are not chemically related, studies have focused on the upstream mechanism of NLRP3 inflammasome activation. For example, the production of ROS, lysosomal damage, ER stress, and ion fluxes have been described to play roles on the activation of the NLRP3 inflammasome (Duewell et al., 2010, Hornung et al., 2008, Menu et al., 2012, Murakami et al., 2012, Pétrilli et al., 2007).

NLRP2 inhibits NF-κB by interacting with the inhibitor of κB kinase (IKK), but can induce IL-1β by interacting with ASC (Bruey et al., 2004). NLRP2 is known to form an inflammasome to regulate IL-1β production in astrocytes (Minkiewicz et al., 2013). NLRP6, in association with ASC, activates the NF-κB pathway and contributes to caspase-1-mediated IL-1β production (Grenier et al., 2002). In contrast, another study indicates that NLRP6 is not involved in IL-1β production, and rather inhibits the NF-κB activation; NLRP6 deficiency can confer resistance against bacterial infection in mice (Anand et al., 2012). A finding that NLRP6 binds viral RNA to activate the IFN signaling in response to enteric viruses in the intestines suggests its role as a viral RNA sensor (Wang et al., 2015c). NLRP7 either negatively regulates caspase-1-mediated IL-1β production (Kinoshita et al., 2005), or inhibits the pro-IL-1β synthesis (Messaed et al., 2011). NLRP7 senses intracellular bacteria, and NLRP7 inflammasome-mediated IL-1β production in response to microbial lipopeptides can limit bacterial replication (Khare et al., 2012). NLRP10, the only NLR member without the LRR domain, inhibits the NF-κB pathway and IL-1β production by associating with ASC and caspase-1 (Wang et al., 2004). According to a mouse study, NLRP10 is critical for antigen presentation by DCs rather than modulation of inflammasome activity (Eisenbarth et al., 2012). Contribution of NLRP10 to the adaptive immunity is also shown by an investigation on NLRP10-mediated helper T cell responses against fungal infection (Joly et al., 2012), while this was not the case for parasite

infection, because inflammation and adaptive immunity were upregulated by NLRP10 deficiency (Clay et al., 2017). Upon bacterial infection, inflammatory cytokine production was positively regulated by NLRP10 and this was regardless of inflammasome activity (Lautz et al., 2012). NLRP12 is defined as a negative regulator of inflammatory cytokines upon bacterial infection (Silveira et al., 2017, Zaki et al., 2014). A contradicting report shows that NLRP12 inflammasome-mediated IL-18 production recruits IFN-γ, thereby conferring protection against *Yersinia pestis* infection (Vladimer et al., 2012). NLRP12 is also responsible for enhanced neutrophil infiltration and contributes to the pathogenesis in IAV-infected mice (Hornick et al., 2018).

Interestingly, implications of NLRP proteins in the downregulation of the IFN signaling have been reported. NLRP4 recruits an E3 ligase responsible for the degradation of TANK (which refers to 'TNF receptor-associated factor family member-associated NF-κB activator')-binding kinase 1 (TBK1), thereby suppressing type I IFN induction in response to dsRNA (Cui et al., 2012). A subsequent study indicates that NLRP4 forms a signaling complex with multiple ubiquitin-modifying enzymes to inhibit the TBK1-mediated IFN induction (Lin et al., 2016). NLRP11 translocates to the mitochondria and inhibits MAVS-mediated IFN signaling upon RNA virus infection (Qin et al., 2017); it also limits TLR signaling by degrading TRAF6 (Wu et al., 2017).

1.3.2.1.2 NLRs with caspase activation and recruitment domain (NLRC)

NOD1 is ubiquitously expressed, while NOD2 is primarily expressed in immune cells (Inohara et al., 2005). NOD1 and NOD2 sense bacterial peptidoglycan after degradation of bacterial cell walls (Caruso et al., 2014). This sensing is followed by self-oligomerization of NODs, and the recruitment of receptor-interacting serine/threonine-protein kinase 2 (RIPK2) mediated by the CARD-CARD interaction between RIPK2 and NODs (Caruso et al., 2014). RIPK2 further activates the NF-κB signaling or mitogen-activated protein kinase (MAPK) signaling that results in the expression of pro-inflammatory cytokines (Caruso et al., 2014). In addition to bacterial components, NODs can also recognize viral RNAs. Upon sensing ssRNA of RNA viruses, NOD2 associates with MAVS, thereby activating the IRF3-mediated type I IFN production (Sabbah et al., 2009), while NOD1 activation upon viral dsRNA detection induces the innate immune responses independent of MAVS (Vegna et al., 2016). The importance of NOD2

in the control of $CD8^+$ T cell or regulatory T cell response upon IAV infection is also revealed (Egarnes and Gosselin, 2018, Lupfer et al., 2014).

NLRC3 was initially known to inhibit T cell functions (Conti et al., 2005). Degradation of TRAF6 by NLRC3 can induce the downregulation of NF-κB pathway and the suppression of pro-inflammatory cytokines (Schneider et al., 2012). NLRC3 also interferes with the type I IFN signaling mediated by the stimulator of interferon genes (STING) in response to cytosolic DNA or a DNA virus, herpes simplex virus 1 (Zhang et al., 2014b). Further, its function to inhibit NLRP3 inflammasome through the interaction with ASC and pro-caspase-1 is also demonstrated (Eren et al., 2017, Gültekin et al., 2015). NLRC5 is a transcriptional activator of MHC I genes (Meissner et al., 2010) and is important for viral clearance (Lupfer et al., 2017). However, its function as a regulator of antiviral and inflammatory response is under debate. Some studies have shown NLRC5 to negatively regulate the RIG-I-mediated type I IFN and inflammatory cytokines (Benko et al., 2010, Cui et al., 2010). In contrast, other studies have shown NLRC5 to enhance antiviral gene expression (Kuenzel et al., 2010), as well as its interaction with RIG-I to promote the IFN response against IAV infection (Ranjan et al., 2015). Interestingly, it is also known to be required for NLRP3 inflammasome activation upon bacterial infection (Davis et al., 2011), showing that its signaling pathway may overlap with other NLRs. NLRC4 functions by forming an inflammasome; its conformation is changed by NAIP that binds to bacterial proteins, and as a result, the NAIP/NLRC4 inflammasome can be activated (Yang et al., 2013, Zhao et al., 2011). As in the case of NLRC5, interplay and association between NLRC4 and NLRP3 upon bacterial infection have also been determined (Man et al., 2014, Qu et al., 2016). Enteric bacteria-induced NLRC4 inflammasome activation can provide protection from rotavirus infection in the intestines (Zhang et al., 2014a), however there is no direct evidence that the NAIP/NLRC4 inflammasome is critical for the inflammation upon viral infection. Several studies demonstrate that NLRC4 is not involved in inflammatory response during IAV infection (Allen et al., 2009, Ichinohe et al., 2009).

1.3.2.1.3 NLR with baculovirus inhibitor of apoptosis protein repeat (NLRB)

NAIP senses bacterial ligands and assembles with NLRC4, which recruits ASC and pro-caspase-1 to form the NAIP/NLRC4 inflammasome. A mouse study suggested that there are variants for NAIP, which determine the specificity to different kinds of bacterial ligands for the

inflammasome activation (Kofoed and Vance, 2011). In contrast, a single NAIP in humans is responsible for inflammasome activation by recognizing different bacterial ligands (Reyes Ruiz et al., 2017). Recently, it is reported that NLRC4 inflammasome-mediated protection against bacterial pathogens requires IRF8 that regulates the transcription of NAIP (Karki et al., 2018).

1.3.2.1.4 NLR with acidic transactivation domain (NLRA)

CIITA is the only member of NLRA. It has a similar structure to NLRC5 and activates the MHC I and II genes (Martin et al., 1997, Steimle et al., 1993). Further, its roles on the negative regulation of cytokines such as IL-4 and IL-10 are described (Gourley et al., 1999, Yee et al., 2005). While its function as an innate sensor is not defined, CIITA is known to inhibit virus-induced NF-κB activation (Forlani et al., 2016).

Table 1.2 NLR members with their activators and modes of action.

Subfamily	Member	Activator	Mode of action
NLRP	NLRP1	LPS, toxin, muramyl dipeptide	Inflammasome formation
	NLRP2	Unknown	Inflammasome formation, negative regulation of NF-κB
	NLRP3	Bacterial/fungal products, toxin, viral/bacterial nucleic acid, particulate matters, ATP, ROS, lysosomal damage, ER stress, ion fluxes	Inflammasome formation
	NLRP4	Unknown	Negative regulation of IFN signaling
	NLRP6	Viral RNA	Inflammasome formation, negative regulation of NF-κB
	NLRP7	Bacterial lipopeptide	Inflammasome formation
	NLRP10	Unknown	Regulation of antigen presentation by DCs
	NLRP12	*Yersinia pestis*	Inflammasome formation, negative regulation of NF-κB
NLRC	NOD1	Bacterial peptidoglycan, viral dsRNA	Positive regulation of NF-κB and MAPK
	NOD2	Muramyl dipeptide, viral ssRNA	Positive regulation of NF-κB and MAPK
	NLRC3	Cytosolic DNA	Negative regulation of NF-κB and IFN signaling
	NLRC4	Bacterial proteins	Inflammasome formation with NAIP
	NLRC5	Unknown	Positive or negative regulation of IFN signaling
NLRB	NAIP	Bacterial proteins	Inflammasome formation with NLRC4
NLRA	CIITA	Unknown	Activation of MHC I and II genes, negative regulation of NF-κB

The nomenclature of NLR subfamilies and members is based on human proteins.

1.3.2.2 Toll-like receptors (TLRs)

TLRs are membrane-associated molecules that sense PAMPs and DAMPs; they are expressed in a broad range of both immune cells and non-immune cells (Leifer and Medvedev, 2016). Three main domains that comprise the TLRs are 1) LRR which is a ligand-binding ectodomain; 2) transmembrane domain; 3) cytoplasmic Toll-interleukin-1 receptor (TIR) domain that mediates downstream signaling (Satoh and Akira, 2016). Among others, TLR3 and TLR7/8 are the main sensors for viral dsRNA and ssRNA, respectively; they function in early endosomes and their signaling induces both pro-inflammatory cytokines and IFNs. TLR3 is expressed in most innate immune cells with the exception of plasmacytoid DCs and neutrophils (Chow et al., 2018). Upon recognition of viral dsRNA or intermediate RNAs, TLR3 recruits its adaptor protein, TRIF (Oshiumi et al., 2003). Binding of TRIF to TRAF3 leads to the activation of transforming growth factor beta-activated kinase 1 (TAK1). Additionally, TRIF associates with TBK1 and IKKε (Sato et al., 2003), which activate the transcription factors, IRF3/7 and NF-κB, respectively. TLR7 is expressed in monocytes, macrophages, plasmacytoid DCs and B cells; and TLR8 is expressed in monocytes, macrophages and myeloid DCs (Chow et al., 2018). Their ligand specificity is slightly different; for example, TLR7 has specificity for GU-rich ssRNA, while TLR8 is specific for AU-rich ssRNA (Vabret et al., 2017). TLR7 and TLR8 are similar in their mode of signaling transduction. Unlike TLR3, activation of TLR7/8 recruits its adaptor protein, myeloid differentiation primary response protein 88 (MyD88). MyD88 associates with TRAF6 that ubiquitinates the NF-κB essential modulator (NEMO) (Chen et al., 2006). Ubiquitinated NEMO recruits IKKα and IKKβ that phosphorylate the inhibitor of κB (IκB). Then, NF-κB translocates to the nucleus for induction of cytokines and IFNs. In addition, MyD88 binds IRF7 to form a complex with TRAF6, TRAF3 and IKKα. Phosphorylated IRF7 dissociates from this complex and translocates to the nucleus for the IFN induction (Kawai et al., 2004).

1.3.2.3 Retinoic acid-inducible gene I-like receptors (RLRs)

RLRs are cytosolic RNA sensors and are expressed in most cell types. RLRs consist of a DExD/H-box helicase domain and C-terminal repressor domain; among them, RIG-I and MDA5 have two tandem CARDs at the N-terminus, additionally (Chow et al., 2018). Expression of RLRs is generally maintained at a low level, but it is upregulated upon viral infection or IFN

stimulation (Chow et al., 2018). RIG-I senses viral 5'-triphosphate dsRNA generated by different RNA viruses, while long dsRNA is preferably recognized by MDA5, whose critical role in detecting picornaviruses is revealed (Chiang et al., 2014, Feng et al., 2012). Upon ligand sensing, RIG-I undergoes a conformational change by exposing its N-terminal CARDs (Takahasi et al., 2008). This domain is activated through lysine 63-linked poly-ubiquitination by E3 ligases, TRIM25 or Riplet (Gack et al., 2008, Gack et al., 2007, Oshiumi et al., 2010). Activated RIG-I interacts with its adaptor protein, MAVS, which further undergoes self-polymerization to form aggregates (Hou et al., 2011). MAVS recruits E3 ligases such as TRAF2, -5 or -6 to activate cytosolic kinases, TBK1 or IKKε (Liu et al., 2013a). As a result, IRF3, IRF7 and NF-κB pathways are activated for the production of IFNs, ISGs and pro-inflammatory cytokines (Beachboard and Horner, 2016, Chatterjee et al., 2016). Independent of the MAVS-mediated signaling cascade, RIG-I can interact with ASC to form an inflammasome without NLRs, thereby leading to production of IL-1β and IL-18 (Poeck et al., 2010, Pothlichet et al., 2013).

1.3.2.4 Other sensors

PKR is an RNA-dependent kinase in the cytoplasm. It is involved in the activation of the NF-κB pathway (Kumar et al., 1994) as well as the stabilization of the *IFN* mRNAs (Schulz et al., 2010). In IAV-infected cells, its activation upon recognition of viral dsRNA leads to the phosphorylation of eIF2α, which suppresses viral protein translation and prevents viral replication (Li et al., 2006). Recent evidence has shown the roles of Z-DNA binding protein 1 (ZBP1), which has been known to sense cytosolic DNA (Takaoka et al., 2007), as an RNA sensor. ZBP1 recognition of genomic RNA of IAV or newly synthesized viral RNA of DNA virus is required for necroptosis and restriction of viral replication (Maelfait et al., 2017, Thapa et al., 2016). Sensing IAV proteins by ZBP1 leads to the RIPK1-RIPK3 mediated NLRP3 inflammasome activation and cell deaths (Kuriakose et al., 2016). STING is known to regulate type I IFN induction in response to RNA viruses with different mechanisms. After sensing viral RNA, RIG-I interacts with MAVS and STING, thereby activating the STING pathway. Another scenario is that cyclic guanosine monophosphate (GMP)-adenosine monophosphate (AMP) synthase (cGAS) senses viral RNA with an elusive mechanism, and produces cyclic GMP-AMP as the second messenger molecule, which binds and activates STING (Maringer and Fernandez-Sesma, 2014). The activated STING is phosphorylated and ubiquitinated. Then, STING

dimerizes and interacts with TBK1 to phosphorylate IRF3, which leads to type I IFN induction (Maringer and Fernandez-Sesma, 2014). Upon IAV infection, viral membrane fusion induces the STING-mediated IFN response, independently of cGAS, whereas the HA fusion peptide counteracts this action by inhibiting STING dimerization (Holm et al., 2016).

1.4 Mitochondria and innate immune responses

1.4.1 Mitochondrial control of the innate immunity

Mitochondria have a variety of functions essential for cell survival, homeostasis and host immune responses. Emerging evidence emphasizes the roles mitochondria play as the key platform for innate immune regulation. MAVS, a protein that is inserted on the outer mitochondrial membrane, is one of the best-known signaling molecules in mitochondria. As an adaptor protein of RLRs, MAVS activates the transcription factors NF-κB and IRFs to induce pro-inflammatory cytokines and IFNs (Kawai et al., 2005, Seth et al., 2005). MAVS can also localize to peroxisomes, and IRF1-mediated IFN-λ is induced when peroxisomal MAVS is activated (Odendall et al., 2014). Mitochondrial ROS and DNA are considered to be DAMPs that trigger innate immunity. ROS released from mitochondria has been shown to activate transcription factors like AP-1 and NF-κB (Sena and Chandel, 2012). Further, mitochondrial ROS can induce MAPK-mediated pro-inflammatory cytokines such as IL-6 and TNF (Bulua et al., 2011). Mitochondrial DNA triggers TLR9-mediated MAPK signaling for cytokine production (Caielli et al., 2016, Zhang et al., 2010); it can also be sensed by cGAS, which activates STING for type I IFN response (Rongvaux et al., 2014, West et al., 2015).

1.4.2 Mitochondrial dynamics modulated by viruses

Mitochondria undergo continuous changes in its dynamics represented by fission, fusion and mitophagy. Dynamin-related protein 1 (DRP1) plays a vital role in fission and fusion events. For fission, DRP1 is phosphorylated at serine 616 (S616) by different kinases including receptor-interacting protein kinase 1 (RIPK1), cyclin-dependent kinase 1 (CDK1), and MAPK1 (Kashatus et al., 2015, Taguchi et al., 2007, Wang et al., 2014c), or dephosphorylated at serine 637 (S637) by the phosphatase, calcineurin (Cereghetti et al., 2008). After DRP1 is recruited to the mitothondrial fission factor, oligomerized DRP1 surrounds and constricts the mitochondrial membranes (Kim et al., 2018). Since fission occurs to process unhealthy mitochondria,

fragmented mitochondria can be subject to mitophagy. In contrast, dephosphorylation at S616 or phosphorylation at S637 on DRP1 leads to fusion. Mitochondrial proteins Mfn1/2 and optic atrophy 1 are involved in the fusion of outer and inner mitochondrial membranes, respectively (Kim et al., 2018).

Viruses can alter the mitochondrial dynamics to regulate the host innate immune responses or the host cell death. Some RNA viruses are reported to induce DRP1-mediated mitochondrial fission to regulate cell deaths. Classical swine fever virus, porcine reproductive and respiratory syndrome virus (PRRSV) and hepatitis C virus (HCV) cause mitochondrial fission to inhibit apoptosis (Gou et al., 2017, Kim et al., 2014, Li et al., 2016c), whereas rotavirus-induced mitochondrial fission stimulates apoptosis (Mukherjee et al., 2018). Downregulation of IFN signaling can be the result of either suppressed or activated mitochondrial fusion upon dengue virus (DENV) infection. DENV proteases cleave Mfn1 to suppress mitochondrial fusion and this leads to the attenuated IFN induction (Yu et al., 2015). In contrast, DENV enhances mitochondrial fusion by disrupting DRP1-mediated mitochondrial fission, which supports viral replication or attenuates the RIG-I-mediated antiviral response (Barbier et al., 2017, Chatel-Chaix et al., 2016). As to DRP1 phosphorylation upon viral infection, RIPK1 phosphorylates DRP1 leading to mitochondrial fission in response to VSV infection (Wang et al., 2014c), while CDK1 is required for DRP1 phosphorylation upon rotavirus infection (Mukherjee et al., 2018). RIPK1 forms a complex with RIPK3 to induce necroptosis and inflammation (Cho et al., 2009), and this complex also plays roles in the host defense and pathogenesis against viral infection (Daniels et al., 2017, Downey et al., 2017, Kuriakose et al., 2016). An initial study on DRP1-mediated NLRP3 inflammasome activation upon viral infection revealed that in response to RNA viruses such as VSV, the RIPK1/RIPK3 signaling can induce necroptosis, while it can also promote the NLRP3 inflammasome-mediated IL-1β production by inducing DRP1-involved mitochondrial damage (Wang et al., 2014c). Regardless of DRP1, a mitochondrial phosphatase, phosphoglycerate mutase family member 5, which mediates mitochondrial fission, can induce the NLRP3 inflammasome activation upon VSV infection (Moriwaki et al., 2016). Upon IAV infection, PB1-F2 protein, which translocates to mitochondria, can cause mitochondrial fission that is correlated with the defective NLRP3 inflammasome activation (Yoshizumi et al., 2014).

1.5 Innate immune evasion

1.5.1 Immune evasion strategies by RNA viruses

Viruses have evolved ways to counteract the host defense at multiple levels. Their countermeasures can target innate immune sensing and signaling pathways, control the production of antiviral or inflammatory molecules, suppress the MHC gene expression, interfere with cytotoxic T cell and B cell responses and modulate apoptosis of infected cells (Alcami and Koszinowski, 2000, Smith, 1994, Tortorella et al., 2000). Among a variety of strategies, how RNA viruses evade the host innate immunity represented by type I IFN production and pro-inflammatory responses is reviewed as following examples:

i) Viruses can sequester viral RNA to escape from host responses. DENV has a strategy to hide its dsRNA in the intracellular membrane to avoid IFN induction (Uchida et al., 2014). An Ebola virus protein binds viral dsRNA to prevent it from being recognized by RIG-I (Cárdenas et al., 2006).

ii) Viral proteins can directly interact with the host PRRs/adaptor proteins. Respiratory syncytial virus (RSV) non-structural proteins interact either with RIG-I to inhibit IFN transcription or with MAVS to dissociate the RIG-I-MAVS interaction (Boyapalle et al., 2012, Ling et al., 2009). Enterovirus 71 protease 3C associates with RIG-I and inhibits the recruitment of MAVS (Lei et al., 2010), while foot-and-mouth disease virus 3A protein interacts with RIG-I and MAVS to inhibit IFN-β signaling (Li et al., 2016b).

iii) Viruses degrade or cleave host PRRs/adaptor proteins. This mechanism is well-established in picornaviruses that express viral proteases. Encephalomyocarditis virus induces the degradation of RIG-I (Papon et al., 2009), while hepatitis A virus proteases cleave MAVS and NEMO to inhibit IFN induction (Wang et al., 2014a, Yang et al., 2007). Cleavage of TRIF by proteases of hepatitis A virus, coxsackievirus B3 and enterovirus 68 is known to suppress IFN induction (Mukherjee et al., 2011, Qu et al., 2011, Xiang et al., 2014). For other viruses rather than picornaviruses, rotavirus and classical swine fever virus proteins induce the proteasomal degradation of IRF3 (Barro and Patton, 2005, Bauhofer et al., 2007).

iv) Viruses inhibit the downstream signaling proteins of PRRs. NS3/4A protease of HCV inhibits the phosphorylation of IRF3 and IRF3-mediated promoter activation (Foy et al., 2003). Non-structural proteins of PRRSV inhibit the phosphorylation and nuclear translocation of IRF3 (Beura et al., 2010, Li et al., 2010), which is also demonstrated by non-structural proteins of

DENV and West Nile virus (WNV) (Angleró-Rodríguez et al., 2014, Dalrymple et al., 2015). HCV NS3 protein interacts with TBK1, thereby inhibiting the association between TBK1 and IRF3 (Otsuka et al., 2005).

v) Viruses can suppress ubiquitination-mediated antiviral responses. Ubiquitination is involved in the activation of the host IFN induction pathway, and cysteine proteases from viruses can function as deubiquitinases to suppress the ubiquitination-dependent IFN induction (Liu et al., 2013b). The cysteine protease domain in NSP2 of PRRSV inhibits the ubiquitination of IκBα and prevents IκBα degradation and subsequently, IFN induction (Sun et al., 2010). Also, hepatitis E virus ORF1 has a cysteine protease domain that functions as a deubiquitinase for RIG-I and TBK1, whose ubiquitination is critical for IFN induction (Nan et al., 2014).

vi) Viruses inhibit the IFN-induced JAK/STAT pathway. Non-structural proteins of RSV mediate the inhibition of STAT2 expression and type I IFN responsiveness (Lo et al., 2005). Chikungunya virus NSP2 inhibits the IFN-stimulated Janus kinases (JAK)/signal transducer and activator of transcription (STAT) signaling by blocking STAT1 phosphorylation and nuclear translocation (Fros et al., 2010). In addition, NSP5 of porcine deltacoronavirus cleaves STAT2 to impair IFN signaling (Zhu et al., 2017). Multiple non-structural proteins of flaviviruses prevent the activation of STAT1 and/or STAT2. NS5 proteins of both DENV and WNV inhibit the accumulation of phosphorylated STAT1 (Laurent-Rolle et al., 2010, Mazzon et al., 2009); NS2A, NS4A and NS4B proteins of DENV inhibit STAT1 phosphorylation, while NS4B of WNV blocks the auto-phosphorylation and activation of JAK1 (Gack and Diamond, 2016); Zika virus NS5 causes the proteasomal degradation of STAT2 (Grant et al., 2016, Kumar et al., 2016).

vii) Viruses can interfere with the cGAS/STING pathway. DENV protease complex cleaves STING to inhibit type I IFN induction (Aguirre et al., 2012). Coronavirus papain-like proteases suppress the dimerization of STING and assembly of the STING-MAVS-TBK1/IKKε complex (Sun et al., 2012). The same protein disrupts the STING-TRAF3-TBK1 interaction and inhibits the ubiquitination of the STING-TRAF3-TBK1 complex (Chen et al., 2014).

viii) Viruses inhibit NF-κB activity and/or secretion of pro-inflammatory cytokines. NF-κB activation is blocked by HCV NS5B (Choi et al., 2006) or enterovirus 68 3C protease (Xiang et al., 2014). Multiple proteins of porcine epidemic diarrhea virus can suppress NF-κB-mediated pro-inflammatory cytokines (Zhang et al., 2017b). Poliovirus 3A protein inhibits the secretion of IL-6 and IL-8 (Dodd et al., 2001), and Rift Valley Fever virus NSs are responsible for blocking

the production of pro-inflammatory cytokines including TNF-α (McElroy and Nichol, 2012).

ix) Viruses inhibit NLRP3 inflammasome-mediated IL-1β production. Viral proteases of enterovirus 71 suppress NLRP3 inflammasome activity by cleaving NLRP3 (Wang et al., 2015b). V proteins of paramyxoviruses (measles virus, Sendai virus, Nipah virus and human parainfluenza virus type 2) interact with NLRP3, thereby inhibiting the NLRP3 inflammasome-mediated IL-1β production (Komatsu et al., 2018, Komune et al., 2011). C protein of another paramyxovirus, human parainfluenza virus type 3 also interacts with NLRP3 to downregulate the NLRP3 inflammasome (Shil et al., 2018).

1.5.2 Immune evasion by non-structural proteins of IAV

1.5.2.1 NS1 protein

1.5.2.1.1 NS1 antagonizing the host immune signaling

NS1 blocks the nuclear accumulation of IRF3 (Talon et al., 2000); this is performed at the pre-transcriptional level and the residues (arginine 38 and lysine 41) critical for NS1's RNA-binding activity are required for this inhibition (Talon et al., 2000). The dsRNA-binding activity of NS1 also suppresses NF-κB-mediated IFN induction (Wang et al., 2000). A cytosolic antiviral protein, OAS that detects viral dsRNA is also targeted by NS1. Activated OAS produces 2'-5'-oligoadenylate that binds and activates RNase L, which can cleave viral RNA (Silverman, 2007); the degraded products activate RIG-I and induce IFNs. Through the dsRNA-binding activity, NS1 sequesters viral RNA from OAS, thereby suppressing the RNase L-mediated restriction of IAV replication (Min and Krug, 2006).

NS1 can also counteract the antiviral responses played by PKR, a dsRNA-dependent antiviral protein (Bergmann et al., 2000). PKR activation by dsRNA leads to the phosphorylation of its target proteins (Hale et al., 2008). Importantly, phosphorylation of eIF2α leads to the inhibition of protein synthesis in infected cells (Hatada et al., 1999). In IAV-infected cells, binding of NS1 to PKR diminishes the PKR activation (Min et al., 2007).

While viral 5'-triphosphate dsRNA is sensed by RIG-I that is important for IFN signaling pathway, NS1 mediates the pre-transcriptional block of IFN-β induction by interacting with RIG-I (Guo et al., 2007, Pichlmair et al., 2006). After binding to viral RNA, RIG-I undergoes a conformational change by exposing its N-terminal CARDs, which are subject to

ubiquitination by E3 ligases, TRIM25 or Riplet; this action is required for IRF3 activation and IFN induction (Ayllon and García-Sastre, 2014). Binding of NS1 to TRIM25 blocks the formation of oligomerized RIG-I and represses the TRIM25-mediated ubiquitination on the CARD of RIG-I (Gack et al., 2009, Mibayashi et al., 2007). Interaction of NS1 with another E3 ligase, Riplet blocks its activation on RIG-I as well (Rajsbaum et al., 2012).

Phosphorylation and nuclear translocation of STAT proteins, which are required for the IFN response, are also blocked by NS1 (Jia et al., 2010). In addition, IFN-induced transmembrane protein 3 (IFITM3) can block the IAV entry and replication; its expression is regulated by the eukaryotic translation initiation factor 4B (eIF4B). NS1 induces the degradation of eIF4B to limit the action of IFITM3 (Wang et al., 2014b).

NS1 participates in the regulation of pro-inflammatory cytokines, with IL-1β that is known to be beneficial for the host (Allen et al., 2009, Schmitz et al., 2005) being one of them. NS1 inhibits NLRP3 inflammasome-mediated IL-1β production by interacting with NLRP3 (Cheong et al., 2015, Moriyama et al., 2016).

1.5.2.1.2 NS1 regulating the host cell death

Apoptosis is one of antiviral strategies used by the host to suppress viral replication, although some pro-apoptotic factors have roles to promote viral propagation (Hale et al., 2008). The suppression of apoptosis during early infection can promote viral replication, while apoptosis during late infection can support the release of progeny viruses, hence the regulation of both pro- and anti-apoptotic mechanisms can be critical for viruses (Hale et al., 2008). NS1 has both pro- and anti-apoptotic roles (Ehrhardt et al., 2006, Lam et al., 2008, Shin et al., 2007a, Zhirnov et al., 2002) and NS1 is thought to contribute to the suppression and induction of apoptosis during the early and late phases of infection, respectively (Hale et al., 2008). PKR plays a role in apoptosis during IAV infection, with the inhibition of PKR by NS1 possibly leading to the establishment of an anti-apoptotic status (Takizawa et al., 1996). Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) produces the messenger molecule, phosphatidylinositol (3,4,5)-trisphosphate (PIP3); PIP3 binding by a serine/threonine protein kinase, Akt is involved in the maintenance of anti-apoptotic status (Hale et al., 2008). During the early phase of infection with IAV, PI3K activation is turned on by the NS1 interaction with the p85β subunit of PI3K, leading to the restriction of apoptosis (Ehrhardt et al., 2006, Li et al.,

2008, Shin et al., 2007c), suggesting that IAV-induced early apoptosis is inhibited by NS1.

1.5.2.2 PB1-F2 and PA-X

While IAV polymerase proteins, PB2, PB1 and PA can downregulate host innate immunity (Graef et al., 2010, Iwai et al., 2010, Yi et al., 2017), non-structural proteins derived from IAV polymerase segments can also exert inhibitory functions. PB1-F2 is encoded by +1 open reading frame (ORF) in the PB1 segment and its presence is strain-dependent (Chen et al., 2001). In addition to being pro-apoptotic, it has multiple functions such as the contribution to virulence, and the regulation of inflammatory or antiviral activities (Klemm et al., 2018). To antagonize the IFN signaling, PB1-F2 interacts with MAVS or inhibits the RIG-I-MAVS complex formation (Dudek et al., 2011, Varga et al., 2012). Dissipated mitochondrial membrane potential by mitochondrial localization of PB1-F2 not only blocks the RIG-I signaling, but also interferes with NLRP3 inflammasome activation (Yoshizumi et al., 2014). PB1-F2, in a strain-dependent way, also modulates the production of pro-inflammatory cytokines (Lee et al., 2017a, Pena et al., 2012). PA-X is expressed from the PA segment as a result of ribosomal frameshifting (Jagger et al., 2012). With its N-terminal endonuclease domain shared with PA, it exerts host shutoff effects by degrading host mRNAs (Desmet et al., 2013, Khaperskyy et al., 2016). PA-X inhibits the host IFN or pro-inflammatory responses, but promotes viral growth or increases virulence (Hayashi et al., 2015, Lee et al., 2017b, Xu et al., 2017). On the contrary, PA-X can repress both host immune responses and viral replication, which lead to decreased pathogenicity (Gao et al., 2015, Hu et al., 2015).

1.6 Conclusions of the literature review

Innate immune responses are critical in determining the consequences of disease outcomes in virus-infected hosts. Among different pathways that regulate the host response, NLRP3 inflammasome-mediated IL-1β production is a key process in inflammation that is required for the elimination of viruses. This literature review has provided knowledge on the immunology of IAV infection and innate immunity focusing on inflammasomes and IL-1β. The mechanisms of NLRP3 inflammasome-mediated IL-1β production, particularly the ones regulated by ubiquitination and the roles of mitochondria on the NLRP3 inflammasome activation were

discussed. In addition, the immune evasion strategies exerted by non-structural proteins of IAVs were explained.

How IAV infection induces NLRP3 inflammasome-mediated IL-1β production in the swine host is elusive, and as a part of immune evasion, whether IAV proteins, particularly NS1 protein, contribute to the regulation of porcine NLRP3 inflammasome activation awaits elucidation. Moreover, how ubiquitination fits in the modulation of the porcine NLRP3 inflammasome requires clarification. Answering these questions further expands the understanding of the host innate immunity upon IAV infection in pigs.

CHAPTER 2 RATIONALE, HYPOTHESIS AND OBJECTIVE

2.1 Rationale

Influenza A virus (IAV) causes annual outbreaks of respiratory infections worldwide and novel reassortant strains can emerge as pandemic viruses. Host responses against the IAV infection, particularly the innate immune responses, determine the outcome of infection. Activation of the innate immune system not only clears the virus, but also leads to the effective protection by adaptive immune responses with virus-specific antibodies. In response to the IAV infection, which initially targets the respiratory epithelium, alveolar macrophages (AMs) play an important role in controlling the IAV-induced inflammation. AMs secrete cytokines such as IL-1β to mediate the inflammation and recruit other immune cells upon recognition of the IAV infection. While IAV-induced IL-1β production is known to be NLRP3 inflammasome-mediated in human and mouse, it is overlooked in swine. Particularly, the underlying mechanisms of the NLRP inflammasome regulation in the swine host, and how viral proteins control the porcine IL-1β signaling are poorly understood. Given the important role of AMs in control of IAV infection and the well-known function of viral NS1 protein in antagonizing the host innate immune responses, I believe that investigation of how IAV infection triggers the NLRP3 inflammasome-mediated IL-1β production in porcine alveolar macrophages (PAMs) is a key to the understanding of the innate immunity to IAV infection. Further, elucidating the roles of viral proteins, specifically NS1, in regulating the porcine NLRP3 inflammasome will provide the essential knowledge on the IAV-host cell interactions. The information obtained through this study will pave the way to a deeper insight into the immunology of influenza infection in pigs.

2.2 Hypothesis and objective

Based on the literature, I hypothesized that NLRP3 inflammasome-mediated IL-1β production occurs in PAMs upon IAV infection and viral NS1 protein contributes to the inhibition of the NLRP3 inflammasome. The main objective of this study is to characterize the NLRP3 inflammasome-mediated IL-1β production in response to IAVs including swine influenza virus (SIV) as well as to identify the signaling pathway involved in IAV-induced IL-1β production, and to define the mechanisms exerted by viral NS1 proteins on the porcine NLRP3 inflammasome. To achieve the objective, I proposed the following specific aims:

Aim 1. Characterization of IL-1β production from PAMs infected with different IAV strains.

Aim 2. Investigation of the mechanisms by which viral NS1 protein regulates porcine NLRP3 inflammasome activation.

Aim 3. Identification of the signaling pathway through which SIV infection induces porcine NLRP3 inflammasome-mediated IL-1β production.

CHAPTER 3 INFLUENZA A VIRUS INDUCES NLRP3 INFLAMMASOME-MEDIATED IL-1β PRODUCTION IN PORCINE ALVEOLAR MACROPHAGES.

Portions of the data presented in this chapter were published in

NS1 Protein of 2009 Pandemic Influenza A Virus Inhibits Porcine NLRP3 Inflammasome-Mediated Interleukin-1 Beta Production by Suppressing ASC Ubiquitination.

HS Park, G Liu, SN Thulasi Raman, SL Landreth, Q Liu and Y Zhou. (2018)

Journal of Virology, 92(8), e00022-18.

3.1 Abstract

The inflammasome is a molecular platform critical for regulating pro-inflammatory cytokine production. NLR-family pyrin domain-containing protein 3 (NLRP3) inflammasome is comprised of NLRP3, apoptosis-associated speck-like protein containing caspase activation and recruitment domain (ASC) and pro-caspase-1. For NLRP3 inflammasome activation, NLRP3 aggregates with ASC to form ASC specks where pro-caspase-1 is recruited, and then caspase-1 is activated for the conversion of pro-IL-1β into IL-1β. Influenza A virus (IAV) infection can induce NLRP3 inflammasome-mediated IL-1β secretion in human and mouse, and multiple N-terminal amino acid clusters of NS1 protein of different IAV strains are reported to suppress the NLRP3 inflammasome. However, whether IAV infection induces NLRP3 inflammasome-mediated IL-1β production in porcine macrophages is not defined. Therefore, in this study, porcine IL-1β production in response to different IAVs including swine influenza viruses (SIVs) and the 2009 human pandemic H1N1 (pdm09) was characterized. The results showed SIV-induced IL-1β production from porcine alveolar macrophages (PAMs) was mediated by toll-like receptor 3 and NLRP3 inflammasome. While SIV and pdm09 induced comparable levels of pro-IL-1β expression and *NLRP3* transcription, the pdm09 induced less IL-1β than SIV did. Further study revealed that NS1 C-terminus of pdm09, but not that of SIV, could significantly inhibit the NLRP3 inflammasome-mediated IL-1β production. This inhibition was attributed to the limited interaction between NLRP3 and ASC, which led to the impaired ASC speck formation. These results showed how IAVs induce IL-1β production in PAMs and revealed a novel mechanism by which NS1 protein of the pdm09 suppresses the NLRP3 inflammasome activation.

3.2 Introduction

Pattern recognition receptors (PRRs) play important roles in host innate immune responses to pathogens (Chen and Ichinohe, 2015). Nucleotide-binding domain and leucine-rich repeat-containing proteins (NLRs) are one of the families of PRRs, among which the NLR-family pyrin domain-containing protein 3 (NLRP3) has been extensively investigated. With apoptosis-associated speck-like protein containing caspase activation and recruitment domain (ASC) and pro-caspase-1, the NLRP3 forms a cytosolic protein complex called NLRP3 inflammasome (Chen and Ichinohe, 2015, Guo et al., 2015). When NLRP3 inflammasome is activated in response to a variety of pathogen-associated molecular patterns (PAMPs), it can then regulate the

secretion of inflammatory cytokines including IL-1β, which is a major mediator for inflammation (Chen and Ichinohe, 2015, Guo et al., 2015). IL-1β is synthesized as an immature form, pro-IL-1β; maturation of pro-IL-1β relies on the NLRP3 inflammasome activation. This activation occurs by the formation of ASC specks and activation of caspase-1, which is converted from pro-caspase-1 through an auto-processing mechanism (Chen and Ichinohe, 2015, Guo et al., 2015).

NLRP3 inflammasome-mediated IL-1β production plays a critical role in modulating host immunity to IAV infection and IAV-induced lung pathology (Allen et al., 2009, Kanneganti et al., 2006a, Thomas et al., 2009). Sensing viral RNAs by toll-like receptors (TLRs) or retinoic acid-inducible gene-I (RIG-I)-like receptors leads to the expression of pro-IL-1β and NLRP3 through the NF-κB pathway (signal 1) (Iwasaki and Pillai, 2014, Ong et al., 2016). Recently, Z-DNA binding protein 1 (ZBP1) is identified as another sensor of IAV for the NLRP3 inflammasome activation and inflammatory responses (Kuriakose et al., 2016). Activation of NLRP3 inflammasome requires the second signal (signal 2) that includes the recognition of viral components or virus-induced intracellular processes through mechanisms that are less clearly understood (Iwasaki and Pillai, 2014, Ong et al., 2016). While viral RNA itself is shown to activate NLRP3 inflammasome (Allen et al., 2009, Thomas et al., 2009), different viral proteins of IAVs are reported to regulate the NLRP3 inflammasome. For example, PB1-F2 protein can activate or inhibit the NLRP3 inflammasome in a viral strain-dependent manner (McAuley et al., 2013, Pinar et al., 2017, Yoshizumi et al., 2014), whereas the M2 protein contributes to the NLRP3 inflammasome-mediated IL-1β production through its ion channel activity (Ichinohe et al., 2010). NS1 protein, which is well known for its interferon (IFN) antagonistic function (Krug, 2015), is reported to inhibit both caspase-1 activation (Stasakova et al., 2005) and NLRP3-mediated IL-1β production (Cheong et al., 2015, Moriyama et al., 2016, Pothlichet et al., 2013). However, there is still a lack of knowledge on how NLRP3 inflammasome-mediated IL-1β production is regulated in porcine cells upon IAV infection.

In this chapter, the NLRP3 inflammasome-mediated IL-1β production in primary porcine alveolar macrophages (PAMs) upon infection with different IAVs was characterized. The negative regulation of porcine NLRP3 inflammasome activity by pdm09 NS1 protein was demonstrated as well.

3.3 Materials and methods

3.3.1 Isolation of porcine alveolar macrophages (PAMs)

Sera from 4- to 7-week-old piglets in SIV-free farms were verified by antigen-specific enzyme-linked immunosorbent assay (ELISA) using purified SIVs as antigens. The pre-screened piglets that were confirmed to be sero-negative against H1N1 and H3N2 subtypes of SIVs were selected for PAM isolation from bronchoalveolar lavage fluid (BALF), where most of the cells are comprised of alveolar macrophages (Ait-Ali et al., 2007, Gordon and Read, 2002). The purity of cells isolated this way was assessed by flow cytometry using mouse anti-pig macrophage antibody conjugated with fluorescein isothiocyanate (MCA2317F, Bio-Rad) as described (Liu et al., 2015). All experimental procedures were in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Saskatchewan Animal Research Ethics Committee. The selected piglets were euthanized and sacrificed by the VIDO-InterVac animal care staff according to the approved protocol. Phosphate-buffered saline (PBS) with antibiotics (PBS-Anti) was prepared by combining PBS with 50 μg/ml gentamicin (BS724, Bio Basic Canada) and 1 × Antibiotic-Antimycotic (15240-062, Thermo Fisher). The BALF from each piglet was collected by adding 50 ml of cold PBS-Anti into trachea and gently squeezing both sides of lungs under sterile conditions. Mucus and debris were excluded from BALF by using 40 μm cell strainers (352340, Corning). After centrifuging at 400 × *g* for 10 min at 4℃, the cell pellets were resuspended in 20 ml cold PBS-Anti on ice. After another centrifugation, the cells were resuspended in 2 ml ammonium chloride lysis buffer (139 mM NH_4Cl, 17 mM Tris, pH 7.2) and left for 1 min to lyse erythrocytes. PBS-Anti was added up to 20 ml and the cells were centrifuged as above. Lysed erythrocytes were removed by micropipetting. The cells were further resuspended with 20 ml PBS-Anti and pelleted down as above conditions once more. The cells were resuspended in complete RPMI 1640 media (HyClone RPMI 1640 [SH30027.01, GE Healthcare], 20% fetal bovine serum [FBS, 16000-044, Thermo Fisher], 10 mM HEPES [15630-080, Thermo Fisher], 50 μg/ml gentamicin, 1 × Antibiotic-Antimycotic) and counted. After another round of centrifugation, the cells were resuspended in complete RPMI 1640 media with 10% dimethyl sulfoxide (DMSO, D2660, Sigma). The cells were frozen at -80℃ overnight in freezing containers (5100-0001, Nalgene) and moved to a liquid nitrogen tank for long-term storage, while small aliquots of the cells from

each individual piglet were seeded to test the background levels of IL-1β. Based on the results of porcine IL-1β ELISA, the cells with less background of IL-1β were selected for further use.

3.3.2 Cell lines and viruses

Madin-Darby canine kidney (MDCK) cells were cultured with minimum essential medium (MEM, M4655, Sigma) supplemented with 10% FBS and 50 μg/ml gentamicin. Human embryonic kidney 293T (HEK293T) cells were cultured with Dulbecco's modified Eagle's medium (DMEM, D5796, Sigma) supplemented with 10% FBS and 50 μg/ml gentamicin. The wild-type (WT) IAVs used were influenza A/swine/Saskatchewan/18789/2002/H1N1 (Sk02), influenza A/swine/Texas/4199-2/1998/H3N2 (Tx98) and influenza A/Halifax/210/2009/H1N1 (Hf09) (Table 3.1). A mutant Sk02 virus (Sk02-NS1/1-99) containing only aa 1 to 99 of the N-terminal NS1 was described previously (Liu et al., 2015). Additional mutant viruses were generated as described in the section 3.3.3. All mutant viruses with truncated NS1 were propagated in MDCK cells stably expressing NS1 protein of influenza A/Puerto Rico/8/1934/H1N1 (PR8) strain (MDCK-NS1), while others were propagated in MDCK cells. Viral titres were determined by plaque assay in either MDCK or MDCK-NS1 cells.

3.3.3 Generation of mutant viruses

Mutant viruses were rescued by the eight-plasmid reverse genetics system as previously established and conducted (Hoffmann et al., 2000, Masic et al., 2009a). This system requires the WT or mutant cDNA of each IAV segment cloned into the pHW2000 vector that is driven by RNA polymerase I and II (Hoffmann et al., 2000). Four mutant viruses were generated in this study as indicated in Table 3.1.

Hf09-816 and Hf09-817 are two isogenic Hf09 viruses that encode truncated NS1, i.e. aa 1-73 or 1-99, respectively. The mutant Hf09 NS segments harbouring the truncated NS1 coding sequence were generated by site-directed mutagenesis. Briefly, three stop codons (for each ORF) were inserted after the 73rd or the 99th aa of NS1 (while not changing the NEP coding sequence) by overlapping PCR using appropriate mutagenic primers (Supplementary Table 1) and pHW-Hf09-NS as the template. The fragments were cloned into *Sph*I and *Nco*I restriction sites of the pHW2000 vector and the positive clones were designated as pHW-Hf09-NS-816 and pHW-Hf09-NS-817 for $NS1_{1-73}$ and $NS1_{1-99}$, respectively. Each of these NS segments and other seven

Hf09 segments in the pHW2000 vector were used to rescue the Hf09-816 and Hf09-817 viruses (Table 3.1).

Sk02-616, an isogenic Sk02 virus that contains the NS segment of Hf09 was rescued using pHW-Hf09-NS and other seven Sk02 segments in the pHW2000 vector. Sk02-930, is another isogenic Sk02 virus, but contains a chimeric NS segment encoding NS1 with aa 1 to 99 from Sk02 and aa 100 to 219 from Hf09. Two PCR products containing either Sk02 $NS1_{1\text{-}99}$ or Hf09 $NS1_{100\text{-}219}$ were amplified and joined by overlapping PCR using mutagenic primers (Supplementary Table 1) with the WT NS segment of Sk02 (pHW-Sk02-NS) or Hf09 (pHW-Hf09-NS) as the templates. The chimeric fragment was cloned into *Sal*I and *Nae*I restriction sites of the pHW2000 vector and the successful clone was designated as pHW-Sk02/Hf09-NS-930. This chimeric NS segment and other seven Sk02 segments in the pHW2000 vector were used to rescue the Sk02-930 virus (Table 3.1).

The viruses were rescued by co-transfection system as follows. On 35 mm × 10 mm dishes (#3294, Corning), 2.2×10^5 cells/dish of MDCK cells and 3.0×10^5 cells/dish of HEK293T cells were seeded in 1.2 ml per dish of complete DMEM. Next day, the cells were transfected by adding a mixture of 1 μg each of the eight pHW2000-derived constructs plus 16 μl of *Trans*IT-LT1 (MIR2300, Mirus Bio) in 240 μl Opti-MEM (31985-070, Thermo Fisher). After 6 h, the media was replaced by 1 ml Opti-MEM. At 24 h post-transfection (hpt), another 1 ml of Opti-MEM containing 2 μl of 1 mg/ml tosyl phenylalanyl chloromethyl ketone-treated trypsin (22725, Affymetrix) and 80 μl of 5% bovine serum albumin (BSA) (A8806, Sigma) in distilled water was added into each dish. When the cells show prominent cytopathic effects (CPEs) between 48 and 72 hpt, the supernatants containing the viruses were harvested. The rescued viruses were passaged twice on MDCK cells and titrated by plaque assay.

Table 3.1 List of WT and mutant viruses used in this study.

Viruses from previous studies		
Virus	NS1 aa in length	Reference
Sk02 (WT)	1 to 230	(Masic et al., 2009b)
Tx98 (WT)	1 to 219	(Masic et al., 2009b)
Hf09 (WT)	1 to 219	(Lin et al., 2012)
Sk02-NS1/1-99	1 to 99	(Liu et al., 2015)

Viruses generated in this study		
Virus	NS1 composition and aa in length	NS construct used to rescue the virus
Hf09-816	Hf09 1 to 73	pHW-Hf09-NS-816
Hf09-817	Hf09 1 to 99	pHW-Hf09-NS-817
Sk02-616	Hf09 1 to 219	pHW-Hf09-NS
Sk02-930	Sk02 1 to 99 and Hf09 100 to 219	pHW-Sk02/Hf09-NS-930

3.3.4 Antibodies and reagents

Rabbit polyclonal antibodies against IAV NP and NS1 were previously generated in our laboratory (Shin et al., 2007b). Goat polyclonal anti-porcine IL-1β antibody (BAF681, R&D Systems), rabbit polyclonal anti-porcine caspase-1 (p20) antibody (PAB592Po01, Cloud-Clone Corp), mouse monoclonal anti-β-actin antibody (3700, Cell Signaling Technology, CST), mouse monoclonal anti-Myc-tag antibody (2276, CST), mouse monoclonal anti-FLAG M2 antibody (F3165, Sigma) and rabbit polyclonal anti-ASC antibody (AG-25B-006, Adipogen) were purchased from different sources. For the secondary antibodies, IRDye 680RD donkey anti-rabbit (926-68073), IRDye 800CW donkey anti-mouse (926-32212) and IRDye 800CW donkey anti-goat (926-32214) antibodies were purchased from LI-COR Biosciences. Transfection was conducted using *Trans*IT-LT1 Transfection Reagent. Ethylenediaminetetraacetic acid (EDTA)-

free protease inhibitor cocktail tablets (4693132001, Roche) were used in cell lysis and immunoprecipitation (IP). An NLRP3 inhibitor, 3,4-methylenedioxy-β-nitrostyrene (MNS) (574713, EMD Millipore) was used as previously reported (He et al., 2014) and DMSO was used as the vehicle.

3.3.5 Plasmid construction

Total RNA was extracted from PAMs stimulated with LPS (L3024, Sigma) and ATP (A1852, Sigma) by using RNeasy Mini Kit (74104, QIAGEN), and then cDNA was synthesized using 2.5 μM oligo (dT), 50 ng random hexamers (N8080127, Thermo Fisher), 0.5 mM dNTP mix (18427088, Thermo Fisher) and SuperScript III Reverse Transcriptase (18080044, Thermo Fisher). The full-length genes of porcine NLRP3, ASC, pro-caspase-1 and pro-IL-1β were amplified using HotStar HiFidelity Polymerase Kit (202602, QIAGEN) and primers that were designed based on the GenBank sequences (accession numbers NM_001256770 [NLRP3], AB873106 [ASC], AK231984 [pro-caspase-1] and NM_214055 [pro-IL-1β]). The genes were cloned into pcDNA3.1 or pCMV-3×Flag (N-terminal tag) vectors and the resulting plasmids were designated as pcDNA-NLRP3, pcDNA-ASC, pCMV-Flag-pro-caspase-1 and pcDNA-pro-IL-1β. Additionally, the full-length genes of porcine NLRP3 and ASC were cloned into pCMV-3×Flag (N-terminal tag) or pcDNA3.1-3×Myc (C-terminal tag) vector by amplifying the genes from pcDNA-NLRP3 or pcDNA-ASC. The resulting plasmids were designated as pcDNA-NLRP3-Myc, pCMV-Flag-NLRP3, pcDNA-ASC-Myc and pCMV-Flag-ASC. WT and truncated NS1 of Hf09 were cloned into pCMV-3×Flag vector and the resulting plasmids were designated as pCMV-Flag-Hf09 NS1, pCMV-Flag-Hf09 $NS1_{1-73}$ and pCMV-Flag-Hf09 $NS1_{1-99}$, encoding the full-length, N-terminal aa 1 to 73 and aa 1 to 99 of NS1, respectively. NS1 C-termini of Hf09 and Sk02 were cloned into pcDNA3.1-3×Myc and designated as pcDNA-Hf09 $NS1_{100-219}$-Myc and pcDNA-Sk02 $NS1_{100-230}$-Myc, respectively. The successful construction of plasmids was confirmed by DNA sequencing. The expression plasmids generated for this chapter are listed in Table 3.2 and the primers are listed in Supplementary Table 2.

Table 3.2 List of expression plasmids used in Chapter 3.

Construct	Template used to clone	Purpose
pcDNA-NLRP3	cDNA from PAMs	NLRP3 inflammasome reconstitution
pcDNA-ASC	cDNA from PAMs	NLRP3 inflammasome reconstitution, NLRP3-ASC interaction
pCMV-Flag-pro-caspase-1	cDNA from PAMs	NLRP3 inflammasome reconstitution
pcDNA-pro-IL-1β	cDNA from PAMs	NLRP3 inflammasome reconstitution
pcDNA-NLRP3-Myc	pcDNA-NLRP3	NLRP3-ASC interaction, ASC speck formation
pCMV-Flag-NLRP3	pcDNA-NLRP3	NLRP3-ASC interaction
pcDNA-ASC-Myc	pcDNA-ASC	NLRP3-ASC interaction
pCMV-Flag-ASC	pcDNA-ASC	ASC speck formation
pCMV-Flag-Hf09 NS1	pcDNA-Hf09-NS1-Myc	NLRP3 inflammasome reconstitution, NLRP3-ASC interaction
pCMV-Flag-Hf09-$NS1_{1-73}$	pcDNA-Hf09-NS1-Myc	NLRP3 inflammasome reconstitution, NLRP3-ASC interaction
pCMV-Flag-Hf09-$NS1_{1-99}$	pcDNA-Hf09-NS1-Myc	NLRP3 inflammasome reconstitution, NLRP3-ASC interaction
pcDNA-Hf09 $NS1_{100-219}$-Myc	pHW-Hf09-NS	NLRP3 inflammasome reconstitution
pcDNA-Sk02 $NS1_{100-230}$-Myc	pHW-Sk02-NS	NLRP3 inflammasome reconstitution

3.3.6 Infection and treatment of PAMs

PAMs were seeded on 24-well plates (#3524, Corning) at 1 × 10^6 cells per well considering the low proliferating capacity of alveolar macrophages (Bowden and Adamson, 1972, Nyunoya et al., 2003). The cells were infected with viruses at a multiplicity of infection (MOI) of 1. At indicated time points, cell-free supernatants were collected for ELISA, while the cells were lysed with 1 × sodium dodecyl sulfate (SDS) sample buffer and boiled at 95℃ for 5 min for Western blotting. For time-course experiments, the supernatants collected at 8, 16 and 24 h post-infection (hpi) were used for both ELISA and plaque assay in MDCK cells. To check the mRNA expression of porcine *NLRP3* upon infection, PAMs were infected with Sk02 or Hf09 at an MOI of 1 for 12 h. As a positive control, the cells were stimulated with 200 ng/ml LPS for 12 h.

3.3.7 Virus purification

MDCK cells were seeded at 3 × 10^6 cells/dish on ten 100 mm × 20 mm dishes (430167, Corning) and were infected with Sk02 or Tx98 at an MOI of 0.001. Viral fluid was harvested at full CPEs at around 48 hpi. After centrifugation at 300 × *g* for 10 min, the supernatants were transferred to ultracentrifuge tubes (Thinwall, Ultra-Clear, 25 × 89 mm, 344058, Beckman Coulter) and centrifuged at 25,000 rpm for 2.5 h using a SW 28 rotor. The supernatants were discarded and 100 μl Tris-buffered saline (TBS) with EDTA (20 mM Tris, pH 7.8, 150 mM NaCl, 2 mM EDTA disodium salt) (TSE) was added to resuspend the pellets followed by incubation at 4℃ overnight. Next day, 2.5 ml 60% sucrose in TSE, 5 ml 30% sucrose in TSE and the resuspended pellets in TSE were added into ultracentrifuge tubes (Thinwall, Ultra-Clear, 14 × 89 mm, 344059, Beckman Coulter) and centrifuged at 25,000 rpm for 2.5 h using a SW 41 rotor. Viral band was collected and transferred to new ultracentrifuge tubes (Thinwall, Ultra-Clear, 14 × 89 mm, 344059, Beckman Coulter) that were filled by 8 ml TSE. After centrifugation at 35,000 rpm for 1.5 h, the supernatants were discarded and 200 μl TSE were added followed by incubation at 4℃ overnight. The resuspended virus was transferred to a microcentrifuge tube and the bottom of ultracentrifuge tubes was further washed with additional 500 μl TSE. The purified viruses were stored at -80℃ until further use.

3.3.8 UV inactivation of viruses

WT SIVs, Sk02 and Tx98 were inactivated by UV irradiation as described previously (Shin et al., 2007b) with modifications. Briefly, 0.5 ml of each virus on a 35-mm dish without a lid was placed on ice. The viruses were left with a distance of 20 cm from a 30-watt UV lamp for 20 min.

3.3.9 RNA interference

Small interfering RNAs (siRNAs) targeting porcine *TLR3* or *TLR7*, and a negative control siRNA were purchased from QIAGEN. PAMs were seeded on 24-well plates at 7×10^5 cells per well. Immediately after seeding, a mixture of 20 nM of each siRNA with 2 μl Lipofectamine RNAiMax Reagent (13778-030, Thermo Fisher) prepared in 50 μl Opti-MEM was added into each well. At 24 hpt, the cells were infected with Sk02 at an MOI of 1. At 24 hpi, cell-free supernatants were collected for IL-1β ELISA. Knockdown efficiency was measured by quantitative PCR as described in the section 3.3.10.

3.3.10 Quantitative PCR

Total RNA from PAMs was extracted with RNeasy Mini kit (74104, QIAGEN) according to the manufacturer's protocol. Two hundred ng of RNA were treated with 1 U of DNase I (18068-015, Thermo Fisher) at room temperature for 15 min and incubated with 1 μl of 25 mM EDTA at 65℃ for 10 min. Half of the above reaction volume was used for the denaturation step using 2.5 μM oligo (dT) and 0.5 mM dNTP mix (18427088, Thermo Fisher) by incubating at 65℃ for 5 min followed by placing on ice. Then, the first-strand cDNA synthesis was completed by adding 5 mM dithiothreitol, 40 U of RNaseOUT RNase Inhibitor (10777-019, Thermo Fisher) and 200 U of SuperScript III Reverse Transcriptase (18080044, Thermo Fisher) and incubating at 50℃ for 50 min followed by 85℃ for 5 min. For quantitative real-time PCR, each cDNA (2 μl from the above reaction) was mixed with 0.2 μM each of forward and backward primers, and Power SYBR Green PCR Master Mix (4367659, Thermo Fisher). Porcine *hypoxanthine phosphoribosyltransferase 1* (*HPRT1*) was selected as the housekeeping gene and primers based on the GenBank sequence (accession number DQ845175) were used as reported (Nygard et al., 2007). Primers for porcine *TLR3*, *TLR7* and *NLRP3* were designed based on the GenBank sequences (accession numbers KC011280, JQ664686 and NM_001256770, respectively). Primer

sequences are shown in Table 3.3. Based on the threshold cycle of the target genes and the housekeeping gene, $2^{-(\Delta\Delta Ct)}$ calculation was used to measure the relative expression of the target genes by normalizing to the expression level of the housekeeping gene.

Table 3.3 List of primers used for quantitative PCR in Chapter 3.

Target	Primer name	Sequence (5'-3')	Amplicon size
Porcine *HPRT1*	HPRT1 Fw	GGACTTGAATCATGTTTGTG	91 bp
	HPRT1 Bw	CAGATGTTTCCAAACTCAAC	
Porcine *TLR3*	TLR3 Fw	GAACCATGCACTCTGTTTGC	101 bp
	TLR3 Bw	GCAGTTTGTGATGAAAGGCA	
Porcine *TLR7*	TLR7 Fw	TCAGAGGCTCATGGATGAAA	143 bp
	TLR7 Bw	GGTGAGCCTGTGGATTTGTT	
Porcine *NLRP3*	NLRP3 Fw	CCTCTTTGGCCTTGTAAACC	144 bp
	NLRP3 Bw	TGGCTGGGCTCAATCTGTAG	

bp, base pairs.

3.3.11 Antigen-specific IgG ELISA

Serum IgG specific for SIVs was determined as described previously (Masic et al., 2009b). Sk02 and Tx98 viruses were purified and UV-inactivated as described in the section 3.3.7 and 3.3.8 to be used as antigens. Immulon 2 HB U plates (3655, Thermo Fisher) were coated with 2.5 μg/ml of antigens in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6) and incubated at 4℃ overnight. All the following steps were performed at room temperature and all reagents or test sera were applied at 100 μl per well. The plates were washed four times with TBS (100 mM Tris, pH 7.5, 170 mM NaCl) with 0.05% Tween 20 (P1379, Sigma) (TBST) between each step. Next, the plates were blocked with 1% skim milk in TBST at room temperature for 1 h. Serum from each piglet that was diluted 1 to 100 in diluent (0.5% skim milk in TBST) was applied and incubated at room temperature for 1.5 h. Positive and negative control sera were also applied. The plates were incubated with alkaline phosphatase-labeled goat anti-swine IgG (H+L) (151-14-06, KPL) that was diluted at the ratio of 1:5,000. Colors were developed by adding 1 mg/ml of p-nitrophenyl phosphate (PNPP) (N3254, Sigma) in diethanolamine buffer (1 M diethanolamine, 0.5 M $MgCl_2$, pH 9.8). The optical density (OD)

was measured by reading at 405 nm with reference at 490 nm using xMark Microplate Absorbance Spectrophotometer (Bio-Rad). The highest dilution at which the OD of the test serum was higher than the cut-off value, which is the mean OD of the negative control sera plus two times the standard deviation (SD), was determined as the titre.

3.3.12 Porcine IL-1β ELISA

Immulon 2 HB U plates (3655, Thermo Fisher) were coated with 2 μg/ml of mouse monoclonal anti-porcine IL-1β antibody (MAB6811, R&D Systems) in PBS at room temperature overnight. All the following steps were performed at room temperature and all reagents or samples were applied at 100 μl per well. The plates were washed four times with TBST between each step. Blocking was performed with 1% BSA (A7030, Sigma) in PBS for 1 h. Two-fold serial dilutions of recombinant porcine IL-1β protein (681-PI-010, R&D Systems) in diluent (0.1% BSA in TBST) was used as the standard. The plates were incubated with samples or the standard for 2 h. The plates were then incubated with 50 ng/ml of goat polyclonal anti-porcine IL-1β biotinylated antibody (BAF681, R&D Systems) in diluent for 1 h, followed by the incubation with alkaline phosphatase streptavidin (016-050-084, Jackson ImmunoResearch) diluted at the ratio of 1:5,000 in diluent for another 1 h. For color development, the plates were incubated with 1 mg/ml of PNPP in diethanolamine buffer until the OD of the first dilution of the standard reached around 2.0. The plates were read at 405 nm with reference at 490 nm using xMark Microplate Absorbance Spectrophotometer (Bio-Rad).

3.3.13 NLRP3 inflammasome reconstitution assay

HEK293T cells were seeded at 1.5×10^5 cells per well on 24-well plates and transfected with pcDNA-NLRP3 (30 ng), pcDNA-ASC (20 ng), pCMV-Flag-pro-caspase-1 (20 ng) and pcDNA-pro-IL-1β (100 ng) along with other constructs (300 ng of Flag-vector or Flag-tagged WT/mutant Hf09 NS1 constructs, or 300 ng of Myc-vector or Myc-tagged mutant Sk02 or Hf09 NS1 constructs). Transfection was conducted by adding the mixture of plasmids and *Trans*IT-LT1 Transfection Reagent. At 16 hpt, cell-free supernatants were collected for porcine IL-1β ELISA and cells were lysed with 1 × SDS sample buffer for Western blotting. To monitor the NLRP3 inflammasome activation levels in response to viral infection, at 12 hpt, the cells were

mock-infected or infected with viruses at an MOI of 5. At 12 hpi, cell-free supernatants were collected and cells were lysed as mentioned.

3.3.14 Western blotting

Cell lysates or IP samples were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto 0.45 μm nitrocellulose membranes (1620115, Bio-Rad). The membranes were blocked with 5% skim milk in TBS with 0.1% Tween 20 for 1 h and incubated with primary antibodies in TBS with 0.1% Tween 20 at 4℃ overnight. After incubation with the secondary antibodies in TBS with 0.1% Tween 20 at room temperature for 1 h, the membranes were scanned with an Odyssey infrared imager (LI-COR Biosciences).

3.3.15 Co-immunoprecipitation (co-IP)

HEK293T cells were seeded at 1×10^6 per well on 6-well plates (#3506, Corning) and were transfected with 1 μg of each plasmid indicated. Transfection was conducted by adding the mixture of plasmids and *Trans*IT-LT1 Transfection Reagent. To examine the effects of viral infection on the NLRP3-ASC interaction, the transfected cells were infected with Hf09 at an MOI of 1 for 12 h. To observe the effects of WT/mutant NS1 of Hf09 on the NLRP3-ASC interaction, the cells were incubated for 48 h after transfection. The cells were lysed with 500 μl lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40 substitute, 1 × protease inhibitor cocktail) and clarified by centrifugation at 12,000 × *g* for 10 min at 4℃. For input, 10% of the cell lysates were stored separately. Dynabeads Protein G (10004D, Thermo Fisher) were first conjugated with 1 μg of the respective antibodies by incubation at room temperature for 1 h and then, were incubated with the cell lysates at 4℃ overnight with agitation. After washed thrice with TBS, the beads were resuspended in 2 × SDS sample buffer and boiled at 98℃ for 5 min to be analyzed by Western blotting.

3.3.16 Immunofluorescence and confocal microscopy

HEK293T cells were seeded at 6×10^4 cells per well on LabTek II CC2 chamber slides (154941, Thermo Fisher). The cells were transfected with 50 ng of pcDNA-NLRP3-Myc or Myc-vector and 30 ng of pCMV-Flag-ASC or Flag-vector for 24 h to validate the specificity of

antibodies. Transfection was conducted by adding the mixture of plasmids and *Trans*IT-LT1 Transfection Reagent. To observe the effects of WT/mutant Hf09 virus on ASC specks, the cells were transfected with pcDNA-NLRP3-Myc (50 ng) and pCMV-Flag-ASC (30 ng) for 12 h, and infected with Hf09 or Hf09-817 at an MOI of 5 for 12 h. Cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. The cells were incubated with 5% BSA in Dulbecco's phosphate-buffered saline (DPBS) (14040-133, Thermo Fisher) for 1 h at room temperature and probed with the primary antibodies (rabbit monoclonal anti-Myc-tag antibody, #2278, CST; goat polyclonal anti-DDDDK tag antibody, ab1257, Abcam; mouse monoclonal anti-IAV NP antibody, MCA400, Bio-Rad) overnight at 4℃ followed by incubation with the secondary antibodies [Alexa Fluor 546 donkey polyclonal anti-rabbit IgG (H+L), A10040, Invitrogen; Alexa Fluor 633 donkey polyclonal anti-goat IgG (H+L), A21082, Invitrogen; Alexa Fluor 488 donkey polyclonal anti-mouse IgG (H+L), A21202, Invitrogen] for 1 h at room temperature. The cells were washed with DPBS between each step. The cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (D1306, Invitrogen) for 5 min and coverslips were mounted with ProLong Diamond Antifade Mountant (P36961, Invitrogen) overnight. Images were visualized by using a confocal laser scanning microscope (TCS SP8, Leica). The numbers of ASC speck-positive cells and IAV NP-positive cells were counted from five randomly selected fields per each condition, and the percentages of speck- and NP-positive cells in NP-positive cells were shown.

3.3.17 Statistical analysis

The data were analyzed using GraphPad Prism 7 by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. Two-way ANOVA with Tukey's multiple comparisons test or Mann-Whitney test was used where specified. The bars indicate the mean ± SD. The *p*-value of less than 0.05 was considered to be statistically significant.

3.4 Results

3.4.1 SIV infection induces IL-1β production in PAMs.

To assess the levels of IL-1β produced from PAMs in response to IAV infection, PAMs were infected with two different subtypes of IAVs isolated from pigs, Sk02 (H1N1) and Tx98

(H3N2) at indicated MOIs (Figure 3.1A). At 24 hpi, cell-free supernatants were collected for porcine IL-1β ELISA and the cells were lysed for Western blotting to measure the expression of pro-IL-1β and viral proteins. Although PAMs from different piglets showed variable levels of IL-1β production in response to SIV infection, the differences in IL-1β levels in response to SIVs at indicated MOIs were reproducible when cells from different piglets were used, and the representative data using PAMs isolated from a piglet with the lowest background level of IL-1β is shown. Pro-IL-1β levels from PAMs infected at an MOI of 1 or 10 were comparable and were significantly higher than those in mock cells or cells infected at an MOI of 0.1 (Figure 3.1A). In contrast, IL-1β levels in virus-infected cells were significantly higher than that of mock infected cells and were viral dose-dependent. Accordingly, the expression of viral NP and NS1 proteins were also viral dose-dependent, confirming that PAMs were infected with SIVs. Based on these results, an MOI of 1 for 24 h was chosen in the following infection experiments with PAMs, except for an experiment to check mRNA expression of *NLRP3*. Since the above results suggested that viral replication is required for IL-1β production in PAMs, experiments were conducted to further confirm this finding by inactivating the two virus strains with UV irradiation (Figure 3.1B). The effect of UV inactivation on virus replication was confirmed by monitoring the NS1 protein expression levels. Compared to WT viruses, UV-inactivated viruses lost the ability to induce IL-1β production in PAMs (Figure 3.1B), indicating viral replication is necessary for the conversion of pro-IL-1β into mature IL-1β.

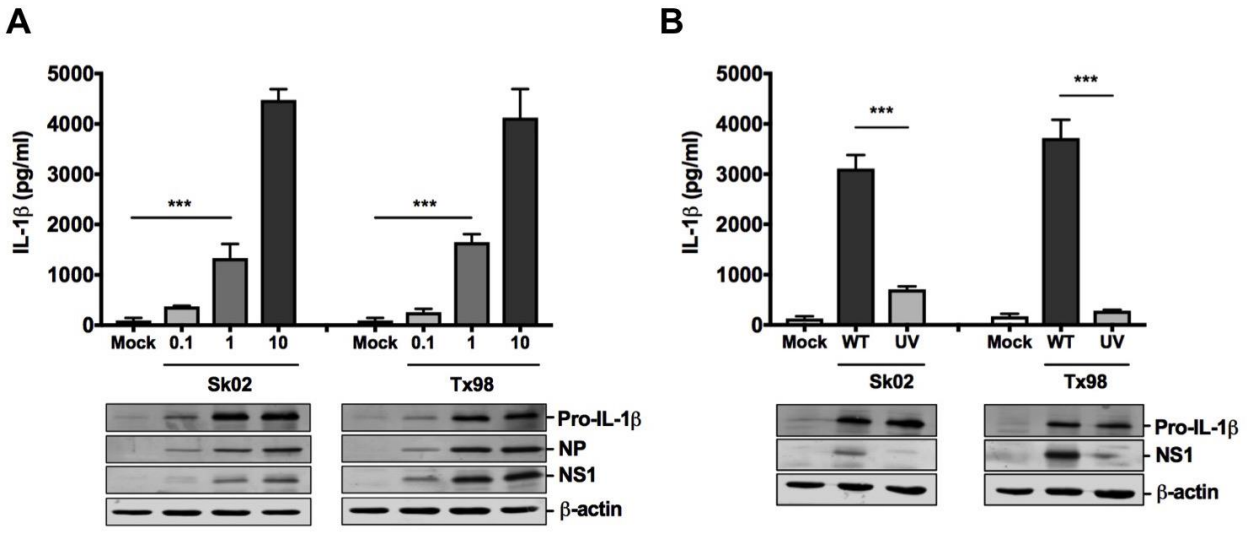


Figure 3.1 SIV infection induces IL-1β production in PAMs.
(A) PAMs were infected with two SIV strains, Sk02 and Tx98 at an MOI of 0.1, 1 or 10. At 24 hpi, cell-free supernatants were collected for porcine IL-1β ELISA and cells were lysed for Western blotting to measure the expression of pro-IL-1β and viral proteins. ***, $p<0.001$. (B) PAMs were infected with WT or UV-inactivated viruses of two SIV strains at an MOI of 1 for 24 h. IL-1β production and pro-IL-1β expression were measured as in (A). ***, $p<0.001$.

3.4.2 SIV-induced IL-1β production is mediated by TLR3.

To identify which cytosolic sensor is mainly involved in IL-1β production from PAMs infected with SIV, endosomal TLRs for dsRNA or ssRNA were knocked down by siRNAs. PAMs were transfected with a negative control siRNA or siRNA specific for porcine *TLR3* or *TLR7* and infected with Sk02. Knockdown efficiency confirmed by comparing the mRNA expression levels of *TLR3* and *TLR7* to that of the housekeeping gene, *HPRT1* was more than 50% (Figure 3.2A). Compared to treatment with a negative control or *TLR7* siRNA, *TLR3* silencing led to reduced IL-1β production upon Sk02 infection (Figure 3.2B), indicating that TLR3 functions as a sensor of SIV infection for porcine IL-1β production.

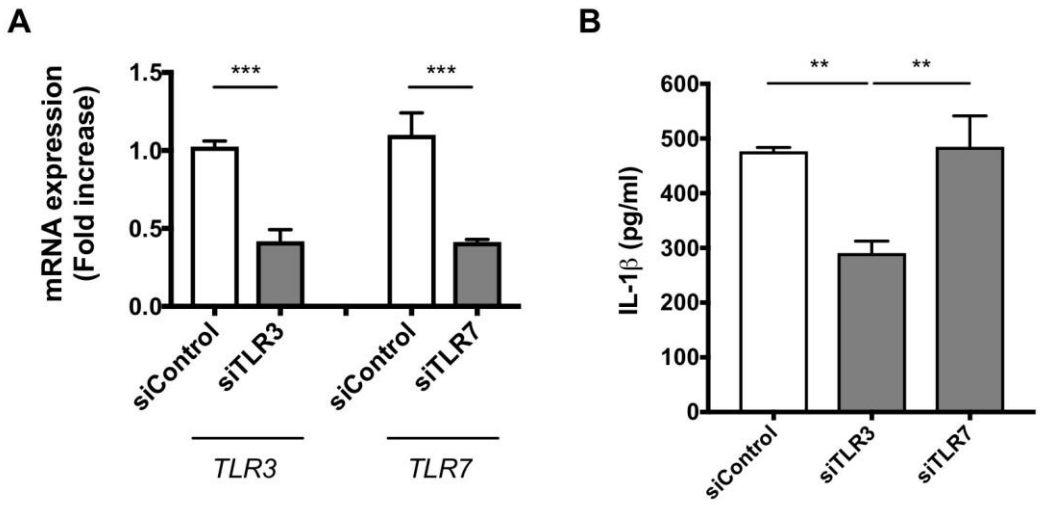


Figure 3.2 SIV-induced IL-1β production is mediated by TLR3.
(A) PAMs were transfected with 20 nM of a negative control siRNA (siControl) or siRNAs targeting porcine *TLR3* (siTLR3) or *TLR7* (siTLR7) for 24 h. Knockdown efficiency was measured by checking the mRNA expression levels of porcine *TLR3* and *TLR7* compared to that of porcine *HPRT1* by quantitative PCR. ***, $p<0.001$. (B) PAMs were transfected with siControl, siTLR3 or siTLR7 for 24 h. The cells were infected with SIV Sk02 at an MOI of 1 for 24 h. Cell-free supernatants were collected for porcine IL-1β ELISA. **, $p<0.01$.

3.4.3 SIV-induced IL-1β production is mediated by NLRP3 inflammasome.

Next, whether the NLRP3 inflammasome is involved in IL-1β production in PAMs upon SIV infection was tested. PAMs were first treated with an NLRP3 inhibitor, MNS, which inhibits the NLRP3 inflammasome activation by binding to NLRP3 (He et al., 2014), and were then infected with Sk02 at an MOI of 1. In virus-infected PAMs, compared to vehicle-treated cells, a dose dependent decrease of IL-1β induction upon the inhibitor treatment was observed (Figure 3.3A), indicating that porcine IL-1β production in SIV-infected PAMs is mediated by NLRP3 inflammasome. At the tested concentration of MNS, cells did not show noticeable cytotoxicity (data not shown). To reaffirm the NLRP3 inflammasome-mediated IL-1β production upon IAV infection, the porcine NLRP3 inflammasome was reconstituted in HEK293T cells that are deficient in endogenous human NLRP3 inflammasome (Bryan et al., 2009). Porcine NLRP3, ASC, pro-caspase-1 and pro-IL-1β genes were cloned from PAMs and the corresponding mammalian expression plasmids were constructed. Co-transfection of all four plasmids led to a moderate level of IL-1β in the supernatant owing to the engagement of NLPR3, ASC and

caspase-1. Only basal level of IL-1β was detected when pro-IL-1β was expressed alone (Figure 3.3B). These results validated the successful setup of the reconstitution system, showing that IL-1β maturation and secretion predominantly rely on the porcine NLRP3 inflammasome. To confirm the dependency of IL-1β production on NLRP3 inflammasome in the context of IAV infection, porcine NLRP3 inflammasome was reconstituted in HEK293T cells for 12 h and then were infected with Sk02 at an MOI of 5 for another 12 h. In the presence of all the NLRP3 inflammasome components and pro-IL-1β, infection with Sk02 induced over 5,000 pg/ml of IL-1β and a noticeable level of cleaved caspase-1, p20, which was much higher than its counterpart in the absence of virus infection (Figure 3.3B). In all samples, viral protein expression and cellular β-actin levels were monitored. Together, these results demonstrate that SIV infection induces porcine NLRP3 inflammasome-mediated IL-1β production.

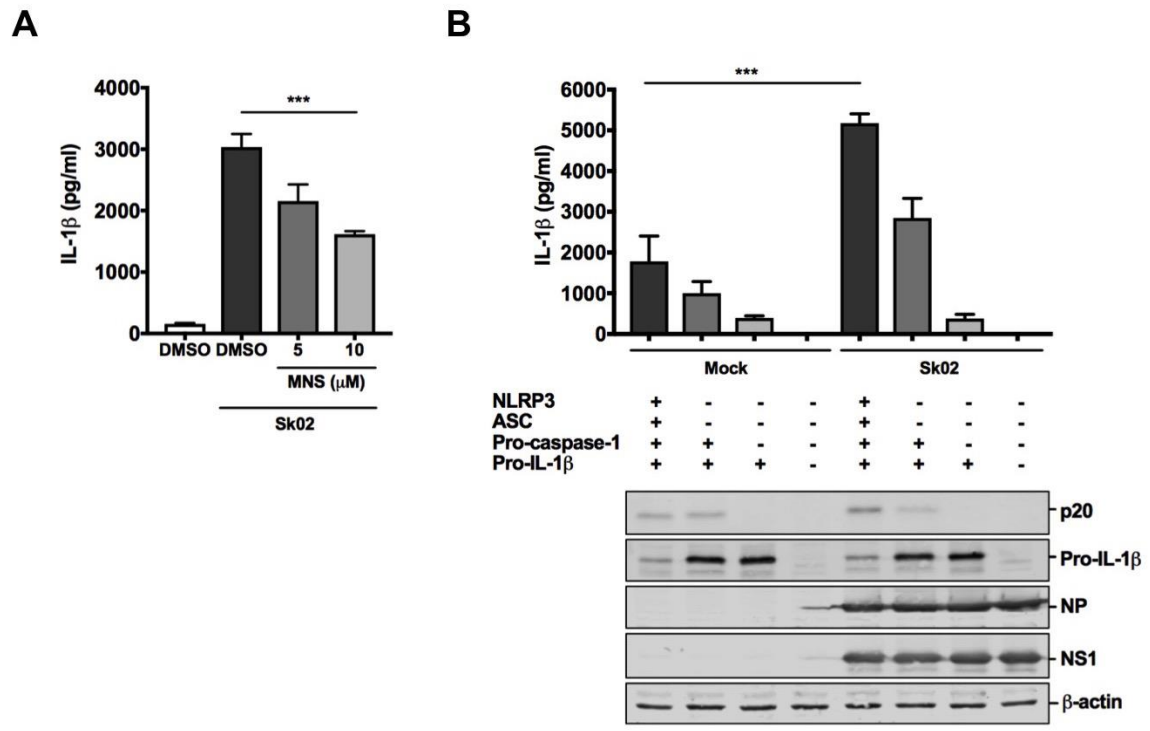


Figure 3.3 SIV-induced IL-1β production is mediated by NLRP3 inflammasome.
(A) PAMs were treated with DMSO or an NLRP3 inhibitor, MNS at indicated concentrations for 2 h and infected with Sk02 at MOI of 1 for 24 h. Porcine IL-1β production was measured by ELISA. ***, $p<0.001$. (B) HEK293T cells were transfected with different combination of the plasmids expressing porcine NLRP3, ASC, pro-caspase-1 and pro-IL-1β as indicated. At 12 hpt, the cells were mock-infected or infected with Sk02 at an MOI of 5. Porcine IL-1β from the cell-free supernatants at 12 hpi was measured by ELISA. The expression of pro-IL-1β, the active caspase-1, p20 and viral proteins was measured by Western blotting with the cell lysates. ***, $p<0.001$.

3.4.4 2009 human pandemic IAV (pdm09) induces less IL-1β than does the SIV.

To examine whether other IAV strains in addition to SIVs also induce IL-1β in PAMs, a pdm09 strain, Hf09 was used. Surprisingly, IL-1β production at 24 hpi from Hf09-infected PAMs was significantly less than that from Sk02-infected PAMs (Figure 3.4A), while the pro-IL-1β level in Sk02-infected PAMs was slightly higher than that in Hf09-infected PAMs. The time course experiments showed that IL-1β production in response to Sk02 increased sharply between 8 and 16 hpi, after the virus established its productive replication at around 8 hpi, as indicated by the viral growth curve (Figure 3.4B). In contrast, this increase in IL-1β was not observed in Hf09-infected PAMs, while the viral titres of Hf09 were comparable to those of

Sk02 during the time course. These results showed that less IL-1β production by Hf09 was not due to the inefficient replication of Hf09 in PAMs, which was also confirmed by viral NS1 expression levels (Figure 3.4A). The signal 1 required for NLRP3 inflammasome-mediated IL-1β production induces the transcriptional upregulation of NLRP3 in addition to pro-IL-1β. Thus, the mRNA expression of *NLRP3* in PAMs infected with Sk02 or Hf09 was also assessed. Porcine *NLRP3* transcription was around 2.5 times increased upon viral infection, while this increase was comparable between Sk02 and Hf09-infected PAMs (Figure 3.4C). LPS-induced upregulation of *NLRP3* expression was shown as a positive control. These indicated that the remarkable difference in IL-1β levels was possibly attributed to the suppression of NLRP3 inflammasome activity in cells infected by pdm09.

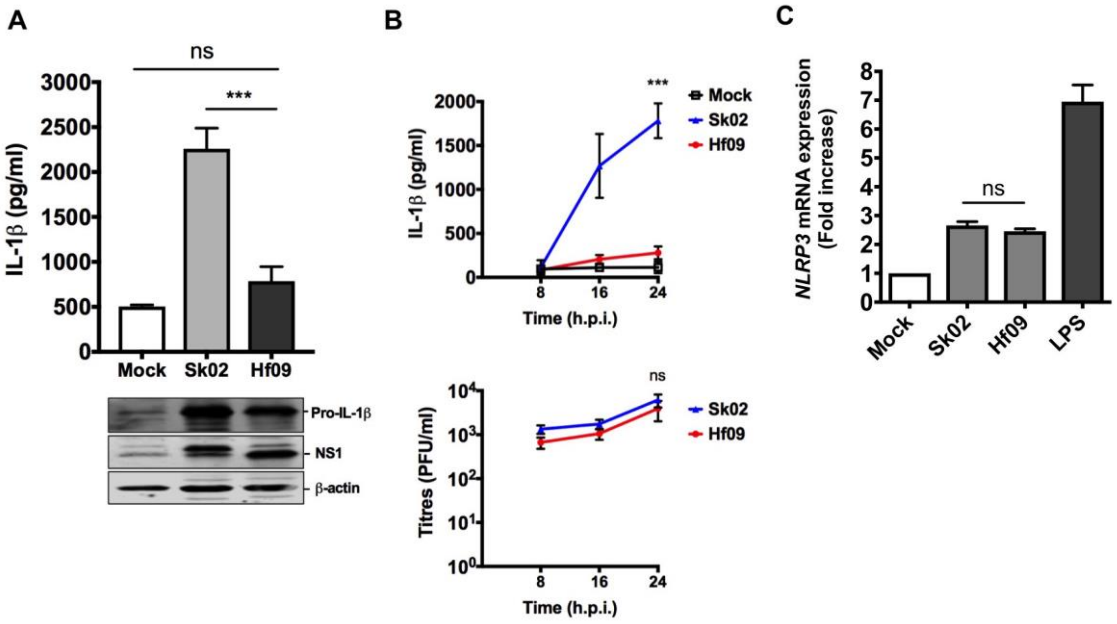
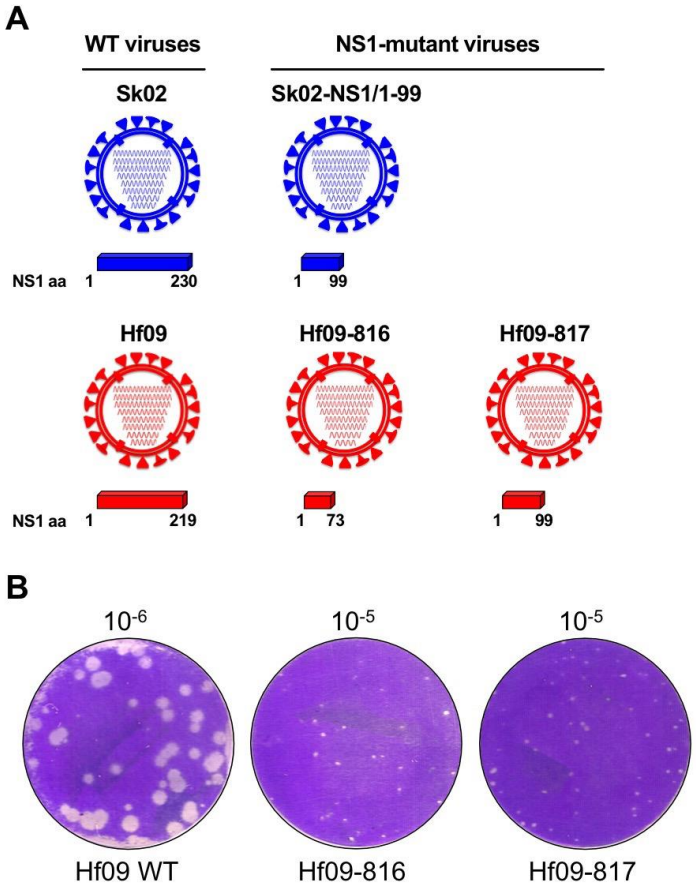


Figure 3.4 pdm09 induces less IL-1β than does SIV.
(A) PAMs were infected with an SIV strain, Sk02 or a human pandemic strain, Hf09 at an MOI of 1. At 24 hpi, cell-free supernatants were collected for IL-1β ELISA and the cells were lysed for Western blotting to measure the expression of pro-IL-1β and viral NS1 protein. ns, not significant, ***, $p<0.001$. (B) PAMs were infected with Sk02 or Hf09 at an MOI of 1, and the supernatants were collected at 8, 16 and 24 hpi. IL-1β was measured by ELISA and viral titres were determined by plaque assay on MDCK cells. Two-way ANOVA with Tukey's multiple comparisons test (B, upper panel), ***, $p<0.001$; Mann-Whitney test (B, lower panel), ns, not significant. (C) PAMs were either infected with viruses (Sk02 or Hf09) at an MOI of 1 for 12 h or stimulated with 200 ng/ml LPS for 12 h. Expression of porcine *NLRP3* mRNA was compared to that of porcine *HPRT1* by conducting quantitative PCR. ns, not significant.

3.4.5 NS1 C-terminus of pdm09, but not that of SIV, inhibits the NLRP3 inflammasome activation.

Based on the above results, viral proteins were postulated to contribute to the regulation of NLRP3 inflammasome activation. Among others, NS1 protein of IAV is well known for its function of antagonizing host immune responses. However, the NS1 protein of pdm09 was reported to be inefficient in controlling the host gene expression (Hale et al., 2010). Unexpected results obtained above that Hf09 has a strong inhibitory effect on porcine IL-1β production led to a further study on whether Hf09 NS1 regulates the NLRP3 inflammasome activity. NS1 C-terminus has been shown to interact with multiple cellular proteins (Hale et al., 2008), which led to a hypothesis that NS1 C-terminus may exert an inhibitory function on the porcine IL-1β production. To investigate this, mutant viruses derived from WT Sk02 or Hf09 containing mutations in the NS1 protein were constructed (illustrated in Figure 3.5A). Sk02-NS1/1-99 virus encoding N-terminal NS1 from aa 1 to 99 was previously generated in our lab; the Sk02-NS1/1-99 induced more type I IFN secretion from PAMs than its WT did (Liu et al., 2015). In addition, two Hf09 mutant viruses encoding truncated NS1 proteins (Hf09-816 and Hf09-817 encoding NS1 aa 1 to 73 and aa 1 to 99, respectively) were generated in the current study. They showed smaller-sized plaques than that of the WT virus (Figure 3.5B) and grew to titers 0.7 to 1 log lower on MDCK-NS1 cells than the WT Hf09 on MDCK cells [titers were 7×10^6 plaque-forming unit (PFU)/ml for Hf09-816 and 1.25×10^7 PFU/ml for Hf09-817 versus 7.5×10^7 PFU/ml for WT Hf09] (Figure 3.5B). Infection of PAMs with WT Sk02 or Sk02-NS1/1–99 virus showed that the deletion of NS1 C-terminal aa 100 to 230 in Sk02 led to a slightly increased pro-IL-1β expression, but did not alter the IL-1β level. Specifically, Sk02-NS1/1–99 virus induced only 1.07-fold more IL-1β than that induced by WT Sk02 (Figure 3.5C). In contrast, upon infection of PAMs, the Hf09-816 and Hf09-817 viruses induced 2.53- and 3.05-fold more IL-1β production than WT Hf09, respectively, yet they did not significantly affect pro-IL-1β expression levels (Figure 3.5D). In the reconstitution system, Hf09-816 and Hf09-817 infection led to a striking increase of IL-1β production above the level by WT Hf09 infection, although they did not markedly alter the pro-IL-1β level (Figure 3.5E). The level of an active caspase-1 subunit, p20, an indicator for NLRP3 inflammasome activation, was also consistently increased in the

cells infected with NS1-mutant viruses. These suggested that NS1 C-terminus of pdm09 suppresses the porcine NLRP3 inflammasome-mediated IL-1β production.



(Figure 3.5 to be continued)

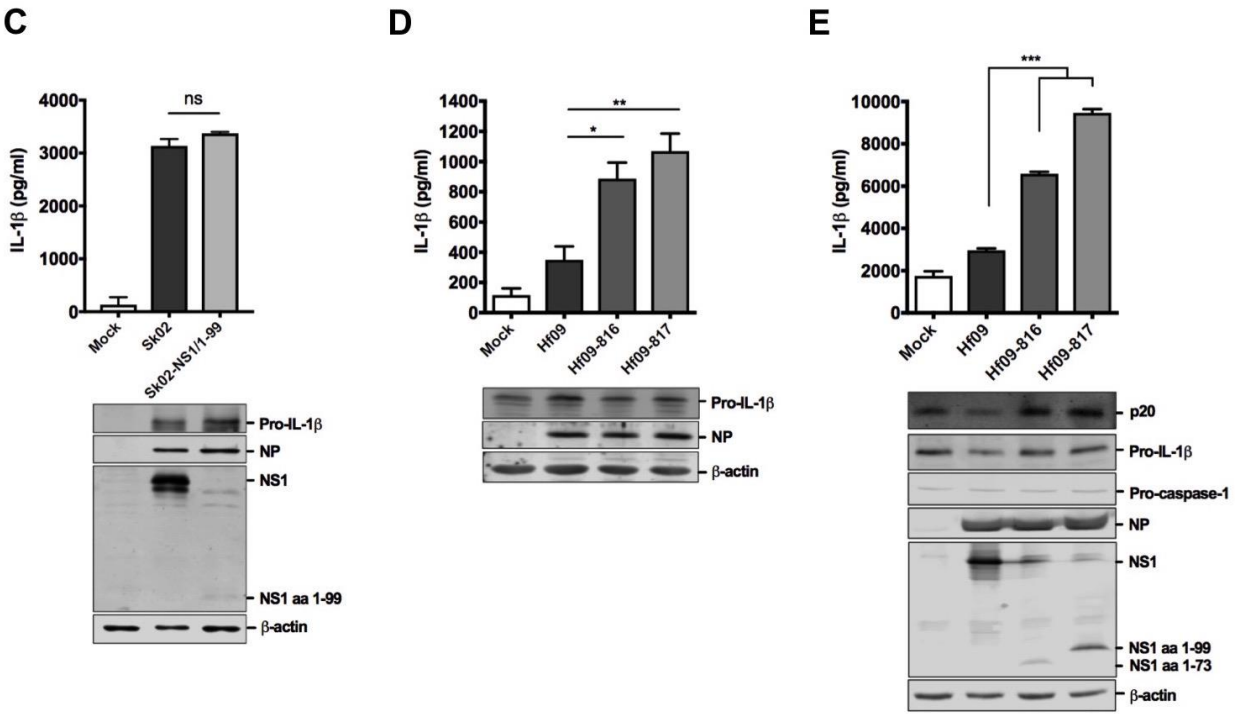


Figure 3.5 NS1 C-terminus of pdm09, but not that of SIV, inhibits porcine NLRP3 inflammasome activation.

(A) A schematic representation of genome composition of WT and mutant viruses. The NS1 protein encoded by different viruses is depicted. (B) Plaque assay results of WT virus in MDCK cells and NS1-mutant viruses in MDCK-NS1 cells at indicated dilutions shown above the plaque images. (C) PAMs were infected with Sk02 WT or Sk02-NS1/1-99 virus at an MOI of 1. At 24 hpi, cell-free supernatants were collected for IL-1β ELISA and the cells were lysed for Western blotting to measure the expression of pro-IL-1β and viral proteins. ns, not significant. (D) PAMs were infected with Hf09 WT or two NS1-mutant virus (Hf09-816 or Hf09-817) at an MOI of 1 for 24 h. IL-1β ELISA and Western blotting were performed as in (B). *, p=0.0201, **, p=0.0015. (E) HEK293T cells were transfected with plasmids expressing porcine NLRP3, ASC, pro-caspase-1 and pro-IL-1β. At 12 hpt, the cells were infected with Hf09 WT or NS1-mutant viruses at an MOI of 5. IL-1β production at 12 hpi was measured by ELISA and the expression of p20, pro-IL-1β and viral proteins was measured by Western blotting. ***, p<0.001.

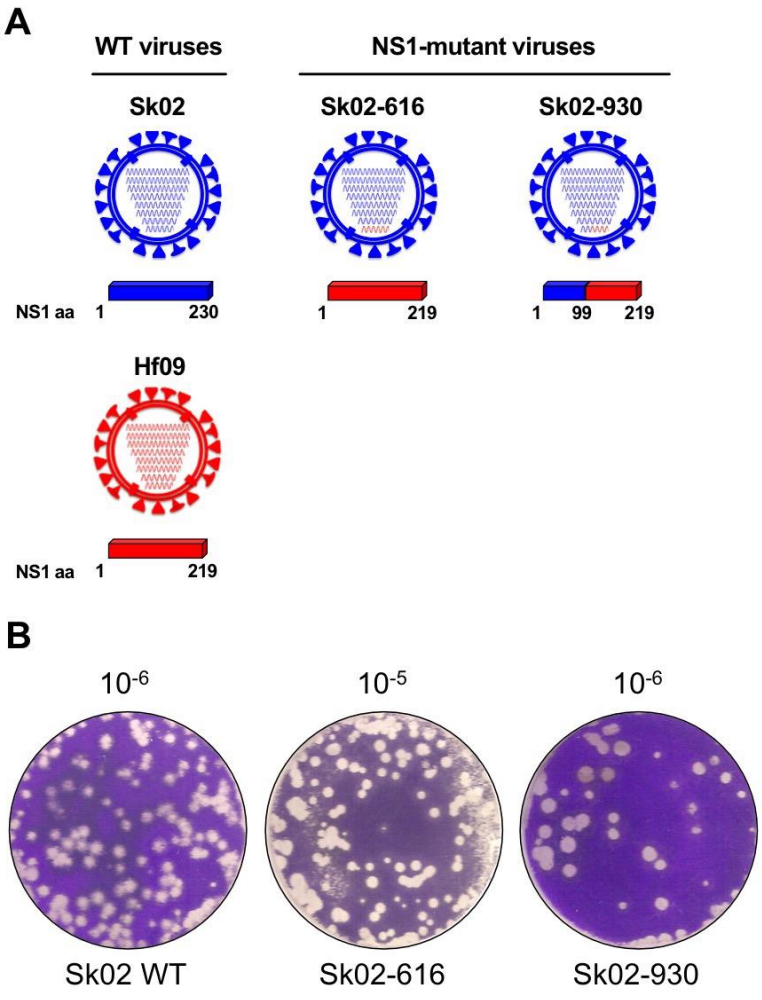
3.4.6 Mutant SIVs with NS1 C-terminus derived from pdm09 inhibits the NLRP3 inflammasome activation.

To demonstrate conclusively that the C-terminus of pdm09 NS1 is responsible for the inhibition of IL-1β production, additional two mutant viruses in the background of Sk02 were generated. Sk02-616 has the entire NS segment from Hf09, and Sk02-930 has a chimeric NS1 with the N-terminus from Sk02 and C-terminus from Hf09 as illustrated in Figure 3.6A. Plaque assay showed that the mutant viruses produced similar-sized plaques to those produced by the WT virus and their titres were comparable to that of WT Sk02: 6.25×10^7 PFU/ml for Sk02-616 and 8.75×10^7 PFU/ml for Sk02-930 versus 4×10^7 PFU/ml for WT Sk02 (Figure 3.6B). Infection of PAMs with Sk02-616 or Sk02-930 led to a reduction of IL-1β, while pro-IL-1β expression was not significantly impaired (Figure 3.6C). To support that the C-terminus of Hf09 NS1 has a greater effect on the inflammasome activation rather than on the pro-IL-1β level, the expression of pro-IL-1β and p20 upon infection with Sk02 WT and Sk02-930 was monitored in the reconstitution system. Indeed, Sk02-930 could inhibit both caspase-1 activation and IL-1β production, but did not affect the pro-IL-1β expression (Figure 3.6D).

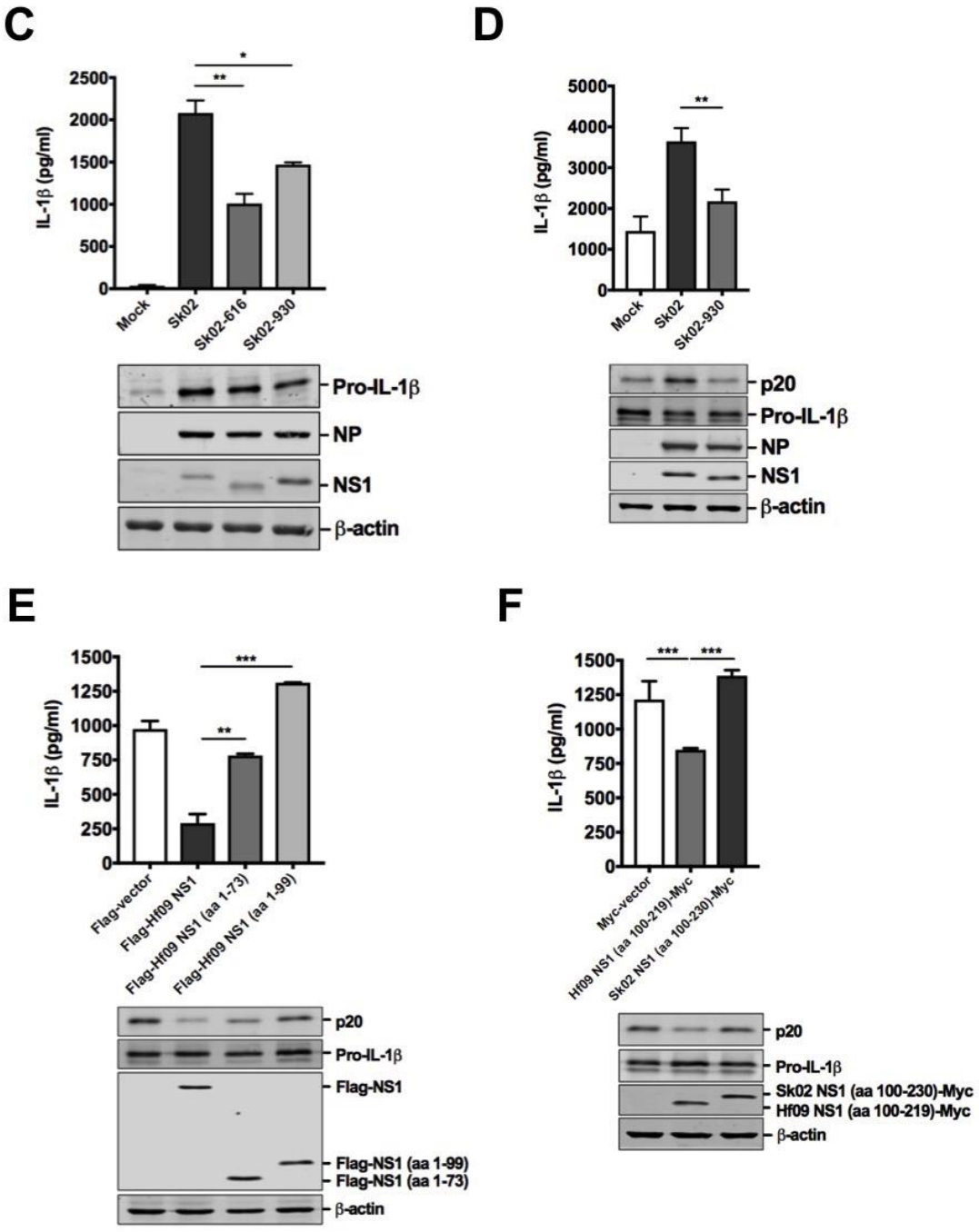
Next, caspase-1 activity and IL-1β production in the reconstitution system were examined by overexpressing Flag- or Myc-tagged WT or truncated NS1. This experiment showed that co-expression of the full-length Hf09 NS1 significantly inhibited caspase-1 activation and IL-1β production, whereas co-expression of the NS1 with C-terminal deletion ($NS1_{1-73}$ or $NS1_{1-99}$) had little or no inhibitory effects (Figure 3.6E). Further, co-expression of the NS1 C-terminal aa 100 to 219 derived from Hf09 led to a reduction of IL-1β due to less caspase-1 activation, whereas this effect was not observed with the NS1 C-terminal aa 100 to 230 derived from Sk02 virus (Figure 3.6F). All of these results evidently demonstrated that the C-terminus of the Hf09 NS1 has an inhibitory effect on NLRP3 inflammasome activation.

Efforts were also made to map the responsible residues in the C-terminus of pdm09 NS1 for the inhibition of IL-1β production. NS1 C-terminal aa sequences among SIVs (Sk02 and Tx98) and a pdm09 strain, Hf09 that induced high and low levels of porcine IL-1β production, respectively, were aligned (Figure 3.6G). In addition, a 1918 pandemic strain, influenza A/Brevig Mission/1/1918/H1N1 (BM18), whose NS1 protein is known to inhibit IL-1β production in primary human cells (Pothlichet et al., 2013), was included. Sk02 and BM18 had 230 aa, while Tx98 and Hf09 had 219 aa in full lengths of NS1 protein, implying that the

presence of additional 11 aa at the C-terminal end did not contribute to the up- or down-regulation of IL-1β production. Identification of unique residues for Hf09 or BM18 NS1 to inhibit the IL-1β production was not successful, because among variable aa residues, those contained by Hf09 NS1 were also harboured by Tx98 NS1, or those contained by BM18 NS1 were shared with Sk02 or Tx98 NS1. It suggested that multiple aa clusters in the NS1 C-terminus of pdm09 are responsible for the inhibition of IL-1β production.



(Figure 3.6 to be continued)



(Figure 3.6 to be continued)

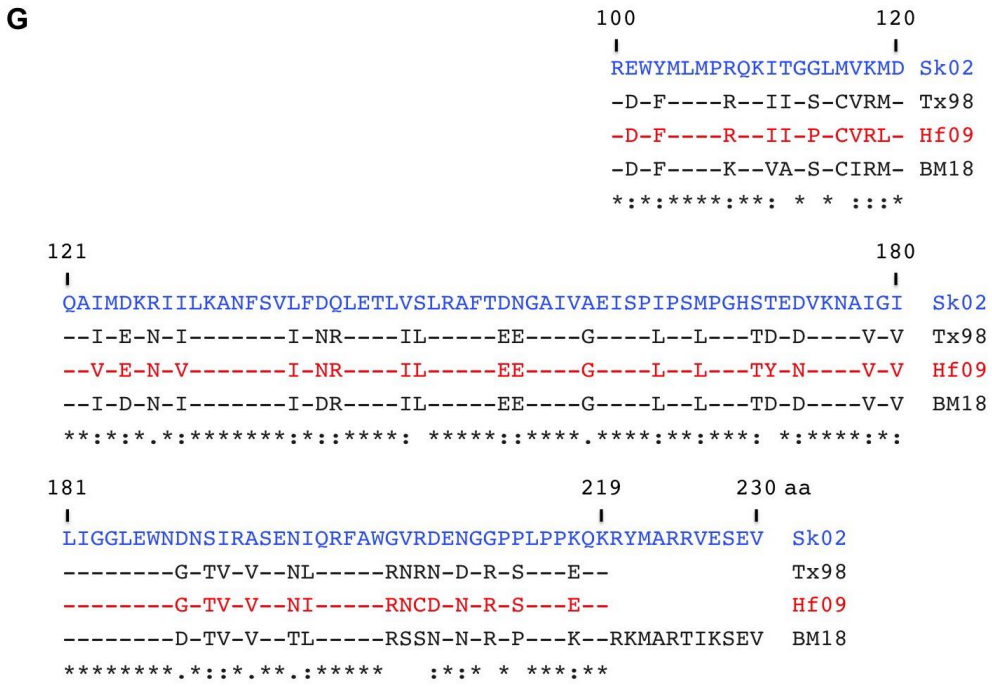


Figure 3.6 Mutant SIVs with NS1 C-terminus derived from pdm09 inhibits the NLRP3 inflammasome.

(A) A schematic representation of genome composition of WT and mutant viruses. The NS1 protein encoded by different viruses is depicted. (B) Plaque assay results of WT and NS1-mutant viruses in MDCK cells at indicated dilutions shown above the plaque images. (C) PAMs were infected with Sk02, Sk02-616 or Sk02-930 at an MOI of 1. At 24 hpi, cell-free supernatants were collected for IL-1β ELISA and the cells were lysed for Western blotting to measure the expression of pro-IL-1β and viral proteins. *, p=0.0104, **, p=0.0012. (D) HEK293T cells were transfected with plasmids expressing porcine NLRP3, ASC, pro-caspase-1 and pro-IL-1β. At 12 hpt, the cells were infected with Sk02 or Sk02-930 at an MOI of 5. IL-1β production at 12 hpi was measured by ELISA and the expression of p20, pro-IL-1β and viral proteins was measured by Western blotting. **, p=0.0056. (E) HEK293T cells were transfected with plasmids expressing porcine NLRP3 inflammasome components and pro-IL-1β along with Flag-vector or Flag-tagged WT/N-terminal aa of Hf09 NS1. IL-1β levels and protein expression at 18 hpt were measured. **, p=0.002, ***, p<0.001. (F) HEK293T cells were transfected with plasmids expressing porcine NLRP3 inflammasome components and pro-IL-1β along with Myc-vector or Myc-tagged NS1 C-terminus from Hf09 or Sk02. IL-1β levels and protein expression at 18 hpt were measured as in (E). ***, p<0.001. (G) Alignment of the C-terminal aa sequences (from aa 100 to 219 or 230) of NS1 among SIVs (Sk02 and Tx98), Hf09 and BM18. Sk02 and Hf09 sequences are shown in blue and red, respectively. Based on the sequence of Sk02, a conserved aa among four strains is shown as a hyphen. Consensus symbols indicate as follows: asterisk (*), fully conserved residues; colon (:), residues that share strongly similar properties; period (.), residues that share weakly similar properties.

3.4.7 NS1 C-terminus of pdm09 inhibits the interaction between NLRP3 and ASC.

In an effort to define the mechanism behind the inhibitory function of pdm09 NS1 on the porcine NLRP3 inflammasome, the NLRP3-ASC interaction was examined. First, whether Hf09 infection can inhibit the NLRP3-ASC interaction was tested by transfecting HEK293T cells with plasmids expressing Myc-tagged ASC and Flag-tagged NLRP3 followed by infection of the cells with Hf09. Co-IP with anti-Myc antibody followed by Western blotting showed that Flag-NLRP3 was not in the IP complex when expressed alone (Figure 3.7A, lane 1), whereas it was successfully pulled down when ASC-Myc was co-expressed (Figure 3.7A, lane 2). However, when comparing between the mock-infected and Hf09-infected conditions, the pulled-down level of Flag-NLRP3 was lower in Hf09-infected cells (Figure 3.7A, lane 4), indicating that the NLRP3-ASC interaction is inhibited by Hf09 infection. Next, the effects of WT and truncated NS1 of Hf09 on the NLRP3-ASC interaction were compared. HEK293T cells were transfected with plasmids expressing Myc-tagged NLRP3 and ASC along with Flag-tagged WT or truncated NS1, and were subjected to co-IP. Association between NLRP3-Myc and ASC was clearly noticeable (Figure 3.7B, lane 2). The NLRP3-ASC interaction was obviously inhibited by the expression of Hf09 WT NS1 (Figure 3.7B, lane 4), while the interaction was restored with the expression of Hf09 $NS1_{1\text{-}73}$ or $NS1_{1\text{-}99}$ (Figure 3.7B, lanes 6 and 8). Therefore, the C-terminal region, particularly aa 100 to 219 of Hf09 NS1 plays a critical role in interfering with the NLRP3-ASC interaction that is essential for the activation of NLRP3 inflammasome.

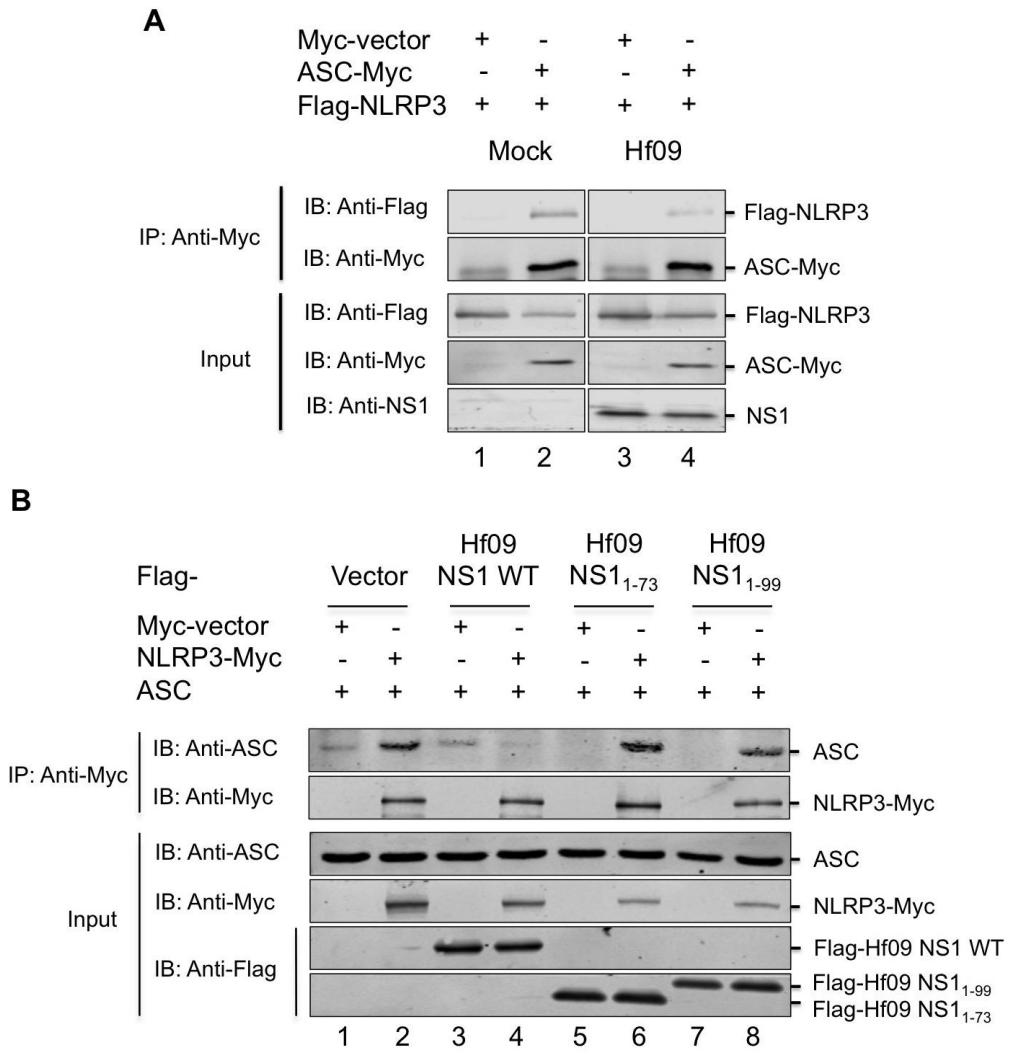


Figure 3.7 NS1 C-terminus of pdm09 inhibits the interaction between NLRP3 and ASC.
(A) HEK293T cells were transfected with Flag-NLRP3 construct along with Myc-vector or ASC-Myc construct, and either mock-infected or infected with Hf09 at an MOI of 1 for 12 h. Cell lysates were subjected to co-IP with anti-Myc antibody. IP and input samples were analyzed with Western blotting using anti-Myc, anti-Flag and anti-NS1 antibodies. (B) HEK293T cells were transfected with ASC construct and Myc-vector or NLRP3-Myc construct in addition to plasmids expressing Flag-tagged WT or mutant NS1 of Hf09. At 48 hpt, the cells were harvested and cell lysates were subjected to co-IP with mouse anti-Myc antibody. IP and input samples were analyzed with Western blotting using anti-ASC, anti-Myc and anti-Flag antibodies. IB, immunoblot.

3.4.8 NS1 C-terminus of pdm09 inhibits the ASC speck formation.

Interference of NLRP3-ASC interaction by pdm09 NS1 C-terminus prompted further investigation on whether ASC specks formed by aggregation of NLRP3 with ASC, a hallmark of NLRP3 inflammasome activation, are affected by NS1 protein. In non-infected cells, transfection of either ASC or NLRP3 alone did not induce a noticeable number of specks (Figure 3.8A, top three rows). In contrast, co-transfection of porcine ASC and NLRP3 led to the aggregation of two proteins, forming the specks in the perinuclear area. Furthermore, in virus-infected cells as indicated by NP staining, Hf09-817 encoding NS1 aa 1-99 led to 2-fold more ASC speck formation than WT Hf09 (Figure 3.8A, bottom three rows). Specifically, while 6.8% of NP-positive cells displayed ASC specks in Hf09-infected cells, 14.3% of NP-positive cells exhibited ASC specks in Hf09-817-infected cells (Figure 3.8B). These results demonstrated that the pdm09 NS1 C-terminus suppresses the ASC speck formation, concomitantly with the disruption of NLRP3-ASC association.

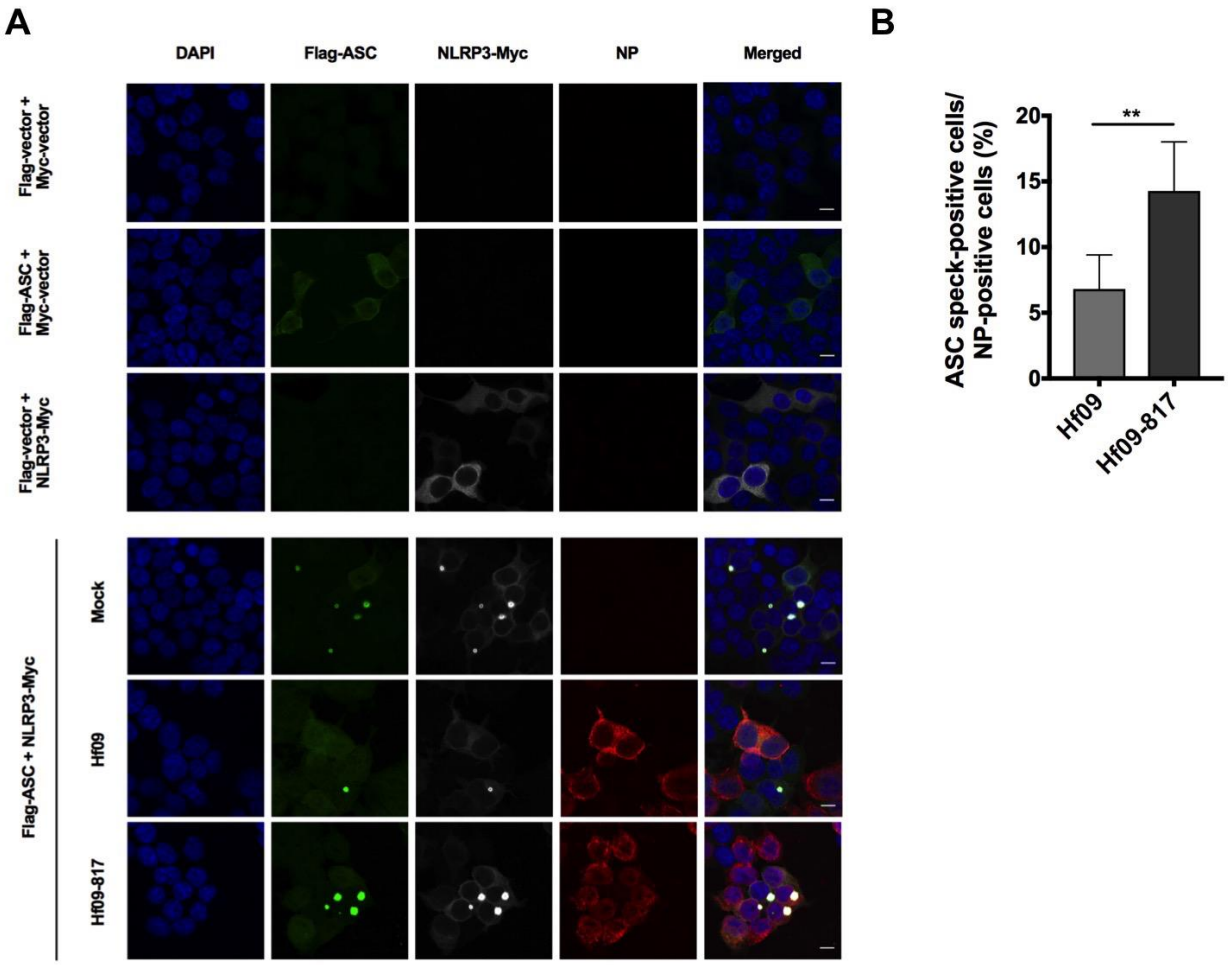


Figure 3.8 NS1 C-terminus of pdm09 inhibits the ASC speck formation.
(A) HEK293T cells were transfected with Myc-tagged NLRP3 or Myc-vector and Flag-tagged ASC or Flag-vector for 24 h and left uninfected (upper three rows). The cells were transfected with Myc-tagged NLRP3 and Flag-tagged ASC for 12 h and infected with Hf09 or Hf09-817 at an MOI of 5 for 12 h (lower three rows). The cells were fixed, permeabilized, blocked and probed with appropriate antibodies followed by DAPI staining. NLRP3-Myc (gray), Flag-ASC (green), viral NP (red) and nuclei (blue) were visualized by confocal microscopy. The scale bars indicate 10 μm. (B) ASC speck-forming cells and NP-stained cells infected by Hf09 or Hf09-817 were quantified from five randomly selected fields and the percentages of ASC speck- and NP-positive cells in total NP-positive cells were shown. Mann-Whitney test, **, p=0.0025.

3.5 Discussion

NLRP3 inflammasome exerts indispensable roles in regulating the innate immunity. In this study, porcine NLRP3 inflammasome regulation in response to IAV infection was investigated. While SIVs generally induce inflammatory responses in pigs (Khatri et al., 2010, Pomorska-Mól et al., 2014), Ma et al. showed pdm09-infected pigs had upregulated expression of genes involved in the inflammatory and immune responses (Ma et al., 2011). In terms of the cytokine response in human cells infected by pdm09, there have been some controversial reports. Both upregulation of inflammatory cytokines (Lee et al., 2011, Sakabe et al., 2011, Yu et al., 2011) and poor inflammatory responses were reported (Osterlund et al., 2010, Zeng et al., 2011). Nevertheless, there is little information concerning the regulation of IL-1β production, which requires the activation of NLRP3 inflammasome upon IAV infection in pigs. Since alveolar macrophages are essential for the regulation of lung inflammation and are required for the protection against IAV infection (Cardani et al., 2017, Kim et al., 2008, Schneider et al., 2014), primary PAMs were utilized to characterize how porcine IL-1β is produced in response to IAV infection.

In this study, swine isolates of IAVs induced IL-1β production, which was mediated by TLR3 and NLRP3 inflammasome, in a viral dose- and replication-dependent manner. In contrast, Hf09, a pdm09 strain, induced much lower level of IL-1β production. The fact that Hf09 and Sk02 replicated to similar titres in PAMs and that they induced similar levels of pro-IL-1β suggested that the NLRP3 inflammasome was less active or was suppressed in Hf09-infected cells. Similar levels of *NLRP3* mRNA transcription induced by Sk02 and Hf09 infection further supported that the intensity of signal 1 provided by the two viruses was comparable.

IAV NS1 protein has been reported to inhibit the NLRP3 inflammasome-mediated IL-1β production in mouse or human macrophages (Cheong et al., 2015, Moriyama et al., 2016). In addition, NS1 from a highly pathogenic, 1918 pandemic strain, BM18 suppresses RIG-I/NLRP3 inflammasome-dependent IL-1β production from primary human lung cells (Pothlichet et al., 2013). Cheong et al. reported that NS1 encoded by human H5N1 or H1N1 strain physically interacts with NLRP3 and suppresses the inflammasome activation in THP-1 cells (Cheong et al., 2015). Based on an early study using PR8 strain, which showed that deletion of NS1 aa 40 to 80 increases human IL-1β production (Stasakova et al., 2005), they tested NS1 from a low pathogenic strain, influenza A/WSN/1933/H1N1 (WSN33) and its mutant with aa 40-80 deleted

(Cheong et al., 2015), however, the mutant NS1 without aa 40-80 could still bind NLRP3. Interestingly, another study with the same virus shows that deletion of 28 aa at the NS1 C-terminal end of the WSN33 virus results in the production of more IL-1β, but less TNF-α and chemokines in mice (Anastasina et al., 2015), suggesting that critical regions for the inhibition of IL-1β production may be strain-dependent and/or host-dependent. Moriyama et al. demonstrated that PR8 NS1 also interacts with NLRP3, thereby inhibiting human or mouse IL-1β production. The inhibitory function was mapped to the RNA-binding domain (R38 and K41) and TRIM25-binding domain (E96 and E97) within the N-terminus of NS1, since PR8 NS1 constructs with mutations on those residues were unable to bind NLRP3 (Moriyama et al., 2016), meaning that multiple regions of NS1 are involved in the NLRP3 inflammasome regulation. In contrary to the aforementioned reports, the current study showed that the mutant pdm09 virus carrying NS1 C-terminal deletion from aa 100 to 219 induced strikingly higher level of IL-1β than did the WT virus. Also, the ectopic expression of the NS1 C-terminal aa 100-219 from Hf09, but not that from Sk02 led to a decreased level of IL-1β secretion. In accordance with it, NS1 C-terminus derived from Hf09 was responsible for disrupting the interaction between NLRP3 and ASC, and suppressing the formation of ASC specks. Thus, the C-terminus of pdm09 NS1 obviously possesses inhibitory functions on the porcine NLRP3 inflammasome activation. Intriguingly, NS1 C-terminal aa 126-225 of avian H5N1 downregulates the RIG-I-mediated IFN signaling by interacting with TRAF3 (Qian et al., 2017). Whether pdm09 NS1 can interact with any of porcine NLRP3 inflammasome components or even other proteins that may participate in the regulation of the inflammasome activity requires further elucidation.

NS1 C-terminus contains multiple motifs critical for binding host cellular partners. For example, by binding to CPSF30 and PABPII, NS1 can repress the polyadenylation of host cellular pre-mRNAs and the nuclear export of cellular mRNAs, respectively (Hale et al., 2008). However, PABPII-binding motif (NS1 aa 223 to 230) is not encoded by pdm09 due to a natural truncation of 11 aa at the C-terminal end (Tu et al., 2011), and pdm09 NS1 is defective in binding CPSF30 (Hale et al., 2010). This prompted an analysis to locate the potential residues in pdm09 NS1 involved in regulating the NLRP3 inflammasome. BM18 NS1 was included in the NS1 aa alignment with a speculation that C-terminus of this protein may be involved in the downregulation of IL-1β production. Unfortunately, aligning the NS1 C-terminal aa 100 to 219 (or 230) of three IAV strains used in the current study (Sk02, Tx98 and Hf09) and BM18 did not

provide any clue in identifying critical residues in Hf09 NS1 for the inhibition of IL-1β. Therefore, it will be required to obtain the porcine IL-1β secretion profiles of other IAV strains and compare the C-terminal aa sequences together with the additional strains.

Collectively, NLRP3 inflammasome-mediated IL-1β production was potently induced in primary PAMs infected with SIVs, while it was suppressed in pdm09-infected cells due to the inhibition of NLRP3 inflammasome activity by NS1. NS1 C-terminus of pdm09 exhibited strong inhibitory functions on porcine IL-1β production by interference with NLRP3-ASC interaction and ASC speck formation, all of which are critical for the NLRP3 inflammasome activation. This unveiled an important mechanism played by pdm09 NS1 to evade the inflammatory responses in pigs.

TRANSITION BETWEEN CHAPTER 3 AND CHAPTER 4

In Chapter 3, IL-1β production from PAMs upon infection with SIVs was found to be TLR3- and NLRP3 inflammasome-mediated. Testing with additional IAV strains led to a finding that a pdm09 strain, Hf09 induced less IL-1β than SIVs. Further study revealed that NS1 C-terminus of Hf09, but not that of SIV, was able to significantly inhibit the NLRP3 inflammasome-mediated IL-1β production. Interfered interaction between NLRP3 and ASC leading to the suppressed ASC speck formation by NS1 C-terminus of Hf09 was suggested as one of the regulatory events behind the inhibition of it. In the following chapter, to dissect the mechanism in depth, further investigation is conducted regarding the modifications on ASC, which functions as a bridge between the sensor protein, NLRP3 and the effector molecule, caspase-1. Aiming to elucidate whether any post-translational modification of ASC is inhibited by pdm09 NS1 and if it is related to the ASC speck phenotypes, the study in Chapter 4 particularly focuses on how ASC ubiquitination is regulated by NS1 C-terminus of Hf09.

CHAPTER 4 NS1 C-TERMINUS OF 2009 PANDEMIC INFLUENZA VIRUS INHIBITS PORCINE NLRP3 INFLAMMASOME BY SUPRESSING ASC UBIQUITINATION.

Portions of the data presented in this chapter were published in

NS1 Protein of 2009 Pandemic Influenza A Virus Inhibits Porcine NLRP3 Inflammasome-Mediated Interleukin-1 Beta Production by Suppressing ASC Ubiquitination.

HS Park, G Liu, SN Thulasi Raman, SL Landreth, Q Liu and Y Zhou. (2018)

Journal of Virology, 92(8), e00022-18.

Acknowledgement specific for this chapter:
The human ASC construct was provided by Dr. Takeshi Ichinohe (University of Tokyo) and the ubiquitin constructs were provided by Dr. Dirk Bohmann (University of Rochester Medical Center) and Dr. Edward Harhaj (Johns Hopkins University School of Medicine).

4.1 Abstract

NLRP3 inflammasome is regulated at multiple levels, with one level being post-translational modification. Among others, ubiquitination of ASC has been reported to be indispensable for the activation of NLRP3 inflammasome. IAV infection induces NLRP3 inflammasome-dependent IL-1β production, which is critical in the host innate immunity. Based on the results that NS1 C-terminus of pdm09, but not that of SIV, inhibited the NLRP3-ASC interaction and ASC speck formation in the previous chapter, the in-depth mechanism behind the inhibitory functions was explored. Studies in this chapter showed that porcine ASC could undergo ubiquitination upon IAV infection; NS1 C-terminus of pdm09 suppressed the ubiquitination on both porcine and human ASC. Moreover, two target lysine residues K110 and K140 that are essential for porcine ASC ubiquitination were identified. The importance of these two residues in regulating the NLRP3 inflammasome-mediated IL-1β production was also demonstrated. These results showed how NS1 protein of the pdm09 suppresses a post-translational modification to downregulate the NLRP3 inflammasome activation.

4.2 Introduction

NLRP3 inflammasome is regulated by post-translational modifications. Among different types of modifications, ubiquitination and deubiquitination play diverse roles in the regulation of inflammasomes (Bednash and Mallampalli, 2016). Ubiquitination is a process by which ubiquitin proteins are conjugated to substrate proteins. In addition to proteasomal degradation of the substrates, this linkage plays crucial roles in a variety of cell signaling pathways. Ubiquitination is a three-step process mediated by E1 activating enzymes, E2 conjugating enzymes and E3 ligases. Ubiquitins can also be recycled after being removed from substrates by deubiquitinases (Kattah et al., 2017). The substrate selectivity is generally determined by E3 ligases that link the C-terminal glycine of a ubiquitin molecule to a lysine (K) on the substrate. Monoubiquitination occurs by binding of a single ubiquitin to each lysine in the substrate; a subsequent linkage of more ubiquitins to lysine residues located on the first ubiquitin leads to different types of polyubiquitination (Davis and Gack, 2015). Additional ubiquitin molecules can be linked on a lysine residue among seven lysines (K6, K11, K27, K29, K33, K48 and K63) or on the first methionine (M1) residue, eventually leading to different consequences. K48-linked ubiquitination can target the substrates for proteasomal degradation, while K63-linked

ubiquitination leads to signal transduction by affecting the stability or localization of target proteins; other linkages are involved in rather diverse activities including cell development, signaling regulation, autophagy or protein degradation (Davis and Gack, 2015, Kattah et al., 2017).

Deubiquitinases are required for inflammasome-dependent IL-1β production (Lopez-Castejon et al., 2013) and more specifically, deubiquitination of NLRP3 is reported to be important for the activation of the NLRP3 inflammasome (Guo et al., 2016, Han et al., 2015, Juliana et al., 2012, Kawashima et al., 2017, Py et al., 2013, Yen et al., 2015). On the contrary, autophagy-mediated or proteasomal degradation of ubiquitinated NLRP3 leads to suppression of the NLRP3 inflammasome (Song et al., 2016, Yan et al., 2015). Upon activation of several different inflammasomes, caspase-1 can be ubiquitinated, which may downregulate the inflammasome activity (Van Opdenbosch et al., 2014). Also, pro-IL-1β undergoes ubiquitination, which leads to either its maturation into IL-1β or its degradation (Ainscough et al., 2014, Duong et al., 2015, Harris et al., 2011). ASC ubiquitination was first reported in a study of AIM2 inflammasome-related autophagy in response to double-stranded DNA (Shi et al., 2012). More recent studies observed ASC ubiquitination upon NLRP3 inflammasome activation. While ASC ubiquitination by an E3 ligase, TRAF6 can limit the NLRP3 inflammasome activity (Chiu et al., 2016), deubiquitination of ASC is required for the NLRP3 inflammasome activation (Lee et al., 2017c). ASC can also be modified by linear ubiquitin assembly complex (LUBAC), which is required for the activation of NLRP3 inflammasome (Rodgers et al., 2014). In the context of RNA virus infection, ubiquitination of ASC by E3 ligase TRAF3 upon infection with vesicular stomatitis virus (VSV) is critical for inflammasome-mediated IL-1β production (Guan et al., 2015). In spite of those studies, it remains unknown how ASC ubiquitination regulates inflammasome activity during IAV infection.

In this chapter, ubiquitin modification on ASC upon WT or NS1-mutant pdm09 virus infection was examined. It showed that the NS1 C-terminus of the pdm09 strain, Hf09 inhibits NLRP3 inflammasome-mediated IL-1β production by suppressing the ubiquitination of porcine ASC. Mutational analyses identified the target lysine residues, K110 and K140 found in porcine ASC to be critical for the NLRP3 inflammasome-mediated IL-1β production. Thus, these results revealed a new mechanism of the innate immune evasion achieved by the NS1 protein of pdm09.

4.3 Materials and methods

4.3.1 Cells and viruses

HEK293T cells were cultured with DMEM supplemented with 10% FBS and 50 μg/ml gentamicin. Human monocytic THP-1 cells were cultured with RPMI 1640 media (30-2001, ATCC) supplemented with 10% FBS, 50 μM 2-mercaptoethanol (21985-023, Thermo Fisher), 1 × penicillin-streptomycin (100 U/ml penicillin, 100 μg/ml streptomycin) (15140122, Thermo Fisher). Hf09, a pdm09 strain, was propagated in MDCK cells. The mutant viruses, Hf09-816 and Hf09-817 are two isogenic Hf09 viruses that encode truncated NS1, i.e. aa 1-73 or 1-99, respectively. Generation and propagation of these viruses were described in the sections 3.3.2 and 3.3.3. THP-1 cells were seeded at 3×10^5 cells/well on 24-well plates and differentiated into adherent macrophages by treatment with 100 ng/ml phorbol 12-myristate-13-acetate (P8139, Sigma) for 24 h. The cells were infected with Hf09 or Hf09-817 at an MOI of 5. At 24 hpi, cell-free supernatants were collected for human IL-1β ELISA.

4.3.2 Antibodies and reagents

Rabbit anti-NP and anti-NS1 antibodies were generated as described (Shin et al., 2007b). Goat polyclonal anti-porcine IL-1β antibody (BAF681, R&D Systems), rabbit polyclonal anti-porcine caspase-1 (p20) antibody (PAB592Po01, Cloud-Clone Corp), mouse monoclonal anti-β-actin antibody (#3700, CST), mouse monoclonal anti-green fluorescent protein (GFP) antibody (#2955, CST), mouse monoclonal anti-Myc-tag antibody (#2276, CST), mouse monoclonal anti-FLAG M2 antibody (F3165, Sigma), rabbit polyclonal anti-HA tag antibody (ab9110, Abcam), mouse monoclonal anti-ubiquitin antibody (sc-8017, Santa Cruz) were purchased from different sources. For the secondary antibodies, IRDye 680RD donkey anti-rabbit (926-68073), IRDye 800CW donkey anti-mouse (926-32212) and IRDye 800CW donkey anti-goat (926-32214) antibodies were purchased from LI-COR Biosciences. Transfection was conducted using *Trans*IT-LT1 Transfection Reagent (MIR2300, Mirus-Bio). EDTA-free protease inhibitor cocktail tablets (4693132001, Roche) were used in cell lysis and IP.

4.3.3 Plasmid construction

Expression plasmids for porcine NLRP3, ASC, pro-caspase-1 and pro-IL-1β used for NLRP3 inflammasome reconstitution and epitope-tagged NLRP3 or ASC were generated as described in the section 3.3.5. WT and truncated NS1 of Hf09 were cloned into pcDNA3.1-3×Myc vector and the resulting plasmids were designated as pcDNA-Hf09-NS1-Myc and pcDNA-Hf09 $NS1_{1-99}$-Myc, encoding the full-length and N-terminal aa 1 to 99 of NS1, respectively. Mutant ASC constructs with lysines replaced by arginines were generated by site-directed mutagenesis using pCMV-Flag-ASC as the template. Truncated ASC with aa 1 to 109 or 1 to 158 was cloned into pCMV-3×Flag vector to generate pCMV-Flag-ASC_{1-109} and pCMV-Flag-ASC_{1-158}. The full-length gene of human ASC was cloned into pCMV-3×Flag vector to generate pCMV-Flag-huASC using pCA7-ASC (Komune et al., 2011) as the template and primers designed based on the GenBank sequence (accession number NM_013258). The HA-tagged ubiquitin construct, pCMV-HA-ubiquitin, with eight tandem sequences of HA-ubiquitin (Treier et al., 1994) was used in ubiquitination assays. pcDNA-HA-ubiquitin (Xiao et al., 2001) was used as the template to generate ubiquitin constructs with a mutation K48R (pcDNA-HA-ubiquitin [K48R]) or K63R (pcDNA-HA-ubiquitin [K63R]). For mutagenesis, primers were designed with the online tool, NEBaseChanger provided by New England BioLabs (NEB), and PCR was performed with Q5 Hot Start High-Fidelity 2 × Master Mix (M0494S, NEB). Purified PCR products were phosphorylated with 10 mM ATP and T4 Polynucleotide Kinase (M0201S, NEB) followed by ligation, *Dpn*I digestion and transformation with standard protocols. Mutagenesis of some constructs was conducted in classical method. PCR was performed with overlapping primer sets containing the mutant sequences using HotStar HiFidelity Polymerase Kit. PCR products were subjected to the digestion of parental plasmids with *Dpn*I before used for transformation. The mutations and truncations were confirmed by DNA sequencing. The expression plasmids generated for this chapter are listed in Table 4.1 and the primers are listed in Supplementary Table 3.

Table 4.1 List of expression plasmids used in Chapter 4.
The constructs for NLRP3 inflammasome components and pro-IL-1β used for NLRP3 inflammasome reconstitution assay were described in Table 3.2.

Construct	Template used to clone	Purpose
pcDNA-NLRP3-Myc	pcDNA-NLRP3	ASC speck formation
pCMV-Flag-NLRP3	pcDNA-NLRP3	Ubiquitination assay
pCMV-Flag-ASC	pcDNA-ASC	ASC speck formation, Ubiquitination assay, NLRP3 inflammasome reconstitution
pcDNA-GFP		Ubiquitination assay
pcDNA-Hf09-NS1-Myc	pHW-Hf09-NS	Ubiquitination assay
pcDNA-Hf09-NS1$_{1-99}$-Myc	pHW-Hf09-NS	Ubiquitination assay
pCMV-Flag-huASC	pCA7-ASC (Komune et al., 2011)	Ubiquitination assay
pCMV-Flag-ASC (K21R/K22R/K24R/K26R)	pCMV-Flag-ASC	Ubiquitination assay
pCMV-Flag-ASC (K55R)	pCMV-Flag-ASC	Ubiquitination assay
pCMV-Flag-ASC (K79R)	pCMV-Flag-ASC	Ubiquitination assay
pCMV-Flag-ASC (K87R)	pCMV-Flag-ASC	Ubiquitination assay
pCMV-Flag-ASC (K91R)	pCMV-Flag-ASC	Ubiquitination assay
pCMV-Flag-ASC (K96R)	pCMV-Flag-ASC	Ubiquitination assay
pCMV-Flag-ASC (K101R)	pCMV-Flag-ASC	Ubiquitination assay
pCMV-Flag-ASC (K106R)	pCMV-Flag-ASC	Ubiquitination assay
pCMV-Flag-ASC (K110R)	pCMV-Flag-ASC	Ubiquitination assay, NLRP3 inflammasome reconstitution
pCMV-Flag-ASC (K140R)	pCMV-Flag-ASC	Ubiquitination assay, NLRP3 inflammasome reconstitution
pCMV-Flag-ASC (K110R/K140R)	pCMV-Flag-ASC (K110R)	Ubiquitination assay, ASC speck formation, NLRP3 inflammasome reconstitution

pCMV-Flag-ASC (K159R)	pCMV-Flag-ASC	Ubiquitination assay
pCMV-Flag-ASC (K175R)	pCMV-Flag-ASC	Ubiquitination assay
pCMV-Flag-ASC (K183R)	pCMV-Flag-ASC	Ubiquitination assay
pCMV-Flag-ASC_{1-109}	pCMV-Flag-ASC	Ubiquitination assay
pCMV-Flag-ASC_{1-158}	pCMV-Flag-ASC	Ubiquitination assay
pCMV-HA-ubiquitin (Treier et al., 1994)		Ubiquitination assay
pcDNA-HA-ubiquitin (Xiao et al., 2001)		Ubiquitination assay, NLRP3 inflammasome reconstitution
pcDNA-HA-ubiquitin (K48R)	pcDNA-HA-ubiquitin	Ubiquitination assay
pcDNA-HA-ubiquitin (K63R)	pcDNA-HA-ubiquitin	Ubiquitination assay

4.3.4 Ubiquitination assay

HEK293T cells were seeded at 1×10^6 cells per well on 6-well plates and were transfected with 1 μg of pCMV-HA-ubiquitin or pcDNA-HA-WT/mutant ubiquitin together with one of the following constructs encoding target proteins: Flag-tagged NLRP3 (pCMV-Flag-NLRP3), WT/mutant ASC (pCMV-Flag-WT/mutant ASC) or human ASC (pCMV-Flag-huASC). At 24 hpt, the cells were infected with viruses at an MOI of 10 for 9 h. For the compensation assay, the cells were transfected with 1 μg of pCMV-HA-ubiquitin and pCMV-Flag-ASC along with either a plasmid expressing GFP, pcDNA-Hf09-NS1-Myc or pcDNA-Hf09-$NS1_{1-99}$-Myc. At 24 hpt, the cells were infected with Hf09-817 as above. Cell lysis and IP were performed as previously described with some modifications (Palazón-Riquelme and López-Castejón, 2016). Briefly, the cells were lysed with 200 μl of SDS-lysis buffer (20 mM Tris, pH 8.0, 250 mM NaCl, 3 mM EDTA, 10% glycerol, 1% SDS, 0.5% Nonidet P-40 substitute) freshly supplemented with 20 mM N-ethylmaleimide (E3876, Sigma), 5 mM 1,10-phenanthroline monohydrate (P9375, Sigma) and 1 × protease inhibitor cocktail, while 5% of the cells were lysed with 1 × SDS sample buffer for input. The clarified cell lysates were mixed with 600 μl of SDS-IP buffer (20 mM Tris, pH 8.0, 250 mM NaCl, 3 mM EDTA, 10% glycerol, 0.1% SDS, 0.5% Nonidet P-40 substitute) freshly supplemented with 20 mM N-ethylmaleimide, 5 mM 1,10-phenanthroline

monohydrate and 1 × protease inhibitor cocktail and incubated with 40 µl slurry of mouse anti-FLAG M2 affinity agarose (A2220, Sigma) at 4℃ for 4 h with agitation. After washed four times with 500 µl of SDS-IP buffer, the agarose was mixed with 60 µl of 2 × SDS sample buffer and boiled at 95℃ for 5 min for elution. The eluted proteins were subjected to SDS-PAGE and Western blotting.

4.3.5 NLRP3 inflammasome reconstitution assay

HEK293T cells were seeded at 1.5×10^5 cells per well on 24-well plates. To examine the effect of ubiquitin on the NLRP3 inflammasome activity, cells were transfected with pcDNA-NLRP3 (30 ng), pcDNA-ASC (20 ng), pCMV-Flag-pro-caspase-1 (20 ng) and pcDNA-pro-IL-1β (100 ng) along with 100 ng of HA-vector or HA-tagged ubiquitin construct. At 24 hpt, cell-free supernatants were collected for porcine IL-1β ELISA and cells were lysed with 1 × SDS sample buffer for Western blotting. To examine the NLRP3 inflammasome activity upon viral infection, the cells were transfected with pcDNA-NLRP3 (30 ng), pCMV-Flag-WT/mutant ASC (10 ng), pCMV-Flag-pro-caspase-1 (20 ng) and pcDNA-pro-IL-1β (100 ng). At 12 hpt, cells were infected with Hf09-817 at an MOI of 5. Cell-free supernatants and cells harvested at 12 hpi were analyzed as mentioned.

4.3.6 Porcine IL-1β ELISA

Porcine IL-1β from cell-free supernatants was measured as described in the section 3.3.12. Briefly, Immulon 2 HB U plates were coated with mouse monoclonal anti-porcine IL-1β antibody in PBS at room temperature overnight. The plates were washed four times with TBST between each step. Blocking was performed with 1% BSA in PBS for 1 h. Serial dilutions of recombinant porcine IL-1β protein in diluent were used as the standard. The plates were incubated with samples or the standard for 2 h. The plates were then incubated with goat polyclonal anti-porcine IL-1β biotinylated antibody in diluent for 1 h, followed by the incubation with alkaline phosphatase streptavidin in diluent for another 1 h. After additional incubation with PNPP in diethanolamine buffer for color development, the plates were read at 405 nm with reference at 490 nm using xMark Microplate Absorbance Spectrophotometer.

4.3.7 Human IL-1β ELISA

IL-1β secreted from THP-1 cells was measured by human IL-1β DuoSet ELISA kit (DY201-05, R&D Systems) according to the manufacturer's protocol. All the procedures were performed at room temperature. A 96-well microplate included in the kit was coated with 100 µl per well of mouse anti-human IL-1β capture antibody (Part #840168) that was reconstituted with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2 µm filtered). After incubation overnight, the plate was washed thrice with 400 µl Wash Buffer (0.05% Tween 20 in PBS, pH 7.2-7.4) per well. Blocking was performed by an hour's incubation with 300 µl Reagent Diluent (1% BSA in PBS, pH 7.2-7.4, 0.2 µm filtered) per well. Recombinant human IL-1β standard (Part #840170) was reconstituted with distilled water and two-fold serial dilution was performed in Reagent Diluent for the seven-point standard curve. Samples or each dilution of the standard were applied at 100 µl per well and left for 2 h. After washing as above, the plate was incubated for additional 2 h with 100 µl per well of biotinylated goat anti-human IL-1β detection antibody (Part #840169) that was reconstituted and diluted in Reagent Diluent. Streptavidin-horseradish peroxidase (Part #843975) diluted in Reagent Diluent was added at 100 µl per well followed by incubation for 20 min in the dark. After washing, the plate was further incubated with 100 µl per well of Substrate Solution (1:1 mixture of H_2O_2 and tetramethylbenzidine) for 20 min in the dark. Upon adding 100 µl Stop Solution (2 N H_2SO_4) to each well, the OD was measured by reading at 450 nm with reference at 540 nm using xMark Microplate Absorbance Spectrophotometer (Bio-Rad).

4.3.8 Cycloheximide (CHX) chase assay

HEK293T cells were seeded at 1.8×10^5 cells per well on 24-well plates and transfected with 200 ng of plasmids expressing Flag-tagged WT ASC or ASC with K110R/K140R mutation. At 24 hpt, the cells were treated with 50 µg/ml CHX (C7698, Sigma) for 2, 4, 6 or 8 h before harvested. Untreated cells were harvested at 24 hpt. The cells were lysed with 1 × SDS sample buffer for Western blotting using anti-Flag and anti-β-actin antibodies.

4.3.9 Western blotting

Cell lysates or IP samples were fractionated by SDS-PAGE and blotted onto nitrocellulose membranes. The membranes were blocked with 5% skim milk in TBS with 0.1% Tween 20 for 1

h and incubated with primary antibodies at 4℃ overnight. After incubation with the secondary antibodies at room temperature for 1 h, the membranes were scanned with an Odyssey infrared imager. Image J software (National Institutes of Health, United States) was used for the normalization of expression levels.

4.3.10 Immunofluorescence and confocal microscopy

ASC specks were visualized as described in the section 3.3.16. Briefly, HEK293T cells were seeded at 6×10^4 cells per well on LabTek II CC2 chamber slides. The cells were transfected with pcDNA-NLRP3-Myc (50 ng) and pCMV-Flag-WT/mutantASC (30 ng) for 24 h. Cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. The cells were incubated with 5% BSA in DPBS for 1 h at room temperature and probed with rabbit anti-Myc and goat anti-Flag antibodies overnight at 4℃ followed by incubation with the secondary antibodies, Alexa Fluor 546 donkey anti-rabbit IgG and Alexa Fluor 633 donkey anti-goat IgG for 1 h at room temperature. The cells were counterstained with DAPI for 5 min and the coverslip was mounted with ProLong Diamond Antifade Mountant overnight. Images were obtained with a confocal microscope.

4.3.11 Statistical analysis

The data were analyzed using GraphPad Prism 7 (version 7.0a) by one-way ANOVA with Tukey's multiple comparisons test. Two-tailed unpaired *t*-test was used where specified. The bars indicate the mean ± SD. The *p*-value of less than 0.05 was considered to be statistically significant.

4.4 Results

4.4.1 Porcine ASC is ubiquitinated and NS1 C-terminus of pdm09 inhibits the ubiquitination.

To examine whether ubiquitin modifications are involved in porcine NLRP3 inflammasome activity, NLRP3 inflammasome reconstitution assay was conducted by co-transfecting HA-vector or HA-tagged ubiquitin. When ubiquitin was co-expressed, there was increased caspase-1 activation and more IL-1β production (Figure 4.1A), indicating that

ubiquitination on the NLRP3 inflammasome components or pro-IL-1β may upregulate the inflammasome activity. Based on this finding, two questions were asked: i) whether NLRP3 or ASC ubiquitination occurs upon viral infection; ii) whether Hf09 NS1 C-terminus contributes to the regulation of this event. HEK293T cells were co-transfected with plasmids expressing HA-tagged ubiquitin and Flag-tagged NLRP3 or Flag-tagged ASC, and were then infected with Hf09 WT, Hf09-816 or Hf09-817 virus. NLRP3 was ubiquitinated in this setting; however, the ubiquitination levels were not affected by infection with the Hf09 WT, NS1-mutant viruses (Figure 4.1B). In contrast, while ASC was slightly ubiquitinated in mock- or WT Hf09-infected cells, the ubiquitination levels of ASC were increased in cells infected with NS1-mutant viruses (Figure 4.1C). The total cellular ubiquitination levels were not considerably altered by viral infection. These results suggested that the NS1 C-terminus of Hf09 plays a role in inhibiting the ubiquitination of ASC, but has no effect on NLRP3 ubiquitination. To further confirm the inhibitory function of NS1 on ASC ubiquitination, a compensation assay was conducted. In this assay, Myc-tagged WT NS1 or truncated NS1 ($NS1_{1\text{-}99}$) was pre-expressed together with ASC and ubiquitin in HEK293T cells. After 24 h, cells were infected with an NS1-mutant virus, Hf09-817, to induce the ubiquitination of ASC. While pre-expression of GFP or $NS1_{1–99}$ protein did not have any impact on ASC ubiquitination, WT NS1 protein could diminish the ubiquitination induced by the Hf09-817 virus (Figure 4.1D), reaffirming that the NS1 C-terminus of Hf09 suppresses porcine ASC ubiquitination.

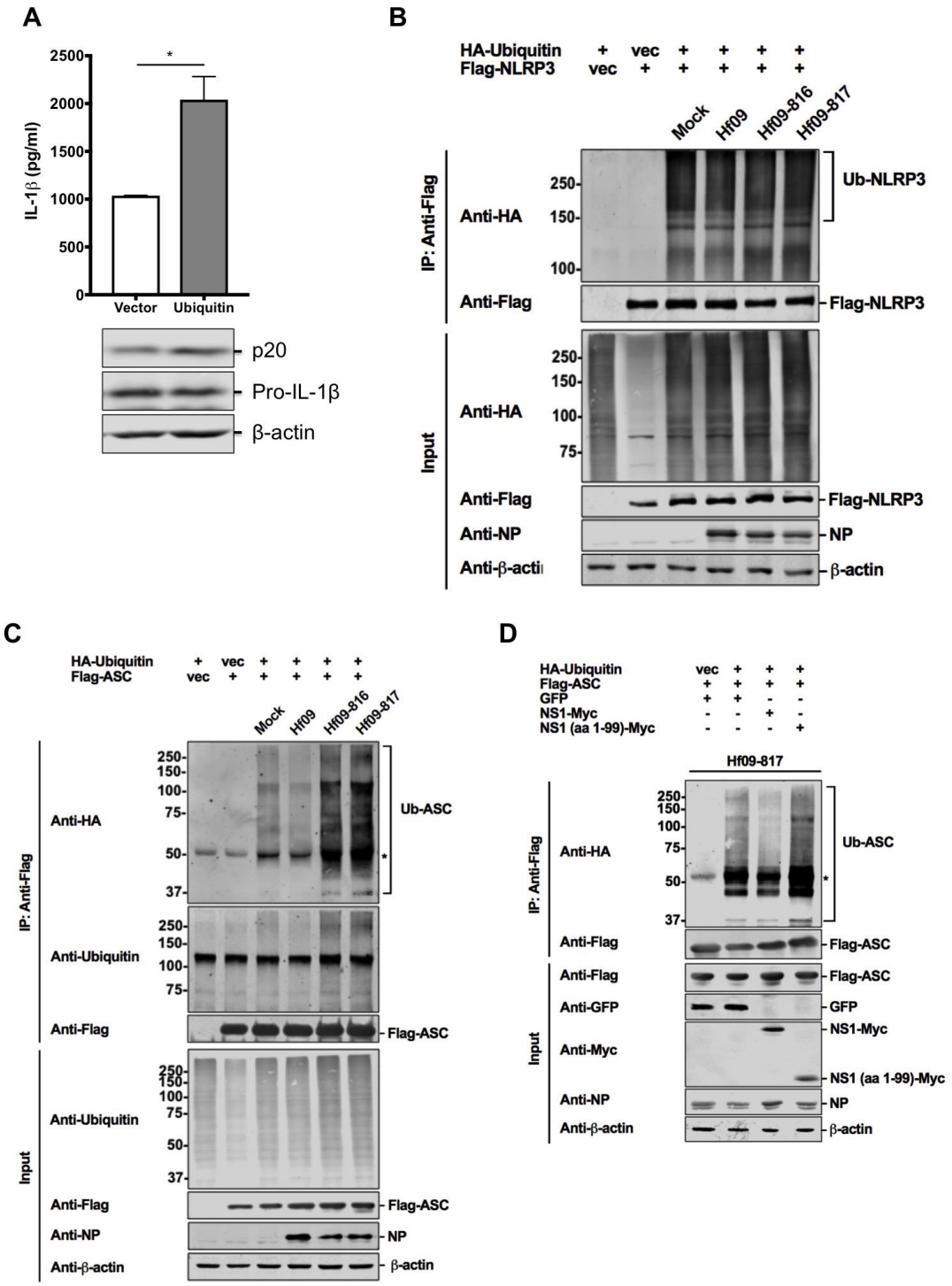


Figure 4.1 Porcine ASC is ubiquitinated and NS1 C-terminus of pdm09 inhibits the ubiquitination.

(A) HEK293T cells were transfected with plasmids expressing porcine NLRP3 inflammasome components and pro-IL-1β along with HA-vector or HA-tagged ubiquitin. IL-1β production at 24 hpt was measured by ELISA and the expression of p20 and pro-IL-1β was measured by Western

blotting. Unpaired *t*-test, two-tailed, p=0.0279. (B and C) HEK293T cells were transfected with HA-vector or HA-ubiquitin and Flag-vector or Flag-NLRP3/ASC constructs. At 24 hpt, the cells were infected with WT or NS1-mutant Hf09 viruses at an MOI of 10 for 9 h. Cell lysates in denaturing condition were subjected to co-IP with beads conjugated with anti-Flag antibody. IP and input samples were used for Western blotting with antibodies indicated. (D) HEK293T cells were transfected with HA-vector or HA-ubiquitin and Flag-ASC constructs along with plasmids expressing GFP, Myc-tagged WT or mutant NS1 of Hf09. At 24 hpt, the cells were infected with Hf09-817 at an MOI of 10 for 9 h. Co-IP was conducted as in (B) and Western blotting was performed with antibodies indicated. The asterisks in (C) and (D) indicate IgG heavy chains.

4.4.2 NS1 C-terminus of pdm09 inhibits the ubiquitination of human ASC.

To explore whether the ability of Hf09 NS1 to suppress ASC ubiquitination is limited to porcine immunity, ubiquitination assay using a Flag-tagged human ASC construct (Flag-huASC) was performed. Interestingly, the ubiquitination level of human ASC was increased in cells infected with Hf09-817 compared to that in mock-infected or WT Hf09-infected cells (Figure 4.2A), showing that Hf09 NS1 C-terminus can inhibit the ubiquitination of both porcine and human ASC. To answer an arising question if human IL-1β production is also suppressed by C-terminal NS1 of Hf09, THP-1 cells were infected by Hf09 or Hf09-817. Indeed, human IL-1β produced from cells infected with Hf09-817 was more than that from Hf09 WT-infected cells (Figure 4.2B), suggesting that the inhibition of human IL-1β production may be attributed to the suppressed ASC ubiquitination by pdm09 NS1 C-terminus.

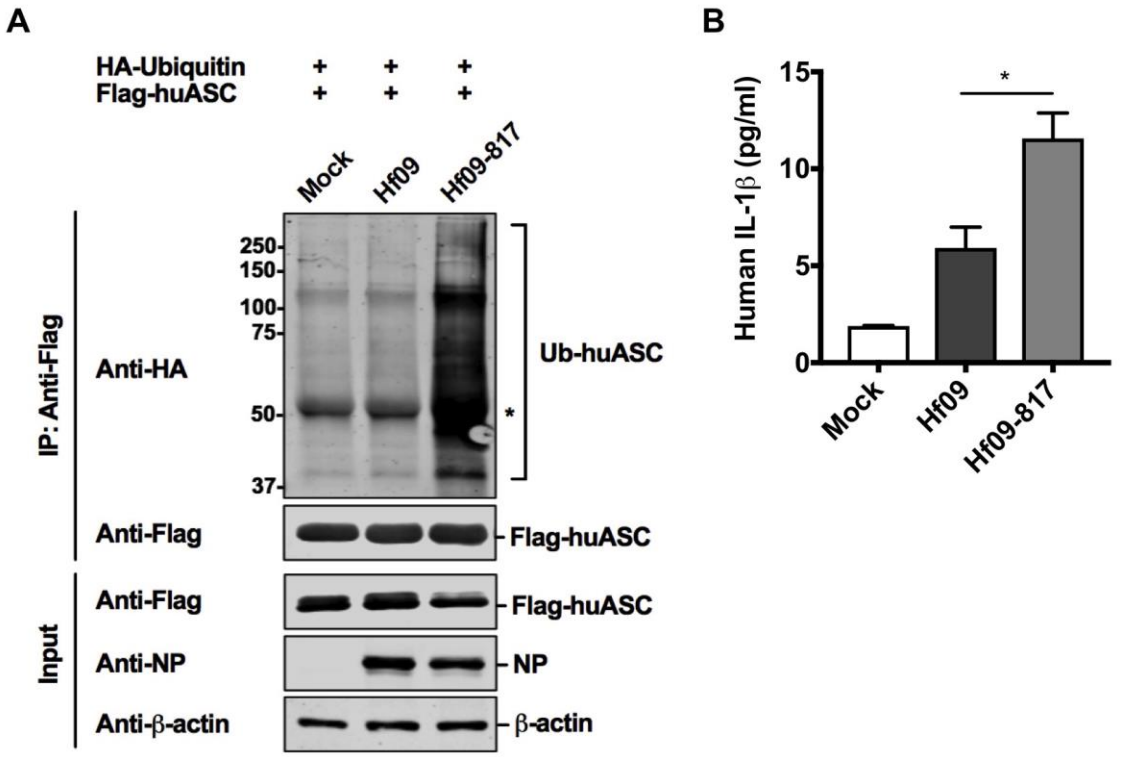


Figure 4.2 NS1 C-terminus of pdm09 inhibits the ubiquitination of human ASC.
(A) HEK293T cells were transfected with HA-ubiquitin and Flag-huASC constructs for 24 h and infected with WT Hf09 or Hf09-817 at an MOI of 10 for 9 h. Cell lysates in denaturing condition were subjected to co-IP with beads conjugated with anti-Flag antibody. IP and input samples were used for Western blotting with antibodies indicated. The asterisk indicates IgG heavy chains. (B) THP-1 cells were infected with Hf09 or Hf09-817 at an MOI of 1 for 24 h. Human IL-1β levels in cell-free supernatants were analyzed by ELISA. *, p=0.021.

4.4.3 ASC undergoes mixed lysine-linked ubiquitination in response to NS1-mutant pdm09 virus.

Which lysine residue(s) among seven in the ubiquitin molecules is involved in the ubiquitination of target proteins may determine the fate of target proteins. Ubiquitination assay was conducted using mutant ubiquitin constructs with each lysine (K) replaced by arginine (R) among seven lysines on a ubiquitin molecule. Ubiquitination levels of porcine ASC upon Hf09-817 infection were not diminished when either K48 or K63 was replaced by arginine in the ubiquitin (Figure 4.3), suggesting that K48 or K63 alone is not linked to ASC ubiquitination, rather, mixed lysines are involved.

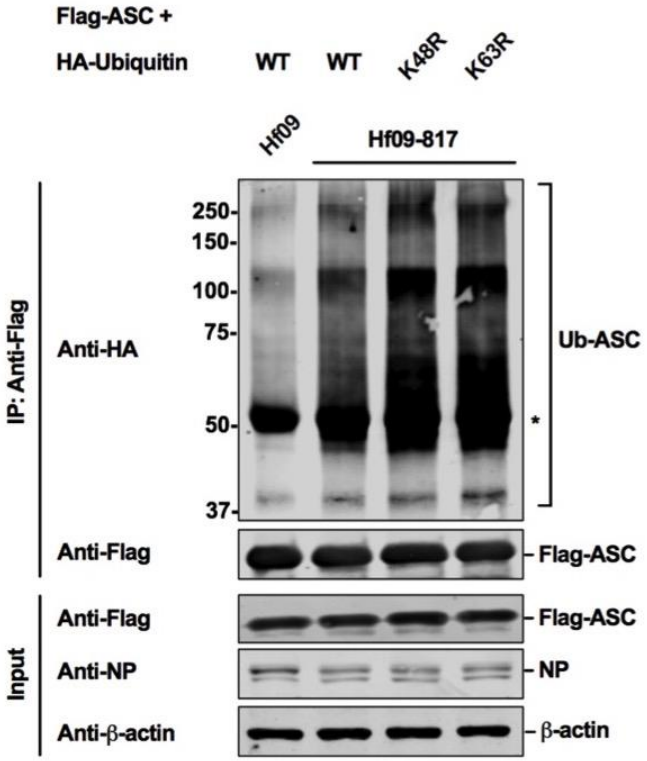
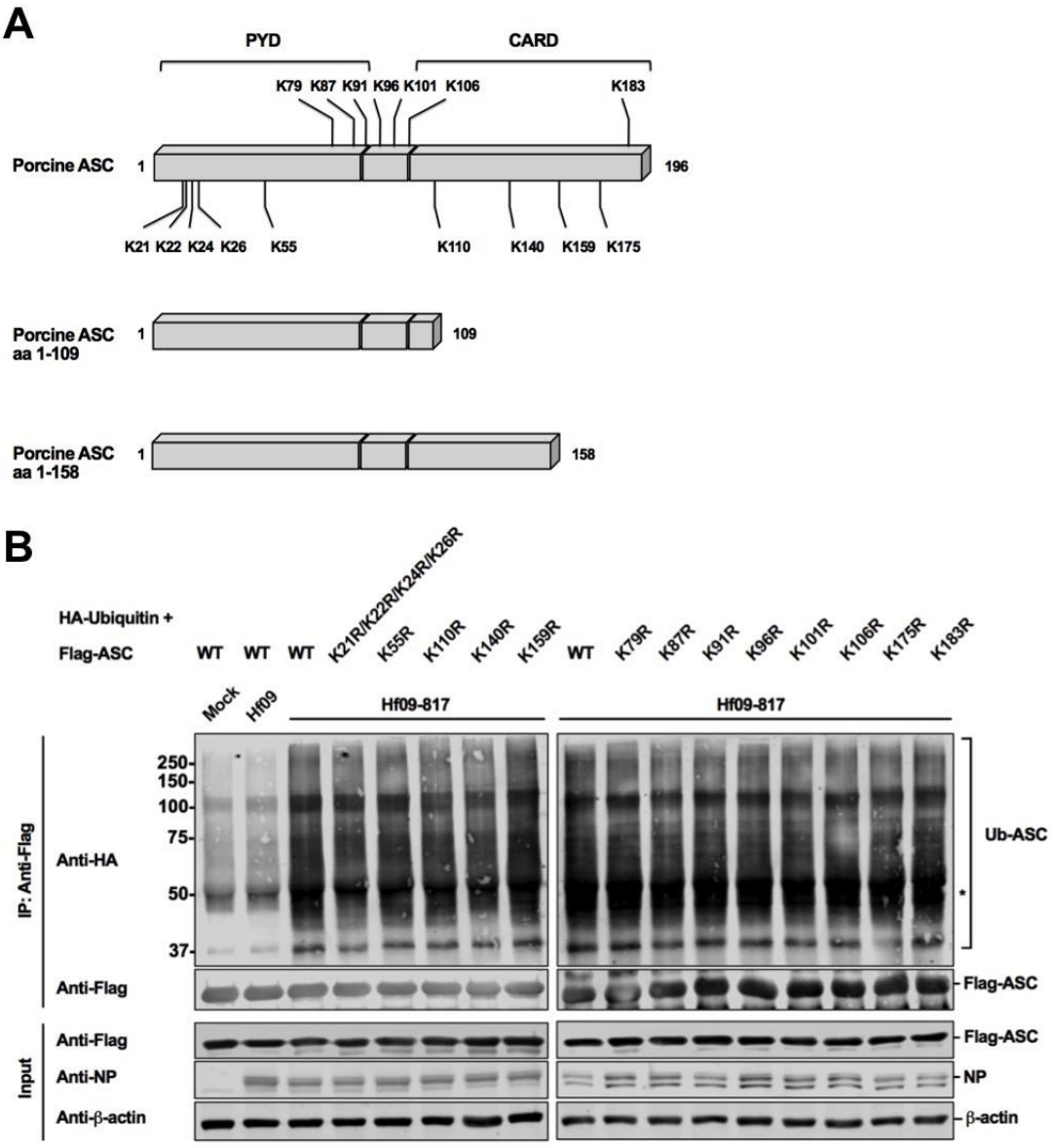


Figure 4.3 ASC undergoes mixed lysine-linked ubiquitination in response to NS1-mutant pdm09 virus.

HEK293T cells were transfected with HA-tagged WT or K-to-R mutant ubiquitin and Flag-ASC constructs. At 24 hpt, the cells were infected with Hf09 WT or Hf09-817 at an MOI of 10 for 9 h. Cell lysates in denaturing condition were subjected to co-IP with beads conjugated with anti-Flag antibody. IP and input samples were used for Western blotting with antibodies indicated. The asterisk indicates IgG heavy chains.

4.4.4 K110 and K140 are ubiquitination sites on porcine ASC.

Ubiquitination generally occurs by conjugation of ubiquitin molecules on the lysine(s) in target proteins. To investigate which lysine(s) in porcine ASC is targeted for ubiquitination, the positions of lysine residues in porcine ASC were compared to those in human ASC. As illustrated in Figure 4.4A, porcine ASC has 16 lysines, among which 7 lysines (K79, K87, K91, K96, K101, K106, and K183) (identified above the bar in Figure 4.4A) are unique for the porcine ASC. The K175 residue in porcine ASC corresponds to K174 in human ASC, which was reported to be the target site for ubiquitination upon an RNA virus infection (Guan et al., 2015). Thus, whether these sites are targeted for ubiquitination was tested first. For that, single lysine-to-arginine (K-to-R) mutation constructs were made; however, none of the mutations led to a reduced level of ASC ubiquitination upon Hf09-817 infection (Figure 4.4B, right panel). The mutations were expanded to the remaining lysines. While individual mutations were made on K55, K110, K140, and K159, a cluster of lysines (K21, K22, K24, and K26) was simultaneously mutated due to their close proximity. However, the replacement of these remaining lysines with arginines did not reduce the ubiquitination levels (Figure 4.4B, left panel). ASC has an N-terminal pyrin domain (PYD) and a C-terminal caspase activation and recruitment domain (CARD). Based on the reports that CARD is targeted for different modifications (Guan et al., 2015, Hara et al., 2013, Lin et al., 2015), whether multiple lysines in the CARD of porcine ASC are targeted for ubiquitination was tested. Thus, two truncated ASCs, ASC consisting of aa 1 to 109 ($ASC_{1–109}$) and ASC consisting of aa 1 to 158 ($ASC_{1–158}$) were constructed (Figure 4.4A). Upon Hf09-817 infection, the ubiquitination level of $ASC_{1–158}$ was not altered, while the level on $ASC_{1–109}$ was significantly reduced compared to that of the WT ASC (Figure 4.4C). This finding suggested that the two lysines, K110 and K140, within aa 110 to 158, may be the target for ubiquitination. Indeed, ASC with a K110R/K140R mutation had an appreciably reduced level of ubiquitination compared to that of WT ASC in response to Hf09-817 infection (Figure 4.4D).



(Figure 4.4 to be continued)

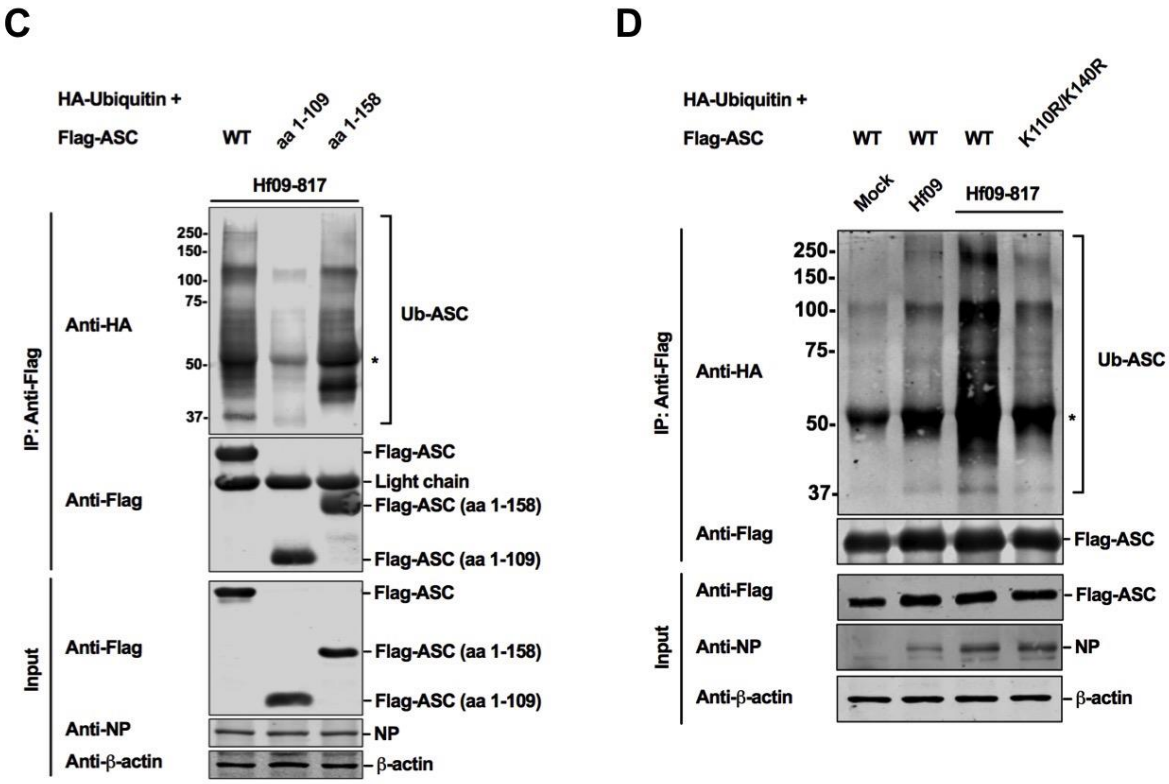


Figure 4.4 K110 and K140 are ubiquitination sites on porcine ASC.
(A) A schematic representation of WT and two mutant ASC constructs (aa 1-109 and aa 1-158) is illustrated with lysine (K) residues. (B, C and D) HEK293T cells were transfected with HA-ubiquitin and Flag-tagged WT or mutant ASC constructs. At 24 hpt, the cells were infected with Hf09 WT or Hf09-817 at an MOI of 10 for 9 h. Cell lysates in denaturing condition were subjected to co-IP with beads conjugated with anti-Flag antibody. IP and input samples were used for Western blotting with antibodies indicated. The asterisks indicate IgG heavy chains.

4.4.5 Inhibition of ASC ubiquitination on K110 and K140 does not impair the ASC speck formation or ASC stability.

To define the effects of ubiquitination at K110/K140 on the ASC protein itself, ASC specks formed by porcine NLRP3 along with either WT or K-to-R mutant ASC were examined by immunofluorescence staining. Elimination of ASC ubiquitination by the K110R/K140R mutation did not alter the overall number or the perinuclear localization pattern of ASC specks (Figure 4.5A). Since ubiquitination may affect the stability of target proteins, the stability of ASC was monitored by treating the cells transiently expressing WT or K-to-R mutant ASC with

CHX, which blocks the translation of new proteins, for different time points before being subjected to Western blotting. The expression intensity of either WT or mutant ASC was not reduced by CHX treatment (Figure 4.5B), suggesting that the ASC stability is not critically affected by preventing the ubiquitination on K110 and K140.

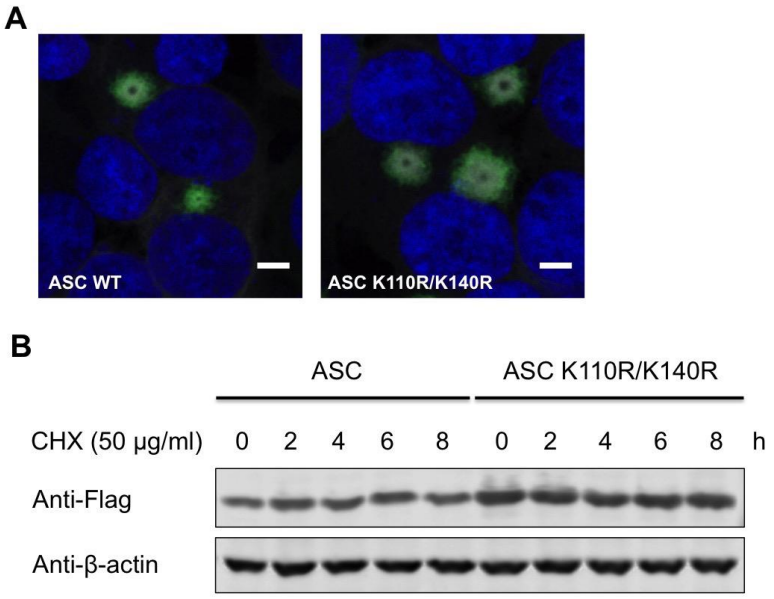


Figure 4.5 Inhibition of ASC ubiquitination on K110 and K140 does not impair the ASC speck formation or ASC stability.
(A) HEK293T cells were transfected with plasmids expressing Myc-tagged NLRP3 and Flag-tagged WT/mutant ASC. At 24 hpt, the cells were fixed, permeabilized, immunostained with appropriate antibodies, and were counterstained with DAPI. ASC specks formed by colocalization of NLRP3-Myc (gray) and Flag-ASC (green) were seen in perinuclear region. Nuclei were visualized in blue. The scale bars indicate 5 μm. (B) HEK293T cells were transfected with 200 ng of plasmids expressing Flag-tagged WT/mutant ASC. At 24 hpt, the cells were treated with CHX for 2, 4, 6 or 8 h before harvested, while untreated cells were harvested at 24 hpt. The cells were lysed and analyzed by Western blotting with antibodies indicated.

4.4.6 Ubiquitination of ASC on K110 and K140 is required for caspase-1 activation in response to NS1-mutant pdm09 virus.

In an attempt to observe the impact of ubiquitination at K110/K140 on the NLRP3 inflammasome activity, NLRP3 inflammasome reconstitution was conducted using WT/mutant ASC. In NLRP3 inflammasome reconstitution followed by Hf09-817 infection, the NLRP3 inflammasome consisting of WT ASC could induce an appreciable level of active caspase-1, p20, and a large amount of IL-1β (Figure 4.6, left panel). In contrast, the inflammasome consisting of ASC K110R/K140R produced lower caspase-1 activity, as evidenced by the reduced level of both p20 and IL-1β. None of the single mutations (K110R or K140R) on ASC had significant effects on caspase-1 activation and IL-1β maturation. The p20 level was almost half diminished in the ASC K110R/K140R condition compared to other conditions, while the pro-IL-1β expression was not significantly impaired as the normalized data showed (Figure 4.6, right panel).

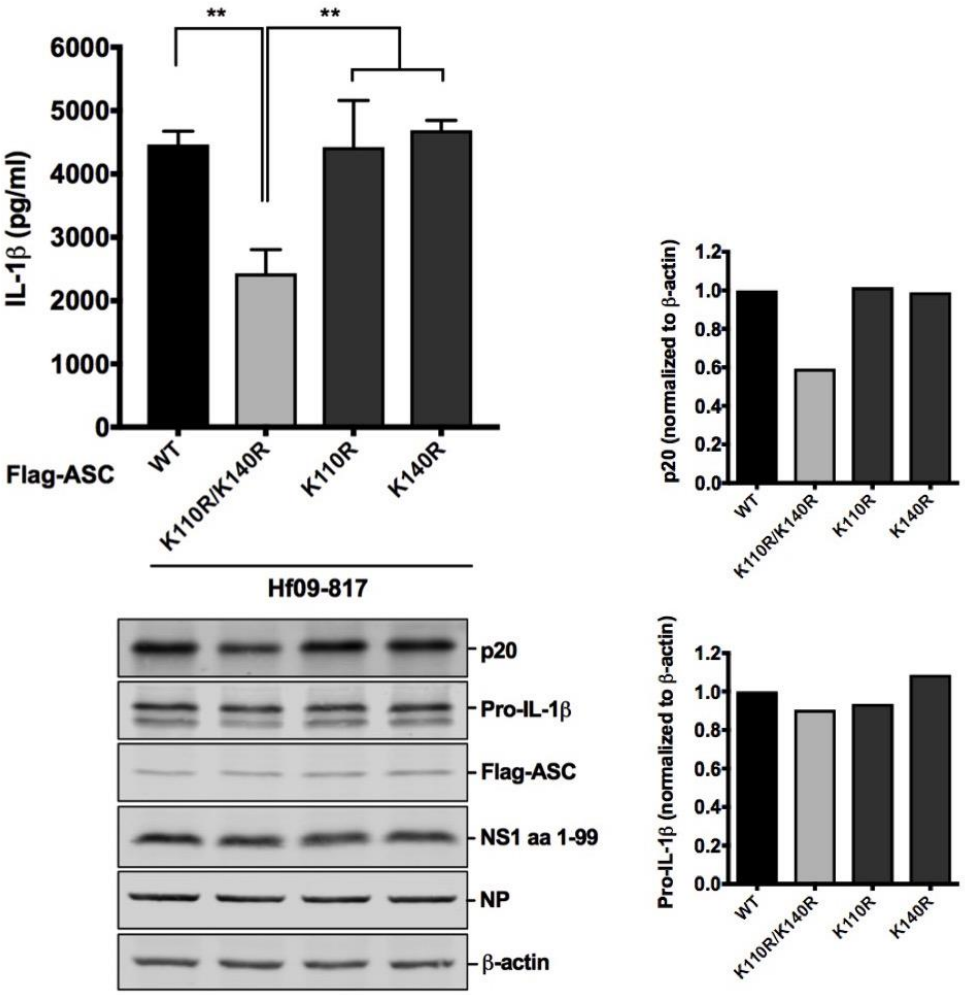


Figure 4.6 Ubiquitination of ASC on K110 and K140 is required for caspase-1 activation in response to NS1-mutant pdm09 virus.

HEK293T cells were transfected with plasmids expressing porcine NLRP3, Flag-tagged WT/mutant ASC, pro-caspase-1 and pro-IL-1β. At 12 hpt, the cells were infected with Hf09-817 at an MOI of 5 for 12 h. Porcine IL-1β was measured by ELISA with cell-free supernatants and the expression of p20, pro-IL-1β, ASC and viral proteins was measured by Western blotting with cell lysates. Expression of p20 and pro-IL-1β was normalized to that of β-actin using Image J. **, p=0.002.

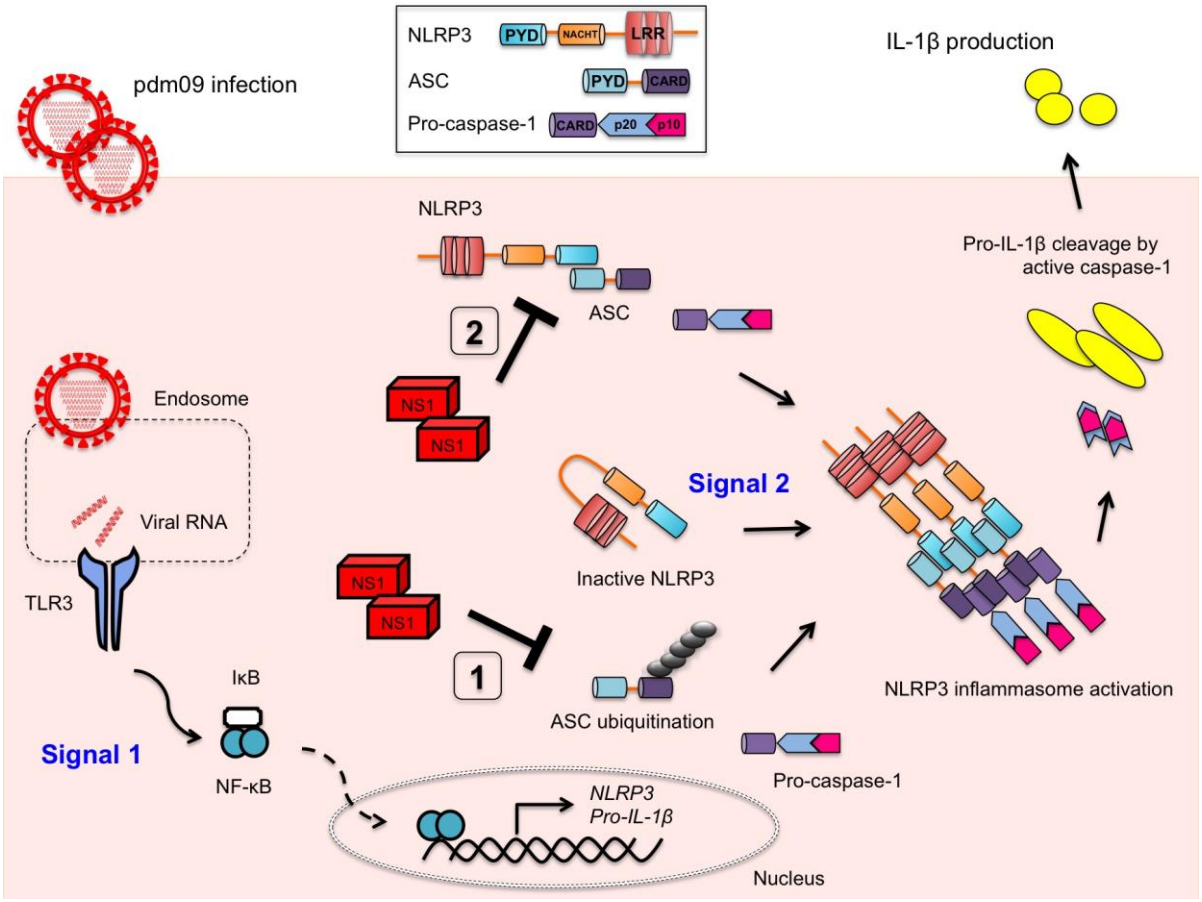


Figure 4.7 A proposed model of the mechanisms by which NS1 C-terminus of pdm09 inhibits the porcine NLRP3 inflammasome.
Viral dsRNA is sensed by TLR3 in the endosome of pdm09-infected alveolar macrophages. This activates the NF-κB pathway, which is required for the transcriptional upregulation of *NLRP3* and *pro-IL-1β* (Signal 1). Cellular stress provoked by viral infection is expected to induce the conformational change of NLRP3 (e.g. oligomerization) (Signal 2), while the roles of pdm09 viral proteins in this process are not fully understood. This results in the PYD-PYD interaction between NLRP3 and ASC leading to the formation of ASC specks. Further recruitment of pro-caspase-1 to ASC by CARD-CARD interaction completes the assembly of NLRP3 inflammasome. Auto-proteolytic cleavage of pro-caspase-1 into active subunits of caspase-1 is required to process the pro-IL-1β, leading to IL-1β secretion. NS1 C-terminus of pdm09 inhibits the porcine NLRP3 inflammasome-mediated IL-1β production with two mechanisms. First, it suppresses the ubiquitination of porcine ASC on CARD, which is crucial for caspase-1 activation, but not involved in the formation of ASC specks, while whether this inhibition occurs before or upon NLRP3 activation is unclear. Second, it disrupts the NLRP3-ASC interaction, thereby impairing the ASC speck formation. The domain structure of NLRP3 inflammasome components is shown in the inset.

4.5 Discussion

Ubiquitination and deubiquitination on NLRP3 inflammasome components mediate the inflammasome activity. To seek for the in-depth mechanism by which pdm09 NS1 suppresses porcine NLRP3 inflammasome activity, whether the NS1 modulates the ASC or NLRP3 ubiquitination was investigated. Infection with mutant Hf09 viruses with NS1 C-terminal deletion caused more ubiquitination of ASC than did the WT virus, but not affecting the ubiquitination of NLRP3, implying an inhibitory role of Hf09 NS1 C-terminus on porcine ASC ubiquitination. A speculation that NS1 C-terminus plays an enhancing role on ASC deubiquitination was eliminated, since WT virus infection induced a low level of ubiquitination, which was similar to that in mock-infected cells. One possible explanation could be that the pdm09 NS1 influences E3 ligase activity required for ASC ubiquitination. This type of regulation is reported in RIG-I ubiquitination by an E3 ligase, TRIM25 that is targeted by NS1 to suppress the IFN production (Gack et al., 2009). NS1 C-terminal aa 126-225 of avian H5N1 is recently reported to downregulate the RIG-I-mediated IFN signaling through interacting with TRAF3 and inhibiting the K63-linked ubiquitination of TRAF3 (Qian et al., 2017). This shows that the inhibitory function of NS1 C-terminus on the host immunity is not limited to the suppression of NLRP3 inflammasome. Guan et al. discovered that ASC undergoes ubiquitination via the E3 ligase TRAF3 by forming the complex of ASC-TRAF3-MAVS upon VSV infection (Guan et al., 2015). In that study, MAVS contributes to the stability of ASC and increases the interaction of ASC with TRAF3. In this context, it will be of interest to examine if NS1 interacts with another adaptor protein that associates with an E3 ligase responsible for the ubiquitination of porcine ASC. In addition to K63-linked or linear ubiquitination as previously reported (Guan et al., 2015, Rodgers et al., 2014), ASC was modified by mixed lysine-linked ubiquitination. This does not seem to be a rare event, since NLRP3 is modified by both K48- and K63-linked ubiquitination, and is also deubiquitinated upon activation (Guo et al., 2016, Kawashima et al., 2017, Py et al., 2013, Song et al., 2016). Different types of ubiquitination are thought to modulate the activities of inflammasomes for tight regulation of the innate immune signalling.

K110 and K140 in porcine ASC were the ubiquitin acceptor sites, suggesting that modifications of the C-terminal CARD of ASC, which interacts with another CARD of pro-caspase-1 (Srinivasula et al., 2002, Wang et al., 2002), are crucial for the inflammasome activation. Removal of ASC ubiquitination by K-to-R mutation on the acceptor sites impaired the

caspase-1 activity during virus infection, but did not affect the stability of ASC, localization and number of ASC specks, suggesting the ubiquitination of porcine ASC contributes to the fully functional activation of NLRP3 inflammasome. What remains elusive is whether the ubiquitination on ASC CARD has any impacts on the CARD-CARD interaction between ASC and pro-caspase-1 upon infection with NS1-mutant virus, or the interaction occurs regardless of the ASC ubiquitination. It is noteworthy that deubiquitination of ASC can lead to the inflammasome activation (Lee et al., 2017c), which contradicts to the observation in the current study. The ambivalence may be due to the diverse interplay between virus and the host signalling pathways that is dissimilar to how conventional agonists stimulate the immune system. IAV infection can manipulate not only the host gene expression, but also the host ubiquitin system (Tisoncik et al., 2011).

In addition to ASC ubiquitination, other post-translational modifications may also occur on any components of the porcine NLRP3 inflammasome to regulate its activation. SUMOylation or deSUMOylation, a process by which small ubiquitin-like modifier (SUMO) proteins are conjugated to or deconjugated from target proteins can be one of them. Unlike ubiquitination, SUMOylation occurs on a lysine residue in the consensus motif as well as other lysines, which do not constitute the motif on the target protein (Wilkinson and Henley, 2010). A recent study shows the elevated activation of NLRP3 inflammasome upon deSUMOylation on NLRP3 (Barry et al., 2018). Interestingly, porcine ASC, but not human ASC, contains the SUMO consensus motif, Ψ-K-x-D/E (where Ψ is a hydrophobic residue and x is any residue). Whether porcine ASC undergoes SUMOylation/deSUMOylation upon IAV infection and if it is relevant to the NLRP3 inflammasome activation are outstanding concerns.

Hf09 NS1 C-terminus not only suppresses ASC ubiquitination on the CARD, but also interferes with the PYD-PYD homotypic interaction between porcine NLRP3 and ASC, which leads to the reduced ASC specks as shown in the previous chapter and in Figure 4.7. Therefore, the two inhibitory effects by pdm09 NS1 exerted on different domains of ASC are thought to be independent each other, and pdm09 NS1 may be equipped with redundant strategies to block NLRP3 inflammasome activation. Considering that ubiquitination of human ASC along with human IL-1β secretion was also repressed by pdm09 NS1 C-terminus, it is suggested that pdm09 has acquired the ability to evade the host immunity of both pigs and humans. Further

investigation is needed to identify the ubiquitination sites on human ASC and to define whether this strategy is also played by other swine-origin human IAVs.

In summary, porcine ASC could be modified by ubiquitination upon IAV infection, and NS1 C-terminus of pdm09 suppressed the ubiquitination on K110/K140, which was critical for NLRP3 inflammasome-mediated IL-1β production. Further, the inhibitory function played by pdm09 NS1 was also executed on human ASC. These provide new insights into NS1-mediated immune evasion in the swine and human hosts infected with IAVs.

TRANSITION BETWEEN CHAPTER 4 AND CHAPTER 5

In Chapter 4, the ubiquitination of porcine ASC was shown to be suppressed by NS1 C-terminus of pdm09. Further analyses identified that K110 and K140 on CARD of porcine ASC were target sites of ubiquitination. Ubiquitination on those residues was indispensable for caspase-1 activation, while it is not directly involved in the ASC speck formation or ASC stability. Along with observations in Chapter 3, this revealed novel mechanisms by which pdm09 NS1 exerts to evade the host inflammatory responses. Considering that a high level of IL-1β was produced upon swine influenza virus (SIV) infection in Chapter 3, an important question about the underlying mechanism for that event is not resolved yet. Thus, the upstream mechanism by which SIV induces NLRP3 inflammasome-mediated IL-1β production is explored in the following chapter. Based on the knowledge that mitochondria play a central role in controlling the innate immunity including the NLRP3 inflammasome activation, how mitochondrial dynamics and its related signalling are involved in SIV-induced IL-1β production is a main subject in Chapter 5.

CHAPTER 5 SWINE INFLUENZA VIRUS INDUCES NLRP3 INFLAMMASOME-MEDIATED INTERLEUKIN-1 BETA PRODUCTION THROUGH THE RIPK1/DRP1 SIGNALING.

The data presented in this chapter were published as

Swine Influenza Virus Induces RIPK1/DRP1-Mediated Interleukin-1 Beta Production.

HS Park, G Liu, Q Liu and Y Zhou. (2018)

Viruses, 10(8), 419.

Acknowledgement specific for this chapter:
The WT and mutant human RIPK1 constructs were provided by Dr. Xin Lin (MD Anderson Cancer Center) and Dr. Francis Chan (University of Massachusetts Medical School), respectively.

5.1 Abstract

Nucleotide-binding domain and leucine-rich repeat-containing protein 3 (NLRP3) inflammasome plays a pivotal role in modulating lung inflammation in response to the influenza A virus infection. Based on the previous finding that swine influenza virus (SIV) infection induced NLRP3 inflammasome-mediated IL-1β production in primary porcine alveolar macrophages (PAMs), the upstream signaling events that are involved in this process were examined. In this study, results showed that SIV infection led to dynamin-related protein 1 (DRP1) phosphorylation at serine 579 (S579) and mitochondrial fission in PAMs. Concomitantly, IL-1β production was dependent on the reactive oxygen species (ROS) production, and NLRP3 inflammasome activity was upregulated by phospho-mimetic mutation at S579 on DRP1. Furthermore, the requirement of the kinase activity of receptor-interacting protein kinase 1 (RIPK1) for the IL-1β production and the association of RIPK1 with DRP1 suggested that RIPK1 is an upstream kinase for DRP1 phosphorylation. These results showed a critical role of the RIPK1/DRP1 signaling axis, whose activation leads to mitochondrial fission and ROS release, in modulating porcine NLRP3 inflammasome-mediated IL-1β production in SIV-infected PAMs.

5.2 Introduction

Interleukin-1 beta (IL-1β) is a pro-inflammatory cytokine that contributes to the effective modulation of the host innate and adaptive immunity upon influenza A virus (IAV) infection (Allen et al., 2009, Ichinohe et al., 2009, Thomas et al., 2009). IL-1β is tightly regulated by pattern recognition receptors (PRRs) such as nucleotide-binding domain and leucine-rich repeat-containing protein 3 (NLRP3), which functions by forming the NLRP3 inflammasome with its adaptor protein, apoptosis-associated speck-like protein containing caspase activation and recruitment domain (ASC), and pro-caspase-1. The activation of NLRP3 leads to the conversion of pro-caspase-1 into caspase-1, which cleaves pro-IL-1β to generate mature IL-1β and also induces pyroptosis (Kuriakose and Kanneganti, 2017, Sarvestani and McAuley, 2017).

Mitochondria are well appreciated for their roles in innate immunity. Mitochondrial proteins are involved in the NLRP3 inflammasome regulation (Ichinohe et al., 2013, Moriwaki et al., 2016, Park et al., 2013b). Pathophysiological changes mediated by mitochondria including the imbalance in mitochondrial dynamics and the release of cytochrome c, mitochondrial DNA

or reactive oxygen species (ROS) can either activate or inhibit the NLRP3 inflammasome (Nakahira et al., 2011, Park et al., 2015, Shi and Kehrl, 2016, Yoshizumi et al., 2014, Zhou et al., 2011). The phosphorylation of dynamin-related protein 1 (DRP1), a guanosine triphosphate (GTP) hydrolase (GTPase), on different residues determines mitochondrial dynamics, namely fission and fusion. For human DRP1 (transcript variant 1), phosphorylation at serine 616 (S616) or dephosphorylation at serine 637 (S637) is linked to mitochondrial fission, whereas phosphorylation at S637 is associated with fusion (Zemirli et al., 2018). In order for fission to occur, DRP1 is recruited to mitochondria upon its phosphorylation at S616 by kinases including receptor-interacting protein kinase 1 (RIPK1) or dephosphorylation at S637 (Cereghetti et al., 2008, Kashatus et al., 2015, Taguchi et al., 2007, Wang et al., 2014c). The association of DRP1 with mitochondrial fission proteins leads to mitochondrial fission, which depends on GTP hydrolysis by DRP1 (Elgass et al., 2013). The suppression of mitochondrial fission by diverse molecules leads to the inhibition of NLRP3 inflammasome activity (Li et al., 2016a, Li et al., 2016d, Zhou et al., 2016), while this is contradicted by a finding that the impaired mitochondrial fission can upregulate the NLRP3 inflammasome activity (Park et al., 2015).

Viruses can alter the mitochondrial dynamics to subvert the host's innate immunity or to modulate the host cell survival. Various RNA viruses are reported to induce DRP1-mediated mitochondrial fission to regulate apoptosis (Gou et al., 2017, Kim et al., 2014, Li et al., 2016c, Mukherjee et al., 2018), whereas the dengue virus is reported to regulate mitochondrial fusion (Barbier et al., 2017, Chatel-Chaix et al., 2016, Yu et al., 2015). An initial study on DRP1-mediated NLRP3 inflammasome activation upon viral infection discovered that in response to vesicular stomatitis virus (VSV), RIPK1 forms a complex with RIPK3 to induce necroptosis, while it also phosphorylates DRP1. The latter event leads to mitochondrial fission, and subsequently, ROS can activate NLRP3 leading to IL-1β production (Wang et al., 2014c). In contrast, another report showed that the NLRP3 inflammasome activation upon VSV infection does not require RIPK1 or DRP1 (Kang et al., 2015). In addition, there are reports showing that cyclin-dependent kinase 1 is required for DRP1 phosphorylation and mitochondrial fission upon infection with RNA viruses such as hepatitis C virus and rotavirus (Kim et al., 2014, Mukherjee et al., 2018). Upon IAV infection, a mitochondrial fusion protein, mitofusin 2, can regulate the NLRP3 inflammasome activation (Ichinohe et al., 2013). The IAV PB1-F2 protein, which translocates to mitochondria, can cause mitochondrial fission that is associated with the defective

NLRP3 inflammasome activation (Yoshizumi et al., 2014). However, there is still a lack of information on how DRP1-mediated mitochondrial fission fits in the innate immune signaling in IAV-infected cells.

In the previous chapter, swine influenza virus (SIV) infection induced NLRP3 inflammasome activation leading to the IL-1β production in primary porcine alveolar macrophages (PAMs). In this chapter, whether DRP1-mediated mitochondrial dynamics are involved in this process was investigated. The results suggest that upon SIV infection of PAMs, mitochondrial fission occurred through the RIPK1/DRP1 signaling axis, which is involved in the NLRP3 inflammasome-mediated IL-1β production.

5.3 Materials and methods

5.3.1 Cells and viruses

PAMs were isolated from lungs of 4-week-old, SIV-negative piglets by collecting bronchoalveolar lavage fluid, where most of the cells are comprised of alveolar macrophages (Ait-Ali et al., 2007, Gordon and Read, 2002). The PAMs were characterized by flow cytometry using mouse anti-pig macrophage antibody conjugated with fluorescein isothiocyanate (MCA2317F, Bio-Rad) (Liu et al., 2015). PAMs were cultured with HyClone RPMI 1640 medium (SH30027.01, GE Healthcare) supplemented with 20% FBS (16000-044, Thermo Fisher), 50 μg/mL gentamicin (BS724, Bio Basic Canada), and 1 × Antibiotic-Antimycotic (15240-062, Thermo Fisher). HEK293T cells were maintained in DMEM (D5796, Sigma) supplemented with 10% FBS and 50 μg/mL gentamicin. The SIV strain, Sk02 was propagated in MDCK cells and titrated by plaque assay as mentioned in the previous chapters.

5.3.2 Antibodies and reagents

Rabbit polyclonal NP and NS1 antibodies were produced as described (Shin et al., 2007b). The commercial antibodies used are as follows. Goat anti-porcine IL-1β antibody (BAF681): R&D Systems; rabbit anti-porcine caspase-1 (p20) antibody (PAB592Po01): Cloud-Clone Corp.; mouse anti-Myc-tag antibody (#2276), mouse anti-β-actin antibody (#3700), rabbit anti-DRP1 antibody (#8570) and rabbit anti-phospho-DRP1 (S616) antibody (#3455): Cell Signaling Technology; mouse anti-FLAG M2 antibody (F3165): Sigma; IRDye 680RD donkey anti-rabbit

(926-68073), IRDye 800CW donkey anti-mouse (926-32212) and IRDye 800CW donkey anti-goat (926-32214) antibodies: LI-COR Biosciences. The following reagents were used: LPS (L3024) and N-acetyl L-cysteine (NAC) (A9165): Sigma; Necrostatin-1 (Nec-1) (BML-AP309): Enzo Life Sciences; Mdivi-1 (ab144589): Abcam.

5.3.3 Plasmid construction

Plasmids expressing porcine NLRP3, ASC, procaspase-1, and pro-IL-1β were generated using cDNA synthesized from the total RNA of PAMs that were stimulated with LPS as described in the previous chapters. Using the same cDNA, the full-length porcine DRP1 was cloned into pcDNA3.1-3×Myc (C-terminal tag) generating pcDNA-DRP1-Myc. Primers were designed based on the GenBank sequence of porcine DRP1 transcript variant X1 (accession number: XM_021092056). Myc-tagged DRP1 constructs with S579D or S579A mutation (pcDNA-DRP1 (S579D)-Myc or pcDNA-DRP1 (S579A)-Myc) were generated by site-directed mutagenesis using pcDNA-DRP1-Myc as the template. Porcine RIPK1 was cloned into pcDNA3.1-3×Myc or pCMV-3×Flag (N-terminal tag) using cDNA from a porcine alveolar macrophage cell line, 3D4/2, generating pcDNA-RIPK1-Myc or pCMV-Flag-RIPK1. Using the above construct as the template, the mutant RIPK1 construct with K41A/K42A (pcDNA-RIPK1 (K41A/K42A)-Myc) was generated by mutagenesis. Myc-tagged human RIPK1 (huRIPK1) (#44159, Addgene) was a gift from Xin Lin (Blonska et al., 2004). HA/GFP-tagged human RIPK1 (K45A) (#41389, Addgene) was a gift from Francis Chan (Cho et al., 2009) and used as the template to generate Myc-tagged human RIPK1 (K45A). All the mutations were confirmed by nucleotide sequencing. The expression plasmids generated for this chapter are listed in Table 5.1 and the primers are listed in Supplementary Table 4.

Table 5.1 List of expression plasmids used in Chapter 5.
The constructs for NLRP3 inflammasome components and pro-IL-1β used for NLRP3 inflammasome reconstitution assay were described in Table 3.2.

Construct	Template used to clone	Purpose
pcDNA-DRP1-Myc	cDNA from PAMs	NLRP3 inflammasome reconstitution, RIPK1-DRP1 interaction
pcDNA-DRP1 (S579D)-Myc	pcDNA-DRP1-Myc	NLRP3 inflammasome reconstitution
pcDNA-DRP1 (S579A)-Myc	pcDNA-DRP1-Myc	NLRP3 inflammasome reconstitution
huRIPK1-Myc (Blonska et al., 2004)		NLRP3 inflammasome reconstitution
huRIPK1 (K45A)-Myc	HA/GFP-huRIPK1 (K45A) (Cho et al., 2009)	NLRP3 inflammasome reconstitution
pcDNA-RIPK1-Myc	cDNA from 3D4/2 cells	NLRP3 inflammasome reconstitution
pcDNA-RIPK1 (K41A/K42A)-Myc	pcDNA-RIPK1-Myc	NLRP3 inflammasome reconstitution
pCMV-Flag-RIPK1	pcDNA-RIPK1-Myc	NLRP3 inflammasome reconstitution, RIPK1-DRP1 interaction

5.3.4 Infection and treatment of PAMs

PAMs seeded at 1×10^6 cells per well on 24-well plates were either infected with the Sk02 virus for 20 h at an MOI of 1 or stimulated with 200 ng/mL LPS for 12 h. The cells were pre-treated with vehicles or inhibitors for 2 h, and were then infected with the virus or stimulated with LPS in the presence of the vehicles or inhibitors. Nec-1 was used at 80 or 160 μM for RIPK1 inhibition; Mdivi-1 was used at 5 or 20 μM for DRP1 inhibition, while DMSO was used as the vehicle. As a ROS scavenger, NAC was used at 2 or 10 mM and distilled water was used as the vehicle. Cell-free supernatants were collected for ELISA. Cells were lysed with 1 × SDS sample buffer and boiled for Western blotting. To detect the phosphorylated DRP1 (phospho-DRP1), the PAMs were seeded at 3×10^6 cells per well on 6-well plates. The cells were infected with the Sk02 virus for 4 h at an MOI of 1 or stimulated with 200 ng/mL LPS for 12 h. The cells in each well were lysed with 120 μL of M-PER Mammalian Protein Extraction Reagent (78503,

Thermo Fisher) containing 1× Halt Protease and Phosphatase Inhibitor Cocktail (78440, Thermo Fisher). After centrifugation, the cell lysates were mixed with 5 × SDS sample buffer and were subjected to Western blotting.

5.3.5 RNA interference and quantitative PCR

siRNA targeting porcine *RIPK1* was designed by the siRNA design tool provided by Dharmacon. The *RIPK1*-targeting siRNA (5′-UGGAAGAGGAUGUGAAGAAUU-3′) and a negative control siRNA (D-001810-01-05, 5′-UGGUUUACAUGUCGACUAA-3′) were purchased from Dharmacon. PAMs seeded at 1×10^6 cells per well on 24-well plates were transfected with 100 nM of the control siRNA or RIPK1-targeting siRNA using Lipofectamine RNAiMAX (13778-030, Thermo Fisher). At 24 hpt, the cells were infected with the Sk02 virus for 16 h at an MOI of 1. To show the knockdown efficiency, the relative mRNA expression of *RIPK1* compared to that of porcine *HPRT1* gene was measured by quantitative PCR using cDNA from total RNA with a standard protocol. Primers for porcine *RIPK1* were designed based on the GenBank sequence (accession number XM_005665536) using an online tool, Primer3 (Untergasser et al., 2012) and those for the housekeeping gene, *HPRT1* were used as previously reported (Nygard et al., 2007). Primer sequences are indicated in Table 5.2.

Table 5.2 List of primers used for quantitative PCR in Chapter 5.

Target	Primer name	Sequence (5'-3')	Amplicon size
Porcine *HPRT1*	HPRT1 Fw	GGACTTGAATCATGTTTGTG	91 bp
	HPRT1 Bw	CAGATGTTTCCAAACTCAAC	
Porcine *RIPK1*	RIPK1 Fw	TTCGGAAAGGTGTCCTTGTG	91 bp
	RIPK1 Bw	CGTTGTACTCATTGCGCTTG	

bp, base pairs.

5.3.6 NLRP3 inflammasome reconstitution assay

The porcine NLRP3 inflammasome reconstitution assay was conducted to examine the effects of ectopically expressed proteins on NLRP3 inflammasome activity as described in the previous chapters. Briefly, the HEK293T cells seeded at 1.5×10^5 cells per well on 24-well plates were co-transfected with expression plasmids for porcine NLRP3 inflammasome

components and pro-IL-1β (pcDNA-NLRP3 (30 ng), pcDNA-ASC (20 ng), pCMV-Flag-procaspase-1 (20 ng), and pcDNA-pro-IL-1β (100 ng)) using *Trans*IT-LT1 Transfection Reagent (MIR2300, Mirus Bio). Other plasmids (50 ng of Myc-vector or Myc-tagged human RIPK1 WT/mutant, 100 ng of Myc-vector or Myc-tagged porcine RIPK1 WT/mutant, 100 ng of Myc-vector or Myc-tagged DRP1 WT/mutant) were co-transfected to study their effects on NLRP3 inflammasome activity. At 16 hpt, supernatants were harvested for IL-1β ELISA and the pelleted cells were lysed for Western blotting.

5.3.7 Co-IP

HEK293T cells were seeded at 9×10^5 per well on 6-well plates and were transfected with 1 μg of Flag-tagged vector or Flag-tagged RIPK1 and Myc-tagged DRP1. At 24 hpt, the cells were lysed with 500 μL lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40 substitute, 1 × protease inhibitor cocktail) and clarified by centrifugation at 12,000 × *g* at 4 ℃ for 10 min. For input, 10% of the cell lysates were mixed with 5× SDS sample buffer and boiled at 95 °C for 5 min. For each sample, 35 μL of Dynabeads Protein G (10004D, Thermo Fisher) were first conjugated with 1 μg of mouse monoclonal anti-FLAG M2 antibody (F3165, Sigma) in 300 μL phosphate-buffered saline (PBS) with 0.02% Tween 20 by agitation at room temperature for 1 h. Then, the beads were incubated with the cell lysates at room temperature for 2 h with agitation. After washed thrice with Tris-buffered saline (50 mM Tris, pH 7.4, 150 mM NaCl), the beads were resuspended in 60 μL of 2× SDS sample buffer and boiled as above to be analyzed by SDS-PAGE and Western blotting.

5.3.8 Porcine IL-1β ELISA

Porcine IL-1β in the cell-free supernatants was determined using ELISA as described in the previous chapters. Briefly, Immulon 2 HB U plates (#3655, Thermo Fisher) were coated with a mouse anti-porcine IL-1β antibody (MAB6811) at 2 μg/mL in PBS overnight. The plates were blocked with 1% BSA (A7030, Sigma) in PBS for 1 h, and were incubated with either samples or the standard for 2 h. Two-fold serial dilutions of the recombinant porcine IL-1β protein (681-PI-010, R&D Systems) in diluent (0.1% BSA in Tris-buffered saline with 0.05% Tween 20) was used for the standard curve. Next, the plates were incubated with goat anti-porcine IL-1β biotinylated antibody (BAF681, R&D Systems) at 50 ng/mL in the diluent for 1 h, and further

incubated for 1 h with alkaline phosphatase-streptavidin (016-050-084, Jackson ImmunoResearch) that was diluted in a ratio of 1:5,000 by the diluent. After incubation with 1 mg/mL of PNPP in the diethanolamine buffer (1 M diethanolamine, 0.5 M $MgCl_2$, pH 9.8), optical densities were measured at 405 nm with a reference at 490 nm using the xMark Microplate Absorbance Spectrophotometer (Bio-Rad).

5.3.9 Western blotting

Cell lysates or IP samples were subjected to SDS-PAGE followed by blotting on nitrocellulose membranes. The membranes were blocked with 5% skim milk (or 5% BSA for the detection of phospho-DRP1) in TBS with 0.1% Tween 20 for 1 h and incubated with primary antibodies at 4 ℃ overnight. The membranes were further incubated with secondary antibodies at room temperature for 1 h and were scanned with an Odyssey Infrared Imager (LI-COR Biosciences).

5.3.10 Confocal Microscopy

To check the mitochondrial integrity, PAMs were seeded at 2×10^5 cells per well on LabTek II CC2 chamber slides (154941, Thermo Fisher). The cells were infected with the Sk02 virus at an MOI of 1 for 7 h or they were stimulated with 200 ng/mL LPS for 16 h. After fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 in DPBS for 5 min, the cells were incubated with 5% BSA in DPBS for 1 h. The cells were probed with mouse monoclonal anti-TOM20 antibody (sc-17764, Santa Cruz) and goat polyclonal anti-IAV antibody (AB1074, EMD Millipore) overnight at 4 ℃, and the secondary antibodies (Alexa Fluor 488 donkey polyclonal anti-mouse IgG_{H+L} (A21202; Invitrogen) and Alexa Fluor 633 donkey polyclonal anti-goat IgG_{H+L} (A21082; Invitrogen)) for 1 h at room temperature. Counterstaining was performed with DAPI (D1306, Invitrogen) for 5 min and the coverslips were mounted with ProLong Diamond Antifade Mountant (P36961, Invitrogen) overnight at room temperature. Images were obtained by using a confocal laser scanning microscope (TCS SP8, Leica).

5.3.11 Statistical analysis

ANOVA with Tukey's multiple comparison tests or unpaired *t*-test with Welch's correction was performed using GraphPad Prism 7. The error bars indicate the mean ± SD. The *p*-values of less than 0.05 were considered to be statistically significant.

5.4 Results

5.4.1 SIV infection induces dynamin-related protein 1 (DRP1) phosphorylation and mitochondrial fission.

It has been reported that virus-induced DRP1 phosphorylation at S616 leads to the mitochondrial fission in the early stages of viral infection (Keck et al., 2017, Mukherjee et al., 2018, Wang et al., 2014c). Amino acid alignment indicated that S616 (location based on the transcript variant X1 of human DRP1) corresponds to S579 of porcine DRP1 (transcript variant X8) and is conserved among different isoforms of human DRP1 and porcine DRP1. It was of interest whether phosphorylation at S579 on porcine DRP1 and the subsequent mitochondrial fission would occur upon SIV infection. PAMs were infected with the Sk02 virus for 4 h at an MOI of 1 and the cell lysates were analyzed by Western blotting with antibodies specific for phospho-DRP1 (S616) or the total form of DRP1. The SIV infection indeed resulted in phosphorylation of DRP1 at S579, while the expression of total form was not changed (Figure 5.1A). As a positive control, PAMs were treated with LPS, which is known to induce DRP1 S616 phosphorylation or DRP1 S637 dephosphorylation-mediated mitochondrial fission in murine cells (Park et al., 2013a, Shen et al., 2018). As expected, LPS stimulation led to DRP1 phosphorylation in the PAMs (Figure 5.1B).

To test whether mitochondrial fission also occurs by SIV infection, the PAMs were infected with Sk02 for 7 h at an MOI of 1. Immunofluorescence staining was conducted by probing the cells with the antibody against translocase of outer mitochondrial membrane 20 (TOM20), to observe the mitochondrial morphology. Confocal imaging revealed that in Sk02-infected cells, mitochondria exhibited short and round forms owing to fragmentation; however, in mock-infected cells, normal tubular and networked mitochondria were observed (Figure 5.1C). Likewise, mitochondrial fission was demonstrated in LPS-treated PAMs (Figure 5.1D). These data show that upon SIV infection, porcine DRP1 phosphorylation at S579 followed by mitochondrial fission occurs in PAMs.

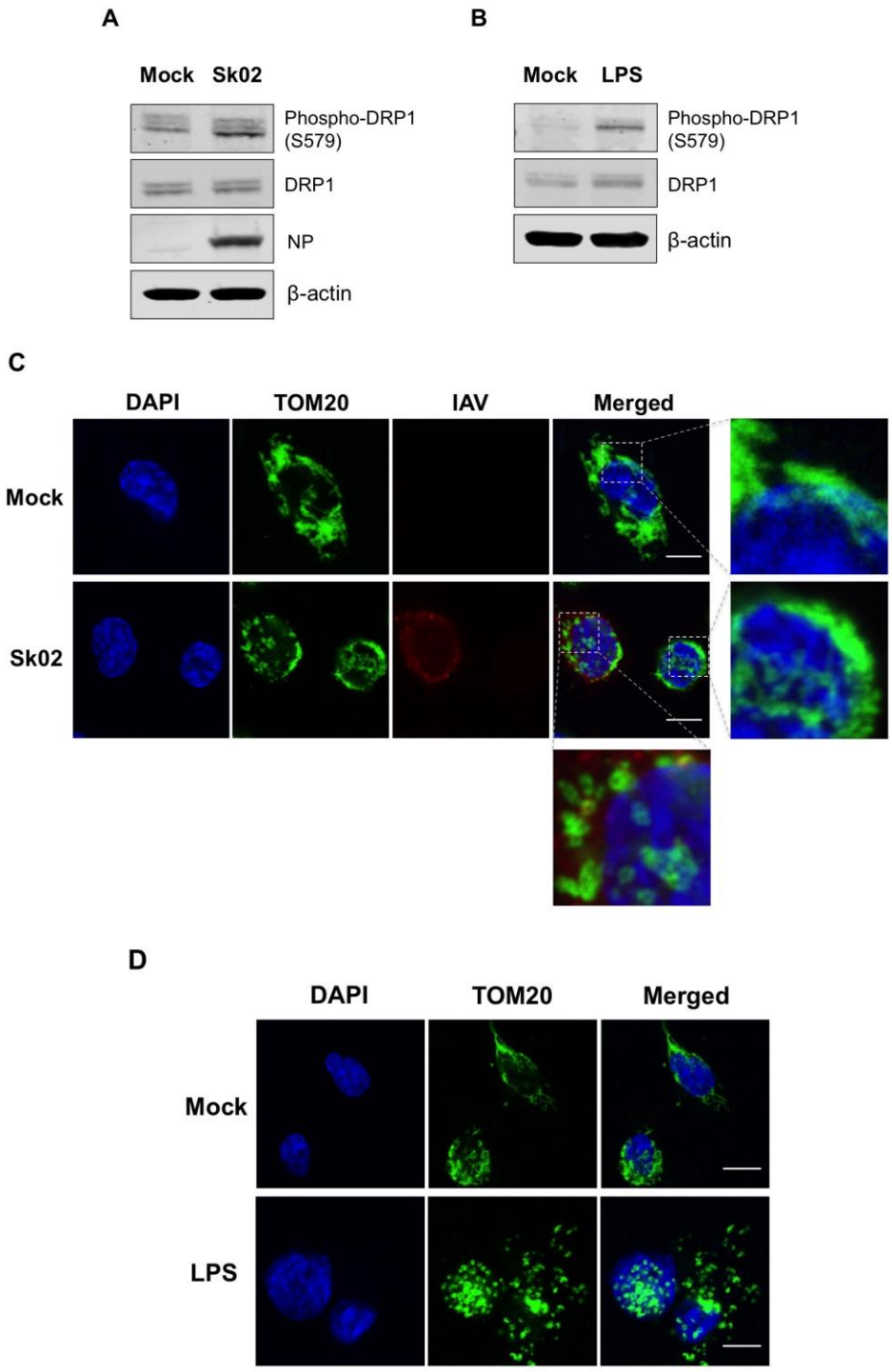


Figure 5.1 SIV infection induces DRP1 phosphorylation and mitochondrial fission.
PAMs were either infected with SIV Sk02 at an MOI of 1 for 4 h (A) or treated with 200 ng/mL lipopolysaccharide (LPS) for 12 h (B). Phospho-DRP1 at S579 and the total form of porcine

DRP1 were detected by Western blotting. The viral NP protein was also monitored by Western blotting in infected cells. PAMs were either infected with Sk02 at an MOI of 1 for 7 h (C) or treated with 200 ng/mL LPS for 16 h (D). Immunofluorescence and confocal microscopy were performed using an antibody against the mitochondrial protein, TOM20, and the influenza A virus (IAV) antibody, when infected. Nuclei were stained by DAPI. Boxed areas were magnified and displayed on the right side (for a mock or un-infected cell) or below (for an infected cell) the corresponding panels in (C). Scale bar, 10 μm.

5.4.2 SIV-induced porcine IL-1β production is dependent on reactive oxygen species (ROS) production.

Mitochondrial ROS from damaged mitochondria can induce NLRP3 inflammasome activation (Allen et al., 2009, Zhou et al., 2011) and whether IL-1β production by SIV infection is ROS-dependent was further examined. PAMs were infected with Sk02 in the presence of a ROS scavenger, NAC, or a vehicle for 20 h. The IL-1β level detected from the supernatant was considerably higher in SIV-infected cells than that in mock-infected cells (Figure 5.2A). Treatment of NAC significantly decreased IL-1β production in a dose-dependent manner. The expression of pro-IL-1β and the viral protein was not impaired, indicating the NAC eliminates a signal required for inflammasome activity. As a control, a decline in IL-1β levels was also achieved when the cells were treated with LPS in the presence of NAC (Figure 5.2B). This suggests that ROS generated from fragmented mitochondria enhances NLRP3 inflammasome-dependent IL-1β production in PAMs upon SIV infection.

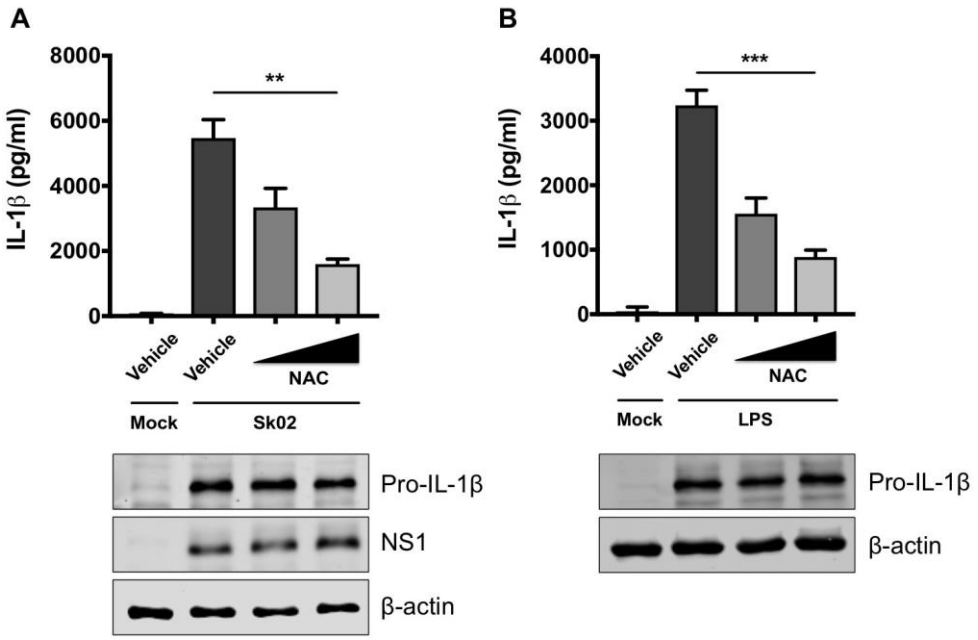


Figure 5.2 SIV-induced porcine IL-1β production is dependent on ROS production.
PAMs were either infected with Sk02 at an MOI of 1 for 20 h (A) or stimulated with 200 ng/mL LPS for 16 h (B) in the presence of vehicle (distilled water) or increasing concentrations (2 and 10 mM) of an ROS scavenger, NAC. Porcine IL-1β levels from the supernatants were measured by ELISA and the expression of pro-IL-1β, viral NS1, and β-actin was analyzed by Western blotting. Statistical analysis was performed with one-way ANOVA. **, $p< 0.01$; ***, $p< 0.001$.

5.4.3 SIV-induced porcine IL-1β production is mediated by DRP1.

The aforementioned data suggested that DRP1-mitochondrial fission axis is involved in the SIV-induced IL-1β production. To further confirm the role of DRP1, PAMs were treated with Mdivi-1, which inhibits the GTPase activity of DRP1 and mitochondrial fission (Cassidy-Stone et al., 2008), and were then infected with Sk02 at an MOI of 1 for 20 h. As seen before, the IL-1β level was appreciably elevated in SIV-infected cells compared to that in mock cells. The treatment with Mdivi-1 led to a decreased IL-1β production upon Sk02 infection in a dose-dependent manner. Pro-IL-1β expression and viral replication as indicated by NS1 expression were not much affected (Figure 5.3A). LPS-induced inflammatory cytokine expression is shown to be mediated by DRP1 activity (Park et al., 2013a), hence the LPS-treated cells were also used as a control. With the pro-IL-1β expression unaffected, the IL-1β production upon LPS was declined by the Mdivi-1 treatment (Figure 5.3B).

To demonstrate the role of S579-phosphorylated DRP1 on pro-caspase-1 cleavage, a hallmark of inflammasome activation, NLRP3 inflammasome reconstitution was performed using HEK293T cells that are deficient for endogenous human NLRP3 inflammasome (Bryan et al., 2009). In this assay, the first signal required for NLRP3 inflammasome activation is provided by the overexpression of NLRP3 inflammasome components and pro-IL-1β; the regulatory effect on the second signal by the proteins and particular amino acids of interest can be examined by monitoring the levels of cleaved caspase-1 and/or mature IL-1β. Despite some limitations, as for all *in vitro* assays, such as excluding the roles of undefined co-factors, the system is especially useful when the cells, in this study PAMs, are difficult to be transfected. Among the different transcript variants of DRP1, porcine DRP1, which was the most identical to the variant X8 (GenBank accession number XM_021092064), was cloned. S579 in porcine DRP1 corresponds to S616 in human DRP1 as explained above and as depicted in Figure 5.3C. Using this as the template, the phospho-mimetic (S579D) and phospho-deficient (S579A) mutants of DRP1 were generated. HEK293T cells were transfected with plasmids expressing NLRP3 inflammasome components and pro-IL-1β along with Myc-tagged vector or WT/mutant DRP1. The co-expression of phospho-mimetic DRP1 (S579D) could significantly increase the caspase-1 activity as shown by the increased level of an active form of caspase-1, p20, while not affecting the expression of pro-IL-1β (Figure 5.3D). In contrast, this was not observed with WT DRP1 or phospho-deficient DRP1 (S579A). Concomitantly, the porcine IL-1β level was also higher in the supernatant of cells co-expressing DRP1 (S579D) compared to other conditions. This data strongly indicate that porcine DRP1 phosphorylation at S579, which is required for mitochondrial fission, enhances the NLRP3 inflammasome activation.

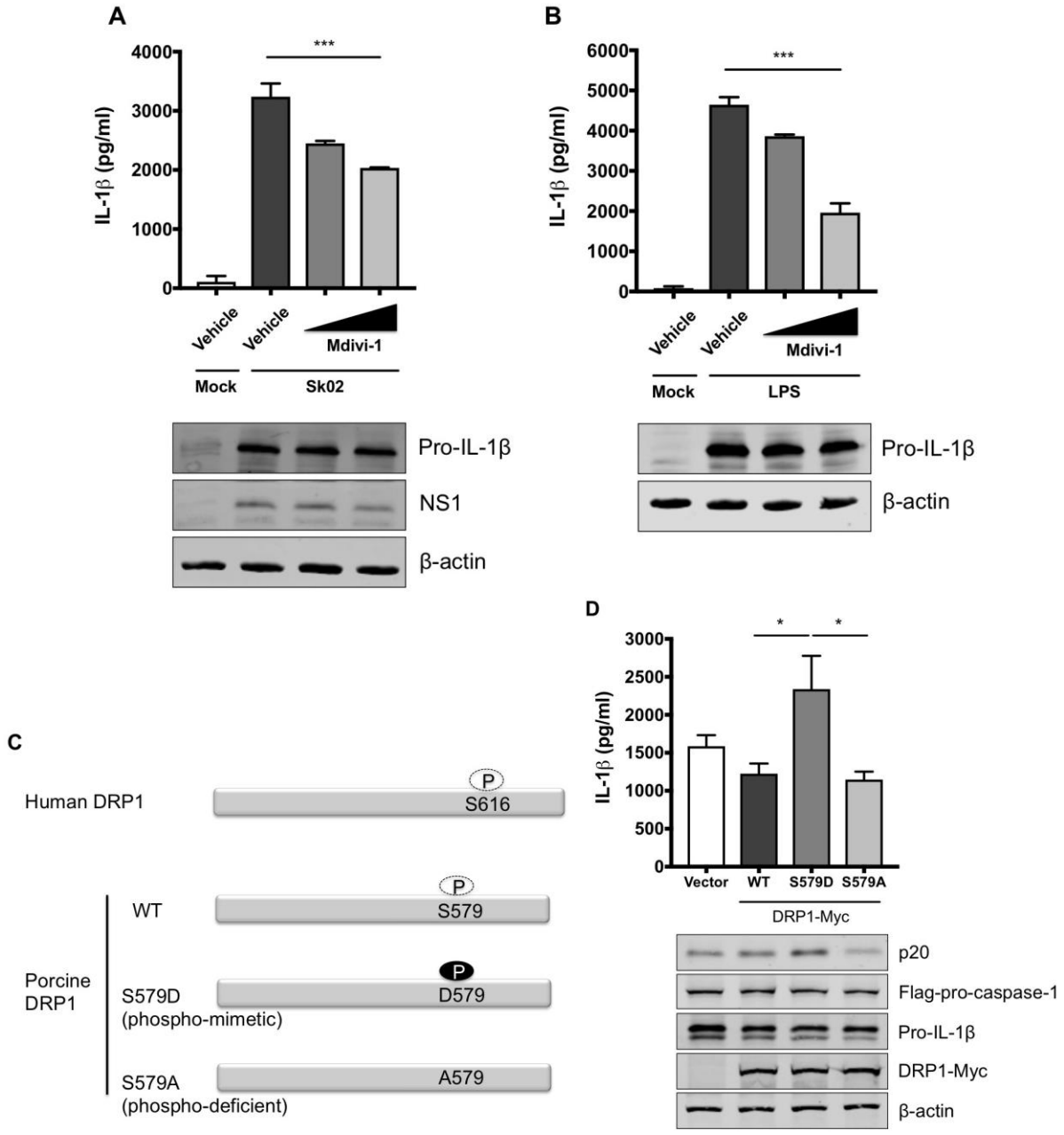


Figure 5.3 SIV-induced porcine IL-1β production is mediated by DRP1.
PAMs were either infected with Sk02 at an MOI of 1 for 20 h (A) or stimulated with 200 ng/mL LPS for 16 h (B) in the presence of vehicle (DMSO) or increasing concentrations (5 and 20 μM) of DRP1 inhibitor, Mdivi-1. Porcine IL-1β levels from supernatants were measured by ELISA and the expression of pro-IL-1β, viral NS1, and β-actin was analyzed by Western blotting. (C) Schematic representation of human DRP1 along with porcine DRP1. S579 in porcine DRP1 WT corresponds to S616 in human DRP1. D579 and A579 harbored by phospho-mimetic and phospho-deficient porcine DRP1 constructs, respectively, are displayed. (D) For NLRP3 inflammasome reconstitution assay, HEK293T cells were co-transfected with plasmids expressing porcine NLRP3 inflammasome components (NLRP3, ASC, and procaspase-1) and

pro-IL-1β along with Myc-vector or Myc-tagged WT/mutant porcine DRP1 for 16 h. Porcine IL-1β levels from supernatants were measured by ELISA. The expression of caspase-1 (active and precursor forms) and pro-IL-1β was analyzed by Western blotting. Statistical analysis was performed with one-way ANOVA. *, $p< 0.05$; ***, $p< 0.001$.

5.4.4 Kinase activity of receptor-interacting protein 1 (RIPK1) is critical for SIV-induced IL-1β production.

Porcine RIPK1 was proposed as a DRP1 kinase upon SIV infection, based on a study showing that DRP1 phosphorylation upon VSV infection is mediated by RIPK1 (Wang et al., 2014c). To demonstrate the requirement of RIPK1 for porcine NLRP3 inflammasome-mediated IL-1β production, the *RIPK1* expression was silenced in PAMs by siRNA. At 24 hpt, the cells were infected with Sk02 for 16 h. IL-1β production from PAMs transfected with *RIPK1* siRNA was significantly decreased compared to the cells transfected with an off-target control siRNA, while the pro-IL-1β expression was not affected by silencing the gene (Figure 5.4A). The knockdown efficiency determined by the mRNA level of *RIPK1* was around 40% compared to that in negative control siRNA-transfected cells. Furthermore, to examine whether the RIPK1 kinase activity is required for upregulation of NLRP3 inflammasome activity, Nec-1, an inhibitor of the RIPK1 kinase activity (Degterev et al., 2008), was used in PAMs. Nec-1 blocks the kinase function by acting on S161 on human RIPK1 (Xie et al., 2013) and this residue is conserved among different transcript variants of porcine RIPK1. Nec-1 treatment drastically reduced IL-1β production in response to the Sk02 infection in a dose-dependent manner, while not impairing the pro-IL-1β expression (Figure 5.4B).

Aiming to confirm the requirement of the porcine RIPK1 kinase function on IL-1β production, the amino acid sequences of the N-terminal ends of the RIPK1 kinase domain from pigs, mice, and humans were aligned (Figure 5.4C). Lysine 45 (K45), indicated with an underline, of human RIPK1 is a critical residue for its kinase activity (Moriwaki and Chan, 2013). While porcine and mouse RIPK1 also contain lysine at the corresponding location to K45 of their human analog, they have two consecutive lysines as underlined, K41/K42 in porcine RIPK1 and K45/K46 in mouse RIPK1. The kinase-dead effects by K45A mutation or K45A/K46T mutation on mouse RIPK1 and their roles in IL-1β production are well documented (Berger et al., 2014, Shutinoski et al., 2016, Zhang et al., 2017c). Considering the higher

sequence identity of porcine RIPK1 to the human one, human RIPK1 constructs were used in the NLRP3 inflammasome reconstitution assay. The co-expression of human RIPK1 WT remarkably increased the porcine IL-1β production. In contrast, the co-expression of kinase-dead human RIPK1 (K45A) led to a decreased level of IL-1β production compared to that of WT RIPK1 (Figure 5.4D). Another experiment after cloning porcine RIPK1 was performed in the same setting as above. Consistently with human RIPK1, porcine RIPK1 WT enhanced IL-1β production, however, the kinase-dead RIPK1 (K41A/K42A) did not (Figure 5.4E). In all samples, the pro-IL-1β expression levels were not affected (Figure 5.4D, E). These suggest that RIPK1 is a positive regulator of NLRP3 inflammasome-mediated IL-1β production and its kinase activity is required for this regulation.

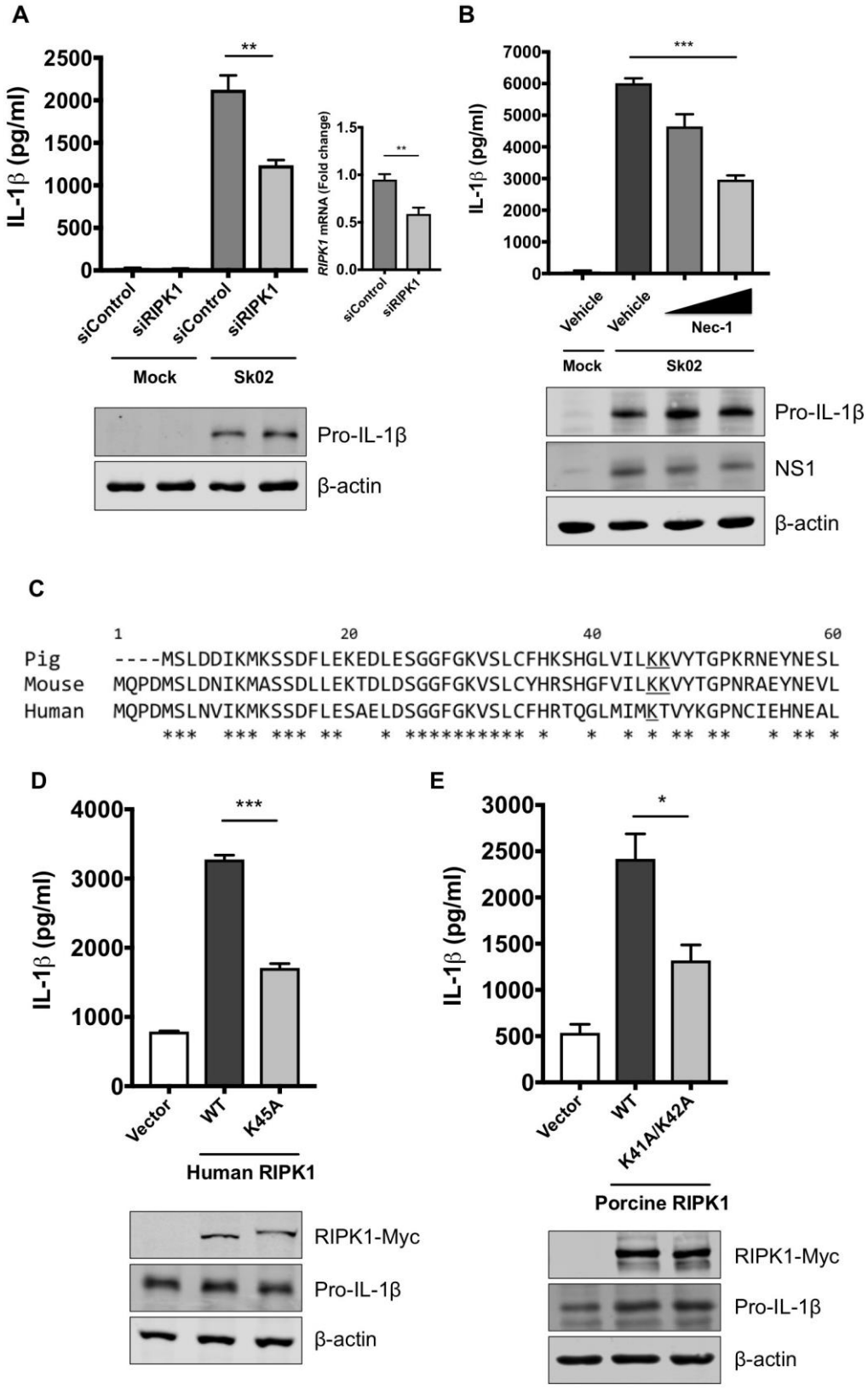

A
IL-1β (pg/ml)
2500
2000
1500
1000
500
0
**
siControl
siRIPK1
siControl
siRIPK1
Mock
Sk02
RIPK1 mRNA (Fold change)
1.5
1.0
0.5
0.0
**
siControl
siRIPK1
Pro-IL-1β
β-actin
B
IL-1β (pg/ml)
7000
6000
5000
4000
3000
2000
1000
0
***
Vehicle
Vehicle
Nec-1
Mock
Sk02
Pro-IL-1β
NS1
β-actin
C
1
20
40
60
Pig ----MSLDDIKMKSSDFLEKEDLESGGFGKVSLCFHKSHGLVILKKVYTGPKRNEYNESL
Mouse MQPDMSLDNIKMASSDLLEKTDLDSGGFGKVSLCYHRSHGFVILKKVYTGPNRAEYNEVL
Human MQPDMSLNVIKMKSSDFLESAELDSGGFGKVSLCFHRTQGLMIMKTVYKGPNCIEHNEAL
*** *** *** ** * ********** * * * * ** ** * ** *
D
IL-1β (pg/ml)
4000
3000
2000
1000
0
***
Vector
WT
K45A
Human RIPK1
RIPK1-Myc
Pro-IL-1β
β-actin
E
IL-1β (pg/ml)
3000
2500
2000
1500
1000
500
0
*
Vector
WT
K41A/K42A
Porcine RIPK1
RIPK1-Myc
Pro-IL-1β
β-actin

Figure 5.4 Kinase activity of RIPK1 is critical for SIV-induced IL-1β production.
(A) PAMs were transfected with a negative control siRNA (siControl) or *RIPK1*-targeting siRNA (siRIPK1) for 24 h and infected with Sk02 at an MOI of 1 for 16 h. Porcine IL-1β levels from supernatants were measured by ELISA. Expression of pro-IL-1β was analyzed by Western blotting. *RIPK1* knockdown efficiency was determined by real-time PCR. (B) PAMs were infected with Sk02 at an MOI of 1 for 20 h in the presence of a vehicle (DMSO) or increasing concentrations (80 and 160 μM) of RIPK1 kinase inhibitor, Nec-1. Porcine IL-1β levels from supernatants were measured by ELISA. Expression of pro-IL-1β and viral NS1 was analyzed by Western blotting. (C) Amino acid sequences of the N-terminal ends of the kinase domains in porcine, murine, and human RIPK1 (based on the GenBank accession numbers XM_005665536, NM_009068, and NM_003804, respectively) were aligned using Clustal Omega. Amino acid positions are based on the human RIPK1 and asterisks indicate conserved residues among the three species. K41/K42 in porcine RIPK1, K45/K46 in murine RIPK1, and K45 in human RIPK1 are underscored. (D) For the NLRP3 inflammasome reconstitution assay, HEK293T cells were co-transfected with plasmids expressing porcine NLRP3 inflammasome components (NLRP3, ASC, and procaspase-1) and pro-IL-1β along with Myc-vector or Myc-tagged WT/mutant human RIPK1 for 16 h. Porcine IL-1β levels from supernatants were measured by ELISA. Protein expression was analyzed by Western blotting. (E) HEK293T cells were transfected as in (D) along with the Myc-vector or Myc-tagged porcine RIPK1 WT/mutant for 16 h. ELISA and Western blotting were performed as in (D). Statistical analysis was performed with one-way ANOVA except for the knockdown efficiency data performed with an unpaired *t*-test. *, < 0.05; **, $p< 0.01$; ***, $p< 0.001$.

5.4.5 RIPK1 interacts with DRP1.

To provide the evidence that DRP1 is regulated by RIPK1, the interaction between the two proteins was examined by co-IP. Since the antibody that is against porcine RIPK1 was not available, proteins overexpressed in HEK293T, instead of endogenous proteins in PAMs, were utilized. HEK293T cells were co-transfected with Flag-vector or Flag-tagged porcine RIPK1 plasmid along with Myc-tagged porcine DRP1 plasmid for 24 h and were subjected to co-IP using the anti-Flag antibody. Association of DRP1 with RIPK1 in IP complex was demonstrated by Western blotting (Figure 5.5), suggesting that RIPK1 and DRP1, by physically interacting, activate the downstream events including the NLRP3 inflammasome activity.

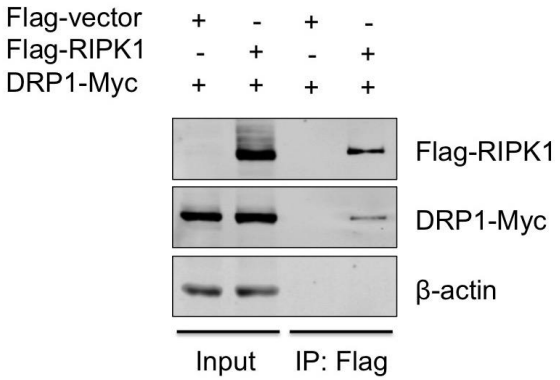


Figure 5.5 RIPK1 interacts with DRP1.
HEK293T cells were co-transfected with the Flag-vector or Flag-RIPK1 construct along with the DRP1-Myc construct for 24 h. Cell lysates were subjected to co-IP with Flag antibody, and the expression of Flag-RIPK1 and DRP1-Myc in input and IP samples was analyzed by Western blotting.

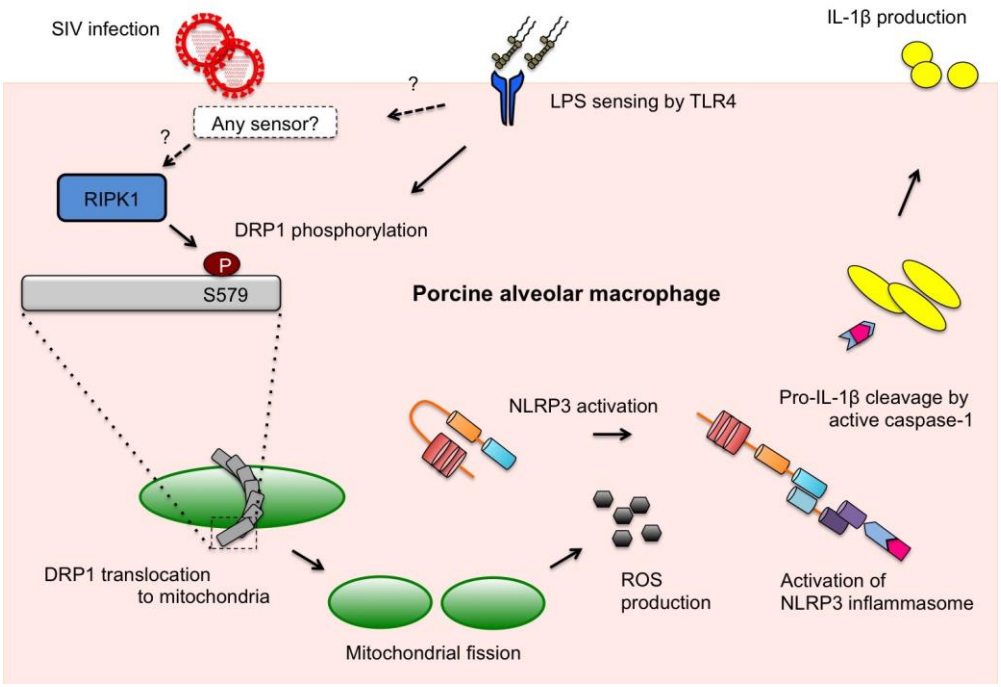


Figure 5.6 A proposed model of RIPK1/DRP1-mediated IL-1β production in SIV-infected PAMs.

Recognition of IAV RNA by endosomal TLRs or RIG-I is known to induce pro-IL-1β synthesis. SIV infection turns on the RIPK1/DRP1 signaling for the NLRP3 inflammasome activation in PAMs. SIV seems to utilize the RIPK1 function, while how RIPK1 is activated by SIV or whether an upstream sensor plays a role in its activation is unclear. By interacting with DRP1 and through its kinase activity, RIPK1 induces the phosphorylation of porcine DRP1 at S579. Upon DRP1 translocation to mitochondria, mitochondrial fission occurs and ROS is generated. This promotes the activation of NLRP3 that is required for the NLRP3 inflammasome assembly and caspase-1 activation. Active caspase-1 converts pro-IL-1β into mature IL-1β, which is critical for SIV-induced lung inflammation. Sensing LPS by TLR4 is known to induce the pro-IL-1β expression, and this also induces the DRP1 phosphorylation leading to the NLRP3 inflammasome activation in PAMs, although it is unclear whether LPS activates any upstream regulator of RIPK1.

5.5 Discussion

NLRP3 inflammasome-mediated IL-1β production is one of the key pathways in inflammatory responses to IAV infection. Alveolar macrophages, as the first responders to lung infection, play critical roles in regulating IAV-induced pulmonary inflammation (Cardani et al., 2017, Kim et al., 2008, Schneider et al., 2014), allowing them to be a useful model to investigate the mechanism of inflammasome activation. The SIV Sk02 strain caused pathogenicity in pigs with typical clinical signs of SIV infection (Masic et al., 2009a), and this virus induced NLRP3 inflammasome-mediated IL-1β production in PAMs in the previous chapter. Here, the upstream mechanism of porcine IL-1β secretion from SIV-infected PAMs was explored.

Changes in mitochondrial dynamics are crucial in modulating the NLRP3 inflammasome activity, and especially, ROS derived from damaged mitochondria is sensed by NLRP3 leading to the activation of NLRP3 inflammasome (Zhou et al., 2011). It was speculated that mitochondrial damage and ROS production would provoke the NLRP3 inflammasome activation upon SIV infection. Indeed, in SIV-infected PAMs, mitochondrial fission and phosphorylation of DRP1, a prerequisite for its fission activity, were observed along with the ROS-dependent IL-1β production. These early events, upon SIV infection, are suggested to provide the signals required for NLRP3 inflammasome activation. These results along with the finding that IL-1β levels were elevated between 8 and 16 h of SIV infection in the previous chapter support the positive regulatory role of DRP1, mitochondrial fission, and the ROS release axis in the inflammasome activation. The suppression of NLRP3 inflammasome activity by mitochondrial fission was reported in a human lung cell line in response to the mouse-adapted IAV, PR8 strain during the late phase of infection (Yoshizumi et al., 2014). Whether this discrepancy is attributed to the differences in cell types and virus strains warrants further investigation.

The current study showed that RIPK1 has critical roles in IL-1β production in PAMs. *RIPK1* silencing or inhibition of the RIPK1 kinase activity by Nec-1 sharply reduced the IL-1β production upon SIV infection. Deficiency of RIPK1 (or its kinase function) did not affect the pro-IL-1β expression, unlike mouse studies demonstrating that the RIPK1 kinase activity is required for the expression of inflammatory cytokines including IL-1β at the mRNA level (Najjar et al., 2016, Saleh et al., 2017, Shutinoski et al., 2016). In VSV-infected murine bone marrow-derived macrophages, RIPK1 promotes the NLRP3 inflammasome-mediated IL-1β production by the phosphorylation of DRP1 (Wang et al., 2014c). According to their study,

cytosolic RNA sensors such as RIG-I, MDA5, DEAH-box helicase 33 (DHX33), and TLR3 do not sense RNA viruses to initiate NLRP3 inflammasome activation (Wang et al., 2014c). Rather, ZBP1 senses viral proteins of IAV to induce RIPK1-mediated NLRP3 inflammasome activation and cytokine production (Kuriakose et al., 2016). In this regard, whether ZBP1 is also an upstream sensor for SIV-induced RIPK1/DRP1 signaling to activate the porcine NLRP3 inflammasome will be an important subject for future investigation.

The present study revealed the intervening role of DRP1 phosphorylation and its fission activity in upregulating the caspase-1 activity, which is required for IL-1β production. Caspase-1 activation, in reverse, can induce mitochondrial damage, leading to pyroptotic cell death (Yu et al., 2014). However, apparent cytotoxicity by the overexpression of the phospho-mimetic DRP1 was not noticeable (data not shown). It is postulated that in addition to phosphorylation, other post-translational modifications on DRP1 (Chang and Blackstone, 2010) may occur and facilitate cell death upon virus infection. While a report describes that cell death is not mediated by RIPK1/DRP1 signaling, which is required for inflammasome activation (Tao et al., 2018), it is hard to fully exclude the possibility that cell death pathways can be involved in RIPK1/DRP1-mediated NLRP3 inflammasome activation in SIV-infected cells. Considering that RIPK1 mediates necroptosis along with inflammasome activation upon virus infection (Kuriakose et al., 2016, Wang et al., 2014c), and the inflammasome activation can lead to pyroptosis, it is possible that both cell death pathways are activated to a certain extent in SIV-infected PAMs. IAV infection induces both RIPK1/RIPK3-mediated NLRP3 inflammasome activation and RIPK3-mediated apoptosis and necroptosis (Kuriakose et al., 2016). It is supposed that there are converging points between necroptotic and pyroptotic pathways. Further studies are required in terms of the crosstalk between the two events mediated by RIPK1. First, whether the RIPK1/RIPK3 complex is formed for necroptosis in SIV-infected cells; and second, whether RIPK1-mediated NLRP3 inflammasome activation also results in pyroptosis, if not, whether SIV interferes with the activation of gasdermin D, a caspase-1 substrate required for pyroptosis execution (Shi et al., 2015) warrant further investigation.

DRP1-mediated mitochondrial fission upon LPS stimulation and LPS-induced IL-1β production mediated by DRP1 activity were shown in this study. In PAMs, IL-1β production upon different ligands may be related to destructive changes in mitochondrial integrity. Although the sensing of IAV ligands and LPS upstream of the NLRP3 inflammasome activation is

different between each other as illustrated in the proposed model (Figure 5.6), the downstream events may partially converge on the DRP1 activation and ROS production stages for IL-1β secretion. It is notable that TXNIP plays as a link between ROS and NLRP3 activation. While TXNIP binds and negatively regulates the antioxidant, thioredoxin, TXNIP is released from thioredoxin upon ROS production, which leads to the binding of TXNIP to NLRP3, thereby activating the NLRP3 inflammasome (Zhou et al., 2010). Hence, it is possible that this pathway is shared for IL-1β production by both SIV infection and LPS stimulation in PAMs.

Taken together, as summarized in the proposed model (Figure 5.6), SIV induces DRP1 phosphorylation at S579 and mitochondrial fission, which leads to porcine NLRP3 inflammasome-mediated IL-1β production in PAMs. The kinase activity of RIPK1, which interacts with DRP1, is found to be critical for this process. While little is defined as to how mitochondrial dynamics fit in the innate immune response upon SIV infection, this study provides valuable information on the mechanism of SIV-induced inflammation.

CHAPTER 6 GENERAL DISCUSSION

6.1 General discussion

NLRP3 inflammasome activation involves the NLRP3-ASC interaction leading to the ASC speck formation and pro-caspase-1 recruitment, which are required for the caspase-1 activation and the maturation of IL-1β. NLRP3 inflammasome-mediated IL-1β production confers host protection in IAV-infected animals, which was demonstrated by using knockout mice (Allen et al., 2009, Ichinohe et al., 2009, Thomas et al., 2009), though its contribution to pathogenesis is also reported (McAuley et al., 2013, Ren et al., 2017). However, the knowledge on IAV-induced IL-1β production mediated by NLRP3 inflammasome in porcine innate immunity has been limited. The overarching aims of my PhD study were to characterize the porcine NLRP3 inflammasome-mediated IL-1β production in IAV-infected alveolar macrophages and to explore the mechanisms of the inflammasome regulated by IAV NS1 protein.

6.1.1 Alveolar macrophages (AMs) as the model for the IL-1β signaling research

AMs are one of the first responders in the immune system upon respiratory viral infection (Joshi et al., 2018, Tumpey et al., 2005) and the indispensability of these cells in immunity to IAV infection has been well evidenced (Cardani et al., 2017, He et al., 2017, Kim et al., 2008, Purnama et al., 2014, Schneider et al., 2014). Data obtained from primary cells can hold more biological relevance to *in vivo* conditions. However, lack of information about the regulation of IL-1β production from primary porcine alveolar macrophages (PAMs) infected with IAVs has hindered our understanding on inflammation in infected pigs and thus, has necessitated the current study using primary PAMs. Moreover, IL-1β was not detected from PAM cell lines such as 3D4 cells infected with SIVs (data not shown). According to previous reports, primary PAMs secrete IL-1β in response to SIV (Zhang et al., 2015) and other swine viruses such as PRRSV and porcine circovirus that also target the PAMs (Han et al., 2017, Li et al., 2013, Qiao et al., 2011). However, the inflammasome research utilizing the cells has been limited to PRRSV (Bi et al., 2014, Li et al., 2015, Wang et al., 2015a, Zhang et al., 2013). Regarding whether IAV replication in AMs is productive or abortive, contradicting reports are available and replication efficiency is believed to be strain-dependent (Cline et al., 2017, Short et al., 2012); H3N2 subtype of SIV can replicate in primary PAMs (Zhang et al., 2015). In the current study, IAV

replication in primary PAMs was productive considering a time-dependent increase of viral titres and a viral dose-dependent NS1 expression.

6.1.2 Signal 1 and 2 activation by SIV and pdm09

The finding that less IL-1β was elicited by pdm09 than SIVs prompted investigation on the capability of pdm09 and SIVs to induce signal 1 and 2 for the NLRP3 inflammasome-mediated IL-1β production. The signal 1 activation by SIV infection was comparable to that by pdm09 infection as displayed by similar levels of pro-IL-1β expression and *NLRP3* transcription. Meanwhile, NLRP3 inflammasome inhibition by pdm09 NS1 C-terminus aa 100-219 was undoubtedly demonstrated by using multiple approaches: PAM infection with WT/NS1-mutant viruses; ectopic expression of WT/mutant NS1 proteins in the NLRP3 inflammasome reconstitution system; examination of the NLRP3-ASC interaction and ASC speck formation. As to the signal 2 induction in SIV-infected PAMs, investigation was performed in Chapter 5, but in pdm09-infected PAMs, how the signal 2 is triggered and whether NS1 protein also controls the signal 2 upstream of NLRP3 inflammasome are unexplored questions. Besides, if other proteins are also responsible for the signal 2 requires further research. At least, PB1-F2, which is known to contribute to NLRP3 inflammasome activation (McAuley et al., 2013, Pinar et al., 2017), is not expected to play that role, since pdm09 does not express the fully functional PB1-F2 protein (Hai et al., 2010). IAV M2 protein-induced ionic perturbation in Golgi network is sensed by NLRP3 and it provides the signal 2 for the NLRP3 inflammasome activation (Ichinohe et al., 2010). Differences in the capability to cause ionic disturbances by M2 proteins from SIV and pdm09 may need investigation. In human DCs, NLRP3 inflammasome activation induced by a naturally emerged, swine-origin human H3N2 virus that acquired the M segment of pdm09 is more robust than H3N2 viruses that did not reassort with the segment (Cao et al., 2015). This points out that the M segment may dictate the capacity of signal 2 by certain IAV strains. However, the same study also demonstrates that a recombinant virus in the background of SIV Tx98 containing HA, NA and M segments from pdm09 does not induce as much IL-1β as another isogenic Tx98 virus containing only M segment of pdm09 (Cao et al., 2015). It implies that HA and NA are also involved in the signal 2, and it may explain why IL-1β production in response to Hf09 viruses with NS1 C-terminus deleted could not be fully restored to the IL-1β level upon Sk02 infection as observed in Chapter 3.

6.1.3 Interaction of NS1 with its host cellular partners for the inhibition of NLRP3 inflammasome activity

Studies on the NS1-mediated inhibition of NLRP3 inflammasome delineate that NS1 proteins derived from either a high or low pathogenic strain interact with NLRP3 (Cheong et al., 2015, Moriyama et al., 2016). Disruption of porcine NLRP3-ASC interaction by pdm09 NS1 C-terminus in the current study is thought to be due to the ability of the NS1 C-terminus that interacts with NLRP3 or ASC, or even both. One may argue that NS1 C-terminus only supports any N-terminal region(s) that can be critical for binding to NLRP3 and/or ASC to downregulate the inflammasome activity. However, this possibility can be negated, since the expression of NS1 C-terminal aa 100-129 from pdm09 was sufficient to suppress the NLRP3 inflammasome activation. Host cellular proteins regulate the NLRP3 inflammasome; NS1 may interact with those proteins to downregulate the NLRP3 inflammasome activation. For example, NIMA (which refers to 'never in mitosis gene A')-related kinase 7 (NEK7) is reported to be a crucial regulator of NLRP3 inflammasome by binding to NLRP3 (He et al., 2016b); MAVS is another NLRP3-binding partner that upregulates NLRP3 inflammasome (Park et al., 2013b, Subramanian et al., 2013); PKR enhances the inflammasome activity by interacting with multiple NLRs including NLRP3 (Lu et al., 2012). Especially, NS1 interaction with PKR is a strategy for IAV to antagonize the host antiviral status, and NS1 C-terminal aa 123-127 constitute the PKR-binding motif (Min et al., 2007), which has variable sequence patterns among IAV strains aligned in the current study. PKR-binding abilities of NS1 proteins from SIV and pdm09 need to be compared in this sense.

6.1.4 Possible engagement of tripartite motif-containing protein 25 (TRIM25) in the IL-1β signaling

Testing with a mouse-adapted PR8 strain shows that NS1 aa 40-80, R38/K41 or E96/E97 are required for inhibiting IL-1β production (Moriyama et al., 2016, Stasakova et al., 2005). Although the pdm09 NS1-mediated inhibition of NLRP3 inflammasome in the current study does not involve the E96/E97 residues, it is worth paying attention to the TRIM25-binding motif, E96/E97. RIG-I signaling that involves MAVS and TRIM25 governs the IL-1β production in human cells, while NS1 from a 1918 pandemic strain targets both RIG-I and NLRP3

inflammasome pathways (Pothlichet et al., 2013), suggesting the interplay between type I IFN pathway and IL-1β secretion. Therefore, limitation of the NLRP3 inflammasome activation is believed to be another consequence of the NS1-TRIM25 interaction, which is already known to limit the IFN induction (Gack et al., 2009). Further work is required to answer whether IFNs positively or negatively regulate the porcine IL-1β production upon IAV infection since the effects of IFNs on NLRP3 inflammasome are disputed (Fernandez et al., 2016, Guarda et al., 2011, Pothlichet et al., 2013).

6.1.5 Post-translational modifications in regulating the NLRP3 inflammasome activity

Ubiquitination of porcine ASC on the C-terminal CARD was critical for NLRP3 inflammasome-mediated IL-1β production upon infection with NS1-mutant pdm09, and the modification was suppressed by pdm09 NS1 C-terminus as shown in Chapter 4. Three scenarios are suggested behind the suppressed ubiquitination of ASC exerted by pdm09 NS1: first, NS1 may directly interact with ASC to prevent the ASC from being ubiquitinated; second, NS1 may interact with an E3 ligase that ubiquitinates the ASC; third, NS1 may interact with another host protein that forms a complex with an E3 ligase responsible for ASC ubiquitination. In either case, identification of the responsible E3 ligase will be critical to better understand the mechanism of porcine NLRP3 inflammasome activation. Given that NS1 C-terminal aa 126-225 of avian H5N1 can interact with an E3 ligase, TRAF3 (Qian et al., 2017), the association of pdm09 NS1 C-terminus with TRAF3 will be a good starting point to examine. For the third scenario, particularly, the MAVS-NS1 C-terminus interaction seems to be plausible, based on the discoveries that MAVS can recruit different E3 ligases (Liu et al., 2013a); MAVS is central in forming E3 ligase-containing signaling complexes that are targeted by viruses (Shi et al., 2014, Zhang et al., 2018); and MAVS promotes the interaction between ASC and TRAF3, an E3 ligase for ASC ubiquitination upon viral infection (Guan et al., 2015).

Other post-translational modifications may regulate the porcine NLRP3 inflammasome. With the SUMO consensus motif, porcine ASC is expected to undergo SUMOylation and deSUMOylation upon IAV infection. SUMOylation/deSUMOylation is involved in different cellular processes including the nuclear-cytoplasmic translocation and the stability of target proteins. Notably, ASC localizes from nucleus to cytoplasm to form perinuclear specks (Bryan et

al., 2009). DeSUMOylation of nuclear ASC may contribute to this step, inasmuch as many SUMO enzymes are located in the nucleus and the nuclear export of target proteins may occur upon inhibition of SUMOylation on them (Ptak and Wozniak, 2017). Moreover, phosphorylation of NLRP3 or ASC (Hara et al., 2013, Lin et al., 2015, Martin et al., 2014, Song et al., 2017, Spalinger et al., 2016), and neddylation of caspase-1 (Segovia et al., 2015) that are known to mediate the NLRP3 inflammasome activity remain to be elucidated.

6.1.6 Additional events involved in the mitochondrial dynamics

RIPK1-mediated DRP1 phosphorylation followed by mitochondrial fission was shown to be a trigger for IL-1β production in SIV-infected PAMs in Chapter 5. In this setting, ROS generated from fragmented mitochondria is thought to be the signal 2 for NLRP3 inflammasome-mediated IL-1β production. In general, upon mitochondrial damage induced by viral infection, host antiviral proteins that interact with mitochondria may better associate with their mitochondrial partners due to the increased surface area of mitochondria, leading to the establishment of antiviral status. At the same time, mitophagy occurs to get rid of the fragmented mitochondria to maintain homeostasis and inhibit apoptosis (Kim et al., 2018); mitochondrial DAMPs including ROS can be eliminated via autophagy (Harris et al., 2017). It is notable that incomplete autophagy in virus-infected cells can support virus propagation (Ding et al., 2017, Gu et al., 2018). In SIV-infected PAMs, the autophagy/mitophagy signaling seems to be initially turned on, but this action may fail to completely remove the NLRP3-activating stimuli such as ROS. Still, mitophagy-mediated limitation of apoptosis is expected to occur, eventually supporting viral replication as in the case of infection with other RNA viruses (Kim et al., 2014, Li et al., 2016c, Mukherjee et al., 2018). Thus, SIV may exploit the mitochondrial dynamics to benefit its replication by turning on the mitophagy signal, but in return, the consequent host response represented by IL-1β production seems to be unavoidable. The capacity of NLRP3-activating effects in SIV-infected cells may not be limited to ROS generated from damaged mitochondria. One such additional effect may be contributed by PB1-F2 that targets mitochondria and induces the dissipated mitochondrial membrane potential, which is reported to activate the NLRP3 inflammasome (Shimada et al., 2012). In view of that, whether PB1-F2 of SIV Sk02 strain is critical in modulating the mitochondrial dynamics can be further addressed. While PB1-F2 of PR8 strain can induce DRP1-meditaed mitochondrial fission (Yoshizumi et al.,

2014), its roles on NLRP3 inflammasome activation are contradictory. Whether apoptosis is inhibited in the early phase of SIV infection in PAMs also requires elucidation. At least, apoptosis induction does not help the promotion of NLRP3 inflammasome activity, as cytochrome c released from apoptotic mitochondria suppresses the NLRP3 inflammasome (Shi and Kehrl, 2016).

6.1.7 Possible sensors upstream of the NLRP3 inflammasome

What plays a role as an upstream sensor for RIPK1/DRP1 signaling and which viral ligands are sensed remain to be examined, particularly focusing on ZBP1. Sensors that are in question also lie upstream of NLRP3. There may be two converging pathways for signal 2 activation: apart from NLRP3 sensing of DAMPs (including ROS and viral M2-mediated ionic disturbance), viral RNA sensors seem to function upstream of NLRP3. Growing evidence suggests that signals are relayed to NLRP3 by upstream sensors for viral RNA; DEAD/H-box helicase (DDX/DHX) family members that sense cytosolic RNA can utilize different adaptor proteins (e.g. MAVS, TRIF or STING) for signal transduction. Two different domains of DHX33 bind viral dsRNA and NLRP3, respectively, to activate NLRP3 inflammasome (Mitoma et al., 2013). A similar model is demonstrated in PRRSV-infected PAMs. DDX19A, which binds to both viral RNA and NLRP3, functions as a sensor for the NLRP3 inflammasome activation (Li et al., 2015). Thus, a comprehensive view suggests that ROS-mediated and/or viral M2- as well as viral RNA-mediated NLRP3 activation may contribute to the NLRP3 inflammasome activation upon IAV infection.

6.1.8 The roles of IL-1β in the swine host

One of the foremost questions is whether IL-1β is beneficial to the swine host. IL-1β is a potent and pleiotropic cytokine. It is generally believed that IL-1β, like other inflammatory cytokines, plays a fundamental role as a mediator of inflammation, thereby protecting the host, but excessive secretion may contribute to pathogenesis. Due to its potency, it seems that a tightly regulated action of this cytokine necessitated the requirement of both signal 1 and 2 as well as the successful assembly of three components of NLRP3 inflammasome. While elevated levels of IL-1β, IL-6 and IFN-α are concurrent with pathological signs in SIV-infected pigs (Masic et al., 2009b), there is not sufficient evidence to support that IL-1β alone is responsible for more

pathogenicity in pigs in response to SIV infection compared to pdm09 infection. Considering the surge in IL-1β release from SIV-infected PAMs observed in the current study, infected AMs in the lung seem to aggressively respond to protect the host, but this drastic action may affect the subsequent stages of inflammation, for example, neutrophil infiltration in a way that causes the detriment to the host. In the IAV-affected areas where pathology is shown, dysregulation and imbalance of cytokines or mediators like histamine (Jang et al., 2018) may also contribute to pathogenesis. Nonetheless, this degree of aggravation seems to be resolved in most cases, seeing that SIV infection is mild or subclinical (Van Reeth et al., 2012). Any mechanism to alleviate the side effects of IL-1β is thought to be co-functioning. For example, cytotoxicity exerted by T cells and natural killer cells upon production of IL-18 (Liu et al., 2004), which is another cytokine dependent on NLRP3 inflammasome, is thought to participate in the alleviation mechanism.

6.1.9 Possible reasons for different IL-1β secretion phenotypes of SIV and pdm09

What would be the reasons that possibly explain the different phenotypes of SIV and pdm09? During its long-time circulation in pigs, SIVs might have acquired abilities conferred on multiple viral proteins to exploit diverse cellular machineries, one of which is mitochondria, thereby productively replicating. Without reassortment, this strategy may cost significantly for SIVs to further expand its measures against the host responses. Hence, its NS1 activity to antagonize the host antiviral status could be minimal or limited to actions against the IFN responses, but its counteraction to the host inflammatory responses represented by IL-1β signalling may not be required to be strong. As a result, moderate but not lethally severe inflammation led by pro-inflammatory cytokines could be unavoidable. For pdm09, while it can replicate optimally as SIVs in pigs (Ma et al., 2011), it may be less equipped with strategies to exploit cellular machineries, rather, more with strategies to maintain its viral fitness in both pigs and its new hosts, humans. To support that, instead, its NS1 may exhibit multiple inhibitory mechanisms that counteract the host inflammatory responses to reduce the host pressure on other viral proteins. This may partially explain how pdm09 can evade the IL-1β response in both swine and human cells as shown in the present study. It will be important to carefully examine whether this is a new strategy gained by recent IAV isolates or an evolutionarily conserved one.

In conclusion, the current study demonstrated the mechanism by which SIV induces NLRP3 inflammasome-mediated IL-1β production in PAMs, and revealed the mechanisms of

NS1-mediated counteraction held by pdm09 to escape the host innate responses represented by NLRP3 inflammasome activation. The obtained knowledge will expand our understanding on the innate immune regulation in IAV-infected hosts.

6.2 Future directions

First, discovering the interacting partners of pdm09 NS1 will enhance the understanding on the NS1-mediated inhibition of NLRP3 inflammasome. The ability of NS1 C-terminus to bind NLRP3 and/or ASC can be tested. Whether NS1 C-terminus associates with other host cellular proteins such as NEK7, MAVS and PKR that promote the NLRP3 inflammasome activity by binding NLRP3 can be accompanied. To determine the critical residues within the C-terminal aa 100-219 of pdm09 NS1 for suppressing the NLRP3 inflammasome is another important area that needs scrutiny. For that, infection of PAMs with other IAV strains to obtain more porcine IL-1β secretion profiles has to be preceded before aligning the NS1 aa sequences.

Second, the E3 ligase, responsible for ubiquitinating ASC remains to be determined. Identifying E3 ligases that are in the same IP complex as ubiquitinated ASC is ideally suggested, but interaction of NS1 C-terminus with TRAF family proteins can be an easier starting point to examine. MAVS is involved in many different signaling events and can recruit multiple TRAFs including TRAF3, which not only interacts with NS1 C-terminus of an avian strain, but also functions as an E3 ligase for ASC ubiquitination upon VSV infection. In case the NS1-MAVS interaction is confirmed, identification of E3 ligases in the same IP complex can be another task.

Third, considering that SIV Sk02 NS1 did not suppress the IL-1β production, NS1 C-terminus of Sk02 is not expected to suppress the ASC ubiquitination, but whether SIV infection also induces ASC ubiquitination on the same acceptor sites, K110 and K140 requires investigation. Other types of post-translational modifications on NLRP3 and ASC, especially SUMOylation/deSUMOylation on porcine ASC, and whether NS1 protein also regulates this modification will also be intriguing subjects to focus.

Fourth, there are pending questions in the regulation of RIPK1/DRP1-mediated mitochondrial fission. Whether incomplete mitophagy followed by limitation of apoptosis takes place in SIV-infected cells, and as a result, viral replication is supported require to be clarified. In addition, an upstream sensor that initiates the RIPK1/DRP1 signaling needs to be identified.

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APPENDIX

A. SUPPLEMENTARY TABLES

Supplementary Table 1 List of primers used to generate NS constructs in the pHW2000 vector in Chapter 3.

Construct	Primer name	Sequence (5'-3')
pHW-Hf09-NS-816	pHW 3538-3559 Fw	CTACGTATTAGTCATCGCTATT
	Hf09 $NS1_{1-73}$ Bw	TCCCTCACTAGTCTAGGATTCCTCTTTCAAGATCCA
	Hf09 NS1 *Spe*I linker Fw	TAGACTAGTGAGGGAGCAATAGTTGGAGA
	pHW 1417-1399 Bw	ATAGAGCCCACCGCATCCC
pHW-Hf09-NS-817	pHW 3538-3559 Fw	CTACGTATTAGTCATCGCTATT
	Hf09 $NS1_{1-99}$ Bw	TCCCTCACTAGTCTATGACATTTCCTCGAGGGTCA
	Hf09 NS1 *Spe*I linker Fw	TAGACTAGTGAGGGAGCAATAGTTGGAGA
	pHW 1417-1399 Bw	ATAGAGCCCACCGCATCCC
pHW-Sk02/Hf09-NS-930	pHW T7 Fw	CGAAATTAATACGACTCACTATAGGG
	$Sk02_{1-99}/Hf09_{100-219}$ NS1 Bw	CATGAGCATGAACCAGTCTCGGCTTATTTCCTCTATGCTCATAT
	$Sk02_{1-99}/Hf09_{100-219}$ NS1 Fw	ATATGAGCATAGAGGAAATAAGCCGAGACTGGTTCATGCTCATG
	pHW hPolI Bw	ACAGGTGTCCGTGTCGCG

Supplementary Table 2 List of primers used to generate expression plasmids in Chapter 3.

Construct	Primer name	Sequence (5'-3')
pcDNA-NLRP3	Porcine NLRP3 *Nhe*I Fw	GCTGGCTAGCATGAGCATGGCAAGCGTCC
	Porcine NLRP3 1784 Bw	GATTTTCTGAGAGATCT TACAAC
	Porcine NLRP3 1784 Fw	GTTGTAAGATCT CTCAGAAAATC
	Porcine NLRP3 *Xho*I Bw	GCCGCTCGAGCTACTGGGAAGGCTCAAAGAC
pcDNA-ASC	Porcine ASC *Nhe*I Fw	GCTGGCTAGCATGGGGTGCACGCGTGAC
	Porcine ASC *Xho*I Bw	GCCGCTCGAGTCAGCTCTGCTCCAGGTCG
pCMV-Flag-pro-caspase-1	Porcine pro-caspase-1 *Not*I Fw	GCTTGCGGCCGCGATGGCTGACAAGATCCTGAAG
	Porcine pro-caspase-1 *Bam*HI Bw	CCCGGGATCCTTAATATCCTGGAAAGAGGTAG
pcDNA-pro-IL-1β	Porcine pro-IL-1β *Nhe*I Fw	GCTGGCTAGCATGGCCATAGTACCTGAACC
	Porcine pro-IL-1β *Xho*I Bw	GCCGCTCGAGTTAGGGAGAGAGGACTTCCATG
pcDNA-NLRP3-Myc	Porcine NLRP3 *Nhe*I Fw	GCTGGCTAGCATGAGCATGGCAAGCGTCC
	Porcine NLRP3 *Not*I Bw	AGTGGCGGCCGCCTGGGAAGGCTCAAAGACAA
pCMV-Flag-NLRP3	Porcine NLRP3 *Eco*RV Fw	TGCAGATATCCATGAGCATGGCAAGCGTCC
	Porcine NLRP3 *Xho*I Bw	GCCGCTCGAGCTACTGGGAAGGCTCAAAGAC
pcDNA-ASC-Myc	Porcine ASC *Nhe*I Fw	GCTGGCTAGCATGGGGTGCACGCGTGAC
	Porcine ASC *Eco*RI Bw	GGTGGAATTCTGCTCTGCTCCAGGTCGGC
pCMV-Flag-Hf09 NS1	pcDNA-Myc *Kpn*I Fw	TATCGGTACCACTCACTATAGGGAGACCCAA
	Hf09 NS1 *Xba*I Bw	GCCCTCTAGATCATTTCTGCTCTGGAGGTA
pCMV-Flag-Hf09-$NS1_{1-73}$	pcDNA-Myc *Kpn*I Fw	TATCGGTACCACTCACTATAGGGAGACCCAA
	Hf09 $NS1_{1-73}$ *Xba*I Bw	ATCCTCTAGATCAGGATTCCTCTTTCAAGATCCA
pCMV-Flag-Hf09-$NS1_{1-99}$	pcDNA-Myc *Kpn*I Fw	TATCGGTACCACTCACTATAGGGAGACCCAA
	Hf09 $NS1_{1-99}$ *Xba*I Bw	ATCCTCTAGATCATGACATTTCCTCGAGGGTCA
pcDNA-Hf09 $NS1_{100-219}$-Myc	Hf09 $NS1_{100-219}$ *Nhe*I Fw	GCTGGCTAGCATGCGAGACTGGTTCATGCTCAT
	Hf09 NS1 *Not*I Bw	AGTGGCGGCCGCTTTCTGCTCTGGAGGTAGTG
pcDNA-Sk02 $NS1_{100-230}$-Myc	Sk02 $NS1_{100-230}$ *Nhe*I Fw	GCTGGCTAGCATGAGGGAATGGTACATGCTCATG
	Sk02 NS1 *Not*I Bw	AGTGGCGGCCGCAACTTCTGACTCAACTCTTCTC

Supplementary Table 3 List of primers used to generate expression plasmids in Chapter 4.

Construct	Primer name	Sequence (5'-3')
pCMV-Flag-ASC	Porcine ASC *Not*I Fw	GCTTGCGGCCGCGATGGGGTGCACGCGTGAC
	Porcine ASC *Bam*HI Bw	CCCGGGATCCTCAGCTCTGCTCCAGGTCG
pcDNA-Hf09-NS1-Myc	Hf09 NS1 *Nhe*I Fw	GCTGGCTAGCACCATGGACTCCAACACCATGTC
	Hf09 NS1 *Not*I Bw	AGTGGCGGCCGCTTTCTGCTCTGGAGGTAGTG
pcDNA-Hf09-NS1$_{1-99}$-Myc	Hf09 NS1 *Nhe*I Fw	GCTGGCTAGCACCATGGACTCCAACACCATGTC
	Hf09 NS1$_{1-99}$ *Not*I Bw	AGTGGCGGCCGCTGACATTTCCTCGAGGGTCA
pCMV-Flag-huASC	Human ASC *Kpn*I Fw	TATCGGTACCAATGGGGCGCGCGCGCGAC
	Human ASC *Xba*I Bw	ATCCTCTAGATCAGCTCCGCTCCAGGTCC
pCMV-Flag-ASC (K21R/K22R/K24R/K26R)	Porcine ASC K21/22/24/26R Fw	AGGATGAGGCTGCTCTCAGTGCCACTG
	Porcine ASC K21/22/24/26R Bw	AAACCTCCTGAGCTCGTCGGCTGTCA
pCMV-Flag-ASC (K55R)	Porcine ASC K55R Fw	CTCACTGACAGGCTCGTCAACTAC
	Porcine ASC K55R Bw	GTCGATGGCATCCAGGGG
pCMV-Flag-ASC (K79R)	Porcine ASC K79R Fw	CTGCGTGACATCGGCATGCGGGAGGTGGCGGAGCAGC
	Porcine ASC K79R Bw	GCTGCTCCGCCACCTCCCGCATGCCGATGTCACGCAG
pCMV-Flag-ASC (K87R)	Porcine ASC K87R Fw	GGAGGTGGCGGAGCAGCTCCAACGGACCCTGCACAAGGGCCC
	Porcine ASC K87R Bw	GGGCCCTTGTGCAGGGTCCGTTGGAGCTGCTCCGCCACCTCC
pCMV-Flag-ASC (K91R)	Porcine ASC K91R Fw	GCAGCTGCAGAAGACCCTCCACAGGGGCCCTGGAGCCAAGC
	Porcine ASC K91R Bw	GCTTGGCTCCAGGGCCCCTGTGGAGGGTCTTCTGCAGCTGC
pCMV-Flag-ASC (K96R)	Porcine ASC K96R Fw	CAAGGGCCCTGGAGCCAGGCCTGCTGGAATCAAAGCCCTTC
	Porcine ASC K96R Bw	GAAGGGCTTTGATTCCAGCAGGCCTGGCTCCAGGGCCCTTG
pCMV-Flag-ASC (K101R)	Porcine ASC K101R Fw	GCCAAGCCAGCTGGAATCAGAGCTCTTCCCCTGAAAGCAGAC
	Porcine ASC K101R Bw	GTCTGCTTTCAGGGGAAGAGCTCTGATTCCAGCTGGCTTGGC
pCMV-Flag-ASC (K106R)	Porcine ASC K106R Fw	CTGGAATCAAAGCCCTTCCGCTGAGGGCAGACAACAAACCAGCAC
	Porcine ASC K106R Bw	GTGCTGGTTTGTTGTCTGCCCTCAGCGGAAGGGCTTTGATTCCAG
pCMV-Flag-ASC (K110R)	Porcine ASC K110R Fw	GCAGACAACAGACCAGCACTG
	Porcine ASC K110R Bw	TTTCAGGGGAAGGGCTTT
pCMV-Flag-ASC	Porcine ASC K140R Fw	CTGTACGGGAGGGTGCTGACG

(K140R)	Porcine ASC K140R Bw	GGCATCCAGCAGCCCGTC
pCMV-Flag-ASC (K159R)	Porcine ASC K159R Fw	AACCCCACCAGAATGAGGAGGCTCTTCAG
	Porcine ASC K159R Bw	GGTGTGCTCCGCCCGCAC
pCMV-Flag-ASC (K175R)	Porcine ASC K175R Fw	CTGACCTGCAGGGACCTGCTC
	Porcine ASC K175R Bw	GTTCCAGGCCGGAGTGAA
pCMV-Flag-ASC (K183R)	Porcine ASC K183R Fw	CTGCTCCTTCAGGCCCTGAGGGACACCCAGCCCTACCTG
	Porcine ASC K183R Bw	CAGGTAGGGCTGGGTGTCCCTCAGGGCCTGAAGGAGCAG
pCMV-Flag-ASC_{1-109}	Porcine ASC *Not*I Fw	GCTTGCGGCCGCGATGGGGTGCACGCGTGAC
	Porcine ASC_{1-109} *Bam*HI Bw	CCCGGGATCCTCAGTTGTCTGCTTTCAGGGGAAG
pCMV-Flag-ASC_{1-158}	Porcine ASC *Not*I Fw	GCTTGCGGCCGCGATGGGGTGCACGCGTGAC
	Porcine ASC_{1-158} *Bam*HI Bw	CCCGGGATCCTCAGGTGGGGTTGGTGTGCTC
pcDNA-HA-ubiquitin (K48R)	HA-Ubi K48R Fw	TTTGCTGGCAGGCAGCTGGAA
	HA-Ubi K48R Bw	GATCAGTCTCTGCTGATCAGGAGG
pcDNA-HA-ubiquitin (K63R)	HA-Ubi K63R Fw	AATATTCAAAGGGAGTCTACTCTTC
	HA-Ubi K63R Bw	GTAGTCAGACAAAGTACG

Supplementary Table 4 List of primers used to generate expression plasmids in Chapter 5.

Construct	Primer name	Sequence (5'-3')
pcDNA-DRP1-Myc	Porcine DRP1 *Xho*I Fw	TAGACTCGAGATGGAGGCACTAATTCCTGTT
	Porcine DRP1 *Bam*HI Bw	GTTCGGATCCCCAAAGATGAGTCTCTCGGA
pcDNA-DRP1 (S579D)-Myc	Porcine DRP1 S579D Fw	CATGCCCGCCGATCCACAAAAAG
	Porcine DRP1 S579D Bw	ATTGGAATTGGTTTTGATTTC
pcDNA-DRP1 (S579A)-Myc	Porcine DRP1 S579A Fw	CATGCCCGCCGCTCCACAAAAAG
	Porcine DRP1 S579A Bw	ATTGGAATTGGTTTTGATTTC
pcDNA-huRIPK1 (K45A)-Myc	Human RIPK1 *Nhe*I Fw	GCTGGCTAGCATGCAACCAGACATGTCCTTG
	Human RIPK1 *Not*I Bw	AGTGGCGGCCGCGTTCTGGCTGACGTAAATCAA
pcDNA-RIPK1-Myc	Porcine RIPK1 *Nhe*I Fw	GCTGGCTAGCATGTCTCTGGATGACATTAAGATG
	Porcine RIPK1 *Not*I Bw	AGTGGCGGCCGCGTTCTGGCTGCTGCGGATC
pCMV-Flag-RIPK1	Porcine RIPK1 *Hind*III Fw	CGACAAGCTTATGTCTCTGGATGACATTAAGATG
	Porcine RIPK1 *Not*I Bw	ATTCGCGGCCGCCTAGTTCTGGCTGCTGCGGAT
pCMV-Flag-RIPK1 (K41A/K42A)	Porcine RIPK1 K41A/K42A Fw	TGTGATTCTGGCGGCGGTGTACACGGGCCC
	Porcine RIPK1 K41A/K42A Bw	AGCCCGTGGGATTTGTGA

B. ACHIEVEMENTS DURING THE STUDY

B.1 Publications

A Gaba, F Xu, Y Lu, **HS Park**, G Liu and Y Zhou. (2018) The NS1 Protein of Influenza A Virus Participates in Necroptosis by Interacting With MLKL and Increasing Its Oligomerization and Membrane Translocation. *Journal of Virology*, http://doi.org/10.1128/JVI.01835-18 (I contributed by examining the effects of necroptosis proteins on the NLRP3 inflammasome activation and conducting IL-1β ELISA.)

HS Park, G Liu, Q Liu and Y Zhou. (2018) Swine Influenza Virus Induces RIPK1/DRP1-Mediated Interleukin-1 Beta Production. *Viruses*, 10(8), 419 https://doi.org/10.3390/v10080419 (I designed most of the experiments, generated most of the tools, performed all the experiments, analyzed the data and wrote the manuscript.)

HS Park, G Liu, SN Thulasi Raman, SL Landreth, Q Liu and Y Zhou. (2018) NS1 Protein of 2009 Pandemic Influenza A Virus Inhibits Porcine NLRP3 Inflammasome-Mediated Interleukin-1 Beta Production by Suppressing ASC Ubiquitination. *Journal of Virology*, 92(8), e00022-18 http://doi.org/10.1128/JVI.00022-18 (I designed some of the experiments, generated most of the tools, performed all the experiments, analyzed the data and wrote the manuscript.)

G Liu, **HS Park**, HM Pyo, Q Liu and Y Zhou. (2015) Influenza A Virus Panhandle Structure Is Directly Involved in RIG-I Activation and Interferon Induction. *Journal of Virology*, 89(11), 6067-6079 http://doi.org/10.1128/JVI.00232-15 (I contributed by isolating primary PAMs and conducting porcine IFN-α and IL-1β ELISA.)

B.2 Presentations

HS Park, G Liu, SN Thulasi Raman, SL Landreth, Q Liu and Y Zhou. NS1 Protein of 2009 Pandemic Influenza A Virus Inhibits Porcine NLRP3 Inflammasome-Mediated Interleukin-1 Beta Production by Suppressing ASC Ubiquitination. Poster presentation at the 37th American Society for Virology Meeting, University of Maryland, College Park, MD, United States, July 14-18, 2018.

HS Park, G Liu, SN Thulasi Raman, Q Liu and Y Zhou. RIP1/RIP3 Signaling Contributes to the Porcine NLRP3 Inflammasome-Mediated Interleukin-1 Beta Production in Response to Swine Influenza Virus Infection. Poster presentation at the 36th American Society for Virology Meeting, University of Wisconsin-Madison, WI, United States, June 24-28, 2017.

HS Park, G Liu, SN Thulasi Raman and Y Zhou. NS1 Protein of Human Pandemic Influenza Virus Contributed to the Down-Regulation of NLRP3 Inflammasome-Mediated Interleukin-1 Beta in Porcine Alveolar Macrophages. Poster presentation at the ImmuNet Research Day 2016, Edmonton, AB, Canada, June 9, 2016.

HS Park, G Liu and Y Zhou. Swine Influenza Virus Induced NLRP3 Inflammasome-Mediated Interleukin-1 Beta Production in Porcine Alveolar Macrophages. Poster presentation at the 34th American Society for Virology Meeting, Western University, London, ON, Canada. July 10-15, 2015.

HS Park and Y Zhou. Characterization of Interleukin-1 Beta Production in Porcine Alveolar Macrophages Infected With Swine Influenza Virus. Poster presentation at the 2nd Li Ka Shing Institute of Virology Symposium, Edmonton, AB, Canada. May 30-31, 2013.

B.3 Scholarships and fellowships

-Graduate Teaching Fellowship ($8,500) from Western College of Veterinary Medicine, 2015.

-Graduate Research Fellowship ($8,000) from Western College of Veterinary Medicine, 2014.

-Graduate Research Fellowship ($8,000) from Western College of Veterinary Medicine, 2013.

-Saskatchewan Innovation and Opportunity Graduate Scholarship ($10,000) from University of Saskatchewan, 2012.

B.4 Awards

-Dr. John Allen Graduate Student Travel Award ($300) from Department of Veterinary Microbiology, Western College of Veterinary Medicine, 2018.

-Graduate Student Travel Award ($200) from Western College of Veterinary Medicine, 2018.

-Graduate Student Travel Award ($450) from University of Saskatchewan, 2018.

-Dr. John Allen Graduate Student Travel Award ($300) from Department of Veterinary Microbiology, Western College of Veterinary Medicine, 2017.

-Graduate Student Travel Award ($200) from Western College of Veterinary Medicine, 2017.

-Graduate Student Travel Award ($450) from University of Saskatchewan, 2017.

-Student Travel Award (US$500) from American Society for Virology, 2017.

-Dr. John Allen Graduate Student Travel Award ($200) from Department of Veterinary Microbiology, Western College of Veterinary Medicine, 2016.

-Dr. John Allen Graduate Student Travel Award ($300) from Department of Veterinary Microbiology, Western College of Veterinary Medicine, 2015.

-Graduate Student Travel Award ($350) from University of Saskatchewan, 2015.

-Student Travel Award (US$500) from American Society for Virology, 2015.

-Graduate Student Travel Award ($250) from Western College of Veterinary Medicine, 2013.