ANTIVIRAL SIGNALING MECHANISMS OF EXTRACELLULAR dsRNA

ROLE OF MACROPHAGE SCAVENGER RECEPTOR 1 AND EXTRACELLULAR DOUBLE-STRANDED RNA IN ANTIVIRAL CELL SIGNALING

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Lay Abstract

Viral infections remain a threat to global health as new diseases continue to emerge. To develop effective vaccines and antivirals to combat viruses and alleviate human disease require a deeper understanding of virus-host interactions. Host cells identify virus-associated molecules to detect viruses and eliminate them whereas, viruses employ tactics to prevent the activation of the immune system. However, virus-induced cell lysis releases viral molecules that can stimulate immune responses in neighbouring uninfected cells. This thesis examines the mechanism by which cells respond to extracellular viral nucleic acids.

We showed that a protein present at the cell surface called ‘class A scavenger receptor 1’ is sufficient to internalize extracellular viral nucleic acids, leading to immune responses. The response is impaired when a channel protein, SIDT2, is absent in the cells. Further work is necessary to understand how this knowledge can be harnessed to develop vaccines and antiviral therapeutics.

Abstract

Recognition of non-self, pathogen-associated molecular patterns is a central component of host immune response to pathogens like viruses. Intracellular detection of viral nucleic acids leads to the production of type I interferons (IFN-I) and subsequent establishment of an antiviral state in infected and neighboring cells. Viruses have evolved multiple mechanisms to counteract IFN-I responses in infected cells, however, viral nucleic acids released from dying cells can stimulate IFN-I production in surrounding or distal uninfected cells. This thesis examines the mechanisms by which cells recognize extracellular viral nucleic acids and the subsequent downstream antiviral signaling. Class A scavenger receptors (SR-As) internalize extracellular viral double-stranded RNA (dsRNA) to mediate IFN-I responses, but little is known about extracellular viral DNA. We observed that extracellular DNA is recognized and internalized by SR-As in a manner like extracellular dsRNA. Furthermore, we established that SR-A1 is sufficient in mediating extracellular dsRNA-induced cellular responses and other nucleic acid receptors like SR-J1 and DEC-205 are dispensable. Finally, a direct interaction of RNA and DNA species was demonstrated with the coiled-coil collagenous domain of SR-A1, but not the scavenger receptor cysteine rich domain of SR-A6.We elaborated the role of SR-A1 by identifying the cellular processes activated through SR-A1 following uptake of extracellular dsRNA. Cytosolic sensors are essential in mediating an antiviral response to the endocytosed dsRNA, but the mechanism of endoplasmic release and cytoplasmic entry of dsRNA remains an enigma. We demonstrated that the lack of a dsRNA-channel,

SIDT2, impaired the ability of the cells to mediate an antiviral response to extracellular dsRNA. Understanding host responses to extracellular viral nucleic acids will enable the development of novel vaccines and antiviral therapeutics against RNA and DNA viruses that efficiently counteract these responses in infected cells.

Acknowledgements

I dedicate this thesis to my mother, father and brother who have been supportive of my decisions to pursue my passion for discovery. Their love and support have enabled me to remain focused at my goal. Thank you mummy, papa and bhaiya for being my pillars. I also thank the ‘power’ above all of us in giving me the strength and energy to persevere.

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Dr. Samuel Tekeste Workenhe, Dr. Arinjay Banerjee and Dr. David Hare have been the source of immense inspiration for me in my pursuit of scientific excellence. Thank you, Sam “THE MAN”, “J” and Dave from the bottom of my heart and I could not have done it without you.

I am fortunate to have made some of the ‘coolest’ friends on my journey to PhD. Thank you, Sam, Nadeem Murtaza, Mathieu Seyfrid, Derek Cummings, and J. You all have left a mark on me and I am looking forward to a lifelong journey of adventures and discoveries with you all.

Last but not the least, I would like to thank my partner, Kristin Ducut and her family who have accepted me as their own and made me feel at home in a new country. Thank you, Kristin, for being there for me through thick and thin over the last 6 years. I am looking forward to our next chapter.

If I have forgotten to thank someone, I apologize but I am truly grateful for each person that has contributed in any way for me to reach this point in my life.

Thank you.

Table of Contents

List of figures

Chapter 4

List of tables

List of abbreviations and symbols

5’-pp/ppp	5’diphosphate/ triphosphate
αMEM	Alpha-modified Eagle's medium
aa	amino acid
acLDL	Acetylated LDL
AMP	Adenosine monophosphate
AP	Affinity purification
BirA	Biotin ligase
BR	Biological replicate
C. elegans	*Caenorhabditis elegans*
cAMP	cyclic adenosine monophosphate
CARD	Caspase activation and recruitment domain
Cas9	CRISPR-associated protein 9
CCHFV	Crimean-Congo haemorrhagic fever virus
cDNA	Complementary DNA
cGAMP	2’3’- cyclic GMP-AMP
cGAS	Cyclic GMP-AMP synthase
CHIKV	Chikungunya virus
CMC	Carboxymethyl cellulose
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Ct	Cycle threshold
CTD	C-terminal domain
DAMPs	Danger associated molecular patterns
DC	Dendritic cells
DENV	Dengue virus
DMEM	Dulbecco’s modified eagle’s medium
DNA	Deoxyribonucleic acid
DOR	δ-opioid receptor
dsRNA	Double-stranded RNA
E	Envelope gene
EBOV	Ebola virus
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FH	Fulminant hepatitis
FLNA	Filamin A
FSC	Forward scatter

GAPDH	Glyceraldehyde-3-phosphate
GFP	Green fluorescent protein
GMP	Guanosine monophosphate
HBV	Hepatitis B virus
HCD	Higher energy collision induced dissociation
HCV	Hepatitis C virus
HEK	Human embryonic kidney
HEL	Human embryonic lung
HIV	Human immunodeficiency virus
hMDDC	Human monocyte–derived dendritic cells
HSV-1	Herpes simplex virus-1
IAV	Influenza A virus
IFN	Interferon
IFNAR	Interferon-α/β receptor
IFN-I	Type I interferons
IKKε	Inhibitor of nuclear factor kappa-B kinase subunit epsilon
IL-1	Interleukin-1
IL-17A	Interleukin 17A
IL-1ß	Interleukin-1ß
IL-21	Interleukin 21
IL-6	Interleukin 6
Indels	insertions/deletions
IRF3	Interferon regulatory factor 3
IRF9	Interferon regulatory factor 9
ISG	Interferon-stimulated genes
ISG56	Interferon, Alpha-Inducible Protein (MW 56kD)
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon-stimulated response elements
JAK	Janus kinase
JNK	c-Jun N-terminal protein kinase
KO	Knockout
LC	Liquid chromatography
LCMV	Lymphocytic choriomeningitis virus
LDL	Low density lipoprotein
LGP2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LRR	Leucine rich repeats
LTA	Lipoteichoic acid

Mac-1	Macrophage-1 Ag
MAPK	Mitogen-activated protein kinases
MAVS	Mitochondrial antiviral-signalling protein
MCMV	Mouse cytomegalovirus
MDA5	Melanoma differentiation-association gene 5
MEF	Murine embryonic fibroblasts
MERS-CoV	Middle east respiratory syndrome coronavirus
Mertk	Tyrosine kinase Mer
miRNA	microRNA
MOI	Multiplicity of infection
MS	Mass spectrometry
MSR1	Macrophage scavenger receptor 1
MVP	Major vault protein
MW	Molecular weight
MyD88	Myeloid differentiation primary response 88
NFκB	Nuclear factor kappa-light-chain enhancer of activated B cells
NK	Natural killer
NLR	NOD-like receptors
NOD	Nucleotide-binding oligomerization domain
NP	Nucleoprotein
NS1	Non-structural protein 1
OAS1	2’-5’-oligoadenylate synthetase 1
oxLDL	Oxidized LDL
PACT	PKR activator
PAMP	Pathogen-associated molecular patterns
pB	PiggyBac
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PFU	Plaque forming units
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PKR	protein kinase R
PL	Proximity labeling
PLC γ1	Phospholipase C gamma 1
POI	Protein of interest
Poly (I:C)	Polyinosinic-polycytidylic acid
Poly A	Polyadenilic acid

Poly G	Polyguanilic acid
Poly I	Polyinosinic acid
Poly U	Polyuridine/ Polyuridylic acid
PRKDC	DNA-Dependent Protein Kinase Catalytic Subunit
PRR	Pattern recognition receptors
PVDF	Polyvinylidene difluoride
qRT-PCR	Real time quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RD	Repressor domain
RIG-I	retinoic acid inducible gene I
RIPA	Radioimmunoprecipitation assay
RLR	RIG-I-like receptors
RNA	Ribonucleic acid
RNAi	RNA interference
RNP	Ribonucleoprotein
RSV	Respiratory syncytial virus
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
rtTA	Reverse tetracycline-controlled transactivator
S. pneumoniae	*Streptococcus pneumoniae*
sgRNA	guide RNA
siRNA	Small interfering RNA
SLE	Systemic lupus erythematosus
SPR	Surface plasmon resonance
SR	Scavenger receptors
SR-A	Class A scavenger receptor
SRCR	Scavenger Receptor Cysteine Rich
SSC	Side scatter
ssRNA	Single stranded RNA
STAT1	Signal transducer activator of transcription 1
STAT2	Signal transducer activator of transcription 2
STING	Stimulator of interferon genes
TANK	TRAF family member-associated NF-kB activator
TBK1	TANK-binding kinase 1
TIR	Toll/ IL-1 receptor domain
TLR	Toll-like receptors
TNF	Tumour necrosis factor
TNFα	Tumour necrosis factor alpha

TRAF2	TNF receptor associated factor 2
TRAF3	TNF receptor associated factor 3
TRAF5	TNF receptor associated factor 5
TRAF6	TNF receptor associated factor 6
TRIF	TIR-domain-containing adapter-inducing interferon-β
TSLP	Thymic stromal lymphopoietin
VSV	Vesicular stomatitis virus
WNv	West Nile virus
WT	Wildtype

Declaration of academic achievement

The manuscript contained in Chapter 3 titled, “Direct binding and internalization of diverse nucleic acid species through the collagenous domain of SR-A1’ was initially submitted 2nd October 2017 and published 26th April 2018 (Immunology and Cell Biology 96(9):922-934; Apr 2018. © 2018 Australasian Society for Immunology Inc.). Dr. Srinivas Nellimarla and I are listed as primary authors with equal contributions. The study was designed by me and Dr. Nellimarla with assistance from Dr. Karen Mossman. Nucleic acid uptake assays in human embryonic lung fibroblasts were performed by Dr. Nellimarla and Susan E. Collins. Dr. Nellimarla generated class A scavenger receptor deficient A549 cells and I identified respective mutations and validated their functional phenotype. Susan assisted with the generation of cell line expressing macrophage scavenger receptor 1 in an inducible manner. Besides flow cytometry, which was done by Dr. Kenneth Mwawasi, I carried out all the experiments in Figure 3.5.3. Melanie Gloyd, and Drs. Angela Hyunh and Stephen Boulton generated and purified recombinant coiled-coil collagenous and scavenger receptor cysteine-rich domains under the supervision of Drs. Alba Guarné and Giuseppe Melacini. Dr. Nellimarla and I optimized the band shift assay to detect protein-nucleic acid interactions and equally shared the experiments in Figures 3.5.4 and 3.5.5. Dr. Kyle Novakowski carried out the flow cytometry experiment in Figure 3.5.5. Dr. Nellimarla and I were responsible for designing and performing majority of the experiments, interpreting the results, and generating figures for the manuscript. The paper was written with assistance from Dr. Nellimarla and Dr. Mossman.

The study described in Chapter 4 titled, “Intracellular signaling of extracellular double stranded RNA” is a study that I designed and executed with guidance from Dr. Yu Lu and Dr. Mossman. The idea to perform BioID for SR-A1 came at the beginning of my graduate studies in 2014 from the then recently published application of BioID (Roux KJ, Kim DI, Burke B. BioID: a screen for protein-protein interactions. Current Protocols in Protein Science 74:19.23.1-19.23.14; Nov 2013. Copyright © 2013 John Wiley & Sons, Inc.) to identify protein-protein interactions for a target protein. I generated the constructs for BioID fusion proteins and validated their expression and function in Figures 4.2.1 and 4.2.2. Under my guidance, Taha Aslam generated a stable cell line engineered to express a BioID fusion protein in an inducible manner and identified a suitable clonal population for further studies by validating expression and function of the BioID fusion protein. The experiment to study ligand internalization by cells expressing BioID fusion protein in Figure 4.2.3 were carried out by myself. I performed all optimizations necessary for a successful BioID screen. I designed and executed the temporal design of the BioID screen with suggestions provided by key lab members and Dr. Mossman. The mass spectrometry of BioID samples and subsequent identification of the proteins were carried out by Dr. Lu. I analyzed the data and identified the list of enriched proteins. The pathway enrichment analyses in Figure 4.5.5 were performed by me with assistance from Nadeem Murtaza. The design and execution of all the experiments in Figure 4.5.6 were carried out by me.

The remainder of the thesis was written by me with editing and suggestions from Dr. Mossman, Dr. Arinjay Banerjee and Dr. David Hare. The findings in this thesis would not have been possible without my collaborators and the teamwork of all members of the Mossman lab.

CHAPTER 1: Introduction

1.1. Innate antiviral immunity

The key characteristic of our immune system is its ability to distinguish self from non-self. The innate immune response constitutes our first line of defense which serves to clear infections (1). The innate immune system consists of evolutionary conserved pattern recognition receptors (PRRs) that survey the extracellular and intracellular spaces for signs of infection, decided by the presence of pathogen-associated molecular patterns (PAMPs). PAMPs are conserved features that are essential for the pathogen's life cycle and distinguishable from 'self' (1). In some cases, abnormal or aberrantly localized cellular products generated in response to infection, inflammation, or other kinds of cellular stress can also activate the innate immune responses (2, 3).

During virus infection, the cellular PRRs recognize viral PAMPs and initiate a cascade of signaling events that culminates in the production of numerous host defense molecules, including type I interferons (IFN-I), proinflammatory cytokines and chemokines (4). IFN-I are key cytokines produced in response to viral infection as mice lacking IFN-I signaling fair poorly against typically harmless viruses (5). Secreted IFN-I and cytokines amplify and broaden the innate immune response through autocrine and paracrine actions that induce the expression of hundreds of interferon-stimulated genes (ISGs). The ISGs encode many factors that disrupt the virus life cycle or modulate host processes such as protein synthesis, cell growth and survival to cumulatively restrict virus

spread (6). Moreover, the cytokines and chemokines produced during these responses such as IFN-I shape an effective adaptive immune response to control the infection and support formation of immunological memory (7). It is important to note that aberrant activation of PRR signaling and inappropriate induction of IFN-I is a major underlying factor in a wide range of immune and autoimmune disorders (8).

1.1.1. Cellular receptors for innate antiviral signaling

The innate immune response relies on the recognition of evolutionarily conserved PAMPs through germline encoded PRRs. These PRRs include the toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and DNA receptors (9, 10). The TLRs and RLRs induce IFN-I and various cytokines whereas NLRs regulate interleukin-1ß (IL-1ß) maturation through activation of caspase-1 (11). As for cytosolic DNA receptors, some have been implicated in IFN-I and cytokine production while others mediate IL-1ß maturation (9).

The TLRs are present largely on cellular surfaces and in endosomal compartments to survey the extracellular space and the contents of endocytosed cargoes. Similarly, cytoplasmic receptors such as RLRs, NLRs and the recently identified DNA receptors serve to recognize PAMPs within the cytoplasm of the cell (9, 10). Several TLRs, RLRs, NLRs and DNA receptors have been identified in humans, each recognizing a particular component of a microorganism (Figure 1.1). Once these PRRs recognize a PAMP, they

initiate signaling cascades that induce the expression of several anti-microbial cytokines and chemokines (1, 4, 10, 12).

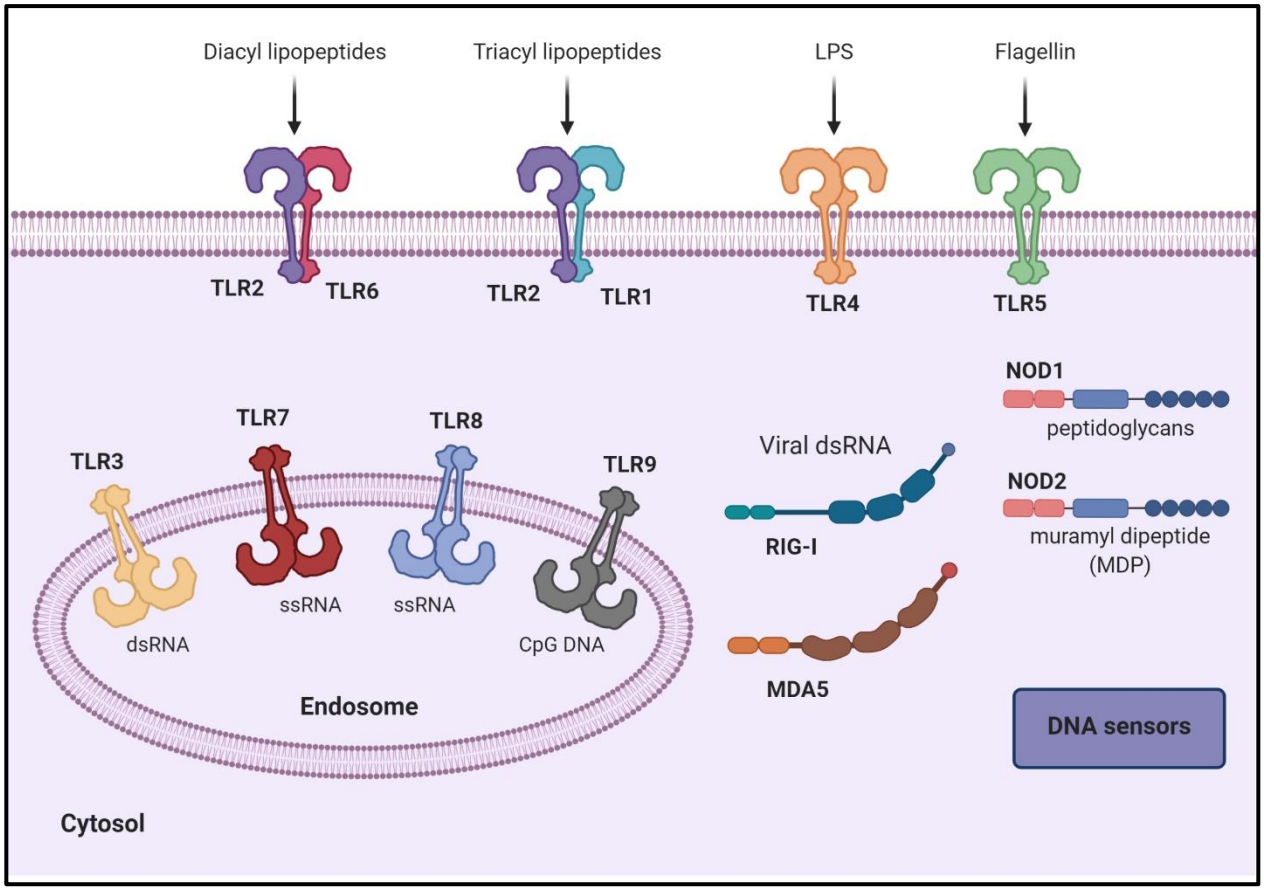

Figure 1.1. Host pathogen recognition receptors (PRRs) survey extracellular and intracellular spaces for conserved microbial structures present in invading pathogens. TLR2-TLR6 and TLR1-TLR2 complex bind diacyl-lipopeptides and triacyl-lipopeptides from bacteria, respectively. TLR4 recognizes bacterial lipopolysaccharide (LPS) and TLR5 is activated by bacterial flagellar protein. TLRs 3 (double stranded RNA (dsRNA)), 7 (single stranded RNA (ssRNA)), 8 (ssRNA) and 9 (CpG DNA) are in endosomes and detect viral and bacterial nucleic acids. RIG-I and MDA5 from the RLR family of PRRs detect viral nucleic acid (dsRNA) in the cytosol; NOD1 and NOD2 belong to the NLR family of PRRs and detect bacterial peptidoglycans and muramyl dipeptide (MDP) respectively. DNA sensors detect viral and bacterial DNA in the cytoplasm. Image created with BioRender.com.

1.2. Sensing virus infection

Cellular PRRs can differentiate the molecular patterns in nucleic acids that constitute the viral genomes and/or the intermediate products of virus replication cycle from host cellular RNA. While structural components of viruses can also be recognized by PRRs during virus entry, nucleic acids are a feature common to all viruses and are found in substantial quantities in infected cells during viral replication therefore, they serve as an ideal viral PAMP (13). The innate immune system has evolved several PRRs that sense viral RNA while maintaining tolerance to self RNA, thus ensuring the integrity of an antiviral response without the undesired activation against the host (14).

Double-stranded RNA (dsRNA) is a key signature of viral infection and is produced by most viruses, if not all, at some point during their replicative cycle (15). Also, the treatment of cells with the synthetic dsRNA analog polyinosinic-polycytidylic acid (poly (I:C)) has been widely used to mimic a viral infection (16). Since my thesis focusses on understanding the mechanisms of action and intracellular trafficking of extracellular viral dsRNA, I have detailed the role and corresponding signaling events that follow recognition of dsRNA by TLR3 and the RLRs in human cells. The role of other TLRs, NLRs and cytosolic DNA receptors have been reviewed in detail elsewhere (9, 10).

1.2.1. Recognition of viral dsRNA by endosomal TLR3

The TLRs are the most extensively studied family of PRRs and were originally discovered based on their homology to the *Drosophila melanogaster* Toll protein (17). The TLRs belong to a conserved family of transmembrane glycoprotein receptors and structurally they are all similarly organized. To date, ten TLRs have been identified in humans, and they each recognize distinct PAMPs derived from various microbial pathogens, including viruses, bacteria, fungi, and protozoa (7).

TLRs can be categorized into subgroups based on the PAMPs they recognize. TLRs 1, 2, 4 and 6 recognize lipids, whereas TLRs 3, 7, 8 and 9 recognize nucleic acids (Figure 1.1). Further, TLRs can also be categorized based on their cellular locations. TLRs 1, 2, 4, 5, 6 and 10 are expressed at the cell surface, whereas TLRs 3, 7, 8, and 9 are located almost exclusively in intracellular compartments such as endosomes and lysosomes (18) suggesting that the nucleic acid sensing TLRs are activated following uptake of virus-infected apoptotic cells or nucleic acid that is taken up by scavenger receptors (13, 19). Antigen presenting cells, such as dendritic cells (DCs) and macrophages express a wide range of TLRs but most cells in the body appear to express at least a subset of them (20). Furthermore, expression of TLRs is not static and is modulated by a variety of cytokines, pathogens, and environmental stresses (10).

TLR3 belongs to a conserved family of transmembrane glycoprotein receptors. The N-terminal PAMP-binding domain is composed of 20–26 leucine rich repeats

(LRRs) and a central transmembrane domain that anchors individual TLRs to either the plasma or the endosomal membranes. The C-terminal region contains a Toll/ interleukin-1 (IL-1) receptor (TIR) domain which mediates downstream signaling after receptor activation (21-23).

TLR3 was the first TLR to be identified as a sensor for viral nucleic acids (24). It recognizes dsRNA, which constitutes the genome of some RNA viruses and is a replication intermediate of single stranded RNA (ssRNA) viruses. DNA viruses also produce dsRNA during their replicative cycle (13). Besides viral dsRNA, TLR3 also recognizes the synthetic RNA analogs poly (I:C) and polyuridine (poly(U)) (24). The proposed mechanism of dsRNA binding is dependent on electrostatic interactions and hydrogen bonds (25). RNA with a minimum length of 40–50 bp is however, necessary for TLR3 activation, with the binding affinity increasing in proportion to dsRNA length (26).

TLRs mediate downstream signaling via a family of adaptor molecules which in part determines the specificity of the response. The cytosolic domain of TLRs recruit different adaptor proteins including myeloid differentiation primary response 88 (MyD88) or TIR-domain-containing adapter-inducing interferon-β (TRIF). Multiple pathways are activated after TLRs bind to their ligands including, (a) nuclear factor kappa-light-chain enhancer of activated B cells (NFκB), (b) mitogen-activated protein kinases (MAPKs), and (c) interferon (IFN) regulatory factors (IRFs). NFκB and MAPK

pathways mediate proinflammatory responses whereas IRFs are key molecules leading to the production of antiviral IFNs (27, 28)

In response to viral dsRNA stimulation, TLR3 signals via adaptor TRIF (18). TRIF interacts with tumour necrosis factor (TNF) receptor associated factor 3 (TRAF3) to form a scaffold to activate the non-canonical IKK-related kinases (TANK)-binding kinase 1 (TBK1), and inhibitor of nuclear factor kappa-B kinase subunit epsilon IKKε. TBK1/IKKε subsequently phosphorylates IRF3 (29, 30); upon phosphorylation, IRF3 dimerizes and translocates to the nucleus to initiate transcription of IFN-I genes (Figure 1.2) (4). Expression of TLR3 is inducible in response to various cytokines like IFN-I, further augmenting the antiviral response (31).

TLR3 is expressed by a broad range of cells (24). TLR3 expression in phagocytic cells such as macrophages, B lymphocytes, and conventional DCs is restricted to intracellular compartments whereas plasmacytoid DCs (pDCs) do not express any TLR3 (32). Therefore, it has been suggested that TLR3 detects viral RNA derived from phagocytosed, virus-infected apoptotic or necrotic cells to mediate cross-priming of T lymphocytes necessary for the induction of virus-specific T cell responses (33). Indeed, self-mRNA released from necrotic cells or generated by *in vitro* transcription stimulated TLR3 signaling in conventional DCs suggesting that sterile tissue damage may also lead to inappropriate activation of innate immune responses (34).

TLR3 can be found both intracellularly and on the cell surface of some non-phagocytic cells such as epithelial cells, and fibroblasts (35, 36); however, optimal dsRNA binding is shown to occur only in acidic subcellular compartments like early phagolysosomes or endosomes leading to receptor aggregation and subsequent downstream antiviral signaling (37). Yet, infection in human airway epithelial cells with either rhinovirus or respiratory syncytial virus (RSV) has shown to increase the expression of TLR3 mRNA and TLR3 protein at the cell surface (38, 39). RSV infection further sensitizes the airway epithelial cells to subsequent stimulation by dsRNA via surface TLR3 (38). Furthermore, poly (I:C) treatment also increases surface TLR3 expression in human kidney epithelial cells (40). Thus, the functional role of TLR3 on the cell surface is still an enigma and an area of intense study. It is proposed that the inducible expression of surface TLR3 may augment the proinflammatory response during viral infections and prime the cells for subsequent exposure to dsRNA (38-41).

TLR3 is an essential mediator of antiviral responses to viruses like herpes simplex virus type-1 (HSV-1) (42) and Epstein Barr virus (EBV) (43) but it is dispensable for pathogenesis and adaptive immune responses in vesicular stomatitis virus (VSV), lymphocytic choriomeningitis virus (LCMV) and reovirus infections (44). Indeed, an IFN-β response was recorded in fibroblasts and conventional DCs derived from TLR3-deficient mice following intracellular administration of poly (I:C) or infection with several RNA viruses like Sendai virus, Newcastle disease virus and VSV (45, 46).

Collectively, these observations implied the existence of additional receptors that detect actively replicating viruses in cytoplasm, including the RNA-binding RLRs.

1.2.2. The RLRs: Sensors of cytoplasmic dsRNA

The RLRs include RIG-I, melanoma differentiation-association gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). They are cytosolic RNA sensors and are crucial for triggering the innate immune response to RNA viruses in most cell types (47, 48). Recent evidence suggests RIG-I may also localize to the nucleus (49, 50). All RLRs have a central helicase domain attached to a C-terminal domain (CTD), otherwise known as the repressor domain (RD). The helicase domain and CTD together mediate dsRNA recognition. At the N-terminus, RIG-I and MDA5 have tandem caspase activation and recruitment domains (CARDs) required for downstream signal transduction (51). LGP2 lacks the N-terminal CARDs but binds dsRNA and has been implicated in differentially regulating RIG-I and MDA5 signaling (14).

RIG-I preferentially binds ligands that are short (10–300 bp), including dsRNA or ssRNA harboring uncapped 5’diphosphate/ triphosphate (5’-pp/ppp) ends and panhandle-like secondary structures composed of double-stranded segments. Host mRNA are commonly capped with a 5’-7-methylguanosine cap and thus avoid RIG-I recognition in the cytosol, maintaining self-tolerance (52, 53). Additionally, RIG-I was shown to activate downstream antiviral signaling in response to *in vitro* transcribed dsRNAs of varying lengths (54). In contrast to RIG-I, MDA5 selectively recognizes long dsRNA

molecules (> 1 kb) such as long dsRNA replicative intermediates of certain viruses (54). However, the natural RNA PAMP ligands of MDA5 remain poorly characterized (14).

The RLRs are expressed at low levels in resting cells but are highly inducible upon virus infection or IFN-I stimulation (51). In unstimulated state, the helicase domain and RD associate with the CARDs which prevents the RLRs from participating in signaling (47, 48, 55). RIG-I binds the 5'- terminal regions of the RNA ligand and oligomerizes to initiate signaling (56). Similarly, MDA5 binds internally to long dsRNA and assembles into 'helical filaments' (54, 57). Ligand binding opens the conformation and exposes the CARD domains to associate with the adaptor molecule mitochondrial antiviral-signalling protein (MAVS) via CARD-CARD interactions. MAVS recruits TRAF2/5, TRAF3 and TRAF6 into a complex and subsequent signaling cascade ultimately culminates in the production of IFN-I and proinflammatory cytokines and chemokines (Figure 1.2) (58).

Prior to the discovery of RLRs, other cytosolic proteins were reported to recognize dsRNA and regulate antiviral responses. These include IFN-inducible 2'-5'-oligoadenylate synthetase 1 (OAS1) and dsRNA-dependent protein kinase R (PKR) (9, 59). When activated by binding to dsRNA (60), OAS1 activates latent ribonuclease RNase L which then catalyzes the degradation of viral and cellular ssRNAs (61). RNase L cleavage products further induce production of IFN-I through the RIG-I pathway (62, 63). PKR is a serine/threonine kinase that is activated by binding to dsRNA.

Activated PKR suppresses translation initiation by phosphorylating the α-subunit of eukaryotic initiation factor 2 (64). However, both OAS1 and PKR do not directly induce production of IFN-I and ISGs (9). Similarly, LGP2 has been shown to modulate RIG-I and MDA5 signaling in response to virus infection (9).

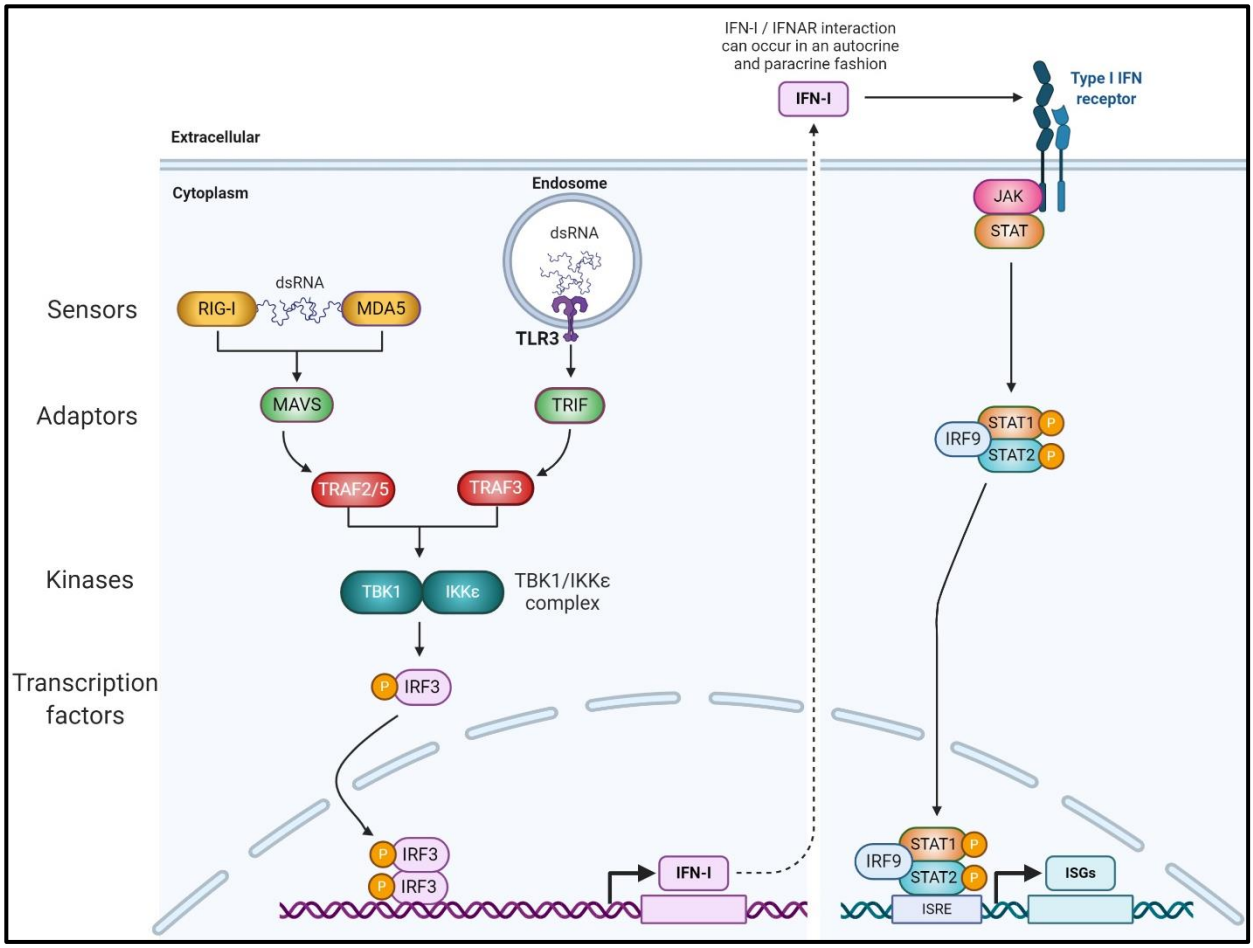

Figure 1.2. Schematic representation of IRF3 mediated antiviral IFN-I expression in response to viral infection. The presence of viral dsRNA in infected cells is detected via conserved pattern recognition receptors. Endoplasmic TLR3 and cytosolic RIG-I and MDA5 are **"Sensors"** of dsRNA. Following recognition of dsRNA, these cellular sensors signal through various **"Adaptors"** such as MAVS and TRIF. The adaptor proteins facilitate formation of a signaling scaffold which leads to the activation of cellular **"Kinases"** such as TBK1 and IKKε. These kinases phosphorylate a key **"Transcription factor"** called IRF3, leading to its dimerization and subsequent translocation to the nucleus of the cell where it initiates the expression of antiviral IFN-I. IFN-I acts in an autocrine or paracrine manner by binding to its cognate receptor on the cell surface. IFN-I binding activates Janus kinase (JAK) which phosphorylates signal transducer and activator of transcription (STAT) proteins. Activated STAT1 and STAT2 dimerize and then combine with IRF9 to form the interferon-stimulated gene factor 3 (ISGF3) transcriptional complex. ISGF3 binds to interferon-stimulated response elements (ISRE) within IFN-I-dependent gene promoters and induces the expression of a series of ISGs. Image created with BioRender.com.

1.2.3. IFN-I signaling

Three types of interferon (IFN) have been identified in humans. IFN-I consist of IFN-β, IFN-ε, IFN-κ, IFN-ω and 13 subtypes of IFN-α. IFN-γ, a type II IFN, is exclusively produced by T cells, natural killer (NK) cells, NKT cells and DCs. The type III IFNs include 4 IFN-λ subtypes and play a role in barrier function (65). Although multiple cytokines and chemokines are produced by several kinds of host cells in response to virus infection, IFN-I are the principal cytokines involved in the antiviral response. IFN-I are constitutively expressed at low levels and their expression is rapidly induced by several innate immune signalling pathways (66).

IFN-I are secreted by responding host cells and then signal in an autocrine and paracrine manner by binding to the IFN-I-receptor (interferon-α/β receptor (IFNAR)). The heterodimer of IFNAR1 and IFNAR2 then activates the intracellular Janus kinase–signal transducer and activator of transcription (JAK–STAT) pathway, particularly STAT1 and STAT2. Once phosphorylated, STAT1 and STAT2 combine with IRF9 to form the interferon-stimulated gene factor 3 (ISGF3) transcriptional complex. ISGF3 binds to interferon-stimulated response elements (ISRE) within IFN-I-dependent gene promoters and induces the expression of a series of ISGs (Figure 1.2) (67, 68). Several ISG products then function together to restrict virus replication via numerous mechanisms that are discussed in greater detail elsewhere (6). The effects of IFN-I are pleiotropic in nature; they can induce apoptosis of infected cells, activate DCs and NK cells and trigger the adaptive immune system (69, 70).

IFN-I have potent immunomodulatory properties which are essential in clearing an infection, but a premature or unregulated innate immune response is harmful to the host. Indeed, inappropriate activation of IFN-I-mediated responses have been associated with autoinflammatory and autoimmune diseases collectively known as interferonopathies (71), such as Aicardi-Goutières syndrome, type 1 diabetes, systemic lupus erythematosus (SLE), Singleton-Merten syndrome, psoriasis, and colitis (71-73). Therefore, the innate immune system has evolved several regulatory mechanisms at various steps before and after activation of innate immune pathways to avoid an exaggerated response to a pathogen and such mechanisms are reviewed in detail elsewhere (14, 74).

1.3. Virus-mediated modulation of innate immune responses

As obligate intracellular parasites, viruses utilize the host cell machinery and resources to replicate and propagate. Humans have evolved elaborate defense mechanisms to detect and restrict viral replication and spread. In turn, this has led to the emergence of viruses that are capable of eluding and manipulating the host immune responses to either generate a productive infection (75) or persist latently from one cell generation to the next without the production of new virus particles (76). Viruses capable of evading and/ or antagonizing the host immune response can also cause severe morbidity and mortality, as illustrated by the swine flu pandemic caused by the H1N1 subtype of influenza A virus (IAV) and the Ebola virus (EBOV) outbreak in West Africa (75).

IFN-I are highly potent cytokines which are key mediators of antiviral responses (66, 77). They influence both innate and adaptive immune mechanisms and induce the expression of restriction factors, which are proteins that directly interfere with the life cycle of a virus (6). In principle, the success of a virus depends on its ability to evade, antagonize or adapt to host antiviral responses including IFN-I and the downstream effects of IFN-I signaling (78). Accordingly, viruses have evolved several strategies to help them propagate within their host (75).

Early viral evasion strategies to circumvent innate antiviral responses fall broadly into two categories, a) avoiding detection through PRRs and, b) inhibiting the activation of PRRs and/or their downstream signalling cascades (75). Viruses can sequester their nucleic acids to escape surveillance by PRRs. For instance, most flaviviruses including dengue virus (DENV) replicate in vesicular structures in the convoluted membranes of the endoplasmic reticulum (ER), which efficiently shield viral nucleic acids from the cytoplasm, thus preventing the activation of RLRs (79). IAVs also avoid being detected by replicating in the nucleus which is atypical for RNA viruses (80). Members of the *Bunyaviridae* family, such as Hantaan virus, and Crimean–Congo haemorrhagic fever virus (CCHFV) encode phosphatases to process the 5′-ppp group on their genomes to 5′-p to escape surveillance by RIG-I (81, 82). Viruses can also impair PRR activity by interfering with the accessory cellular components required for PRR activation (83). For example, PKR-activator (PACT) is a cellular dsRNA-binding protein that augments the IRF3-dependent production of IFN-I following dsRNA-induced activation of the RLRs

(84, 85). Some viral proteins like VP35 from EBOV, Middle East respiratory syndrome coronavirus (MERS-CoV) 4a protein and non-structural protein 1 (NS1) from IAV directly bind PACT to disrupt its interaction with RIG-I and/or MDA5 and thereby, supress the production of IFN-I (75, 83, 86).

Many viruses commonly engage multiple evasion strategies such as degradation or cleavage of PRRs, their adaptor proteins or downstream molecules like TBK1, IRF3 and NFκB; interference with specific post-translational modifications of PRRs or their adaptor proteins; and blocking IFNAR receptor signalling or the function of specific antiviral effector proteins. These mechanisms disrupt the IFN-I responses to promote successful replication of viruses and associated pathology (75). How such evasion strategies are mediated by individual viruses or virus families are beyond the scope of this thesis and have been discussed extensively by others including examples of viruses of clinical relevance (75, 87, 88).

1.4. Importance of extracellular viral dsRNA in the innate immune response to viruses

It is abundantly clear that viruses develop mechanisms to inhibit, evade and even hijack the immune system to further their replication and persistence within the infected host (75, 88, 89). Accordingly, the host cells also adopt measures to counter viral evasion tactics. A phenomenon commonly referred to as “bystander immunity”, is mediated by the transfer of ‘danger signals’ from the infected cell to the surrounding non-infected cells

to facilitate activation of innate immunity, and thereby bypassing viruses' defences (Figure 1.3A-B). Bystander activation likely represents a host mechanism whereby an initial stimulation from an infected cell results in activation of multiple bystander cells to mount a self-sustaining and often amplified innate immune responses (90).

Viral RNAs are an important PAMP (danger signal) which facilitate the detection of viral pathogens (16, 91). Infected host cells can transfer viral RNAs to neighbouring cells via exosomes to overcome virus's defense strategies and control the infection. Indeed, exosomes from infected cells have been shown to contain viral mRNAs and microRNAs (miRNAs) which activate bystander immunity through the recognition of viral RNA by corresponding PRRs (Figure 1.3A) (92-94).

Hepatitis C virus (HCV) consists of a ssRNA viral genome and has been shown to effectively inhibit IFN-I responses in hepatic cell lines *in vitro*. In contrast, *in vivo* challenge with HCV induces a potent IFN-I response in the infected liver (95-97) and the IFN-I responses are mediated by the neighbouring uninfected pDCs in a TLR7-dependent manner (98). Takahashi *et al.* (2010) further demonstrated that IFN-I responses are dependent on active HCV RNA replication but independent of the assembly and release of new virus particles (98). Ensuing work confirmed that the activation of bystander immunity in neighbouring uninfected pDCs was mediated via the uptake of exosomes containing HCV RNA released from infected hepatocytes (99). Similar observations have

also been made with other clinically relevant viruses like EBV and human immunodeficiency virus (HIV) (90).

Besides viral RNA, the host cells have also been shown to transfer viral DNA and secondary messenger molecules like 2'3'-cyclic GMP-AMP (cGAMP) (90). Cyclic guanosine monophosphate (GMP)–adenosine monophosphate (AMP) synthase (cGAS) is a cytosolic sensor for many DNA viruses and HIV-1. In response to cytosolic viral DNA (100), cGAS catalyses the synthesis of the second messenger cGAMP. cGAMP activates ER-resident stimulator of interferon genes (STING) (101, 102) leading to a robust IFN-I response and a subsequent antiviral state. Recent studies have reported that cGAMP can be transferred to nearby cells to activate STING-mediated antiviral pathways (103-105). Indeed, HIV-1 propagated in human kidney epithelial cells reconstituted with wildtype cGAS, induces expression of IFN-I in human monocyte–derived dendritic cells (hMDDCs) upon infection. HIV-1 virions produced by cells lacking cGAS are however, unable to induce IFN-I responses in hMDDCs. Mass spectrometry (MS) analysis of the HIV-1 virus stocks obtained from cGAS expressing cells confirmed the presence of cGAMP within the virions (104). The authors of the study further observed similar results following mouse cytomegalovirus (MCMV) infection (104), suggesting that the mechanism of intercellular transfer of cGAMP packaged within virus particles may apply to multiple viruses.

It is important to note that while exosomes rich in host and/ or viral proteins and nucleic acids have been recovered from the sera of infected individuals. their physiological role in the pathogenesis of viral disease remains unclear (92). Indeed, contrasting studies suggest that the exosomal transfer of viral proteins and/ or nucleic acids is a pro-viral mechanism and contributes to the pathology of viral disease (92). Moreover, there is limited evidence to suggest that exosome-transferred viral nucleic acids or cGAMP can achieve effective activation of bystander innate immunity at a systemic level (90).

1.4.1. Viral dsRNA: A potent extracellular signaling molecule

Viral dsRNA is a powerful inducer of both innate and adaptive immune responses and is also the only known cytokine-inducing component common to all viruses (15, 106). Within infected host cells, genomes of dsRNA viruses and intermediates generated during replication of ssRNA and DNA viruses are the sources of viral dsRNA (15). Under normal conditions, host cells produce minute amounts of short stretches of dsRNA ($\leq$ 20 bp), but substantial quantities of long dsRNA molecules are generated in virally infected cells (107). The length of dsRNA molecules generated in infected cells correlate with the viral genome length (54, 108) and are commonly sufficient to trigger the dsRNA sensors which recognize dsRNAs >30 bp in length (37). As for localization, viral dsRNA typically cumulates within the cellular compartment in which the virus replicates but in the case of viruses replicating within the nucleus, dsRNA can be found in both the nucleus and cytoplasm (109).

Many acute viral infections are characterized by toxic symptoms despite highly localized viral replication. Like bacterial toxins such as LPS, a soluble 'viral toxin', independent of the virus particle was intensively sought in the 1940s (110) which could induce strong systemic responses. In 1963, it was found that viruses produced dsRNA (111) but it was not until the 1970s that the intrinsic toxicity of dsRNA was being compared with the pathological effects of 'viral toxin' of the 1940s (112). Both viral or synthetic dsRNA like poly (I:C) were demonstrated to induce IFN-I when added exogenously (either *in vitro* or *in vivo*) (113). Indeed, animals administered viral dsRNA or poly (I:C) systemically showed 'flu-like' symptoms like those infected with influenza virus (114). Moreover, extracellular dsRNA could be detected *in vivo* from the lungs of influenza-infected mice (115) and *in vitro* from influenza-infected cell cultures (116). Therefore, it is apparent that there are two ways in which viral dsRNA contributes to the pathogenesis of a viral infection: (a) the viral dsRNA generated within the infected cell induces the production of cytokines including IFN-I and (b) the extracellular dsRNA released from dying cells can also induce antiviral responses in neighbouring or distal cells (bystander immunity) (Figure 1.3B). The direct cytotoxicity of viral dsRNA and its cytokine induction capacity are amplified in the surrounding bystander cells (neighbouring cells and infiltrating immune cells) which are primed and activated by the cytokines (particularly IFN-I) released from the infected cells. Activated bystander cells produce more cytokines ultimately, leading to the systemic flu-like syndrome (106, 117).

DsRNA has characteristics of an ideal signaling molecule. It is remarkably potent at inducing antiviral activity; a single long dsRNA molecule within a cell is capable of triggering a response (118). Besides potent signaling ability, nuclease-resistance is a critical factor determining extracellular dsRNA's ability to mediate local and systemic effects (106). Whilst tissue and serum ribonucleases are known to preferentially digest ssRNA, they also display nucleolytic activity against dsRNA, albeit the digestion of dsRNA is less efficient and occurs at a significantly lower rate relative to the digestion of ssRNA (106, 119-121). The relative resistance of dsRNA to degradation by many RNases is also a result of its a) structure, b) high melting temperature and c) interactions with viral and/or host proteins (106, 119, 122). Poly (I:C) forms an A-form helix with a deep, narrow major groove which restricts interactions with macromolecules such as nucleases and the high melting temperature of dsRNA directly influences RNase resistance as it takes longer to destabilize the duplex (119). 'Native' dsRNA generated during viral replication associates with viral and/or host proteins and such interactions serve to stabilize dsRNA within the cell as well as outside the cell by interfering with nuclease-mediated degradation (106). Indeed, viral dsRNA and poly (I:C) are capable of resisting digestion by RNases *in vitro* and *in vivo* and induce antiviral responses (106, 115, 116). Lastly, while it is possible that the electronegative properties of dsRNA can limit its ability to circulate within the host, systemic responses have repeatedly been observed following *in vivo* challenges with dsRNA (122). Indeed, both intraperitoneal (123) and intravenous (124) injections of poly (I:C) have shown to affect cells in organs such as the spleen and liver of mice. Therefore, a small amount of nuclease-resistant extracellular

viral dsRNA is likely sufficient to induce local bystander immunity and may also contribute to systemic effects observed during viral infections.

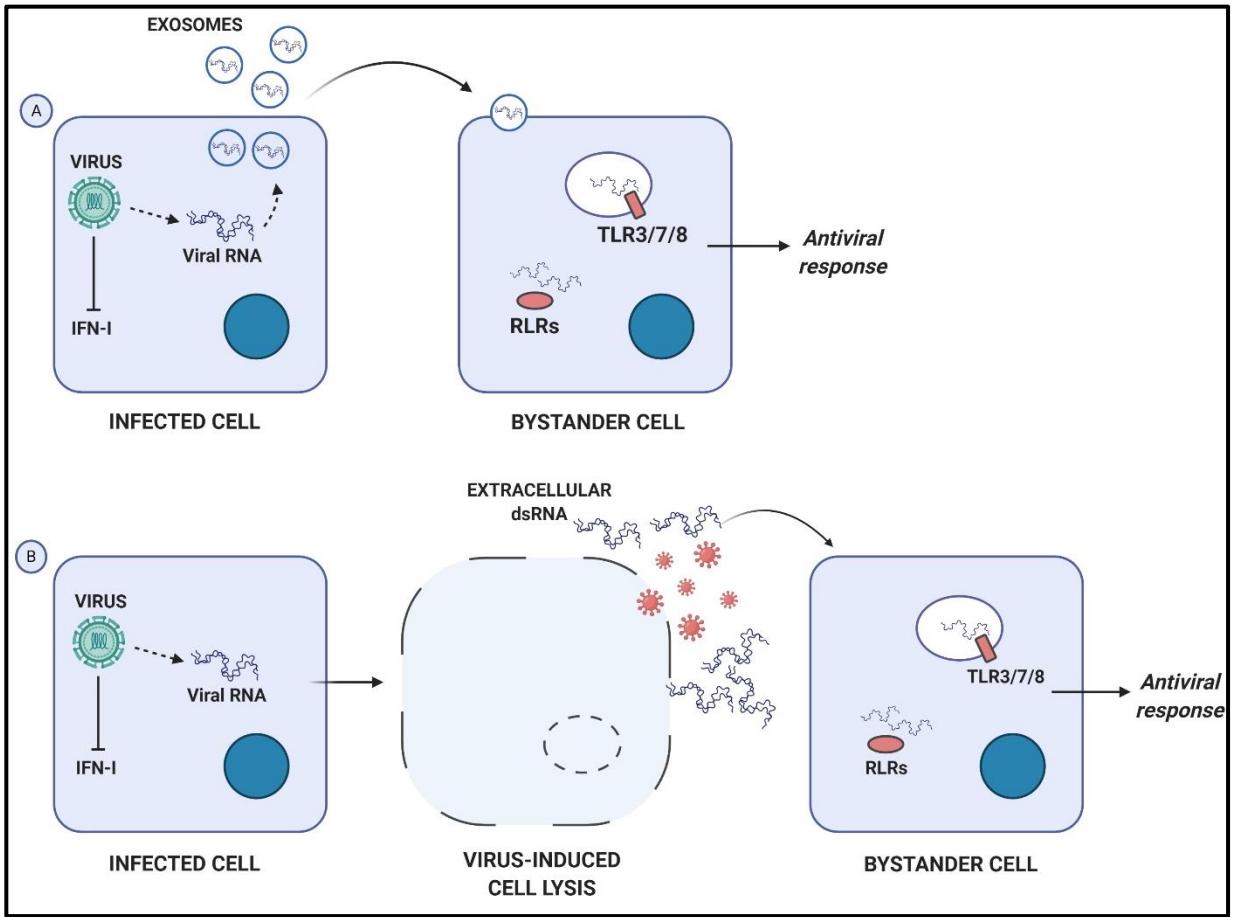

Figure 1.3. Intercellular transfer of pathogen derived PAMPs leads to the activation of "bystander immunity". Virus derived RNAs are produced within the cytoplasm of infected cells and are released into the extracellular space (A) incorporated into exosomes or following (B) virus-induced cell lysis. Pathogenic viral RNA contained within exosomes or present in the extracellular milieu are subsequently internalised by uninfected bystander cells via endocytosis. Uptake of extracellular RNAs activate endosomal TLRs and cytoplasmic RLRs, leading to the production of IFN-I and proinflammatory cytokines constituting an antiviral response. Image created with BioRender.com.

1.4.2. Role of dsRNA in adaptive immunity

Much of the effects of dsRNA has been studied in the context of triggering innate antiviral responses following viral infection. However, dsRNA can link innate and adaptive immunity by mediating either direct or indirect effects on adaptive immune cells (122). Typically, the innate immune cells like macrophages, DCs or the non-immune cells such as epithelial or stromal cells sense dsRNA via classic dsRNA sensors, such as TLR3 and RLRs (33, 125) and produce proinflammatory cytokines and chemokines in response. These cytokines, including IFN-I are sensed by adaptive immune cells. It has been shown that poly (I:C)-mediated production of IFN-I by the intermediary cells (phagocytes, fibroblasts, or stromal cells) result in DC maturation and subsequent CD4+ T cell immunity (126), CD8+ T cell differentiation (127) and generation of memory CD8+ T cells (125). Invariably, IFN-I are key mediators of the indirect effects of dsRNA (128).

The direct effects of dsRNA on adaptive immunity have been evaluated based on expression of dsRNA sensors in adaptive immune cells. For instance, T cells express TLR3 and RLRs and can respond to viral dsRNA (129, 130). Poly(I:C) treatment has shown to induce the synthesis of IL-17A and IL-21 in human naive CD4+ T cells (131). It was also found that the loss of suppressive function of Treg cells during encephalomyocarditis virus (EMCV) infection was dependent on MDA5 (129). In response to dsRNA, human upper respiratory mucosa B cells initiate class switching from IgM to IgG and IgA through an NF-B-dependent signaling pathway requiring TLR3 and TRIF (132). Similarly, differentiated plasma cells express TLR3 and produce elevated

levels of immunoglobulins in response to poly (I:C) treatment (133). Notably, the effects of dsRNA on adaptive immune cells contrast with the effects seen in innate immune cells where production of IFN-I and ISGs are the most prominent outcome. In adaptive immune cells, dsRNA treatment often induces the expression of co-stimulatory molecules, enhances antigen presentation, polarizes T cell responses, and reverses suppressive Treg activity (117, 128).

1.4.3. Role of dsRNA in autoinflammatory and autoimmune conditions

In addition to the 'toxin' effects, dsRNA has also been implicated as a potent stimulator of sterile inflammation. A single intranasal administration of poly (I:C) induces inflammation in lungs of mice comparable to inflammation in the lungs of patients with chronic obstructive pulmonary disease (134). Similarly, thymic stromal lymphopoietin (TSLP) plays a key role in allergic diseases and it was confirmed that treatment with extracellular poly (I:C) triggers the release of TSLP in primary human keratinocytes. Whether keratinocytes can be stimulated by endogenous dsRNA released from damaged skin tissue or leaked viral dsRNA remains unclear (135). DsRNA can also contribute to the pathogenesis of autoimmune conditions as poly (I:C) treated T cells express high levels of IL-17A and IL-21, two cytokines associated with regulating autoimmunity (131). Recent studies have implicated dsRNA in several autoimmune diseases like myasthenia gravis (136), rheumatoid arthritis (RA) (137), and type 1 diabetes (138, 139).

TLRs appear to play a role in regulating autoimmunity; their activation is associated with chronic inflammation in cases of RA. Indeed, necrotic synovial fluid from patients with RA triggered TLR3-mediated production of IFN-I (140). TLR3, in combination with TLR7 and TLR9, mediates generation of anti-nucleic acid autoantibodies in SLE (141). Multiple studies have also shown that TLR3 can be activated by endogenous dsRNA released during necrosis, independent of a viral infection resulting in proinflammatory responses and production of IFN-I (34, 142). Therefore, uncontrolled cell death or any other non-homeostatic condition is likely to trigger TLR3-mediated responses in surrounding cells (34, 142).

A causal link between viral infections and autoimmunity has also been studied for a long time and the role of some viruses in the induction or exacerbation of SLE has been proved. For example, EBV, parvovirus B19, human endogenous retroviruses, and human cytomegaloviruses have shown to be involved in SLE pathogenesis (143). The underlying trigger for SLE remains elusive but it is suggested that a complex interplay of multiple environmental and genetic factors likely contributes to the onset and perpetuation of the disease (144-147). Likewise, EBV infection is also associated with other systemic autoimmune diseases like RA, and mixed connective tissue disease (148). Consequently, high levels of IFNs and ISGs, an ‘‘IFN signature’’, is observed in several autoimmune conditions like SLE and RA (149). Indeed, genome-wide association studies have identified a common gene set involved in the IFN-I pathway that are unanimously upregulated in patients with SLE, RA, polymyositis, and systemic scleroderma (150). In

contrast, most patients with multiple sclerosis, an autoimmune condition, have low levels of serum IFN and instead, IFN-β is used to treat multiple sclerosis patients (151). Viral infections can interact with the host immune system through several mechanisms such as molecular mimicry, altered apoptosis and the innate immune activation leading to the production of IFN-I which ultimately contributes to the loss of immune tolerance (143). Importantly, circulating endogenous and exogenous (viral) nucleic acids such as dsRNA have been shown to contribute to the pathogenesis of some autoimmune conditions including SLE (152-154) thus, highlighting the importance to uncover the trafficking and signaling mechanisms of cell free dsRNA.

1.5. DsRNA sensing at the cell surface

Typically, the PRRs like TLR3 and the RLRs sense dsRNA within intracellular compartments such as endosomes and the cytoplasm, respectively (37, 74). The extracellular dsRNA must therefore by-pass the phospholipid cell membrane which presents a barrier to its passive diffusion and be internalized for it to mediate its effects such as triggering bystander immunity in surrounding cells during a viral infection. Although mechanisms of RNA uptake by mammalian cells are not well understood, it has been shown that extracellular RNAs can enter mammalian cells by natural processes such as direct cell-to-cell contact, channels, and membrane receptors (155). Over the years, several plasma membrane proteins have been shown to uptake extracellular nucleic acids, including dsRNA (156). Among them, class A scavenger receptors (SR-As) are of

particular interest given their ability to bind microbial PAMPs including dsRNA and influence host immune responses via activation of intracellular signaling cascades (157).

1.5.1. Scavenger receptors

In 1979, Michael Brown and Joseph Goldstein first identified scavenger receptors (SRs) in macrophages and described their activity in the uptake of modified low-density-lipoprotein (LDL) such as oxidized LDL (oxLDL) or acetylated LDL (acLDL) (158). The first SRs to be cloned were the prototype SR-As, then named type I and type II macrophage scavenger receptors (159). With additional SR family members identified over the years, SRs are currently grouped into 11 classes (A–L) based on sequence homology or shared structural features (160). Notably, different classes of SRs bear little or no primary sequence similarity (161, 162). Also, only *Drosophila melanogaster* exhibits class C SRs and there are currently no known mammalian class C SRs (162).

The range of ligands recognized by different classes of structurally heterogenous SRs is extremely diverse and includes several conserved microbial PAMPs such as bacterial lipopolysaccharide (LPS) and lipoteichoic acid (LTA) as well as DAMPs like modified endogenous proteins and lipoproteins (157, 163, 164). SRs are known to mediate the removal of degraded or harmful substances from circulation via endocytosis or phagocytosis and subsequent signaling (165). As such, SRs are considered an important subclass of the membrane bound PRRs in innate immunity (162, 166).

As mentioned earlier, there are currently 11 classes of SRs and new members continue to be identified (160). However, due to several inconsistencies in nomenclature there had finally been a push to form a unified naming system to avoid scenarios where a single receptor is cited in the literature under multiple aliases like MSR1, SR-AI, CD204, and SCARA1. In addition, there are proteins that exhibit scavenger activity but have been named under a different nomenclature such as RAGE, CD163, and CXCL16. Therefore, a standard set of rules to name current and newly identified SRs will enable clear communication and decrease the number of redundant studies in the SR field. The standardized SR nomenclature currently follows a system of SR-J1.1, where SR stands for scavenger receptor and J represents the class of SR. The first number identifies the order in which the molecules were identified. Splice variants of a specific receptor are designated as 1.1, 1.2 and so on (160). I have prepared this thesis based on the new standardized SR nomenclature (160) and recommended changes are outlined in Table I. Please note that the consensus nomenclature of only those SRs have been summarized in Table I that are mentioned in this thesis.

Table I. Summary of the current and consensus nomenclature of human SRs

Gene name	Alternative names	Consensus nomenclature	Accession number
MSR1	SR-AI, CD204, SCARA1	SR-A1	NM_138715
Splice variant #1 of MSR1	SR-AII*	SR-A1.1	NM_002445
Splice variant #2 of MSR1	SR-AIII	SR-A1.2	NM_138716
MARCO	SCARA2	SR-A6	NM_006770
SCARA3 (isoform 1)	MSRL1	SR-A3	NM_016240
SCARA3 (isoform 2)	MSRL1	SR-A3.1	NM_182826
COLEC12	SCARA4, SRCLI, SRCLII, CL-P1	SR-A4	NM_130386
SCARA5	TESR, NET33	SR-A5	NM_173833
SCARF1	SREC-1	SR-F1	NM_003693
RAGE (membrane form)	AGER	SR-J1	NM_001136

* There will be no scavenger receptor designated as SR-A2 to avoid confusion with the current SR-AII (new designation is: SR-A1.1)

1.5.2. Class A Scavenger receptors

The SR-As are highly conserved, multi-functional type II transmembrane glycoproteins with homotrimeric structures. The SR-A family currently consists of five members: SR-A1, SR-A3, SR-A4, SR-A5 and SR-A6, each encoded by distinct and unrelated genes. Historically, the expression of SR-A1 and SR-A6 was thought to be restricted to myeloid cells like macrophages and dendritic cells but they are now known to be more widely expressed. SR-A3 is ubiquitously expressed while SR-A4 is enriched in placenta and vascular endothelial cells, but not in macrophages; expression of SR-A5 is localized to epithelial cells (160).

The members of SR-A family share structural homology. All SR-As comprise of a short N-terminal cytoplasmic domain, followed by a transmembrane and a spacer domain which serve to anchor the receptor into the cell membrane and stabilize the receptor, respectively. Next, an α-helical coiled-coil domain is important in the receptor trimerization and dissociation from ligand in endosomes (167). A common feature of all the SR-As is a distinct collagenous domain (159, 168) whose length varies from approximately 75 residues in SR-A5 to 250 residues in SR-A6 (161, 169). All SR-As share a conserved lysine-rich motif within the collagenous domain that has been implicated in ligand recognition by SR-A1, SR-A4 and SR-A5 but not by SR-A6 (170-172). The C-terminus domain is heterologous between SR-A members: SR-A1, SR-A5 and SR-A6 contain a terminal Scavenger Receptor Cysteine Rich (SRCR) domain; SR-A3 terminates at the collagenous domain, and SR-A4 possesses a C-type lectin domain (161).

The SR-As exhibit unusually broad ligand specificity, including chemically modified or altered self-molecules, bacterial surface components and apoptotic cells. By virtue of their functional versatility and selectivity for a wide range of ligands, they are involved in both the maintenance of homeostasis and in the pathogenesis of various diseases (157). Both SR-A1 and SR-A6 have been implicated in the clearance of apoptotic cells and modified self-molecules like oxLDL and β-amyloids to maintain homeostasis and somewhat paradoxically play a role in the pathogenesis of atherosclerosis and neurodegeneration, respectively (157, 173). They also mediate host

defense against invading microorganisms via the direct recognition and subsequent endocytosis of pathogens and the modulation of cytokine production (157, 166, 173). SR-A3 is intracellular and has been associated with protecting cells from the detrimental effects of reactive oxygen species (174). SR-A4 mediates the degradation of oxLDL in vascular endothelial cells (175) while both SR-A4 and SR-A5 have been shown to bind bacteria *in vitro* and may play an important role in host defense (161, 176, 177). SR-A5 has been also shown to mediate the scavenging of serum ferritin (178). Importantly, the roles of SR-A1 and SR-A6 in innate immunity has been more extensively studied than other members of the SR-A family. Specifically, SR-A1 has been demonstrated to play a key role in extracellular dsRNA-mediated induction of pro-inflammatory cytokines including IFN-I (19, 179).

1.5.3. Ligand specificity of SR-A1

SR-A1 is a type II homotrimeric transmembrane glycoprotein whose structure can be divided into six distinct domains: 50-amino acid (aa) cytoplasmic domain; a 25-aa transmembrane region; a 75-aa spacer domain which may be N-glycosylated; a 121-aa α-helical coiled-coil domain; a 69-aa collagenous domain and a 110-aa C-terminal conserved SRCR domain. SR-A1 exists as multiple splice variants with differences in their C-terminus; SR-A1 expresses the SRCR domain while SR-A1.1 and SR-A1.2 have a short or truncated C-terminal region (159). SR-A1.2 is not functional since it remains trapped in the ER and thus is often considered as a dominant negative isoform of SR-A1 (180).

SR-A1 and SR-A1.1 (lacking the SRCR domain) bind modified lipoproteins with similar efficiency and affinity suggesting that SRCR domain is dispensable for ligand binding in SR-A1 (168). Analysis using truncation mutants of the extracellular domains of SR-A1 suggested that the ligand binding region lies within the collagenous domain and, a highly conserved lysine-rich motif was shown to be critical for binding polyanionic ligands like acLDL and polyribonucleotides (170). Subsequently, it was shown that complex interactions with residues along the entire collagenous domain may be required for ligand binding (181).

All of the SR-As share a highly conserved collagenous domain implicated in ligand binding for SR-A1, SR-A1.1, SR-A4 and SR-A5 but it is dispensable for SR-A6 (161). Instead, the SRCR domain of SR-A6 has been proposed to be the primary ligand binding site based on two highly conserved arginine residues, termed the RxR or RGRAEVYY motif (171, 172). While the collagenous domain is highly conserved amongst SR-A members, a differential preference for similar polyanionic ligands is observed. Unlike SR-A1 and SR-A4, SR-A5 is unable to bind modified LDL while SR-A1, SR-A4 and SR-A5 can all bind bacteria (176, 177). Also, the SRCR domain in SR-A5 is dispensable as is in SR-A1/ 1.1 (176), although some recent studies have implicated the SRCR domain of SR-A1 in recognizing spectrin from dead cells and mediating their uptake (182). Despite structural similarities and overlapping functions of SR-As (183), the differences in their ligand binding mechanisms remain intriguing.

The binding between SR-A1 and its polyanionic ligands reflect an ionic interaction, yet the preference of SR-A1 for certain polynucleotides like poly (I:C), polyinosinic acid (poly I) or polyguanilic acid (poly G) and its failure to bind polyadenilic acid (poly A) or polyuridylic acid (poly U) suggest that there may be additional factors that contribute to ligand binding specificity (184, 185). Indeed, studies have shown that the high affinity recognition is dependent upon conformational interactions between negatively charged phosphates on polynucleotide quadruplexes and the positively charged surface of the collagenous domain of SR-A1 (184, 185). Such a conformational requirement explains the preferential polyribonucleotide-binding specificity of SR-A1. However, there is no evidence suggesting whether viral nucleic acids preferentially bind the collagenous domain of SR-A1 or the SRCR domain of SR-A6 and if such binding is dependent on nucleic acid species, sequence, or length.

1.5.4. Signaling ability of SR-A1

The cellular function of surface receptors such as SR-A1 is often determined by the presence of motifs within the cytoplasmic domain and the specific interaction of these motifs with intracellular proteins (186). While specific residues and regions required for membrane trafficking and recycling, ligand internalization, and adhesion have been identified (187-190), SR-A1 lacks any conventional signaling motifs or sequences within its short cytoplasmic tail. Instead, it is suggested that the SR-A1 activates signaling pathways either by protein-protein interactions between signaling effector molecules or through recruitment of adaptor proteins to the cytoplasmic tail (191). Other SR-A

members have not yet been implicated in activation of intracellular signaling pathways in part due to the lack of conservation between their cytoplasmic domains (191).

SR-A1 ligand binding activates multiple intracellular signaling pathways through interaction with other cellular proteins (183). Indeed, incubation of AcLDL with human THP-1-derived macrophages rapidly activates the non-receptor tyrosine kinase Lyn, which then activates phospholipase C gamma 1 (PLC γ1) and downstream signaling pathways involving protein kinase C (PKC) and phosphoinositide 3-kinase (PI3K) (192). Importantly, Lyn and SR-A1 co-precipitate with each other, indicating that they may be physically associated (193). Moreover, lipoprotein and non-lipoprotein ligands have been shown to induce production of inflammatory cytokines via SR-A1 associated signaling cascades. AcLDL up-regulates TNFα production in J774A.1 macrophage cell line whereas fucoidan (SR-A1 ligand) was shown to induce both TNFα and IL-1β production (194). A subsequent study confirmed SR-A1 interaction with major vault protein (MVP) to stimulate TNFα secretion via the pro-apoptotic p38/c-Jun N-terminal protein kinase (JNK) signaling pathway (195). Similarly, SR-A1 ligands poly (I:C) and LTA have also been shown to induce TNFα in RAW 264.7 macrophage cell line through the activation of MAPK pathway (196).

The studies above suggest that the SR-A1 mediates downstream signaling following the internalization of its ligands but it can also function as either a co-receptor or as part of a multimeric signaling complex (157). For example, SR-A1 interacts with

receptor tyrosine kinase Mer (Mertk) to form a functional complex that enables clearance of apoptotic cells. The association with SR-A1 facilitates optimal phosphorylation of Mertk and PLC γ2 (197). Mertk possesses signaling abilities through a multi-substrate docking site which can activate multiple downstream signaling intermediates essential for uptake of apoptotic cells (157). Similarly, SR-A1-TLR4 cooperation following LPS treatment induces expression of inflammatory cytokines while simultaneously inhibiting the pro-survival IFN-I signaling pathway in macrophages (198). In contrast, SR-A1 attenuates TLR4-mediated NFκB signaling by inhibiting the activation of TRAF6 via direct interaction in mouse bone-marrow derived DCs suggesting that SR-A1 modulates signal transduction in a ligand and cell-type dependent manner (199).

Although the earlier studies clearly demonstrate that SR-A1 ligands induce a range of intracellular signaling pathways, the presence of other SRs, including members of the SR-A family, which display overlapping ligand specificity must be taken into consideration (157). Fucoidan and acLDL are extensively used as SR-A specific ligands yet class B and class F SRs bind both fucoidan and acLDL (157) and SR-F1, a class F SR binds poly (I:C) like SR-A1 (200). Evidently, Kim *et al.* (2003) confirmed that fucoidan and LTA activated cellular pathways independent of SR-A1 and instead CD14 was involved in the production of TNFα in SR-A1$^{-/-}$ mice (201). However, in this case the possibility of differential upregulation of other SR-A members in the absence of SR-A1 cannot be disregarded since they are all functionally redundant and bind similar ligands, albeit with distinct mechanisms (19, 157, 173). Besides, CD14 has been shown to form a

signaling complex with SR-A6 and TLR2 to mount an optimal inflammatory response to *Mycobacterium tuberculosis* bacteria (202) and thus, may function in a similar capacity with other SR-As. Perhaps a cell line lacking all known SR-A members (ΔSR-A) shall provide a more specific approach to delineating the presence of signaling cascades whose activation is dependent on SR-A1. ΔSR-A cells would also serve as an ideal model to characterize individual SR-A members by introducing them back one at a time.

1.5.5. SR-A1 and innate immunity

The role of SR-A1 in innate immunity includes maintenance of tissue homeostasis by clearance of modified self-components and apoptotic cells, maintenance of the architecture of lymphoid organs, regulating immune responses to cancer and host defense against invading microorganisms (203). SR-A1 mediates non-opsonic uptake and clearance of *Neisseria meningitides, Listeria monocytogenes* and *Staphylococcus aureus* (157). SR-A1 has also been shown to interact with TLR4 and TLR2 to promote phagocytosis of Gram-positive and Gram-negative bacteria, respectively and is required for LPS-induced TLR4 signaling (157). Although, in some cases SR-A1 may also play a detrimental role in the pathophysiology of LPS-induced sepsis caused due to an exaggerated inflammatory response (173). Nonetheless, SR-A6 shares with SR-A1 the ability to clear bacterial infections, albeit by recognizing overlapping but distinct microbial ligands. Such subtle differences in ligand specificity indicate an evolutionary benefit by increasing the repertoire of innate immune recognition (157).

However, the evidence for the physiological role that SR-A1 plays during viral infections is inconsistent. SR-A1 mediates uptake of extracellular viral nucleic acids like dsRNA and presents them to endosomal TLR3/9, thereby triggering the production of IFN-I in bystander uninfected cells to restrict viral spread (204, 205). Haisma *et al.* (2009) demonstrated that adenovirus type 5 was internalized via SR-A1 and degraded by macrophages (206). SR-A1$^{-/-}$ mice also have significantly lower survival than wildtype mice following a lethal challenge with HSV-1 (207). Similarly, SR-AI expression on liver macrophages including tissue resident Kupffer cells and monocyte-derived macrophages, has shown to promote recovery from adenovirus type 5-induced hepatic inflammation and fibrosis by mediating a switch to a pro-resolving and anti-inflammatory M2 polarization state (208). In contrast, SR-A1 promotes the pathogenesis of murine hepatitis virus-induced fulminant hepatitis (FH) by enhancing induction of neutrophil-mediated complement activation (209). SR-A1 has also been shown to impair the production of IFN-I in response to hepatitis B virus (HBV) by limiting activation of TRAF3 (210). More recently however, SR-A1 has been shown to restrict the spread of chikungunya virus (CHIKV) by mediating autophagy of the infected cells via interaction with core autophagy complex ATG5-ATG12 and this interaction is enhanced by CHIKV nsP1 protein (211). The contradictory observations above suggest that the physiological functions of SR-A1 during viral infections vary with viral species and disease models (211). However, the molecular mechanisms underlying SR-A1's antiviral behaviour remain elusive.

It is now abundantly clear that SR-A1 plays a dichotomous role in modulating immune responses to microbial pathogens. On one hand, SR-A1 can form a signaling complex with TLR4 to mediate LPS-induced inflammatory response (212) but on the other, it scavenges TLR4 ligands from the cell surface to limit its activation (213). Similarly, SR-A1 interacts with TRAF3 or ATG12, resulting in a pro-viral and an antiviral outcome, respectively (210, 211). Perhaps an explanation for the dual role of SR-A1 might be the various ways in which SR-A1 can influence innate immune responses such as, **(a)** endocytosis of microbial PAMPs or autoantigens to either present it to the intracellular PRRs or scavenge it away to limit the activation of corresponding signaling pathways, **(b)** activation of signaling cascades following ligand binding and, **(c)** interactions with proteins involved in innate immune signaling. It is also important to note that SR-A1-mediated immune modulation has mostly been studied in the context of murine and human macrophage cells since SR-A1 expression was thought to be restricted to myeloid cells such as DCs and macrophages (173). Macrophages from various organs and circulating blood monocytes are different from each other and their microenvironment plays a key role in shaping their responses to different stimuli (214). Besides, it is now appreciated that SR-A1 is more widely expressed (173) and mechanisms of innate immune modulation via SR-A1 may indeed vary between different cell types. Further studies are required to explain the complex interactions of SR-A1 with its ligands and activation of a host of both pro-survival and pro-death cell signaling pathways.

1.6. Extracellular dsRNA, SR-A1 and the cytosolic RLRs

As discussed in section 1.4, it is evident that viral dsRNA can behave as an efficient extracellular signaling molecule and participates in the immune sequelae associated with infection and autoimmunity (16). Also, our understanding of the host innate immune signaling pathways which curb viral infections comes largely from the use of poly (I:C) as a viral mimetic in diverse experimental models (16). Several cell surface receptors like SR-As, CD14, SR-J1 and Macrophage-1 Ag (Mac-1) have been reported to participate in extracellular nucleic acid uptake, including dsRNA (156). But these receptors often contribute only partially to extracellular dsRNA-mediated responses likely because, **(a)** these studies have been performed in different species and do not account for potential evolutionary differences between species, **(b)** these studies are often restricted to specific cell types such as macrophages and DCs which typically express several receptors with 'scavenger' activity or whose properties have not been completely characterized that can compensate for other dsRNA receptors and **(c)** multiple mechanisms might participate in dsRNA internalization. Thus, the precise mechanisms underlying extracellular dsRNA uptake and its intracellular fate remain largely elusive.

It is abundantly clear that SR-A1 can modulate innate immune responses upon ligand binding by either activating or inhibiting intracellular signaling pathways (173). The SR-As mediate extracellular dsRNA entry via clathrin-mediated endocytosis, delivering dsRNA to the intracellular dsRNA-sensors. The loss of SR-A1, both *in vitro* and *in vivo*, significantly lowers the levels of extracellular dsRNA-induced

proinflammatory cytokines (179) and IFN-I responses (19). Although SR-A1 has been previously shown to induce proinflammatory responses to poly (I:C) via activation of MAPK and PKC pathways (179, 196), whether it can modulate IFN-I responses is still an enigma. Our lab has previously demonstrated that SR-As lack the signaling ability to trigger IFN-I production in response to extracellular poly (I:C) independent of the canonical dsRNA-sensing pathways (215). The above findings raise a central question: Whether SR-A1 functions mainly as a 'carrier' to deliver dsRNA to intracellular sensors or can SR-A1 modulate extracellular dsRNA-induced cellular pathways including IFN-I responses in one or more ways?

Importantly, while SR-A1 is preferentially expressed on macrophages, its expression has been described in several cell types including vascular smooth muscle cells, endothelial cells, human lung epithelial cells, microglia, astrocytes, and murine embryonic fibroblasts (MEFs) (173). SR-A1 expression is also inducible following stimulation with TNFα and IL-6 (173, 205). Therefore, the ubiquitous expression of SR-A1 broadens the scope of its pathophysiological importance in mediating systemic effects of nuclease-resistant, circulating viral dsRNA.

Extracellular dsRNA also activates the RLR-sensing pathway within the cytoplasm both *in vitro* and *in vivo* (19). Indeed, it is the cytoplasmic dsRNA pathway that is responsible for much of IFN-I responses upon systemic administration of poly (I:C) in mice (216) which implies the existence of a mechanism by which extracellular

dsRNA enters the cytoplasm. Such a mechanism may involve a previously undetermined function of SR-A1 or other specialized protein/s either at the cell surface or within endosomes. As for SR-A1, the current understanding is that it can endocytose extracellular dsRNA (19) but whether it can mediate transport of dsRNA into the cytoplasm remains unclear. Alternatively, SIDT1 and SIDT2, the mammalian orthologs of the *Caenorhabditis elegans (C. elegans)* SID-1 dsRNA transporter are two potential candidates.

RNA interference (RNAi) is an antiviral defense mechanism prominent in non-mammalian systems and entails the systemic spread of dsRNA molecules from the site of infection. In *C. elegans*, this spread requires SID-1, a broadly expressed transmembrane protein that mediates the import of dsRNA into the cytoplasm of the cells (217, 218). Orthologs of SID-1 are present in most animals with mammals encoding two closely related paralogs, SIDT1 and SIDT2, suggesting that the mammals may have evolved additional specialized RNA transport functions (155). *Sidt2* is more broadly and abundantly expressed than *Sidt1* in humans and mice (219). More importantly, expression of both *Sidt1* and *Sidt2* is stimulated by IFN-I/ II to different degrees (220) and thus, may potentially play a role in the antiviral response to viral infections. Indeed, SIDT2 has recently been implicated in the transport of extracellular dsRNA from endosomes to the cytoplasm for innate immune recognition and subsequent induction of bystander immunity during HSV-1 infection in MEFs (221). SIDT1 appears to share dsRNA transport capability with SIDT2, although SIDT1 is not essential for viral clearance

following infection *in vivo*, suggesting that its dsRNA transport activity is likely to be functionally redundant in the presence of SIDT2 (222). It is important to note that the current literature about the physiological role of SIDT1 and SIDT2 in viral infections is limited and remains controversial regarding their cellular localization, dsRNA specificity and whether they are functionally redundant (221, 222).

1.7. Thesis objectives and discovery

Viral infections remain a considerable health threat today. Despite rapid advances in the development of antiviral drugs for some viruses such as HIV and HCV, the lack of antiviral drugs for numerous clinically important viral pathogens is alarming. The continued emergence of new diseases such as swine flu and COVID-19, along with the resurgence of existing diseases, including West Nile, Ebola hemorrhagic fevers and the occurrence of drug-resistant variants, highlights the urgent need for the development of novel strategies for more effective vaccines and antivirals to combat viruses and mitigate human disease (75). Therefore, more must be learned about the mechanisms of host-virus interactions, including evasion strategies of viruses that permit them to evade host immune responses in infected cells as well as the counter strategies exerted by host immune system which shall enable us to identify novel targets and/ or strategies for developing antiviral therapeutics (12, 75).

Bystander immunity is a counter strategy adopted by the host immune system to clear viruses that are adept at antagonizing antiviral response and evading detection through cellular PRRs in the infected cell. Bystander immunity is quite simply the transfer of 'danger signals', including viral nucleic acids, from infected cells to the neighbouring cells. While there are a few regulated mechanisms to activate bystander immunity through the transfer of danger signals via exosomes or gap junctions between cells, virus-induced cell lysis is an uncontrolled event and abruptly releases host and virus specific danger signals (90). Viral dsRNA in the extracellular space is extremely stable against nuclease digestion and can induce the production of protective IFN-I and proinflammatory cytokines in surrounding cells to limit the spread of the virus. Extracellular dsRNA can also have unnecessary pathophysiological effects through unregulated production of potent cytokines such as IFN-I leading to inflammatory disorders (16, 122). Therefore, it is important to understand the molecular mechanisms of extracellular dsRNA-mediated signaling to be able to enhance its antiviral capabilities while limiting its deleterious effects on human health.

The central objective of my doctoral research was to dive deeper into the signaling mechanisms of cell-free viral dsRNA which entailed a better understanding of its recognition, uptake at the cell surface and intracellular fate. Our knowledge of the cell surface receptors like the SR-As and the intracellular sensors involved in response to extracellular dsRNA has grown substantially since the early 2000's. However due to the continued discovery of new scavenger receptors which showcase overlapping ligand

specificity and whose physiological roles remain poorly characterized, the mechanisms that dictate SR-A1 functions in dsRNA binding and modulation of antiviral responses to extracellular dsRNA remain controversial. Furthermore, the requirement of cytoplasmic RLRs add another layer of complexity in understanding the signaling mechanisms of extracellular dsRNA. Therefore, to broaden our understanding of underlying mechanisms of extracellular dsRNA signaling and, in many ways, validate and expand on the role of SR-A1 considering new discoveries in the scavenger receptor field continue to question published literature led us to two central hypotheses:

1. As SR-A1 can influence intracellular signaling pathways besides its function as a 'carrier' for a plethora of extracellular ligands, we hypothesized that, ***"SR-A1 plays a key role in modulating antiviral responses to extracellular dsRNA independent of other cell surface nucleic acid receptors, including SR-A family members.***"

2. We hypothesized that, "***the activation of cytoplasmic RLRs is mediated through the transport of extracellular dsRNA via a specialized protein either at the cell surface or within the endo-lysosomal membranes.***"

With the help of my colleagues, mentors, and collaborators, the work during my doctoral research has addressed some gaps in the field of extracellular viral nucleic acid signaling, including that of viral dsRNA. The key findings of my research are the *in vitro* characterization of the nucleic acid binding domain of SR-A1 and a clear illustration of the necessity of SR-A1 in response to extracellular dsRNA stimulation in a cell culture-based model. I have also generated preliminary evidence to support the role of SIDT2 as a putative dsRNA channel in response to extracellular dsRNA in human cells although further work is warranted to validate my observations. The biggest achievement throughout my doctoral research has been the undertaking of a challenging project to identify the host of intracellular pathways that become activated via SR-A1 following extracellular dsRNA uptake. The project included a newly discovered experimental technique and presented us constantly with several expected and unexpected challenges but ultimately it has helped me grow professionally and personally. I strongly believe that my work takes us a step forward in identifying novel strategies to develop antiviral therapeutics and vaccines against viral infections.

CHAPTER 2: Materials and methods

2.1. Cells and materials

Human embryonic lung fibroblasts (HEL; ATCC® CCL-137™) and 293T (ATCC® CRL-3216™) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Carlsbad, CA, USA) supplemented with 10% FBS (Gibco; Gaithersburg, MD, USA) and 2 mmol L^{-1} L-glutamine (Gibco). A549 lung epithelial cells (ATCC® CCL-185™) were maintained in α-modified Eagle's medium (αMEM; Invitrogen) supplemented with 10% FBS and 2 mmol L^{-1} L-glutamine. Cells were incubated at 37°C in a humidified 5% CO2 incubator. Fucoidin and fetuin were purchased from Sigma (Oakville, ON, Canada). Trypan blue was purchased from Gibco. Oligomers were purchased from Sigma-Aldrich and Integrated DNA technologies (Coralville, IA, USA). Lipofectamine 3000 transfection reagent was purchased from Invitrogen Life technologies (Catalog number: L3000001). Puromycin dihydrochloride (Catalog number: A1113803) and blasticidin S (Catalog number: A1113903) was purchased from Thermo Scientific. The anti-human SR-A1 antibody was kindly provided by Dawn Bowdish, McMaster University. Poly I:C was purchased from GE Healthcare. Green fluorescent protein (GFP) expressing VSV (VSV-GFP; kindly provided by Brian Lichty, McMaster University) was propagated on Vero cells (ATCC® CCL-81TM). Doxycycline hyclate (Catalog number: D9891) and biotin (Catalog number: B4501) were purchased from Sigma. Real-time quantitative PCR TaqMan probes for human ISG56,

SR-J1, DEC-205 and GAPDH were purchased from Applied Biosystems. Construct with SR-J1 cDNA sequence was kindly provided by Eicke Latz, University of Bonn.

2.2. Nucleic acid synthesis

DNA of different lengths and sequences were synthesized by PCR amplifying regions of the cloned West Nile virus (WNv) envelope (E) gene using primers as described in Table II. 1µg of PCR fragments were used as template to synthesize RNA. DsRNA and ssRNA were synthesized by *in vitro* transcription using the T7 RiboMAX™ Express RNAi System (Promega; Catalog number: P1700). The primers contained a T7 sequence tag used by the T7 polymerase during RNA synthesis. The average length for the poly(I:C) was ~4000 bp as determined by marker size comparison using agarose gel electrophoresis and a 1kb Plus DNA ladder.

Table II. Primers used for generating *in vitro* transcribed RNA and DNA species of different lengths using cloned Envelop gene of WNv as template.

Length	Primer sequences*
200	F: TCCTCCAACTGCGAGAAACGTG R: AAAGGAGCGCAGAGACTAGCCG
300	F: GCATTGGTGTCAATCCCTGACC R: ACACATGCGCCAAATTTGCC
500	F: TCCTCCAACTGCGAGAAACGTG R: TGGCACGGATGGACCTTG
600	F: GTACTGCAATTCCAACACCACAG R: ACACATGCGCCAAATTTGCC
1100	F: TCCTCCAACTGCGAGAAACGTG R: ACACATGCGCCAAATTTGCC

*Primers included a T7 sequence tag (5' taatacgactcactataggg 3') used by the T7 polymerase during RNA synthesis; F, forward primer; R, reverse primer.

2.3. Nucleic acid uptake assay

A quantity of 1µg 1100 bp dsRNA and DNA, each similar in sequence was purified and labeled using a Ulysis^{TM} Alexafluor 488 Nucleic Acid Labeling Kit (Invitrogen; Catalog number: U21650). Excess labeling reagent was removed using Micro Bio-Spin P-30 columns (Bio-Rad; Catalog number: 7326250). 1×10^4 cells/well were seeded into 96-well plates and treated the next day with Alexafluor 488 labeled dsRNA or DNA. 30 mins post treatment, total fluorescence was measured using a fluorescence plate reader (SpectraMax i3). Following another 30 mins of incubation, unbound nucleic acid was removed, and cell associated (bound and internalized) fluorescence was measured. Subsequently, cells were washed with PBS, and 0.025% trypan blue was added to quench extracellular, surface-bound fluorescence to measure only intracellular fluorescence. Results were reported as a percentage of total fluorescence.

2.4. Live cell fluorescence microscopy

HEL cells were seeded into 12-well plates at a concentration of 1×10^5 cells/well. The cells were treated with Alexafluor 488 labeled 1100 bp dsRNA or DNA. Following incubation, unbound nucleic acid was washed away. Cell associated (surface-bound and intracellular) nucleic acids were directly observed under the microscope while internalized nucleic acid was observed upon washing the cells with PBS and adding 0.025% trypan blue to quench extracellular, surface-bound fluorescence. All images were

captured using a Leica DM-IRE2 inverted microscope and analyzed using Openlab software (Improvision).

2.5. Reverse transcription PCR (RT-PCR)

Total RNA was harvested from cells using TRIzol reagent (Thermo Scientific: Catalog number: 15596026). A quantity of 1µg of total RNA was DNase treated to remove contaminating genomic DNA and subjected to cDNA synthesis using iScript™ gDNA Clear cDNA synthesis kit (Bio-Rad; Catalog number: 1725035). 2 µl of resulting cDNA was used as template for subsequent PCR reactions using each primer set (Table III) and 1U of Q5® High-Fidelity DNA Polymerase (New England Biolabs; Catalog number: M0491). The PCR products were visualized on a 1.0% agarose gel followed by sanger sequencing at the MOBIX facility (McMaster University) to confirm the identity of the PCR product.

Table III: Primer sequences used for amplifying gene transcripts by RT-PCR.

Gene name	Accession #	Primer sequences
SR-A1	NM_138715	F: GACATGGAAGCCAACCTCAT R: CCAAGCTCCTACAGACGACC
SR-A1.1	NM_002445	F: TCGAGGACTCCCAGGATATG R: GGCAGAGAACTGAGGACTGG
SR-A6	NM_006770	F: CAACAAGCTGCTTTTCACCA R: ACATCCCTGGGTTCTGAGTG
SR-A3	NM_016240	F: ACGAGATTGAAATTGGCACC R: CCCTCATTGGAATCAGAGGA
SR-A3.1	NM_182826	F: TGCAGCTGGATAACATCTCG R: CTTGGTCATCCTGGGCTTTA
SR-A4	NM_130386	F: CTGCGGACGCTGACCAGCAA R: GTGAGGCGGGCAGCCATTGT
SR-A5	NM_173833	F: CTCTTGAACATGTGCTCCGA R: TCACTTGACGTTGCCTCTTG
SR-J1	NM_001136	F: CAATGAACAGGAATGGAAAG R: TCCTCTTCCTCCTGGTTTT
SIDT2	NM_001040455	F: CCCTTCTTGGTGCTCTTGGT R: CGGAGCTGGTATGTGGTGTT

2.6. Generation and validation of CRISPR knockout cells

A549 SR-A3-SR-A5 double knockout cells (A549 ΔSRA) were generated by sequentially knocking out SR-A3 and SR-A5 in parental A549 cells. To generate guide RNA (sgRNA) targeting *SR-A3* and *SR-A5*, exon sequence of each of the genes were submitted to an online software (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design; see Table IV for gRNA sequences). The sgRNA hits were screened for high on-target and low off-target specificity. One sgRNA sequence for each *SR-A3* and *SR-A5* were chosen for plasmid construction. pSpCas9(BB)-2A-Puro (PX459), a gift from Feng Zhang (Addgene plasmid # 48139) was used to express Cas9 and gene specific sgRNA. The pSpCas9(BB)-2A-Puro SR-A3 plasmid was used as a vector as previously

published (223). Parental A549 cells were seeded in 6-well plates at a concentration of 8×10^5 cells/well and transfected with 2.5 µg of plasmid using Lipofectamine 3000 as per manufacturer's recommendation. 24 hours after transfection, cells were selected with 1 µg mL^{-1} puromycin for 2-3 days. Selected clones were collected and seeded in 96-well plates at a concentration of approximately 3 cells/well. One week later, single colonies of cells were selected and cultured over time. To characterize A549 SR-A3 knockout cells, we were unable to validate the knockout of SR-A3 by immunoblots since commercially available antibodies did not provide reliable results. To overcome this limitation, we performed mutagenesis analysis to demonstrate the loss of SR-A3. Briefly, the genomic DNA from selected clonal cell populations was extracted using DNeasy Blood & Tissue Kit (Qiagen; Catalog number: 69504) and genetic modifications at or around sgRNA binding site were verified by PCR, followed by sanger sequencing as previously described (224) (see Table V). The clone containing insertions/deletions (indels) at the SR-A3 genomic locus resulting in a premature 'stop' codon was further used to generate SR-A5 knockout using same strategy (pSpCas9(BB)-2A-Puro SR-A5) as above to generate A549 ΔSRA cells.

A549 SIDT2 knockout cells were generated by knocking out SIDT2 in A549 ΔSRA cells that were engineered for inducible expression of SR-A1 (ΔSRA +SR-A1). To generate sgRNA targeting SIDT2, exon sequence of *SIDT2* of the genes were submitted to an online software (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design; see Table IV for sgRNA sequences). The sgRNA hits were screened for high on-

target and low off-target specificity. Two sgRNA sequences were chosen for plasmid construction. lentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid # 52961). The puromycin antibiotic resistance cassette was replaced with a blasticidin antibiotic resistance cassette by a colleague in the Mossman lab (personal communication). Lentivirus transduction was used to express Cas9 and sgRNA targeting SIDT2 in ΔSRA +SR-A1 cells. Briefly, transfer vector (lentiCRISPR v2 blast SIDT2 sgRNA #1/ #2) containing SIDT2 sgRNA and Cas9, and packaging plasmids psPAX2 (Addgene) and pMD2.G (Addgene) were transfected into 293T cells using Lipofectamine 3000. 6 h post transfection, the transfection mixture was replaced with complete media containing 1% BSA. 60 h post transfection, the supernatant was harvested, and virus was concentrated by ultracentrifugation. ΔSRA +SR-A1 cells were infected with the lentivirus in complete media containing 0.1% polybrene. The following day, cells were passaged and seeded in 10 cm dishes in complete media containing 20 μg ml^{-1} blasticidin. Following selection, clones were collected and seeded in 96-well plates at a concentration of approximately 0.5-1 cells/well. 10 days later, single cell clones were selected and cultured over time. To characterize SIDT2 knockout cells, we were unable to validate the knockout of SIDT2 by immunoblots since commercially available antibodies did not reliably detect human SIDT2. To overcome this limitation, we performed mutagenesis analysis to demonstrate the loss of SIDT2 in ΔSRA +SR-A1 cells. Briefly, the genomic DNA from selected clonal cell populations was extracted using DNeasy Blood & Tissue Kit (Qiagen; Catalog number: 69504) and genetic modifications at or around sgRNA binding site were verified by PCR, followed by sanger sequencing as previously described (224) (see Table V).

Two clones, CR1.1 and CR2.2 containing insertions/deletions (indels) at the SIDT2 genomic locus resulting in a premature 'stop' codon were used for subsequent studies.

Table IV: Primer sequences for sgRNA constructs to generate CRISPR knockout cell lines.

Target Gene	Target construct	Primers
SR-A3	pSpCas9(BB)-2A-Puro SR-A3	F: caccgCAAGGCATCTCCATCGCCGC R: aaacGCGGCGATGGAGATGCCTTGc
SR-A5	pSpCas9(BB)-2A-Puro SR-A5	F: caccgCACAGTCGCTGACGGTGTGT R: aaacACACACCGTCAGCGACTGTGc
SIDT2	lentiCRISPR v2 blast SIDT2 sgRNA#1	F: caccgCGAGTTTGAGCGCACCTACG R: aaacCGTAGGTGCGCTCAAACTCGc
SIDT2	lentiCRISPR v2 blast SIDT2 sgRNA#2	F: caccgCCTCGGTCGAGAGCCATCTG R: aaacCAGATGGCTCTCGACCGAGGc

Note: The sequences in UPPERCASE correspond to the gene specific sgRNA sequence

Table V: Primer sequences to verify indels in CRISPR edited KO cells.

Cell line	Target gene	Primers
A549 ΔSR-A3	SR-A3	F: ATGTGTCTTTCCCAGCGTGT R: CCGTGAACTCTGCCCTTTCA
A549 ΔSRA	SR-A5	F: GCAAGTGGACTCTGTACCCC R: TCCTCACCTGAGAGGGTCTG
ΔSRA +SR-A1 (CR1.1 and CR2.2)	SIDT2	F: GCTCTTGGGAGGGGACATTTC R: CAGTAATAAGCCTGTTTCCTC

2.7. Virus infection and quantification

2 x 10^5 cells/well were seeded in 12-well plates and incubated overnight at 37°C with 5% CO2. Following mock or poly (I:C) stimulation, cells were either mock infected or infected with VSV-GFP (virus engineered to express GFP during viral replication) in serum-free media at a multiplicity of infection (MOI) of 0.1 PFU/cell. The duration of poly (I:C) treatments are specified in the figure legends. Infected cells were incubated at

37°C for 1 h with gentle rocking every 15 mins. After 1 h, virus inoculum was aspirated and minimum essential medium with Earle's salts (Sigma) containing 2% FBS and 1% carboxymethyl cellulose (CMC; Sigma) was added on the cells. The plates were incubated for 19 h at 37°C and GFP levels were measured using a Typhoon Trio scanner (GE Healthcare). The GFP intensity was quantified using Image Quant TL software.

2.8. Generation and validation of cells engineered with tetracycline-inducible expression

SR-A1 expressing cells were generated by introducing a tetracycline-ON system in A549 ΔSRA cells (ΔSRA +SR-A1). The vector construction, transfection, single cell screening and validation were done as previously described (215). Briefly, the coding sequence of SR-A1 was PCR amplified from pcDNA3.1-SR-AI (a generous gift from Dawn Bowdish) and cloned into the piggyBac (pB) vector (pB-TET) using pDONR221 as an intermediate to yield pB-TET-SR-AI (harbours a tetracycline response element upstream of *SR-A1*) using the standard Gateway recombination protocol by Life Technologies (see Table VI for primers). The reverse tetracycline-controlled transactivator (rtTA) was expressed by pB-CAG-rtTA. Both, the pB-TET and pB-CAG-rtTA vectors were graciously provided by Dr Jonathan Draper. The transposase vector pCyL43 PBase was obtained from Sanger (http://www.sanger.ac.uk/technology). A549 ΔSRA cells were seeded in 6-well plates at a concentration of 8×10^5 cells/well and co-transfected with 2.5 μg of a mixture of pB-TET-SR-AI, pB-CAG-rtTA and pCyL43 plasmids in a 10:5:2 ratio using Lipofectamine 3000 as per manufacturer's recommendation. 24 hours after transfection, cells were selected

with 1 µg mL^{-1} puromycin for 2-3 days. Selected clones were collected and seeded in 96-well plates at a concentration of approximately 3 cells/well. One week later, single colonies of cells were selected and cultured over time. To characterize ΔSRA +SR-A1 clones, 1×10^5 cells/well were seeded in a 12-well dish in duplicates. Following mock or 1 µg ml^{-1} doxycycline treatment for 24 h, cells were harvested and subjected to immunoblot analysis for SR-A1 expression.

Cells expressing biotin ligase (BirA) and SR-A1 fusion protein were generated by introducing a tetracycline-ON system in parental A549 cells (A549 BioID). First, two BioID vectors were generated with BirA ligated to either N-terminus or C-terminus of SR-A1 for performing expression and functional analysis using transient transfection. The coding sequence of SR-A1 was cloned into pcDNA3.1 mycBioID (Addgene; Catalog number: 35700; N-BirA*) and pcDNA3.1 MCS-BirA(R118G)-HA (Addgene; Catalog number: 36047; C-BirA*) using EcoRI and BamH1 restriction sites, resulting in pcDNA3.1-N-BioID and pcDNA3.1-C-BioID, respectively (see Table VI). pcDNA3.1-N-BioID was chosen for subsequent studies and the complete coding sequence of N-BioID was sub-cloned into pB-TET using Gateway recombination protocol to yield pB-TET-N-BioID vector (see Table VI). Parental A549 cells were seeded in 6-well plates at a concentration of 8×10^5 cells/well and co-transfected with 2.5µg of a mixture of N-BioID, pB-CAG-rtTA and pCyL43 plasmids in a 10:5:2 ratio using Lipofectamine 3000 as per manufacturer's recommendation. 24 hours after transfection, cells were selected with 1 µg mL^{-1} puromycin for 2-3 days. Selected clones were collected and seeded in 96-well

plates at a concentration of approximately 3 cells/well. One week later, single colonies of cells were selected and cultured over time. To characterize A549 BioID clones, 1×10^5 cells/well were seeded in a 12-well dish in duplicates. Following mock or 1 µg ml^{-1} doxycycline treatment for 24 h, cells were harvested and subjected to immunoblot analysis for BirA-SR-A1 expression.

Table VI: Primer sequences to generate tetracycline inducible vectors.

Target plasmid	Primers
pB-TET-SR-A1	F: [1]ggggacaagtttgtacaaaaaagcaggctcaccATGGAGCAGTGGGATCACTTT R:[2]ggggaccactttgtacaagaaagctgggtTTACTTATCGTCGTCATCCTTGTAATCT AAAGTGCAAGTGACTCCAGC
pcDNA3.1-N-BioID	F: aagaattcATGGAGCAGTGGGATCACTTT R: aaggatccTAAAGTGCAAGTGACTCCAGCA
pcDNA3.1-C-BioID	F: aagaattcATGGAGCAGTGGGATCACTTT R: aaggatccTTATAAAGTGCAAGTGACTCCAGCA
pB-TET-N-BioID	F: [1]ggggacaagtttgtacaaaaaagcaggctcaccATGGACAAGGACAACACCGTGC R:[2]ggggaccactttgtacaagaaagctgggtTTACTTATCGTCGTCATCCTTGTAATCT AAAGTGCAAGTGACTCCAGC

Note: The sequences in UPPERCASE correspond to the gene specific sequence.
1- attB1 sequence; 2- attB2 sequence, these sequences facilitate recombination into Gateway vectors.

2.9. Preparation of protein lysates

For whole cell extracts, cells were washed twice with ice-cold PBS and scraped into radioimmunoprecipitation assay (RIPA) buffer [10 mmol L^{-1} Tris-HCl (pH 7.2), 150 mmol L^{-1} NaCl, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mmol L^{-1} EDTA, and 3X Halt™ Protease Inhibitor Cocktail (Thermo Scientific; Catalog number: 78430)]. Lysates were incubated on ice for 10 min, passed through a 22-gauge needle, and

centrifuged at 13000xg for 15 min at 4°C. Extracts were quantified using a Bradford assay (Bio-Rad).

2.10. Immunoblots

Cells were seeded at a concentration of 2×10^5 cells/well in 12-well plates. The cells were harvested following transfection or treatment with doxycycline as indicated in the figure legends. Samples were denatured in a reducing sample buffer and analyzed on a reducing gel. Proteins were blotted from the gel onto polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore) and detected using primary and secondary antibodies. Primary antibodies used were: 1:10000 mouse anti-GAPDH (EMD Millipore; Catalogue number: AB2302), 1:2000. mouse anti-SR-A1. Secondary antibodies used were: 1:5000 donkey anti-rabbit 800 (LI-COR Biosciences; Catalogue number: 926-32213) and 1:5000 goat anti-mouse 680 (LI-COR Biosciences; Catalogue number: 925-68070). Blots were observed and imaged using Image Studio (LI-COR Biosciences) on the Odyssey CLx imaging system (LI-COR Biosciences).

2.11. Flow cytometry analysis of SR-A1 expression

SR-AI expression was induced using different concentrations of doxycycline for 24 h. The cells were then harvested. 5×10^5 cells were counted for each treatment and stained for SR-AI expression using anti-SR-AI antibody at 1:200 dilution, followed by Alexafluor 488 conjugated anti-mouse secondary antibody (Invitrogen) at 1:400 dilution.

Cells were gated based on their light scatter properties and expression of SR-AI, while gating out cell debris. A low forward scatter (FSC) and increased granularity, as denoted by a high side scatter (SSC) were hallmarks of dying/dead cells. Thus, optimal SR-AI expression was defined as FSC high and increased cellular toxicity was defined as FSC low. Staining was performed at RT for 30 mins. Flow cytometry was conducted on a BD LSRII cytometer (BD Bioscience) and the data was analyzed using FlowJo vX software (FlowJo, LLC, Ashland, OR).

2.12. Ligand internalization assay

Alexafluor 488 labeled acLDL was purchased from Thermo Scientific (Catalog number: L23380) and Alexafluor 488 labeled 1100bp dsRNA was generated as mentioned above. 2×10^4 cells/well were seeded on glass coverslips in 12-well plates and treated with fluorescently labeled 1100bp dsRNA (1 µg/mL) or acLDL (2.5 µg/mL). Following 1 h incubation, cells were fixed with 4% paraformaldehyde and blocked with blocking solution (3% goat serum, 3% BSA, and 0.02% Tween 20). Cells were stained for SR-A1 with 1:200 primary anti-SR-A1 antibody followed by 1:400 anti-mouse Alexafluor 594 secondary antibody (Invitrogen). Nuclei were stained with Hoechst 33258 at 1:10000 dilution (Sigma; Catalog number: 94403). Images were captured using a Leica DM-IRE2 inverted microscope and analyzed using Openlab software (Improvision). All antibody dilutions were performed in blocking solution.

2.13. Real time quantitative RT-PCR (qRT-PCR)

Total RNA was harvested from treated cells using TRIzol reagent. A quantity of 1µg of total RNA was DNase treated to remove contaminating genomic DNA and subjected to cDNA synthesis using iScript^{TM} gDNA Clear cDNA synthesis kit. Resulting cDNA was diluted 1:10 times prior to performing qRT-PCR. The qRT-PCR was performed with TaqMan^{TM} Universal PCR Master Mix and gene-specific oligomers (Applied Biosystems) in a total reaction volume of 25 µL using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). GAPDH was used for normalization of cDNA input. Data analysis was done using ΔΔCt method and gene expression was expressed as fold-change over the control group. The experiments were repeated at least three times.

2.14. Production of recombinant collagenous domain of SR-A1

A portion of human SR-AI (residues 110 to 341) that includes α-helical coiled-coil and collagenous domains was sub-cloned in a modified pET15b vector including removable hexa-histidine and SUMO tags. The integrity of the resulting construct was confirmed by DNA sequencing (MOBIX, McMaster University). The plasmid encoding collagenous domain was transformed into *E.coli* BL21 Star Rosetta 2 (DE3) cells (Life Technologies). Bacterial cell cultures were grown at 37^{o}C to mid-exponential phase, and protein expression was induced by addition of 0.5mM isopropyl β-D-1-thiogalactopyranoside. Cells were incubated for 5 h at 25^{o}C with orbital agitation

and harvested by centrifugation at 3300 g for 15 minutes. Cell pellets were washed in phosphate buffer saline and resuspended in lysis buffer (20 mM Tris pH 7.5, 0.5 mM PMSF, 5% glycerol, 100 mM NaCl, 5 mM DTT and 10 mM EDTA) containing protease inhibitors (1 mM PMSF, 5 µg mL^{-1} leupeptin, 0.7 µg mL^{-1} pepstatin A and 1 mM benzamidine) and 0.03% LDAO. Cells were lysed by sonication and the lysates clarified by centrifugation at 39000 g for 40 minutes. The collagenous domain was then precipitated with 35% (w/v) ammonium sulphate over 3 hours at 4^{o}C. The pellet was resuspended in 10 mL of lysis buffer and dialyzed overnight at 4^{o}C. The protein was further purified over a MonoS (5/50) column (GE Healthcare) equilibrated with 20 mM Tris pH 7.0, 0.5 mM EDTA, 5 mM DTT, 0.5 mM PMSF, 5% glycerol and 150 mM NaCl and eluted with a linear gradient to 500 mM NaCl. The collagenous domain was eluted at approximately 300 mM NaCl. Purified protein was concentrated and stored in 50 mM Tris pH 7.4, 0.1 mM EDTA, 0.1 mM DTT and 150 mM KCl. The protocol for producing recombinant SRCR domain from SR-A6 has been previously described (172).

2.15. Electrophoretic Mobility Shift Assay

Aliquots of DNA, ssRNA and dsRNA fragments of corresponding sequence and length were incubated with purified proteins (coiled-coil collagenous domain or SRCR domain) in binding buffer (50 mM Tris, pH 7.4, 0.1 mM EDTA, 0.1 mM DTT, 150 mM KCl) in a final reaction volume of 20 µl. The reaction mix was incubated for 30 minutes at 4^{o}C without agitation. The reaction products were then mixed with gel loading buffer (10 mM HEPES, 1 mM EDTA, 50% (v/v) glycerol, 0.1% bromophenol blue and 0.1%

xylene cyanol) and fractionated on 1% or 2% (w/v) agarose gels at 4^{o}C. The reaction products were visualised on the agarose gel using ethidium bromide staining. For competition assay, nucleic acids (DNA, ssRNA and dsRNA) were labeled with Ulysis^{TM} Alexafluor 488 using nucleic acid labeling kit. Excess labeling reagent was removed using Micro Biospin P-30 columns. The labeled nucleic acids were then used in the binding reactions with purified proteins as described above. The reactions were resolved on 1% (w/v) agarose gel at 4^{o}C and detected using Typhoon Trio.

2.16. Whole bacteria binding to recombinant SRCR and coiled-coil collagenous domain

Streptococcus pneumoniae (S. pneumoniae) was grown and prepared as described previously. Bacteria (1.25 x 10^{8}) were resuspended in a folding buffer (50 mmol L^{-1} Tris pH 8.0, 800 mmol L^{-1} L-arginine and 10 mmol L^{-1} β-mercaptoethanol) and incubated with 40 µg recombinant SRCR or coiled-coil collagenous domain for 2 h at room temperature (RT). Bacteria were then washed twice with PBS and stained using an anti-His antibody (Abm Goods) at 1:200 dilution in FACS buffer (0.5% BSA, 2 mmol L^{-1} EDTA in PBS) for 30 min at RT. Cells were washed once with FACS buffer and stained with Alexafluor 633 goat anti- mouse IgG (Invitrogen) at 1:1000 dilution for 30 min at RT in a dark room. Cells were washed twice with PBS and analyzed on a LSR II flow cytometer (BD Biosciences). Data were gathered using FACSDiva software (BD Biosciences) and analyzed using FlowJo version 7.6.2 software (TreeStar, Ashland, OR USA). To ensure the population being analyzed was *S. pneumoniae*, bacteria were stained for 30 min with

1 µM CFSE at RT in a dark room. Bacteria were then washed twice with PBS. Following observation of unstained bacteria by the forward and side-scatter analysis, a shift in CFSE-fluorescence confirmed the population being observed was *S. pneumoniae*. To ensure no cross-reactive binding between secondary antibodies and our recombinant proteins or the bacteria, samples were probed with recombinant SRCR or collagenous constructs and secondary antibody alone. A negligible (< 1%) amount of nonspecific shift in fluorescence was observed.

2.17. Surface staining for SR-A1

Parental A549 cells were seeded on glass coverslips at a concentration of 2×10^4 cells/well in 12-well plates and transiently transfected with specified expression construct for 24 h. Subsequently, cells were fixed with 4% paraformaldehyde and blocked with blocking solution (3% goat serum, 3% BSA, and 0.02% Tween 20). Cells were stained for SR-A1 with 1:200 primary anti-SR-A1 antibody followed by 1:400 anti-mouse Alexafluor 488 secondary antibody (Invitrogen). Nuclei were stained with Hoechst 33258 at 1:10000 dilution. Images were captured using a Leica DM-IRE2 inverted microscope and analyzed using Openlab software (Improvision). All antibody dilutions were performed in blocking solution.

2.18. Biotinylation assay

Cells were seeded at a concentration of 2×10^5 cells/well in 12-well plates. Following expression of biotin ligase and SR-A1 fusion protein as specified in the figure legends, the growth media was supplemented with 50μM biotin for indicate lengths of time. Whole cell protein extracts were harvested, separated by SDS-PAGE, and transferred to PVDF membrane. Membrane was blocked in blocking buffer (1% BSA, 0.02% Triton X-100 in PBS). The membrane was probed with streptavidin-HRP (Thermo Scientific; Catalog number: 21130), diluted at 1:40000 in blocking buffer. ECL reagent was used to develop the blots on to an x-ray film.

2.19. BioID pulldowns

For large-scale BioID pulldowns, ten 15 cm dishes were seeded with 5×10^6 cells (A549 BioID) each to reach 100% confluency (~20×10^6 cells) the following day. 12 h post seeding, the cells were treated with 0.01 μg ml^{-1} doxycycline for 12 h to induce optimal levels of N-BioID expression. The cells were then pre-incubated with 50 μM biotin for 1 h to mediate generation of reactive biotinyl-AMP intermediates. Subsequently, the cells were either mock treated or treated with 10 μg ml^{-1} of extracellular dsRNA poly (I:C) in media supplemented with 50 μM biotin for indicated lengths of time. The cells were lysed in RIPA lysis buffer containing 3X Halt™ Protease Inhibitor Cocktail and the cell lysates were then incubated with benzonase (Sigma; Catalog number: E1014) for an hour at 4°C and sonicated to further shear DNA. Lysates

were cleared by ultracentrifugation for 30 mins and then incubated with Streptavidin Sepharose High Performance beads (GE Healthcare; Catalog number: 17511301) for 3 h at 4^{o}C for the biotinylated proteins to bind the beads. The streptavidin sepharose beads were washed six times with wash buffer (50mM ammonium bicarbonate) and resuspended in 200 µl wash buffer. The proteins on the beads were then subjected to 16 h (overnight) digestion with 2 µg MS sequencing grade trypsin (Promega; Catalog number: V5111) to cleave the proteins into peptides and release them from the beads. Following digestion, the beads were further washed with wash buffer to remove any residual peptides and the BioID samples were lyophilized in a speed vacuum centrifuge for 3-3.5 h. The samples were stored at -80^{o}C until MS analysis.

2.20. Liquid chromatography Mass spectrometry (LC-MS)

BioID samples were resuspended with 20 µl 0.1% formic acid, 2 µl out of 20 µl was injected for LC-MS/MS analysis. Liquid chromatography was conducted using a home-made trap-column (5 cm x 200 µm inner diameter) and a home-made analytical column (50 cm x 50 µm inner diameter) packed with Reprosil-Pur 120 C18-AQ 5 µm particles (Dr. Maisch), running a 2-hour reversed-phase gradient at 70 nl/min on a Thermo Scientific Ultimate 3000 RSLCNano UPLC system coupled to a Thermo QExactive HF quadrupole-Orbitrap mass spectrometer. A parent ion scan was performed using a resolving power of 120,000 and then up to the 30 most intense peaks were selected for MS/MS (minimum ion counts of 1000 for activation), using higher energy collision induced dissociation (HCD) fragmentation. Dynamic exclusion was activated

such that MS/MS of the same m/z (within a range of 10ppm; exclusion list size=500) detected twice within 5s were excluded from analysis for 30s.

2.21. BioID data analysis

Mass spectrometric raw files from the Thermo QExactive HF quadrupole-Orbitrap were searched using Proteome Discoverer, against the UniProt Human database (Version 2017-06-07), in addition to a list of common contaminants maintained by MaxQuant (225). The database parameters were set to search for tryptic cleavages, allowing up to 2 missed cleavage sites per peptide, with a parent MS tolerance of 10 ppm for precursors with charges of 2+ to 4+ and a fragment ion tolerance of ±0.02 amu. Variable modifications were selected for oxidized methionine. The results from each search were statistically validated within Proteome Discoverer, with 1 unique peptide and a false discovery rate (FDR) cut-off at 0.01 required for protein identification. Student's t test was used to calculate the probability of each potential proximal-protein by comparing mock treated and extracellular poly (I:C) treated samples using a *p-value* cut-off of $p < 0.05$. Proteins were classified as candidate interactors if they were identified in all three triplicate samples and abundances were at least 1.2-fold greater compared to respective controls. Pathway enrichment analysis was performed using the REACTOME Functional Interaction (Reactome FI) Network and an FDR cut-off at 0.05 was applied to identify enriched biological pathways. The pathway enrichment was visualized on Cytoscape v3.8.0.

2.22. Quantification and statistical analysis

A) Immunoblot quantification: Immunoblot bands were quantified using Image Studio (LI-COR Biosciences).

B) Statistical Analysis: Data analysis were performed using GraphPad Prism package (Version 6). All data are shown as Mean ± SEM. Statistical analysis was performed using Student's t test with two-tailed, 95% confidence. P values less than 0.05 were considered statistically significant (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). 'n' represents number of experimental replicates that were carried out and are specified in the figure legends.

CHAPTER 3: Direct binding and internalization of diverse nucleic acid species through the collagenous domain of SR-A1

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Baid, K., Nellimarla, S., Huynh, A., Boulton, S., Guarne, A., Melacini, G., Collins, S. E., Mossman, K. L. (2018). Direct binding and internalization of diverse extracellular nucleic acid species through the collagenous domain of class A scavenger receptors. Immunol Cell Biol. *96 (9)*, 922-934.

3.3. Abstract

Nucleic acids are potential pathogen-associated or danger-associated molecular patterns that modulate immune responses and the development of autoimmune disorders. SR-As are a diverse group of pattern recognition receptors that recognize a variety of polyanionic ligands including nucleic acids. While SR-As are important for the recognition and internalization of extracellular dsRNA, little is known about extracellular DNA, despite its association with chronic infections and autoimmune disorders. In this study, we investigated the specificity of and requirement for SR-As in binding and internalizing different species, sequences, and lengths of nucleic acids. We purified recombinant coiled-coil collagenous domain of SR-A1 and SRCR domain of SR-A6 that have been implicated as potential ligand binding domains. We detected a direct interaction of RNA and DNA species with the coiled-coil collagenous domain, but not the SRCR domain. Despite the presence of additional surface receptors that bind nucleic acids, SR-A1 was found to be sufficient for nucleic acid binding and uptake in human A549 lung epithelial cells. Moreover, these findings suggest that the coiled-coil collagenous domain of SR-A1 is sufficient to bind different nucleic acid species independent of sequence or length.

3.4. Introduction

Innate immunity is the first line of defense against invading pathogens and includes distinguishing self from non-self. Pathogen sensing by structural and immune cells is mediated by germ line encoded PRRs present either on the cell surface or within distinct intracellular compartments. These receptors recognize PAMPs and host-derived danger signals, resulting in the induction of innate immune mediators, including IFN-I (1). Over the past 10 years, significant progress has been made in the identification of host receptors that recognize pathogen and host-derived nucleic acids, revealing the powerful role for nucleic acid sensing in triggering primary immune responses.

Both DNA and RNA have been implicated as important modulators of innate antiviral responses. Innate immune sensors of nucleic acids in mammalian cells include endosomal members of the TLR family and multiple cytosolic sensors. While TLR3 plays a crucial role in antiviral responses against viruses by its ability to sense dsRNA (226, 227), TLR9 recognizes unmethylated CpG DNA motifs that are common in bacteria, but rare in mammalian genomes (228). RIG-I and its homolog MDA5 are sensors of specific viral RNA structures in the cytoplasm while multiple cytosolic DNA sensors have been identified (229-232). Under certain conditions, these PRRs also sense endogenously derived nucleic acids as danger associated molecular patterns (DAMPs), leading to autoimmunity and inflammatory disorders (9, 16, 233-238). Restricting the expression of certain sensors to intracellular compartments was found to be critical for the

discrimination between self and non-self nucleic acid (239). Indeed, TLRs are trafficked to nucleic acid-containing endosomes through an elaborate process involving the chaperon protein UNC93B1 and further undergo proteolysis for efficient activation to induce specific immune responses. Such mechanisms avoid unwanted detection of self-nucleic acids and prevent self-directed inflammatory responses, while not compromising the ability of cells to sense pathogenic nucleic acids (233, 239).

As these nucleic acid sensors are restricted to the endosome or cytoplasm, the mechanism by which circulating nucleic acid is sensed remained elusive until SR-As were identified as sensors of extracellular dsRNA (19, 179). SRs constitute a structurally diverse group of PRRs that recognize a wide array of pathogen-associated molecular motifs (240, 241). The SR-As are highly conserved trimeric molecules containing a short cytoplasmic tail, a transmembrane domain, an α-helical coiled-coil domain and a collagenous domain, while several members also contain a C-terminal SRCR domain (183). The SR-A family has five known members: SR-A1, SR-A3, SR-A4, SR-A5 and SR-A6 (157). SR-A1 has multiple splice variants; full-length (SR-A1), lacking the SRCR domain (SR-A1.1), and a dominant negative isoform trapped in the ER (SR-A1.3) (172, 180). Unlike other cell surface receptors, the SR-As exhibit unusually broad ligand specificity, including chemically modified or altered molecules, bacterial surface components, apoptotic cells and polyribonucleotides, such as polyinosinic acid and polyguanilic acid, but not polyadenilic acid or polycytidilic acid (158, 242-244). SR-A-s

are of particular interest given their ability to uptake nucleic acids (183, 245-248) and modulate innate immune responses to both RNA and DNA viruses (204-206, 210, 249).

We previously demonstrated that SR-As function to bind and internalize extracellular dsRNA, leading to the production of IFN-I and establishment of an antiviral state (19). Deletion of TLR3 or the cytoplasmic RLRs caused a similar reduction in the antiviral response to the synthetic dsRNA poly (I:C) (19). However, simultaneous deletion of the adaptors for TLR3 and the RLRs abolished antiviral state induction, indicating that the SR-As do not mediate induction of antiviral IFN-I independent of established TLR3 and RLR pathways (215). Besides SR-As, other cell surface receptors have also been identified such as DEC-205 (250), SR-J1 (commonly known as receptor for advanced glycation end-products; RAGE) (238, 251) and Mac-1 (252, 253), that bind and internalize nucleic acids. The presence of other nucleic acid receptors complicates delineating the role of SR-As against extracellular nucleic acids. Indeed, in our previous study, successive depletion of SR-A family members using siRNA technology coordinately decreased binding of extracellular dsRNA, but residual dsRNA binding and activity remained (19). Moreover, studies suggesting that SR-As bind DNA are based on competitive inhibition experiments using SR-A ligands which can be bound by other receptors with similar ‘scavenger’ properties as SR-As (238, 251-255).

Analysis of SR-A family members is further challenging due to their functional redundancy (19) which is believed to be a result of their ability to bind similar polyanionic ligands, albeit using distinct mechanisms (256-259). For instance, although SR-A1, SR-A5 and SR-A6 contain a C-terminal SRCR domain, it is the two highly conserved arginine residues (RxR motif) present only in SR-A6 that enable ligand binding (161, 171). The SRCR domain of SR-A1 does not contain a RxR motif and is consequently not involved in ligand recognition. Instead, SR-A1 has been shown to primarily bind ligands such as modified LDL within the proximal collagenous domain (161, 168). Yet, both SR-A1 and SR-A6 carry out similar functions of recognizing and clearing modified host components, apoptotic cells and pathogens (157). While all the SR-As have a collagenous domain (albeit of variable length), SR-A5 is unable to bind modified LDL but binds bacterial components (176). Similarly, the collagenous domain of SR-A4 has been identified as a putative ligand binding site for bacteria and oxLDL but not acLDL (177). While binding between SR-A and its polyanionic ligands reflects an ionic interaction, the preference of SR-As for certain nucleic acids (e.g. poly I/G) and their failure to bind polyanions such as chondroitin sulphate or poly A/U suggest that there may be additional factors that contribute to ligand binding specificity (184, 185). Specifically, we observed that poly (I:C) is more effective than poly dA:dT in blocking acLDL binding to SR-As (19).

A positively charged lysine cluster within the collagenous domain of SR-A1 has been implicated in conformation-dependent polyanionic ligand binding (164, 170, 183) whereas, the SRCR domain is dispensable for ligand binding as SR-A1.1 (splice variant lacking the SRCR domain) binds ligands with similar specificity to SR-A1 (169, 260). Similarly, a positively charged motif within the SRCR domain of SR-A6 is essential for ligand binding (172). Despite structural and functional similarities of SR-A1 and SR-A6 (183), the differences in their ligand binding mechanisms remain intriguing. To our knowledge, there is no evidence suggesting whether nucleic acids preferentially bind the collagenous domain of SR-A1 or the SRCR domain of SR-A6 and if such binding is dependent on nucleic acid species, sequence, or length.

Therefore, the goals of this study were 1) to determine whether SR-As bind nucleic acid of different species, 2) determine whether additional nucleic acid binding receptors play a redundant role in extracellular dsRNA-mediated antiviral responses and 3) to identify the domain(s) of SR-As responsible for nucleic acid binding. In this study, we verified that DNA uptake, like dsRNA uptake, is also SR-A dependent and utilizing a standard gain-of-function analysis, showed that SR-A1 is required for mediating extracellular dsRNA-induced antiviral responses in the absence of detectable expression of additional nucleic acid receptors such as SR-J1 and DEC-205. Since specificity of SR-As for nucleic acids of different species, sequence and length remains unclear, we assessed binding specificity using recombinant SR-A1 collagenous domain and SR-A6 SRCR domain. We established that the collagenous domain of SR-A1 but not the SRCR

domain, can directly bind to different nucleic acid species, irrespective of their length or sequence.

3.5. Results

3.5.1. SR-As mediate binding and internalization of extracellular DNA in human lung fibroblasts

SR-As mediate the entry of immunostimulatory dsRNA, which can be blocked with specific competitive ligands (19, 215), but little is known about the binding and internalization of DNA; our previous studies found that poly dA:dT bound less efficiently than poly (I:C) (19). HEL fibroblasts were chosen for this study as they express several members of the SR-A family including SR-A1 (19). A ligand uptake assay was performed to determine the binding and internalization capacity of HEL cells for DNA. Cell associated (surface bound and internalized) and intracellular dsRNA and DNA molecules were quantified using a standard plate reader. HEL fibroblasts bound and internalized both dsRNA and DNA in a similar manner (Figure 3.5.1A). The finding was further validated using live cell fluorescence microscopy (Figure 3.5.1B). Furthermore, DNA binding and internalization were assessed in the presence of fucoidin and fetuin, competitive and non-competitive SR-A ligands, respectively. Analogous to dsRNA (19), fucoidin reduced DNA binding and internalization while fetuin did not (Figure 3.5.1C). These results indicate that in human embryonic fibroblasts, extracellular DNA binding and uptake occurs in a SR-A dependent manner.

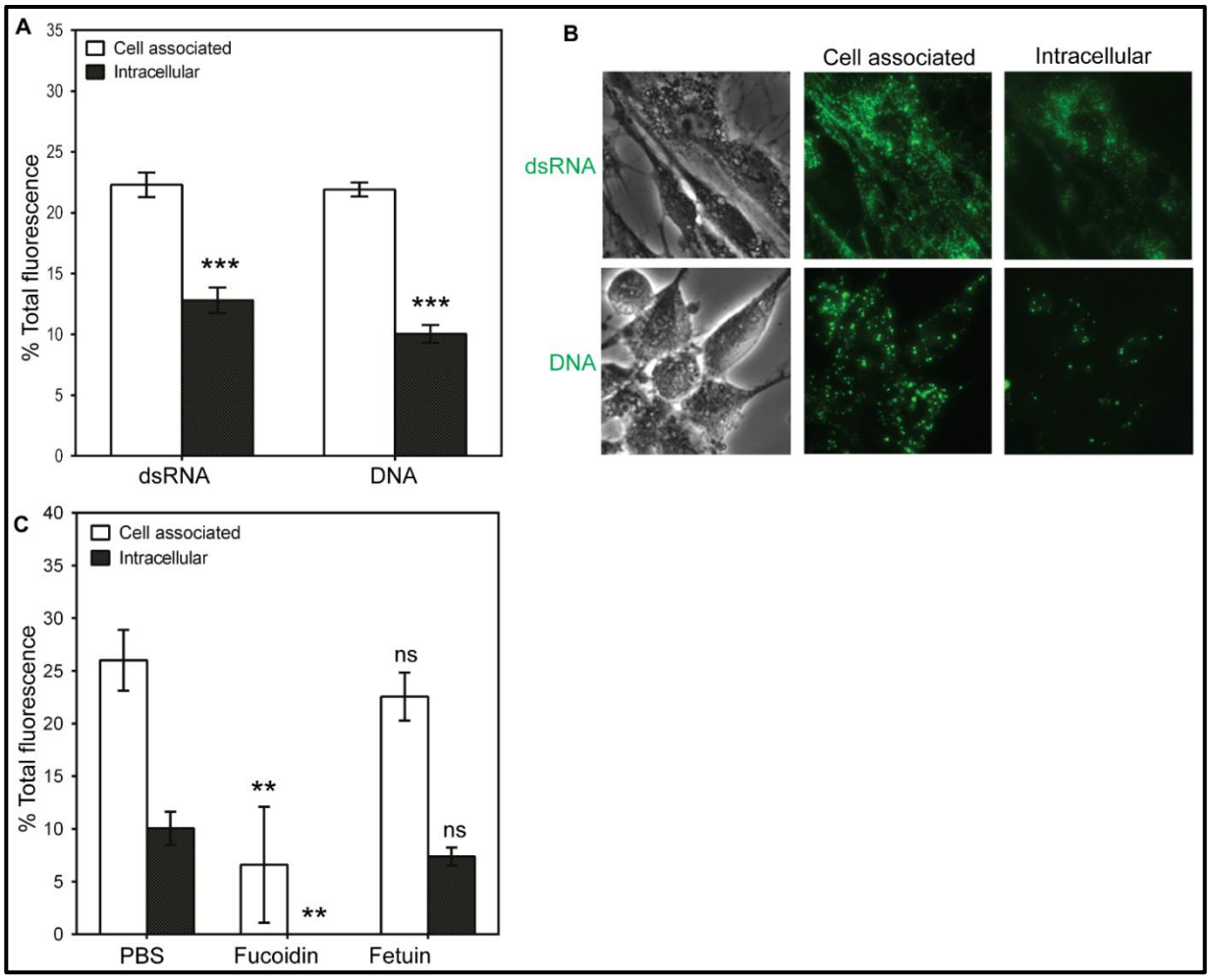

Figure 3.5.1. HEL fibroblasts mediate binding and internalization of extracellular DNA in a SR-A dependent manner.

(A) Nucleic acid uptake observed in HEL fibroblast cells treated with 1 µg ml^{-1} Alexafluor 488 labeled 1100 bp dsRNA or DNA for 1 hr (n=3).

(B) Cell associated and intracellular nucleic acid observed using live cell fluorescence microscopy in HEL fibroblast cells treated with 1 µg ml^{-1} Alexafluor 488 labeled 1100 bp dsRNA or DNA.

(C) Nucleic acid uptake observed in HEL fibroblast cells treated with Alexafluor 488-labeled 1100 bp DNA or dsRNA for 1 h in the presence of fucoidin or fetuin (100 µg mL^{-1}) (n=3). PBS treated cells were used as control.

Cell associated and intracellular fluorescence were reported as a percentage of total fluorescence. Statistical significance was calculated by two-way ANOVA with a **(A)** Sidak's and **(C)** Dunnett's post-test, respectively. Data are represented as mean ± SEM.**$p < 0.01$, ***$p < 0.001$. ns, not significant.

3.5.2. Cells lacking SR-As mediate a defective antiviral response to extracellular dsRNA

We demonstrated that the SR-As function to bind and internalize extracellular immunostimulatory dsRNA, leading to the production of type I interferon and an antiviral response (19). However, analysis of SR-A function is complicated by the functional redundancy amongst SR-A family members, their ubiquitous expression across many cell types and the existence of other cell surface receptors that can bind RNA and DNA, whose activity can be potentially inhibited by SR-A specific ligands (19, 250, 252, 253, 261-263). Therefore, in the context of a virus infection, to determine if cells are proficient at responding against extracellular viral dsRNA in the absence of SR-As, we compared the differences in antiviral response to extracellular poly (I:C) stimulation in human lung epithelial cells. Wildtype (WT) A549 (A549 WT) cells expressed only SR-A3 (NM_016240), SR-A3.1 (NM_182826) and SR-A5 (NM_173833) at the transcript level (Figure 3.5.2A); using CRISPR gene-editing technology, we generated SR-A deficient cells (A549 ΔSRA) to perform loss-of-function analysis. Due to the lack of reliable antibodies for SR-A3 and SR-A5, specific Cas9-mediated indels were verified by sequencing the respective genomic loci of SR-A3 and SR-A5 in A549 ΔSRA cells (Figure 3.5.2B).

To quantify the antiviral response in A549 WT and A549 ΔSRA cells, we performed bioassays using VSV that was genetically engineered to express GFP (VSV-GFP). VSV is known to infect cells from multiple species of mammals and is very

sensitive to IFN signaling, making it ideal for antiviral studies in cells from diverse mammalian species (264). To determine if SR-A deleted cells lose their ability to respond to extracellular dsRNA, we treated A549 WT and A549 ΔSRA cells with extracellular poly (I:C) as described in our previous studies (19, 215) and compared the extent of virus replication in A549 WT and A549 ΔSRA cells by quantifying the amount of GFP expressed by replicating VSV-GFP. A549 ΔSRA cells that lacked SR-As, displayed significantly reduced antiviral protection compared with A549 WT (Figure 3.5.2C).

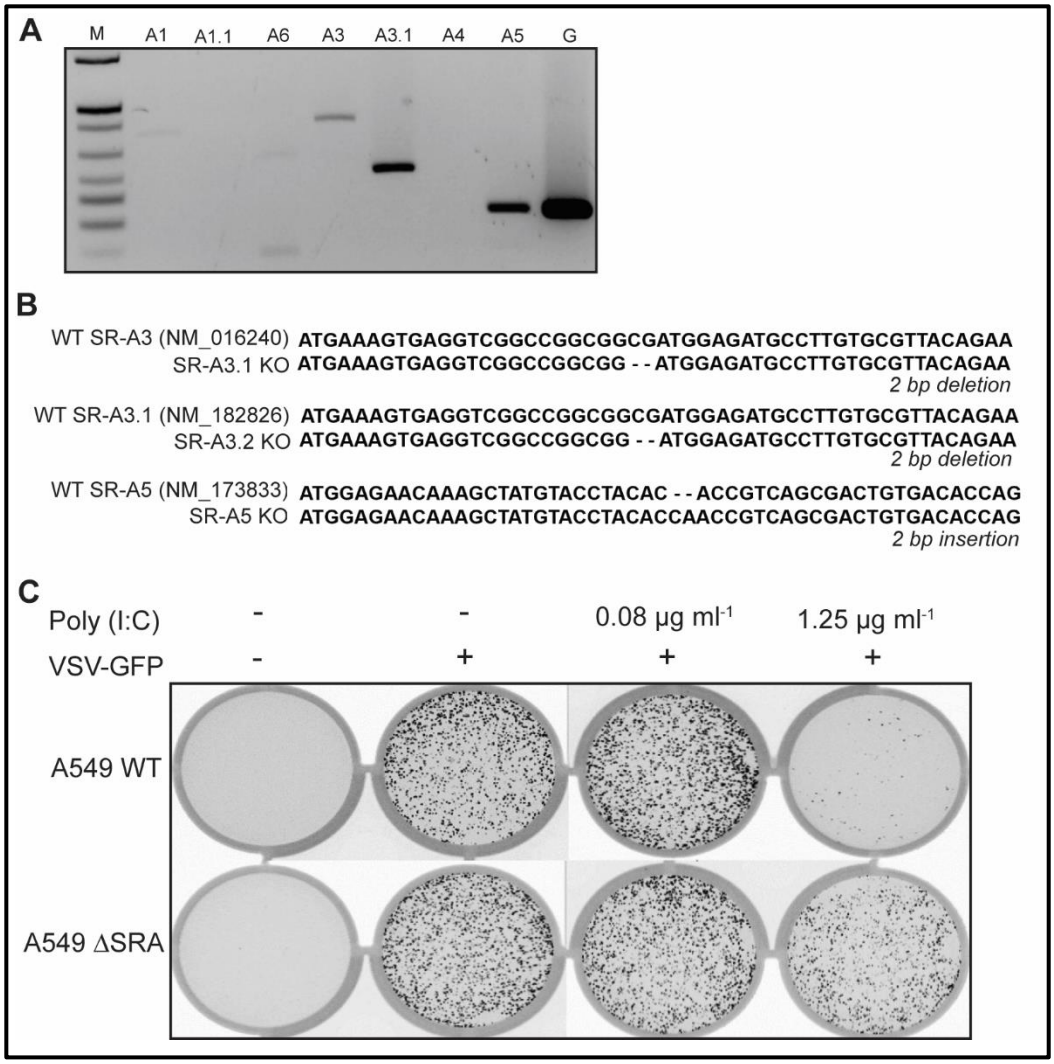

Figure 3.5.2. SR-A deficient A549 lung epithelial cells are impaired in their ability to mediate an antiviral response against extracellular dsRNA.
(A) Conventional RT-PCR analysis to detect mRNA transcripts of SR-A1 (A1), SR-A1.1 (A1.1), SR-A6 (A6), SR-A3 (A3), SR-A3.1 (A3.1), SR-A4 (A4) and SR-A5 (A5) in A549 WT lung epithelial cells. GAPDH (G) was used as a control.
(B) Sanger sequencing analysis of CRISPR-Cas9 mediated indels generated at SR-A3 and SR-A5 genomic loci in A549 WT and A549 ΔSRA cells.
(C) Antiviral responses measured in A549 WT and A549 ΔSRA cells stimulated with extracellular poly (I:C) (n=3) suspended in 50 µg ml^{-1} DEAE-dextran for 24 h, followed by infection with VSV-GFP (MOI = 0.1). Twenty hours post infection, GFP expression was measured as a surrogate for virus replication.
M, marker; KO, knockout, bp, base pair; WT, wild type.

3.5.3. Expression of SR-A1 is sufficient to rescue antiviral protection in SR-A deficient A549 cells

To support the finding in Figure 3.5.2C, we introduced back a single SR-A family member and tested if the capability of A549 ΔSRA cells to respond to extracellular dsRNA is restored. SR-A1, also termed MSR1 (macrophage scavenger receptor 1), is a prototype member of the SR-A family that is not expressed at the transcript level in A549 WT cells (Figure 3.5.2A). We designed a tetracycline-inducible gene expression system to regulate SR-A1 expression in A549 ΔSRA cells. Utilizing this system, a control line expressing the regulatory protein rtTA (ΔSRA rtTA) only and an experimental cell line expressing both, rtTA and SR-A1 (ΔSRA +SR-A1) were generated. Immunoblot analysis confirmed the inducible expression of SR-A1 (~50 kDa) and its post-translationally modified forms (Figure 3.5.3A). As expected, ΔSRA rtTA cells showed no evidence of SR-A1 expression upon induction with doxycycline (Figure 3.5.3B). Consistent with our inability to select stable cell lines constitutively overexpressing SR-A1 *(unpublished work from Mossman lab)*, increasing cytotoxicity was detected by flow cytometry analysis following induction of SR-A1 expression with increasing concentrations of doxycycline (Figure 3.5.3C).

As SR-A deficient cells were unable to develop antiviral responses to immunostimulatory extracellular dsRNA, likely due to the inability of the cells to uptake extracellular dsRNA, we assessed whether SR-A1 restores the ability to respond to extracellular dsRNA in ΔSRA +SR-A1 cells. In this study, dsRNA uptake was visualized

using fluorescence microscopy. Doxycycline induced ΔSRA +SR-A1 cells and uninduced controls were treated with Alexafluor 488 labeled 1100bp dsRNA and a punctate staining was observed in induced cells compared with uninduced control cells indicating dsRNA uptake being mediated by SR-A1 (Figure 3.5.3D). We further validated if SR-A1 would mediate extracellular dsRNA-induced antiviral protection in SR-A deleted cells. We treated ΔSRA +SR-A1 cells with extracellular poly (I:C) following induction of SR-A1 expression with doxycycline and performed bioassays using VSV-GFP. We compared the extent of virus replication in uninduced and induced ΔSRA +SR-A1 cells by quantifying the amount of GFP expressed by replicating VSV-GFP. Cells induced with doxycycline, i. e., the cells expressing SR-A1 displayed a robust extracellular poly (I:C)-induced antiviral protection relative to the uninduced cells, leading to undetectable virus replication (Figure 3.5.3E). Likewise, extracellular poly (I:C)-mediated increase in the transcripts of ISG56 in ΔSRA +SR-A1 cells induced with doxycycline compared with ΔSRA rtTA cells, further attesting the role of SR-A1 in restoring antiviral potency of SR-A deficient cells (Figure 3.5.3F).

In addition, we also investigated if other cell surface nucleic acid receptors can either substitute for SR-As in their absence or act in concert with SR-A1. We failed to detect SR-J1 at the mRNA level using conventional RT-PCR analysis (Figure 3.5.3G) despite reports suggesting that A549 WT cells express SR-J1 (265). Using a more sensitive assay like qRT-PCR, we further evaluated SR-J1 expression in parental A549 cells. Results indicated that expression levels of SR-J1 were extremely low (cycle

threshold value (Ct value) > 30) (Figure 3.5.3H). While lung tissue also potentially expresses DEC-205 (266), its expression in HEL fibroblasts and A549 WT cells at the mRNA level was found to be either below the limit of detection (Ct > 35 cycles) or extremely low (Ct >30 cycles) respectively, using qRT-PCR analysis (Figure 3.5.3H). Together, these observations suggest that SR-A1, independent of other receptors with similar properties, is likely sufficient in restoring antiviral potency in SR-A deficient cells.

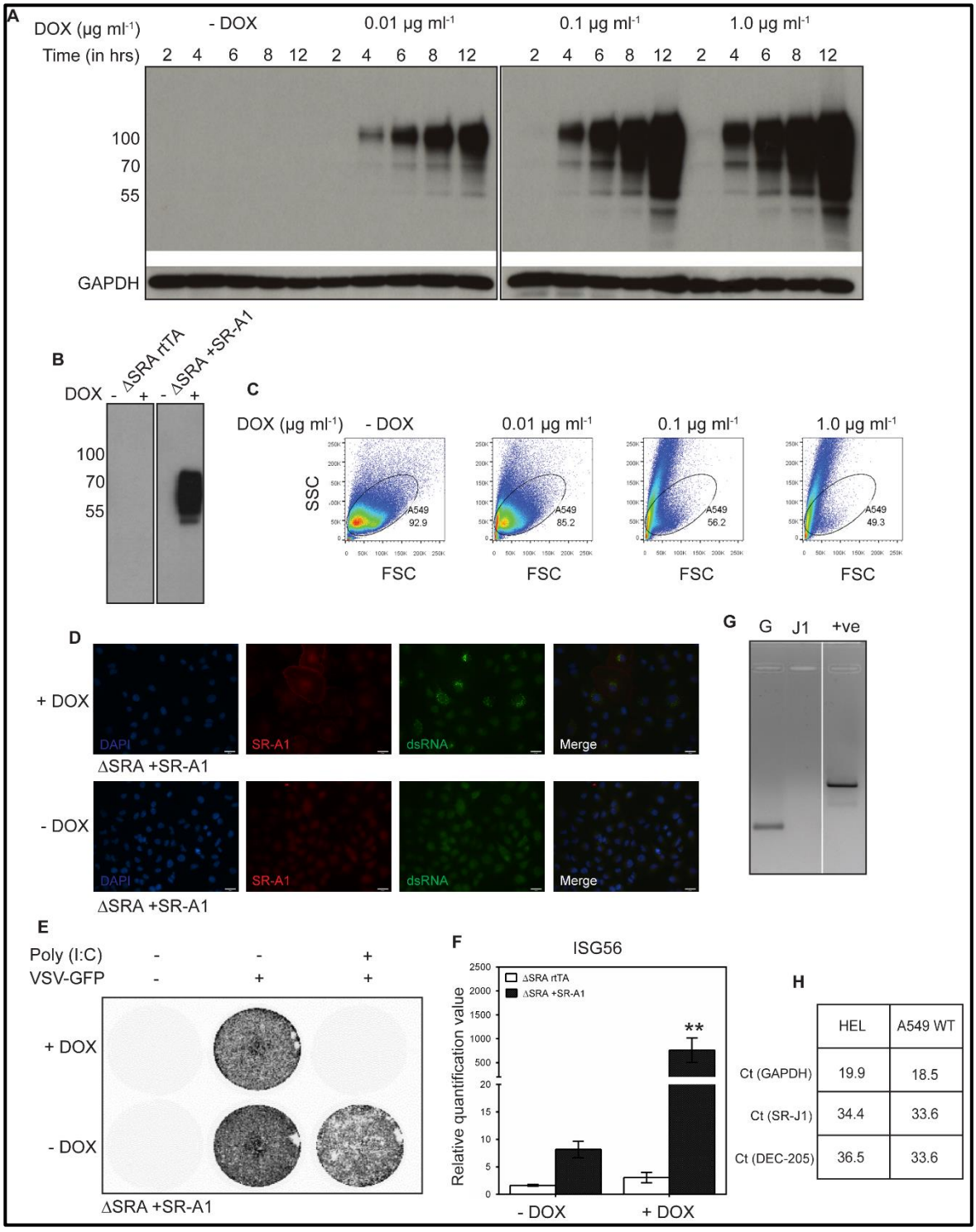
A
DOX (μg ml-1)
- DOX
0.01 μg ml-1
0.1 μg ml-1
1.0 μg ml-1
Time (in hrs)
2 4 6 8 12 2 4 6 8 12 2 4 6 8 12 2 4 6 8 12
100
70
55
GAPDH
B
DOX - + - +
ΔSRA rtTA
ΔSRA +SR-A1
100
70
55
C
DOX (μg ml-1)
- DOX
0.01 μg ml-1
0.1 μg ml-1
1.0 μg ml-1
SSC
FSC
A549
92.9
A549
85.2
A549
56.2
A549
49.3
D
+ DOX
- DOX
DAPI
SR-A1
dsRNA
Merge
ΔSRA +SR-A1
G
G J1 +ve
E
Poly (I:C) - - +
VSV-GFP - + +
+ DOX
- DOX
ΔSRA +SR-A1
F
ISG56
ΔSRA rtTA
ΔSRA +SR-A1
Relative quantification value
**
- DOX
+ DOX
H
HEL
A549 WT
Ct (GAPDH) 19.9 18.5
Ct (SR-J1) 34.4 33.6
Ct (DEC-205) 36.5 33.6

Figure 3.5.3. Expression of SR-A1 in SR-A deficient A549 lung epithelial cells can rescue antiviral potency.
Tightly regulated expression of SR-A1 at the protein level observed by immunoblot analysis in **(A)** ΔSRA +SR-A1 and **(B)** ΔSRA rtTA cells treated with doxycycline (n=3).
(C) SR-A1 expression induced toxicity detected by flow cytometry analysis in ΔSRA +SR-A1 cells treated with increasing concentrations of doxycycline for 24 h (n=3).
(D) SR-A1 dependent dsRNA uptake using fixed cell fluorescence microscopy in ΔSRA +SR-A1 cells treated with Alexafluor 488 labeled 1100bp dsRNA for 1 h (n=2). The brightness is enhanced equally for all images by +40%.
(E) VSV-GFP replication in ΔSRA +SR-A1 cells mock induced or induced with 0.01 μg ml^{-1} doxycycline for 12 h and treated with 0.5 μg ml^{-1} extracellular poly (I:C) (n=3) for 24 h.
(F) ISG56 transcript levels measured by quantitative RT-PCR in ΔSRA +SR-A1 and ΔSRA rtTA cells mock induced or induced with 0.01 μg ml^{-1} doxycycline for 12 h and treated with 0.5 μg ml^{-1} extracellular poly (I:C) (n=3) for 24 h.
(G) Detection of SR-J1 (J1) mRNA transcripts using conventional RT-PCR analysis in A549-WT cells. GAPDH (G) was used as an internal control. Plasmid encoding SR-J1 cDNA was used as a positive control (+ve) (n=3)
(H) Expression of SR-J1 and DEC-205 at the mRNA level using quantitative RT-PCR in human HEL fibroblasts and A549 WT cells. GAPDH was used as an internal control.
Statistical significance was calculated by a two-way ANOVA with a Sidak's post-test.
Data are represented as mean ± SEM. $^{**}p < 0.01$.
DOX, doxycycline; ns, not significant; Ct, cycle threshold.

3.5.4. Collagenous domain of SR-A1 binds dsRNA in a sequence and length independent manner

Collagenous domain and SRCR domain of SR-As have been implicated in ligand binding based on the presence of a positively charged region or motif in each domain (170, 172, 242, 256, 257, 267). To determine dsRNA binding specificity to either domain, we synthesized and purified recombinant coiled-coil collagenous domain and SRCR domain (Figure 3.5.4A) and performed electrophoretic mobility shift assay or band shift assay to detect protein-nucleic acid interactions. The α-helical coiled-coil region was retained as it was essential for trimerization, but has no noted ligand-binding function (268). Two major peaks eluted from the size-exclusion gel chromatography column: peak

1 was eluted on the void volume of the column and did not contain the coiled-coil collagenous domain ; conversely, peak 2 eluted within the separation volume of the column at a molecular weight (MW) approximating 160 kDa, corresponding to the trimerized collagenous domain. The purity of each peak was confirmed by immunoblotting, where under reducing conditions, coiled-coil collagenous domain was detected at the expected monomer size of ~ 45 kDa (Figure 3.5.4B, inset). Synthesis and purification process of the SRCR domain has been previously described by Novakowski *et al.* 2016 (172). DsRNA molecules of different lengths and sequences were derived by *in vitro* transcription using the envelop gene sequence of WNv as the template (Figure 3.5.4C). DsRNA molecules were incubated together with either recombinant collagenous domain or SRCR domain and subsequently ran on an agarose gel to observe the protein-nucleic acid interaction. Irrespective of the length or sequence of dsRNA, they formed stable ribonucleoprotein (RNP) complexes with recombinant coiled-coil collagenous domain but not with the SRCR domain (Figure 3.5.4D).

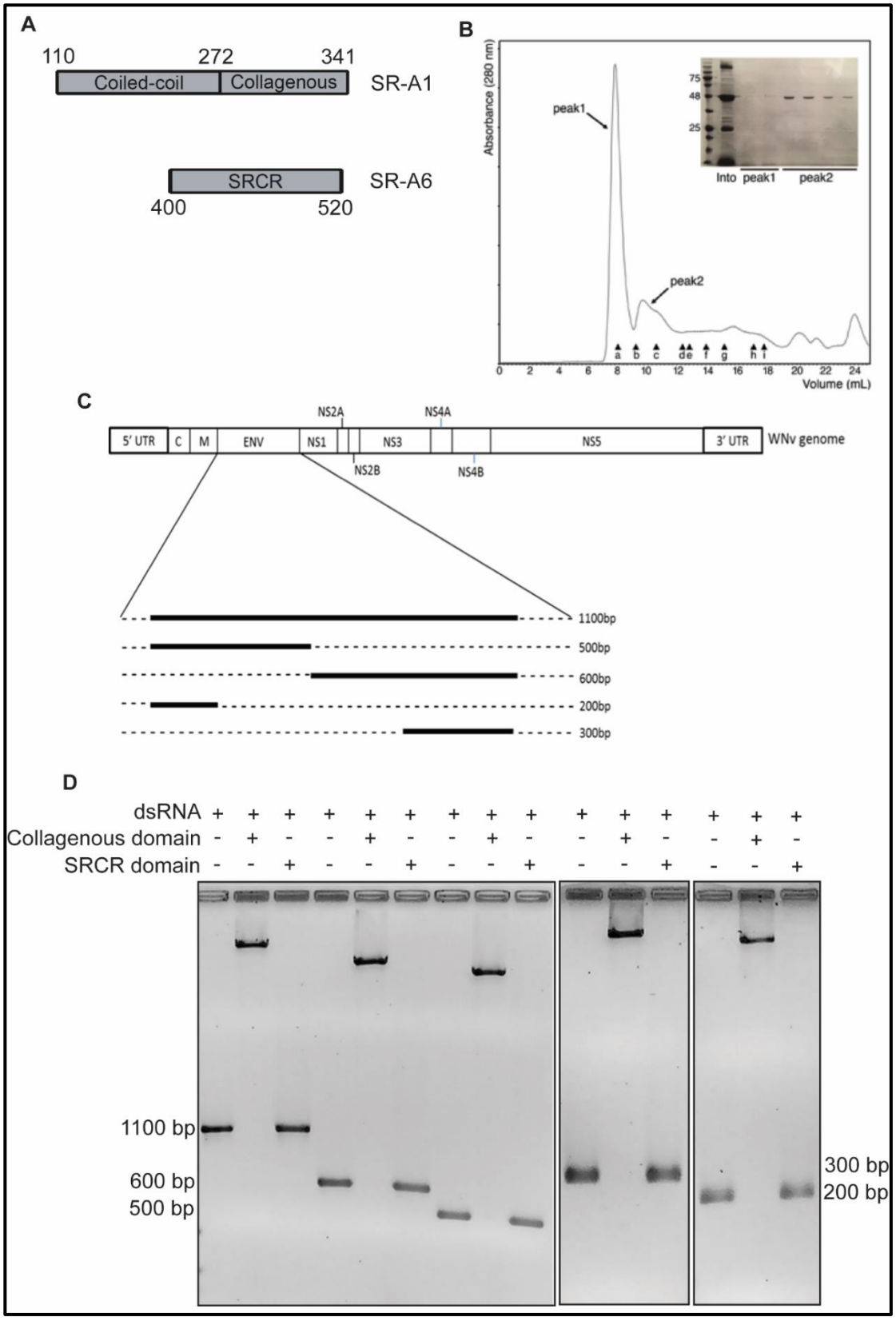
A
110
272
341
Coiled-coil
Collagenous
SR-A1
SRCR
SR-A6
400
520
B
Absorbance (280 nm)
peak1
peak2
75
48
25
Into
peak1
peak2
a b c de f g h i
0 2 4 6 8 10 12 14 16 18 20 22 24
Volume (mL)
C
NS2A
NS4A
5' UTR
C
M
ENV
NS1
NS3
NS5
3' UTR
WNv genome
NS2B
NS4B
1100bp
500bp
600bp
200bp
300bp
D
dsRNA + + + + + + + + + + + + + + +
Collagenous domain - + - - + - - + - - + - - + -
SRCR domain - - + - - + - - + - - + - - +
1100 bp
600 bp
500 bp
300 bp
200 bp

Figure 3.5.4 Collagenous domain of SR-A1 binds dsRNA independent of length and sequence.
(A) Schematic representation of the purified recombinant coiled coil collagenous domain of SR-A1 (110 aa - 341 aa) and SRCR domain of SR-A6 (400 aa - 520 aa).
(B) Purification of recombinant coiled coil collagenous domain using a Superdex200 size-exclusion chromatography column. Two major peaks eluted: peak 1 eluted on the void volume of the column did not contain coiled coil collagenous domain; conversely, peak 2 eluted within the separation volume of the column at a molecular weight around 160 kDa, corresponding to trimerized coiled coil collagenous domain. The purity of each peak was confirmed by immunoblotting (inset), where under reducing conditions, coiled coil collagenous domain was detected as a monomer at ~45 kDa.
Elution volumes for molecular weight standards used to calibrate the column are indicated with arrows: (a) void volume; (b) thyroglubulin (669 kDa); (c) ferritin (440 kDa); (d) catalase (232 kDa); (e) aldolase (158 kDa); (f) albumin (67 kDa); (g) ovoalbumin (43 kDa); (h) chymotrypsinogen A (25 kDa);
(C) A schematic illustration of the dsRNA fragments synthesized *in vitro* using Envelop gene of WNv genome encoded in a plasmid as template.
(D) Interaction of dsRNA with purified recombinant coiled coil collagenous domain and SRCR domain using EMSA (n=3).
aa, amino acid residue; WNv, west nile virus.

3.5.5. The collagenous domain of SR-A1 binds different forms of nucleic acids

We subsequently determined whether the collagenous domain is sufficient to bind additional forms of nucleic acid. While our previous study suggested poly (I:C) binds SR-As more efficiently than poly dA:dT (19), data in Figure 3.5.1 suggest similar binding of DNA and RNA species to SR-As. We performed similar experiments as in Figure 3.5.4D using DNA and ssRNA of different lengths and sequences. We observed that upon incubating DNA or ssRNA with coiled-coil collagenous domain, they efficiently formed RNP complexes but not with the SRCR domain (Figure 3.5.5A-B) suggesting the coiled-coil collagenous domain can bind multiple forms of nucleic acids. To validate this observation, a ligand binding competition assay was performed. We labeled 1100 bp long

dsRNA with Alexafluor 488 and co-incubated increasing amounts of unlabeled dsRNA of the same length and sequence with purified coiled-coil collagenous domain. Consistent with Figure 3.5.4D, labeled dsRNA bound to the coiled-coil collagenous domain and formed an RNP complex, but the interaction was competed away with increasing amounts of unlabeled dsRNA (Figure 3.5.5C). We then replaced unlabeled dsRNA with unlabeled DNA and repeated the competition assay. DNA was able to efficiently compete away dsRNA binding to the coiled-coil collagenous domain in a similar manner (Figure 3.5.5D).

Since SRCR domain of SR-A6 is critical for binding bacterial ligands (172), we were interested in determining if bacterial ligands would also bind to the coiled-coil collagenous domain. We examined the ability of *S. pneumoniae*, a bacterium that is cleared from the murine nasopharynx in a SR-A6-dependent manner (269), to bind purified coiled-coil collagenous domain. Following the incubation of *S. pneumoniae* with either coiled coil collagenous or SRCR domains, flow cytometry analysis indicated preferential binding of bacteria to the SRCR domain, with low levels of binding to the coiled-coil collagenous domain (Figure 3.5.5E). Together, these *in vitro* observations provide evidence of direct interaction of different forms of nucleic acids with the SR-A coiled-coil collagenous domain while bacteria can bind both domains, with a preference for the SRCR domain.

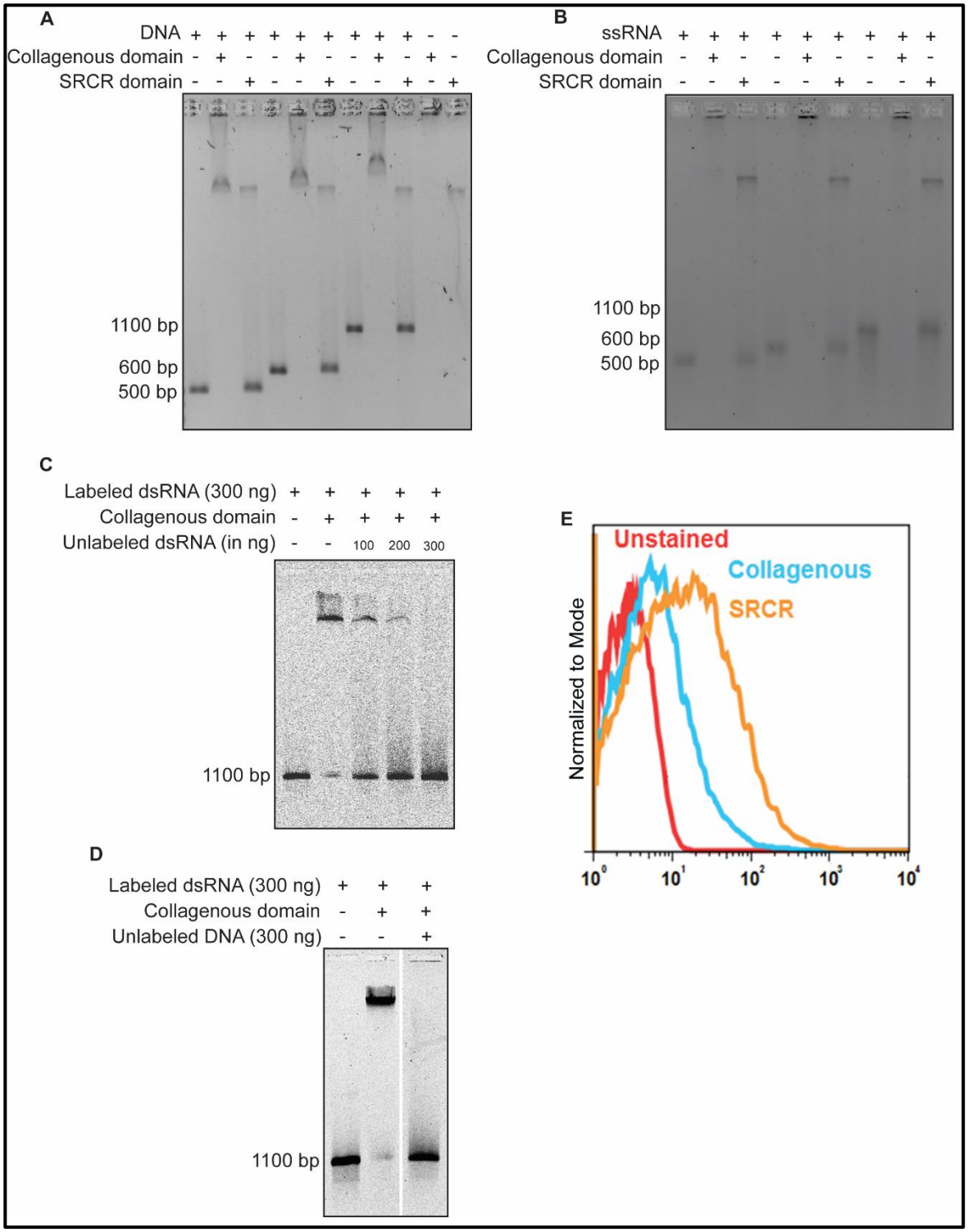
A
DNA + + + + + + + + - -
Collagenous domain - + - - + - - + - + -
SRCR domain - - + - - + - - + - +
1100 bp
600 bp
500 bp
B
ssRNA + + + + + + + + +
Collagenous domain - + - - + - - + -
SRCR domain - - + - - + - - +
1100 bp
600 bp
500 bp
C
Labeled dsRNA (300 ng) + + + + +
Collagenous domain - + + + +
Unlabeled dsRNA (in ng) - - 100 200 300
1100 bp
D
Labeled dsRNA (300 ng) + + +
Collagenous domain - + +
Unlabeled DNA (300 ng) - - +
1100 bp
E
Unstained
Collagenous
SRCR
Normalized to Mode
10^0
10^1
10^2
10^3
10^4

Figure 3.5.5. Collagenous domain of SR-A1 can bind different forms of nucleic acid.
Interaction of **(A)** DNA and **(B)** ssRNA with purified recombinant coiled-coil collagenous domain and SRCR domain observed by EMSA (n=3). A nonspecific band observed in samples incubated with the purified recombinant SRCR domain.
Binding specificity to coiled coil collagenous domain observed using competition assays. 300 ng of Alexafluor 488-labeled 1100 bp dsRNA and **(C)** increasing amounts of unlabeled dsRNA (100 – 300 ng; in increments of 100 ng) or **(D)** 300 ng of unlabeled DNA of the same length co-incubated with coiled coil collagenous domain.
(E) Binding specificity of coiled coil collagenous domain and SRCR domain for whole bacteria determined using flow cytometry (n=3).

3.6. Summary of results and transition statement

Extracellular nucleic acids contribute to the pathogenesis of bacterial and viral infections and in the development of autoimmune and inflammatory disorders. In some cases, extracellular nucleic acids also act as early prognostic marker for diseases like cancer. In the above study, we demonstrated that extracellular nucleic acids like DNA and dsRNA are recognized and internalized in a class A scavenger receptor-dependent manner. The importance of SR-A1 in mediating the effects of extracellular dsRNA was further elucidated as cells lacking the SR-As responded poorly to extracellular dsRNA while expressing other cell surface nucleic acid receptors such as SR-J1 and DEC-205, albeit at low levels. Upon exogenous expression of SR-A1, the ability of the cells lacking the SR-As to respond to extracellular dsRNA was restored (Figure 3.6). Furthermore, we also demonstrated that the collagenous domain of SR-A1 directly binds different species of nucleic acids and SRCR domain of SR-A6 does not (Figure 3.6). However, our studies in the above chapter did not fully elucidate the signaling mechanisms of extracellular dsRNA. We were interested in determining whether SR-A1 acts only as a ‘carrier’ or can

it influence the antiviral responses to extracellular dsRNA as well and thus decided to study the protein interactions of SR-A1 induced by extracellular dsRNA binding which may help discover a broader role for SR-A1 during virus infections. We were also interested in determining the intracellular fate of extracellular dsRNA which may provide insights into its intracellular sensing and subsequent antiviral response. We thus decided to study its trafficking following SR-A1-mediated uptake, which is the focus of Chapter 4.

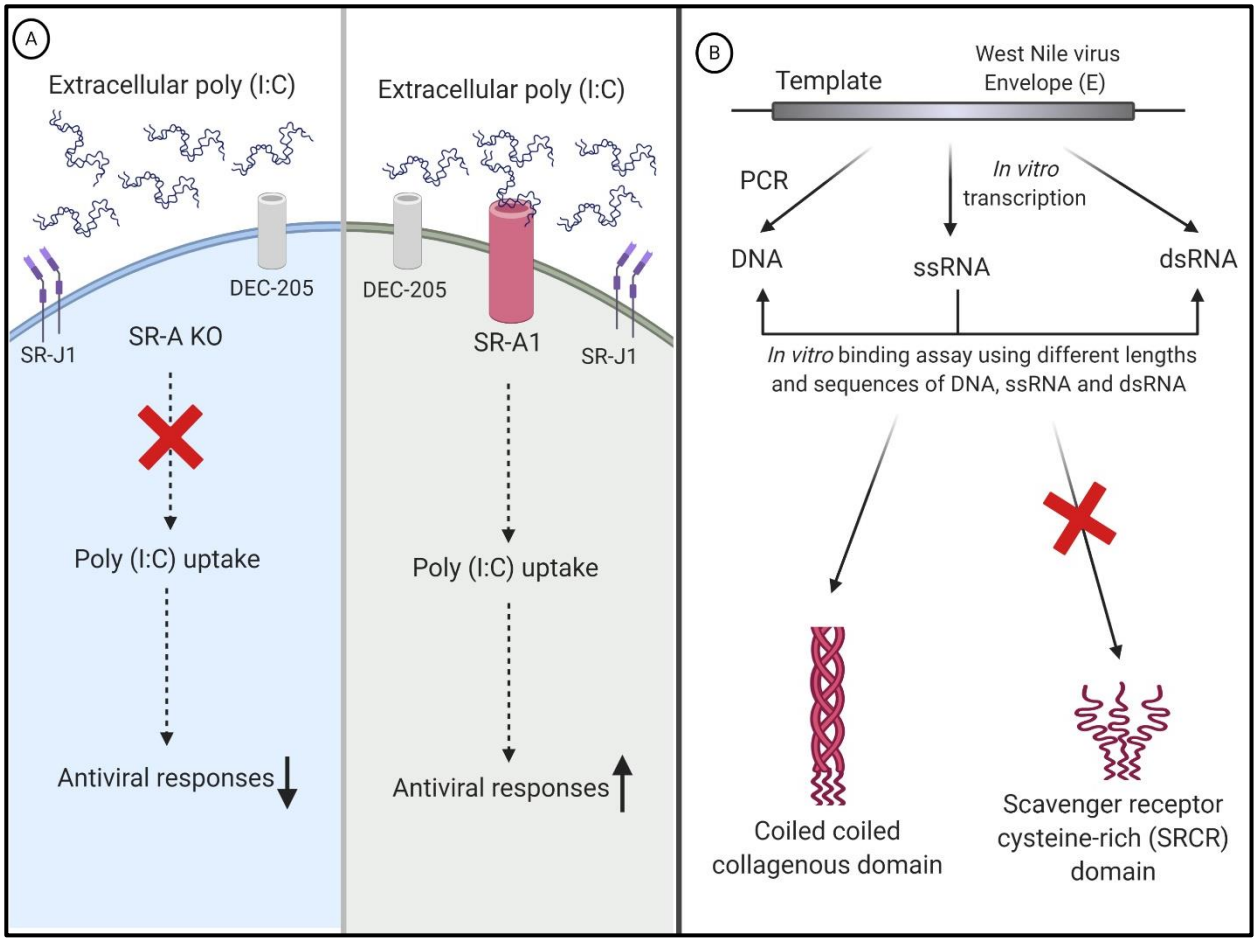

Figure 3.6. Summary of Chapter 3.
(A) In absence of all SR-As, human lung epithelial cells fail to respond to extracellular poly (I:C) despite expressing low levels of other nucleic acid receptors like SR-J1 and DEC-205. Expression of SR-A1 is necessary to mediate the effects of extracellular dsRNA in human lung epithelial cells.
(B) The coiled-coil collagenous domain of SR-A1 binds different nucleic acids like DNA, ssRNA and dsRNA generated from West Nile virus envelope gene sequence in a length and sequence independent manner whereas, the SRCR domain of SR-A6 does not.
Image created with BioRender.com.

CHAPTER 4: Intracellular signaling of extracellular dsRNA

4.1. Introduction

DsRNA is a common by-product of viral replication (15) and acts as a potent PAMP. The innate immune system selectively recognizes viral dsRNA through germline encoded PRRs like the TLRs and RLRs. Members of the TLR and RLR family are implicated in innate antiviral responses and they survey distinct intracellular locations for viral dsRNA; TLR3 and the RLRs (RIG-I, MDA5 and LGP2) sense viral dsRNA present within the endosomal lumen or the cytoplasm, respectively. Aside from LGP2 which lacks signaling ability (270), the sensors then initiate a signaling cascade which culminates in the production of immunomodulatory cytokines such as IFN-I and the subsequent establishment of an antiviral state (271).

The triggering of intracellular responses is only part of dsRNA's influence on the host. During virus-induced cell lysis, dsRNA molecules are released into the extracellular environment and while their immunostimulatory properties are often dampened in the infected cell due to the viruses' immune evasion tactics, 'extracellular' viral dsRNA can mediate its effects unhindered in surrounding or distal bystander cells (16). Circulating self dsRNA is also a potent inducer of sterile inflammation leading to the production of cytokines like IFN. Inappropriate activation of IFN leads to an array of interferonopathies, including autoimmune disorders such as lupus, and renders individuals more susceptible to infections (8, 272). Thus, nucleic acids and their sensors

are being targeted to control infection and autoimmunity (234, 235, 273, 274). Conversely, dsRNA molecules such as poly (I:C) and siRNA are being developed for systemic delivery as adjuvants and targeted therapeutics, respectively (236, 275, 276).

The role of extracellular dsRNA in health and disease is evident yet, little is known about its trafficking and mechanism of action. Indeed, the process by which extracellular dsRNA is sensed by intracellular sensors remained elusive until SR-As, particularly SR-A1 was identified as a surface receptor for extracellular dsRNA (19, 179). The SR-As constitute a family of PRRs with a broad ligand specificity, including nucleic acids. We previously confirmed that the SR-As uptake extracellular dsRNA through receptor-mediated endocytosis (19, 183, 277). Importantly, both endosomal TLR3 and the cytoplasmic RLRs were required for dsRNA-mediated IFN production (19, 33, 204, 216, 278, 279), consistent with a partial reduction in the antiviral response to extracellular poly (I:C) in cells lacking RLRs (19). In addition, simultaneous deletion of the adaptors for TLR3 and the RLRs abolished antiviral state induction, indicating that the SR-As do not mediate antiviral signaling independent of the established TLR3 and RLR pathways (215). Such cooperation between the endosomal and cytoplasmic dsRNA-sensing pathways contrasts with other studies where intracellular and extracellular nucleic acids mediate distinct responses (280, 281).

SR-As have been shown to activate a range of diverse intracellular signaling pathways, including the PI3K, PKC and MAPK pathways (183) following ligand binding

whilst containing only a few discernible signaling motifs in their cytoplasmic domain (157). Likewise, SR-As also cooperate with other PRRs such as TLR2, TLR4 and CD14 to mediate clearance of bacteria suggesting that they are capable of functioning as components of signaling complexes (157). We previously confirmed that the human lung epithelial cells lacking SR-As are unable to mediate antiviral responses to extracellular dsRNA and the response is rescued by putting back SR-A1 in those cells (282). Yet, whether SR-A1 can modulate the innate antiviral response through interaction with other co-receptors or cellular proteins following dsRNA binding remains elusive. A direct interaction between the cytoplasmic domain of SR-A1 with TRAF3 or TRAF6 attenuates the immune response to HBV and bacterial LPS respectively (199, 210), while SR-A1 has shown to activate autophagy to restrict CHIKV replication by directly interacting with the core autophagy complex ATG5-ATG12 (211). Thus, determining relevant protein interactions and/or networks of SR-A1 will allow us to evaluate the extent of its role in response to extracellular dsRNA and uncover novel biological pathways of extracellular dsRNA trafficking and/or signaling.

While both endosomal TLR3 and the cytoplasmic RLRs are required for extracellular dsRNA-mediated antiviral responses, we previously found that the response to short dsRNA species (~200bp) was completely dependent on cytoplasmic RIG-I (19) suggesting that extracellular dsRNA somehow enters the cytoplasm. Mechanisms such as pore formation using cell-penetrating peptides, pH-buffering effect of polycationic reagents, and lipid-based fusion of endosomal membranes are primarily utilized to

mediate the endosomal release of oligonucleotides but require the addition of synthetic peptides or chemical agents. In contrast, transmembrane proteins can switch their topology, as in 'flip' within the lipid bilayer based on the changes in their surrounding phospholipid environment. The membrane protein-lipid environment changes rapidly through lateral movement within a membrane or during trafficking in response to a ligand (283, 284). Therefore, it is plausible that the SR-As might flip to deliver extracellular dsRNA into the cytoplasm to provide access to the RLRs but whether they possess such capacity remains unclear.

An alternate mechanism for the transport of extracellular dsRNA into the cytoplasm is the existence of a dsRNA-specific channel that would allow easy movement of hydrophilic dsRNA molecules across the hydrophobic barrier of either the plasma membrane or endosomal membrane. Two potential candidates are SIDT1 and SIDT2, the mammalian orthologs of the *C. elegans* SID-1 dsRNA transporter (217, 218). Recently, both SIDT1 and SIDT2 have been shown to transport extracellular dsRNA from endo-lysosomal vesicles into the cytoplasm (221, 222) albeit SIDT1 appears to be functionally redundant in the presence of SIDT2 in response to virus infections (222). Since SIDT2 is more widely and abundantly expressed than SIDT1 (219), we therefore chose to focus on SIDT2 and its potential role in the innate immune response to viral dsRNA in human cells. Since SR-As interact with other proteins as part of signaling complexes (157), whether they collaborate with SIDT2 to mediate the effects of extracellular dsRNA remains to be tested.

In part of this study, we investigated extracellular dsRNA-induced protein interactions for SR-A1 using a proximity-dependent protein labeling assay known as BioID. BioID is a unique technique that harnesses a promiscuous biotin ligase, BirA*, to biotinylate proteins based on proximity. The ligase is fused to a protein of interest and expressed in cells, where it biotinylates proximal endogenous proteins. Because it is a rare protein modification in nature, biotinylation of these endogenous proteins by BioID fusion protein enables their selective isolation and identification with standard biotin-affinity capture (285-287). By coupling proximity-labelling (PL) with MS, one can identify weak, transient protein interactors along with the strong, robust interactors. This method is especially useful to study proteins such as membrane receptors that are difficult to purify by immunoprecipitation (285-288).

Therefore, the goals of this study were to 1) elaborate the role of SR-A1 in response to extracellular dsRNA and 2) identify the mechanism/s that facilitates the involvement of the cytoplasmic RLRs in extracellular dsRNA-mediated antiviral responses. In this study, we performed a proximity ligation assay to identify extracellular dsRNA-induced protein interactions for SR-A1 in human lung epithelial cells. We captured snapshots of SR-A1 interactions at different times following extracellular dsRNA treatment and observed an enrichment for RNA and protein metabolism pathways. We then investigated the role of a putative dsRNA channel, SIDT2 in extracellular dsRNA-mediated antiviral responses by using standard loss-of-function

analysis. We verified that the cells lacking SIDT2 were significantly impaired in their ability to mediate antiviral responses to extracellular dsRNA relative to transfected dsRNA. While these findings are preliminary, it provides a framework to further elucidate SR-A1-dependent and/or SR-A1-independent mechanisms of extracellular dsRNA trafficking and signaling.

4.2. Results

4.2.1. Attachment of BirA* on C-terminus or N-terminus does not impact expression and targeting of SR-A1 in human lung epithelial cells

BioID harnesses a promiscuous biotin ligase, BirA*, fused to a protein of interest to biotinylate proteins based on proximity. The fundamental component of this system is the BioID fusion protein. An ideal fusion protein will represent a functional replacement of the original protein (287). SR-A1 is a type II transmembrane protein, with its C-terminal domain targeted to the extracellular space, while the N-terminus faces the cytoplasmic side (183). SR-A1 executes its ligand binding activity through the collagenous (282) and SRCR domains (182) in the C-terminal region and the N-terminal cytoplasmic tail is essential for receptor targeting and ligand uptake function (187-190). Therefore, to determine if the addition of BirA* to either termini of SR-A1 affected its cellular localization and function, we generated two expression plasmids in parallel by ligating BirA* on either the N- or C-terminus (N-BioID and C-BioID) of SR-A1 (as shown in schematic 4.2.1A). We then introduced either N-BioID or C-BioID in WT

human A549 lung epithelial cells (A549 WT) and confirmed the expression of each fusion protein and their post-translationally modified forms by immunoblot analysis (Figure 4.2.1B). As expected, parental plasmids encoding only BirA* (N-BirA* and C-BirA*) showed no evidence of SR-A1 expression (Figure 4.2.1B). We further validated proper targeting of the fusion proteins using fixed cell immunofluorescence microscopy in A549 WT cells. Surface staining of cells expressing either N-BioID or C-BioID displayed a SR-A1-specific signal compared with mock transfected A549 WT cells (Figure 4.2.1C). Together, these findings suggest ligation of BirA* on either terminus of SR-A1 does not compromise its expression or cellular localization.

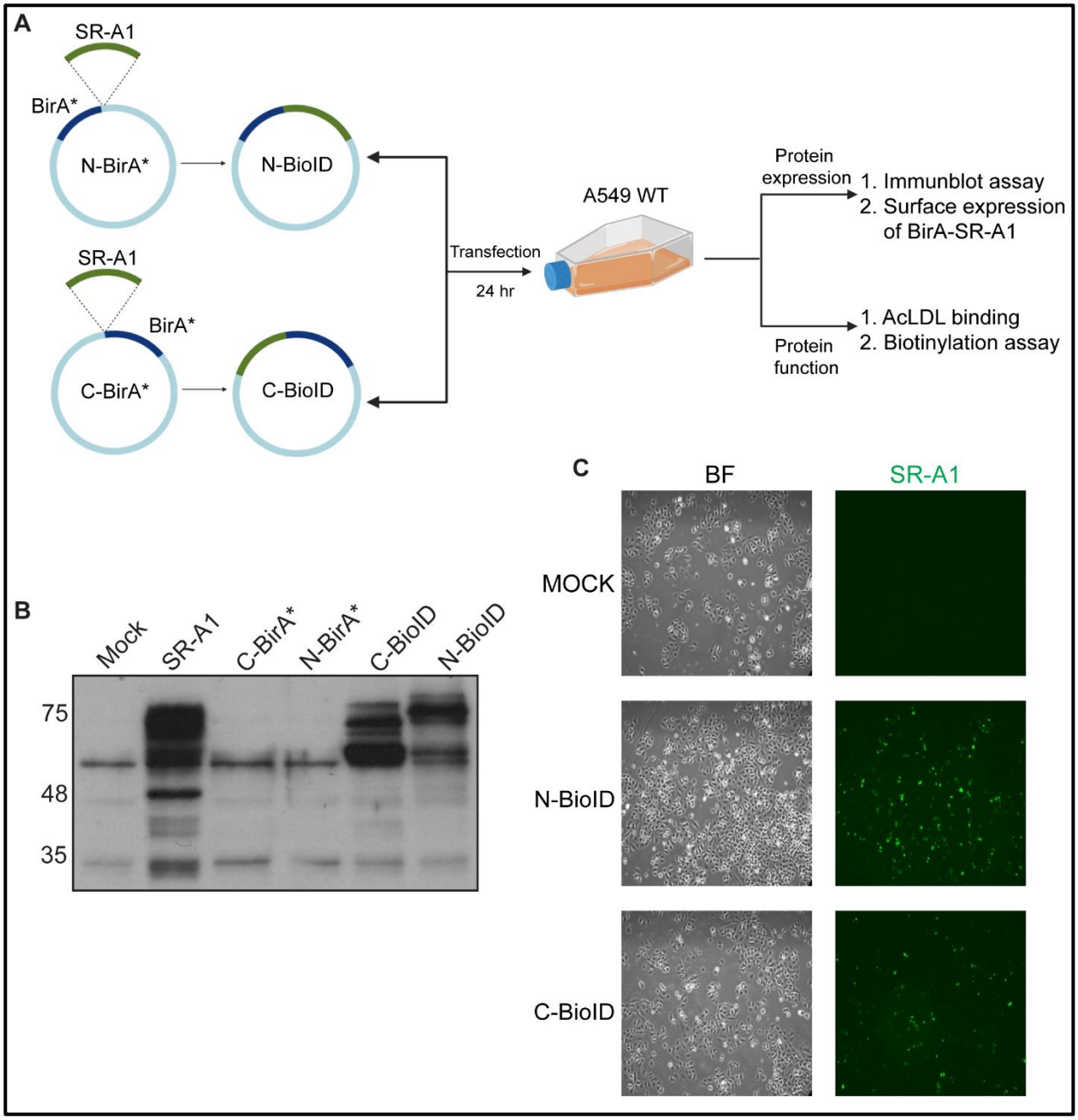
A
SR-A1
BirA*
N-BirA*
N-BioID
SR-A1
BirA*
C-BirA*
C-BioID
Transfection
24 hr
A549 WT
Protein expression
1. Immunblot assay
2. Surface expression of BirA-SR-A1
Protein function
1. AcLDL binding
2. Biotinylation assay
B
Mock
SR-A1
C-BirA*
N-BirA*
C-BioID
N-BioID
75
48
35
C
BF
SR-A1
MOCK
N-BioID
C-BioID

Figure 4.2.1. Attachment of BirA* on C- or N-terminus does not impact expression and targeting of SR-A1 in human lung epithelial cells.
(A) Schematic representation of the experimental strategy. A549 WT cells were transfected with N-BioID or C-BioID expression plasmids for 24 h. The cells were then subjected to expression and functional analyses for BirA-SR-A1 fusion protein.
(B) BirA-SR-A1 fusion protein expression observed by immunoblot analysis in A549 WT cells transfected with N-BioID or C-BioID expression plasmids for 24 h. N-BirA* and C-BirA* plasmids were used as empty vector controls. Plasmid encoding SR-A1 was used as a positive control (n=3).
(C) Surface localization of SR-A1 using fixed cell fluorescence microscopy in A549 WT cells transfected with N-BioID and C-BioID expression plasmids for 24 h (n=3).
WT, wild type; BF, bright field.

4.2.2. BirA* and SR-A1, constituting the BioID fusion protein retain their activity in human lung epithelial cells

Following protein expression analyses of the fusion proteins, we then determined their functionality. Functional SR-A1 was determined by investigating acLDL binding (Schematic 4.2.1A). AcLDL is a well-characterized ligand for SR-A1, SR-A1.1 and SR-A6 (183) but not SR-A5 (176). SR-A3 is expressed intracellularly and its ability to bind acLDL has yet to be elucidated (19). A549 WT cells expressing either the N-BioID or the C-BioID fusion protein were able to bind fluorescently labeled acLDL but with a lower efficiency than cells expressing SR-A1 (Figure 4.2.2A), likely due to differences in the expression levels. As expected, the mock transfected cells did not bind acLDL (Figure 4.2.2A) as they express only SR-A3 and SR-A5 (282).

Similarly, the function of BirA* was confirmed using a biotinylation assay (Schematic 4.2.1A). We supplemented exogenous biotin to A549 WT cells expressing the fusion proteins and observed the biotinylation of proteins through a biotin immunoblot

(Figure 4.2.2B). C-BioID biotinylated with lower efficiency than N-BioID (Figure 4.2.2B). Also, N-BirA* displayed substantial biotinylation but on the contrary, we observed minimal biotinylation intensity from C-BirA* (Figure 4.2.2B). Although attaching BirA* on either the C-terminus or N-terminus of SR-A1 did not affect expression, localization, or function of SR-A1, ligating BirA* on the C-terminus of SR-A1 decreased the number of biotinylation events (Figure 4.2.2B), possibly due to its extracellular position. The extracellular location of BirA* in C-BioID also limits the detection of relevant protein interactions to proteins present only within the endosomal lumen and other proximal transmembrane proteins. Therefore, to identify protein interactions for the cytoplasmic domain of SR-A1 during trafficking of extracellular dsRNA, N-BioID was chosen as a more suitable candidate fusion protein to perform the BioID screen.

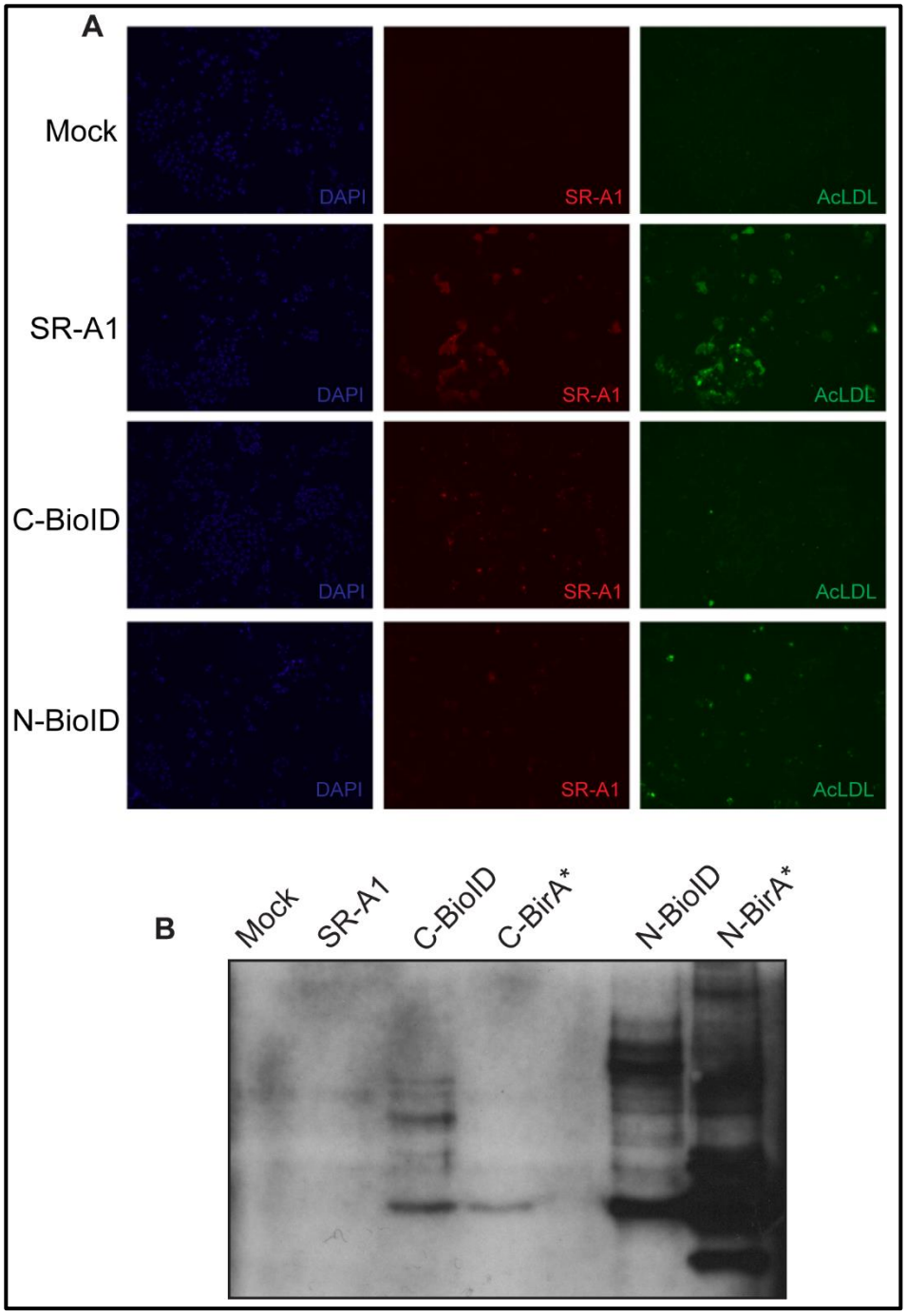
A
Mock
SR-A1
C-BioID
N-BioID
DAPI
SR-A1
AcLDL
B
Mock
SR-A1
C-BioID
C-BirA*
N-BioID
N-BirA*

Figure 4.2.2: BirA* and SR-A1, constituting BioID fusion protein retain their activity in human lung epithelial cells.
(A) Functional SR-A1 assessed by fixed cell fluorescence microscopy in A549 WT cells transfected with N-BioID and C-BioID expression plasmids for 24 h. The cells were then treated with Alexafluor 488 labeled acLDL for 1 h. Plasmid encoding SR-A1 was used as a positive control for AcLDL binding (n=3). The brightness (+40%) and contrast (-20%) are enhanced equally for all images.
(B) Functional BirA* assessed using biotinylation assay in A549 WT cells transfected with N-BirA*, C-BirA*, N-BioID, and C-BioID expression plasmids for 24 h and treated with exogenous biotin (50µM) for 24 h. Plasmid encoding SR-A1 was used as a control for endogenous biotinylation (n=3).

4.2.3. Functional validation of inducible N-BioID in human lung epithelial cells

Based on the transient expression analyses of N-BioID, both SR-A1 and BirA* retained their functional abilities while being expressed as a fusion protein (Figure 4.2.2A-B) but the cells bound AcLDL with varying intensities (Figure 4.2.2A), likely due to the differential expression of N-BioID. Typically, expression of BioID fusion protein at low levels (at or below the level of the endogenous protein) are sufficient for the identification of candidate proteins (287). Therefore, to circumvent undesirable overexpression of N-BioID and obtain similar levels of expression in each cell, we designed a tetracycline-inducible gene expression system to regulate the expression of N-BioID in human A549 WT cells. Utilizing this system, a control cell line expressing the regulatory protein rtTA only (A549 rtTA) and an experimental cell line expressing both, rtTA and N-BioID (A549 BioID) were generated (Schematic 4.2.3A). Immunoblot analysis confirmed the inducible expression of full-length N-BioID (~85 kDa) and its

post-translationally modified forms (Figure 4.2.3B) and A549 rtTA showed no evidence of N-BioID expression upon induction with doxycycline (Figure 4.2.3B).

Functional SR-A1 was assessed using an AcLDL binding assay. We treated A549 BioID cells with fluorescently labeled AcLDL, following induction of N-BioID expression with doxycycline. Cells expressing N-BioID bound AcLDL efficiently relative to control rtTA cells (Figure 4.2.3C). Functional SR-A1 restores antiviral capacity in SR-A deleted cells (282). Therefore, to determine whether SR-A1 retains its dsRNA-associated activity when expressed as the N-BioID fusion protein, we compared the differences in antiviral response to extracellular poly (I:C) stimulation in human lung epithelial cells. To quantify the antiviral response in A549 rtTA and A549 BioID cells, we performed bioassays using VSV-GFP. We treated A549 rtTA and A549 BioID cells with extracellular poly (I:C) following the induction of N-BioID expression with doxycycline and compared the extent of virus replication in A549 rtTA and A549 BioID cells by quantifying the amount of GFP expressed by replicating VSV-GFP. The cells expressing N-BioID displayed a robust extracellular poly (I:C)-induced antiviral protection relative to A549 rtTA and uninduced A549 BioID cells, leading to undetectable virus replication (Figure 4.2.3D).

Next, the ligase activity of N-BioID was validated using a biotinylation assay, as in Figure 4.2.2B. Following the induction of N-BioID expression with doxycycline, exogenous biotin was added to A549 rtTA and A549 BioID cells. In cells expressing N-

BioID, we observed a small amount of promiscuous biotinylation without exogenous biotin, and a much higher biotinylation intensity when biotin was supplied (Figure 4.2.3E). Mock induced A549 BioID cells displayed background biotinylation alongside A549 rtTA cells representing the naturally biotinylated proteins (Figure 4.2.3E) (287).

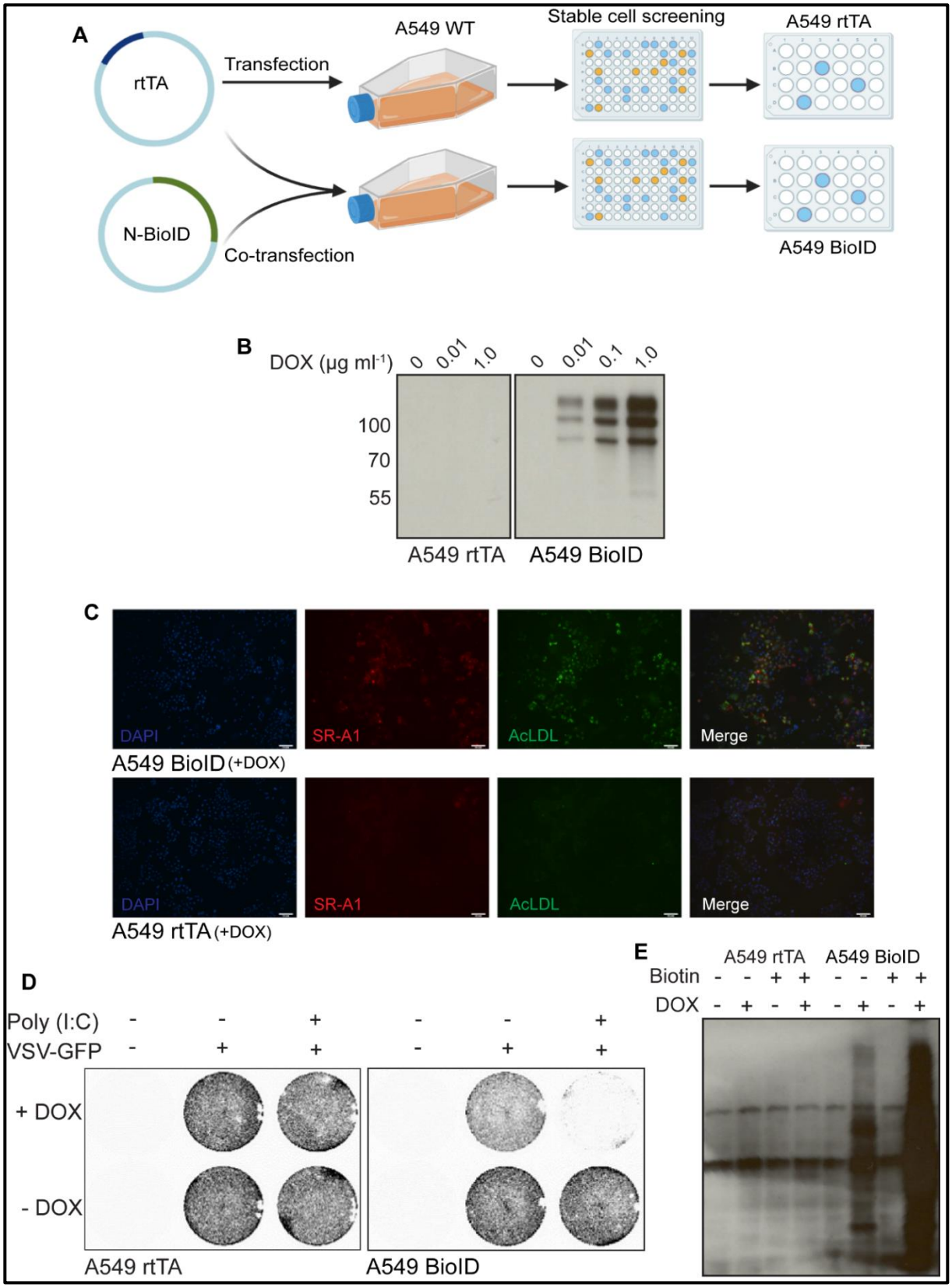
A
rtTA
Transfection
A549 WT
Stable cell screening
A549 rtTA
N-BioID
Co-transfection
A549 BioID
B
DOX (μg ml-1)
0
0.01
1.0
0
0.01
0.1
1.0
100
70
55
A549 rtTA
A549 BioID
C
DAPI
SR-A1
AcLDL
Merge
A549 BioID (+DOX)
DAPI
SR-A1
AcLDL
Merge
A549 rtTA (+DOX)
D
Poly (I:C)
VSV-GFP
+ DOX
- DOX
A549 rtTA
A549 BioID
E
A549 rtTA
A549 BioID
Biotin
DOX

Figure 4.2.3. Functional validation of inducible N-BioID in human lung epithelial cells.
(A) Schematic representation of the experimental strategy. Human A549 WT lung epithelial cells were transfected with rtTA or co-transfected with rtTA and N-BioID plasmids and performed clonal selection and expansion of stable cells.
(B) Tightly regulated expression of N-BioID at the protein level observed by immunoblot analysis in A549 BioID and A549 rtTA cells induced with doxycycline for 24 h (n=3).
(C) Functional SR-A1 assessed by fixed cell fluorescence microscopy in A549 rtTA and A549 BioID cells induced with 0.1 µg ml^{-1} doxycycline for 24 h and treated with Alexafluor 488 labeled acLDL for 1 h (n=3). The brightness is enhanced equally for all images by +40%.
(D) Antiviral responses measured in A549 rtTA and A549 BioID cells mock induced or induced with 0.1 µg ml^{-1} doxycycline for 24 h and treated with 0.5 µg ml^{-1} extracellular poly (I:C) (n=3) for 24 h, followed by infection with VSV-GFP (MOI = 0.1). 24 h post infection, GFP expression was measured as a surrogate for virus replication.
(E) Functional BirA* assessed using biotinylation assay in A549 rtTA and A549 BioID cells mock induced or induced with 0.1 µg ml^{-1} doxycycline for 24 h and treated with exogenous biotin (50µM) for 24 h (n=3).
DOX, doxycycline.

4.2.4. Optimizing the protein expression level and biotin-labeling efficiency of N-BioID

Overexpression of BioID fusion proteins can often lead to inappropriate localization within the cell and eventually result in the biotinylation of proximal proteins that otherwise would not have been in the local intracellular environment of the target protein, contributing to non-specific interactions. Ideally, low level expression of BioID fusion proteins enriches specific interactions for a target protein (287, 289). Therefore, to determine the optimal expression level of N-BioID sufficient to mediate its dsRNA-associated activity, we compared the differences in antiviral responses to extracellular poly (I:C) stimulation in human lung epithelial cells. To quantify the antiviral response in A549 BioID cells, we performed bioassays using VSV-GFP. We treated A549 BioID cells with extracellular poly (I:C) following the differential induction of N-BioID

expression with doxycycline and compared the extent of VSV-GFP replication in A549 BioID cells. VSV-GFP replication was inhibited in cells expressing N-BioID relative to uninduced A549 BioID cells and the potency of the antiviral response increased with the dose and duration of doxycycline treatment (Figure 4.2.4A). Importantly, we noted comparable antiviral responses in A549 BioID cells induced with 0.1 μg ml^{-1} doxycycline for 6 hours or 0.01 μg ml^{-1} doxycycline for 12 hours (Figure 4.2.4A) with minimal visible toxicity associated with overexpression of SR-A1 (282).

Receptor-mediated endocytosis following ligand binding is often a dynamic process that occurs on the timescale of minutes or just a few hours and is applicable to SR-A1 mediated uptake of extracellular dsRNA as well (290). Additionally, longer periods of biotin labeling saturate proximal nucleophiles with biotin, enabling BioID-generated biotinyl-AMP (active form) to travel farther and biotinylate distal, non-specific proteins. Therefore, to reduce the biotin labeling period for N-BioID, a shorter window of relevant biological activity pertaining to uptake and trafficking of extracellular dsRNA was identified in human lung epithelial cells. We evaluated the kinetics of extracellular dsRNA-induced antiviral responses by using quantitative RT-PCR. Following the induction of N-BioID expression with doxycycline, we treated A549 BioID cells with extracellular poly (I:C) for increasing periods of time and quantified transcripts of interferon-stimulated gene 56 (ISG56). We observed an increase in ISG56 transcripts with time and, importantly, noted induction of ISG56 as early as 2 hours following extracellular poly (I:C) treatment (Figure 4.2.4B). As sensing of extracellular dsRNA is

upstream of production of ISGs, the period prior to induction of ISGs represents the relevant biological activity for N-BioID.

The optimization of biotin labeling efficiency was based upon the catalytic activity of BirA*. Following the addition of biotin, BirA* begins to produce reactive biotinyl-AMP (active form) with ATP and reaches its peak biotinylation activity at 15-24 hours. Extended periods of biotin-labeling also lead to biotinylation of non-specific proteins, adding to background noise. Therefore, to determine sufficient catalytic activity of BirA* required to capture relevant interactions for SR-A1, a temporal analysis of biotinylation activity was performed using a biotinylation assay. Following the induction of N-BioID expression with doxycycline, we treated A549 BioID cells with exogenous biotin and assessed biotinylation intensity using a biotin immunoblot. A549 BioID cells displayed increasing biotinylation activity over time relative to untreated cells (Figure 4.2.4C). Particularly, a subtle increase in biotinylation activity was noted between the first and the second hour following biotin treatment indicative of the presence of reactive biotinyl-AMP (Figure 4.2.4C). Therefore, the protein expression levels and biotin labeling efficiency of N-BioID were optimized to minimize non-specific interactions for SR-A1.

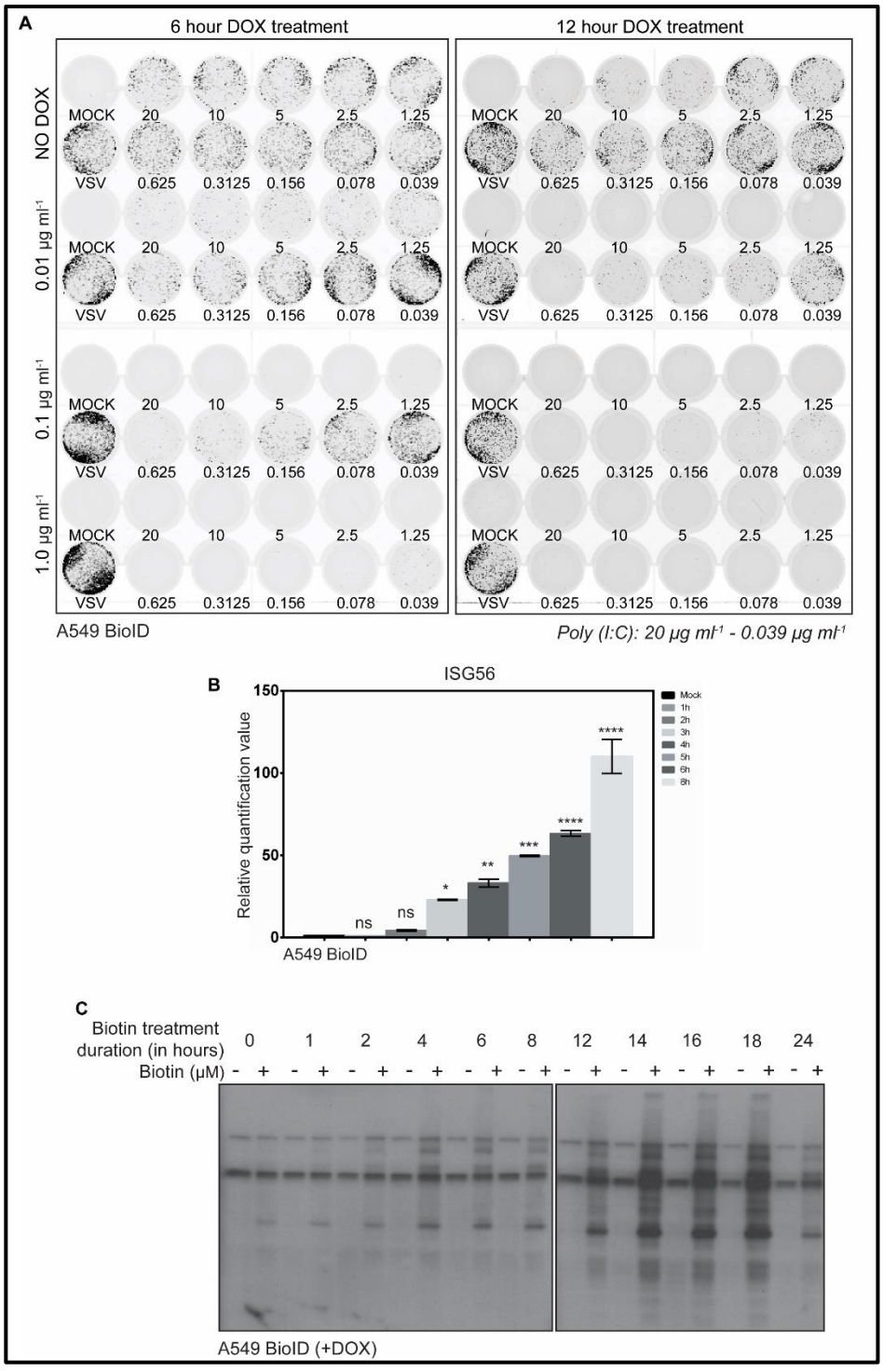
A
6 hour DOX treatment
12 hour DOX treatment
NO DOX
0.01 µg ml-1
0.1 µg ml-1
1.0 µg ml-1
MOCK
VSV
20
10
5
2.5
1.25
0.625
0.3125
0.156
0.078
0.039
A549 BioID
Poly (I:C): 20 µg ml-1 - 0.039 µg ml-1
B
ISG56
Relative quantification value
150
100
50
0
Mock
1h
2h
3h
4h
5h
6h
8h
ns
ns
*
**
***
****
****
A549 BioID
C
Biotin treatment duration (in hours)
0 1 2 4 6 8 12 14 16 18 24
Biotin (µM)
A549 BioID (+DOX)

Figure 4.2.4. Optimizing protein expression of N-BioID for optimal SR-A1 and BirA* activity for BioID screen.
(A) VSV-GFP replication in A549 BioID cells mock induced or induced with doxycycline for indicated lengths of time and treated with extracellular poly (I:C) (n=3) for 24 h.
(B) ISG56 transcript levels were measured by quantitative RT-PCR in A549 BioID cells mock induced or induced with 0.01 µg ml^{-1} doxycycline for 12 h and treated with 0.5 µg ml^{-1} extracellular poly (I:C) for indicated lengths of time (n=3).
(C) Biotin immunoblotting to observe biotinylated proteins in A549 BioID cells induced with 0.01 µg ml^{-1} doxycycline for 12 h and treated with exogenous biotin (50µM) (n=3) for indicated lengths of time.
Statistical significance was calculated by a two-way ANOVA with a **(A)** Dunnett's post-test. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
ns, not significant; DOX, doxycycline.

4.2.5. Pathway enrichment of SR-A1 interactions provide no direct evidence of *bona fide* antiviral processes

Biological processes rely on precise spatial organization and dynamic temporal reshaping of local protein interaction networks within cells (291). While affinity purification (AP) coupled with MS (AP-MS) captures the immediate biochemical environment of a target protein, it is merely a snapshot of underlying protein networks at a given time (290, 292-295). Thus, it was important to capture the evolution of SR-A1's underlying local protein interaction networks over time to obtain temporal resolution of its extracellular dsRNA-associated activity. To determine protein interactions for SR-A1 over time, we implemented findings from Figure 4.2.4 in the experimental design for the BioID screen. The expression of N-BioID fusion protein was induced with 0.01 µg ml^{-1} doxycycline for 12 h (for optimal N-BioID expression) in A549 BioID cells, followed by incubation with exogenous biotin for 1 h (for generating reactive biotinyl-AMP) prior to the addition of extracellular poly (I:C). A549 BioID cells not treated with extracellular

poly (I:C) served as a control. Protein lysates were harvested at different times post extracellular poly (I:C) treatment and AP-MS was performed to determine the identity of the biotinylated proteins. A student's t-test was applied to identify the significant interactions by comparing the average log2 fold change of the proteins in cells treated with extracellular poly (I:C) relative to control cells. Only proteins that had a *p value* < 0.05 with the student's t-test and a fold enrichment score of at least 1.2 were considered as significant interacting proteins (Table VII). The SR-A1 protein interaction network contained a cumulative total of 125 proteins over different time points (Table VII). Temporal enrichment analysis using the REACTOME functional (Reactome FI) network database identified multiple biological processes including RNA metabolism, endocytosis, protein targeting, post-translations modifications and more (based on the highest enriched pathways for each protein cluster) (Figure 4.2.5A). Pathways related to RNA metabolism were enriched at each time point but importantly, we observed a temporal shift in SR-A1 specific protein interaction networks (Figure 4.2.5A). We identified endocytosis and proteasome linked processes soon after the addition of extracellular poly (I:C) on A549 BioID cells (15 mins). Over time, processes linked to protein metabolism such as Asparagine-N-linked glycosylation (30 mins) and SRP-dependent cotranslational protein targeting to membranes (120 mins) were enriched. At later times (240 mins), we observed processes linked to RNA metabolism and antigen presentation. Despite the lack of enrichment of well characterized antiviral processes in extracellular poly (I:C) treated cells, the possibility of SR-A1 specific protein interaction networks modulating antiviral processes such as transcription and translation of viral and

host proteins cannot be disregarded. While pathway and network analysis provide a global picture of various signaling processes that are triggered via SR-A1 in response to extracellular dsRNA, it is critical to validate the physical interaction of SR-A1 and other candidate proteins through various molecular techniques.

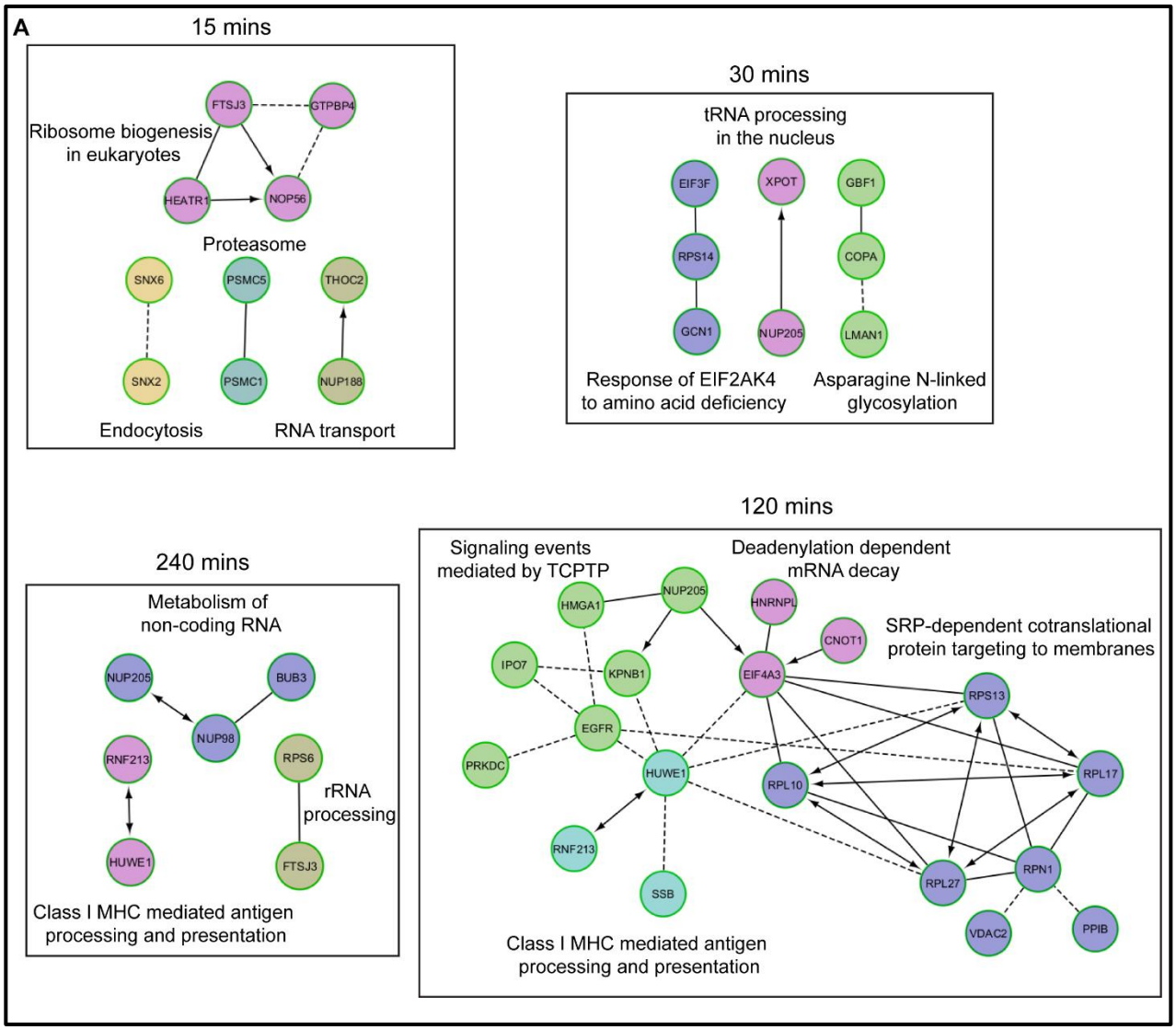

Figure 4.2.5. SR-A1 interactions do not constitute *bona fide* antiviral processes.
A549 BioID cells were mock treated or treated with 10 µg ml^{-1} of extracellular poly (I:C) for indicated lengths of time (in the presence of 50 µM biotin) following induction of N-BioID expression with 0.01 µg ml^{-1} doxycycline for 12 h and 1 h pre-treatment with biotin (50 µM). Biotinylated proteins pulled down using streptavidin beads were run through the mass spectrometer. Extracellular poly (I:C) treated A549 BioID (n=3) protein abundances were compared to the untreated control protein abundances and only proteins that were enriched by 20% ($p < 0.05$ by Student's t-test) were used to perform **(A)** enrichment analysis with the REACTOME Functional Interaction (Reactome FI) Network (FDR <0.05) and visualized on Cytoscape _v3.8.0. Each protein cluster represents a specific biological pathway and is distinguishable by color. Edges are displayed based on FI direction attribute values; "→" for activating/catalyzing, " − " for FIs extracted from complexes, and "---" for predicted FIs

Table VII: List of candidate interactors for SR-A1

Time	Accession	Mock treated			Poly (I:C) treated			Foldchange	*p value*
		*BR1	BR2	BR3	BR1	BR2	BR3		
15 mins	P46013	2	3	3	5	12	8	3.125	0.025483
	Q9P287	1	1	1	4	2	2	2.66666667	0.033383
	Q86T24	2	1	1	3	4	2	2.25	0.033383
	P62195	1	2	2	4	3	4	2.2	0.006618
	Q9Y2T2	2	1	2	3	5	3	2.2	0.02752
	Q9UNH7	1	2	4	4	6	5	2.14285714	0.032338
	P53396	2	2	3	3	6	5	2	0.034294
	Q01844	1	1	1	2	2	2	2	0
	Q6P2H3	2	2	1	3	4	3	2	0.012055
	Q86VP6	5	5	7	8	14	12	2	0.019869
	Q8NI27	1	1	1	2	2	2	2	0
	Q9H583	4	1	4	6	5	7	2	0.030085
	Q9BZE4	4	4	3	8	6	7	1.90909091	0.003745
	Q5SRE5	6	5	4	6	11	11	1.86666667	0.034967
	P62191	4	4	3	6	7	7	1.81818182	0.001563
	Q8IY81	3	3	2	6	4	4	1.75	0.02752
	P22102	4	5	6	9	10	7	1.73333333	0.012693
	O94905	4	4	3	6	7	6	1.72727273	0.002406
	Q92538	3	2	2	3	4	5	1.71428571	0.033383
	O00469	2	4	4	7	5	5	1.7	0.034294
	P40763	3	4	3	7	6	4	1.7	0.034294
	P56192	3	6	3	6	7	7	1.66666667	0.032338
	O43747	4	3	4	7	6	5	1.63636364	0.012448

	Q7Z6Z7	9	4	6	11	10	10	1.63157895	0.02752
	Q7KZF4	3	3	2	4	5	4	1.625	0.012055
	Q04637	3	4	3	6	5	5	1.6	0.006618
	O00567	4	4	5	6	8	6	1.53846154	0.017584
	P30041	6	3	4	7	7	6	1.53846154	0.034294
	P46940	14	11	11	19	20	14	1.47222222	0.027388
	O60749	6	6	5	10	7	8	1.47058824	0.02371
	P17655	5	4	4	7	6	6	1.46153846	0.006618
	P60891	4	5	3	5	6	6	1.41666667	0.033383
	P54136	6	6	8	9	9	10	1.4	0.011608
	Q14914	7	7	4	8	8	9	1.38888889	0.04563
	P62906	7	8	6	9	11	8	1.33333333	0.04563
	P63244	12	17	15	17	19	22	1.31818182	0.042812
	Q8WVV4	4	4	5	5	6	6	1.30769231	0.02371
	Q8TBA6	13	12	13	18	17	14	1.28947368	0.021197
	Q86Y07	8	7	8	10	9	9	1.2173913	0.012055
	Q15643	15	13	14	19	17	15	1.21428571	0.0404
	P23396	25	22	22	25	29	29	1.20289855	0.024406
30 mins	O00303	4	4	3	8	5	5	1.63636364	0.04563
	O00425	6	2	6	7	8	9	1.71428571	0.041737
	O43592	8	6	6	9	8	8	1.25	0.044505
	O95816	3	4	3	6	4	5	1.5	0.033383
	P18583	19	13	18	22	24	22	1.36	0.019153
	P22102	4	7	6	7	10	9	1.52941177	0.036963
	P49257	4	3	4	8	7	10	2.27272727	0.003881
	P49915	4	3	1	4	6	5	1.875	0.04563
	P53621	9	3	7	12	9	15	1.89473684	0.041823
	P62263	4	6	3	8	7	7	1.69230769	0.016736
	Q14914	6	5	6	6	9	9	1.41176471	0.04563
	Q15046	3	2	3	5	5	3	1.625	0.044505
	Q3KR37	1	1	1	3	2	4	3	0.012861
	Q86VP6	6	6	7	9	10	8	1.42105263	0.008065
	Q8TBA6	10	9	10	12	15	17	1.51724138	0.01423
	Q8TCT9	3	1	1	3	4	3	2	0.044505
	Q92538	1	2	3	3	4	4	1.83333333	0.033383
	Q92616	21	17	17	21	24	22	1.21818182	0.033306
	Q92621	8	7	14	14	14	16	1.51724138	0.046952
	Q96EP5	1	1	1	2	3	3	2.66666667	0.003745
	Q9BQG0	7	8	5	12	13	8	1.65	0.034967

	Q9BUJ2	1	1	2	4	3	2	2.25	0.033383
	Q9NTJ3	4	5	4	7	6	9	1.69230769	0.016736
	Q9P0L0	3	1	2	4	5	5	2.33333333	0.008065
	Q9Y277	6	4	5	10	6	9	1.66666667	0.033383
60 mins	O00469	4	4	2	4	7	8	1.9	0.047223
	P17096	2	2	2	3	3	3	1.5	0
	P61619	3	2	3	4	4	4	1.5	0.008065
	Q8N7C3	1	1	2	2	4	3	2.25	0.033383
	Q8TC12	1	1	1	2	2	2	2	0
	Q92604	1	1	1	2	2	2	2	0
	Q9Y6Y8	5	6	4	7	8	8	1.53333333	0.008065
120 mins	A5YKK6	1	1	1	2	2	2	2	0
	P54886	14	12	14	19	18	21	1.45	0.002795
	P17096	2	2	2	4	4	3	1.83333333	0.003745
	Q9NZN4	3	2	2	5	4	5	2	0.003881
	O15269	2	3	3	4	4	4	1.5	0.008065
	P23284	2	2	2	3	4	3	1.66666667	0.008065
	Q7Z6Z7	7	5	9	12	11	14	1.76190476	0.010689
	P61353	5	5	4	6	7	8	1.5	0.012448
	P14866	6	7	8	10	10	14	1.61904762	0.020321
	Q9Y6M7	9	8	5	13	10	12	1.59090909	0.021907
	Q92621	12	13	13	14	17	15	1.21052632	0.02371
	P02545	35	30	30	35	40	39	1.2	0.024371
	Q63HN8	20	12	14	29	23	41	2.02173913	0.02717
	P62277	2	3	2	3	5	5	1.85714286	0.02752
	P27635	5	4	6	8	6	8	1.46666667	0.028618
	P45880	5	6	4	8	6	8	1.46666667	0.028618
	Q6PKG0	2	2	4	5	4	6	1.875	0.028618
	Q8NB16	3	1	1	4	5	3	2.4	0.028618
	O60701	14	15	16	16	20	20	1.24444444	0.032554
	O95373	3	4	2	4	7	8	2.11111111	0.033383
	P05455	1	1	2	2	3	4	2.25	0.033383
	Q86T24	4	3	3	5	4	6	1.5	0.033383
	P04843	6	7	5	7	8	9	1.33333333	0.035242
	P78527	95	81	85	103	96	118	1.21455939	0.036339
	P38919	8	8	10	11	10	13	1.30769231	0.036694
	P18621	6	8	6	9	8	8	1.25	0.044505
	P00533	3	5	5	6	7	11	1.84615385	0.046326
	P27708	18	23	22	24	27	34	1.34920635	0.046326

	Q14974	4	6	2	6	7	10	1.91666667	0.046326
	Q03169	2	2	4	5	4	8	2.125	0.047223
240 mins	P16070	1	1	1	2	2	2	2	0
	Q7Z6Z7	4	5	5	11	15	13	2.78571429	0.001136
	P56192	2	3	2	5	6	7	2.57142857	0.002664
	P62753	5	6	5	7	8	8	1.4375	0.003881
	O43684	2	1	1	4	3	4	2.75	0.003881
	Q63HN8	15	13	15	22	26	30	1.81395349	0.004159
	Q13643	2	2	1	3	3	3	1.8	0.008065
	Q6NZI2	1	5	1	7	10	10	3.85714286	0.008065
	Q6WCQ1	3	3	2	4	5	4	1.625	0.012055
	Q9Y5B9	1	3	2	6	4	4	2.33333333	0.01951
	Q14566	5	3	2	8	6	6	2	0.019676
	Q86TB9	4	4	5	5	6	6	1.30769231	0.02371
	Q9Y6N5	1	1	2	2	3	3	2	0.02371
	P12956	3	6	3	6	8	8	1.83333333	0.025075
	Q86UP2	2	3	4	5	8	11	2.66666667	0.02599
	P47897	3	3	6	7	6	8	1.75	0.030085
	Q8IY81	3	7	5	8	8	8	1.6	0.030085
	Q9ULH0	4	7	5	9	8	7	1.5	0.032338
	P53621	11	14	11	15	15	14	1.22222222	0.032338
	Q14444	2	2	3	5	4	3	1.71428571	0.033383
	P52948	9	10	12	12	18	14	1.41935484	0.046461
	Q92621	13	8	11	14	13	16	1.34375	0.048593

Note: *BR - biological replicate

4.2.6. Human lung epithelial cells lacking SIDT2 mediate an impaired antiviral response against extracellular dsRNA

Besides endosomal TLR3, the cytoplasmic RLRs are also implicated in response to extracellular dsRNA (19) and are responsible for much of IFN-I responses upon systemic administration of poly (I:C) in mice (216). We also found that the response to short dsRNA species (~200bp) was completely reliant on cytoplasmic RIG-I (19) in MEFs suggesting that dsRNA somehow enters the cytoplasm. SIDT2 is a mammalian

ortholog of the *C. elegans* SID-1 dsRNA transporter (217, 218) which has been shown to transport dsRNA from late endo-lysosomal compartments into the cytoplasm for innate immune recognition in MEFs (221). Therefore, to determine whether SIDT2 plays a similar role in human cells, we compared the differences in antiviral responses to extracellular poly (I:C) stimulation in human lung epithelial cells. We determined SIDT2 (NM_001040455.2) expression at the transcript level by amplifying a region within SIDT2 mRNA using conventional RT-PCR (Figure 4.2.6A). Using CRISPR gene-editing technology, we deleted SIDT2 in ΔSRA +SR-A1 cells (refer to section 3.5.3) to perform loss-of-function analysis. Due to the lack of reliable antibodies for SIDT2, specific Cas9-mediated indels were verified by sequencing the corresponding genomic locus of SIDT2. Two separate clonal populations were identified, CR1.1 and CR2.2, each with an insertion of the nucleotide 'T' at positions 116 and 58, respectively, causing a frame shift and a premature stop codon at positions 369-372 bp and 105-108 bp, respectively (Figure 4.2.6B).

We noted variable expression of SR-A1 between ΔSRA +SR-A1 (Control), CR1.1 and CR2.2 cells following induction with doxycycline, likely a result of the clonal selection and expansion processes (Figure 4.2.6C). Since the role of SIDT2 is likely downstream of extracellular dsRNA uptake (221), it was essential to induce relatively similar levels of SR-A1 in Control, CR1.1 and CR2.2 cells to decrease variability in our experimental assays. Based on relative quantification of SR-A1 expression, CR1.1 and CR2.2 cells induced with 0.1 μg ml^{-1} doxycycline for 6 h showed similar expression

levels compared with Control cells induced with 0.1 μg ml^{-1} doxycycline for only 4 h (Figure 4.2.6D).

To determine whether SIDT2 plays a role in response to extracellular dsRNA in human lung epithelial cells, we compared the differences in the antiviral response to extracellular poly (I:C) stimulation in SIDT2 deleted cells. To quantify the antiviral response to extracellular dsRNA, we performed bioassays using VSV-GFP. We treated Control, CR1.1 and CR2.2 cells with increasing concentrations of extracellular poly (I:C) following induction of SR-A1 expression with doxycycline (Figure 4.2.6D) and compared the extent of virus replication in Control, CR1.1 and CR2.2 cells by quantifying the amount of GFP expressed by replicating VSV-GFP. CR1.1 and CR2.2 cells that lacked SIDT2, displayed impaired antiviral protection relative to Control cells as evident from VSV-GFP replication (Figure 4.2.6E)

Importantly, we noted disparity between CR1.1 and CR2.2 cells' ability to respond to extracellular poly (I:C) relative to Control cells (Figure 4.2.6E). Since clonal variability for SR-A1 expression was noted earlier (Figure 4.2.6C), we tested the ability of CR1.1, CR2.2 and Control cells to respond to transfected dsRNA using quantitative RT-PCR. We transfected increasing quantities of poly (I:C) into Control, CR1.1 and CR2.2 cells and quantified ISG56 transcripts. Control and CR2.2 cells displayed a concurrent increase in ISG56 transcripts with poly (I:C) amounts while CR1.1 cells were unable to respond as efficiently (Figure 4.2.6F). Together, these findings suggest that

deleting SIDT2 negatively impacts the cells' ability to respond to extracellular dsRNA and further investigation is necessary to establish the role of SIDT2 in human cells with certainty.

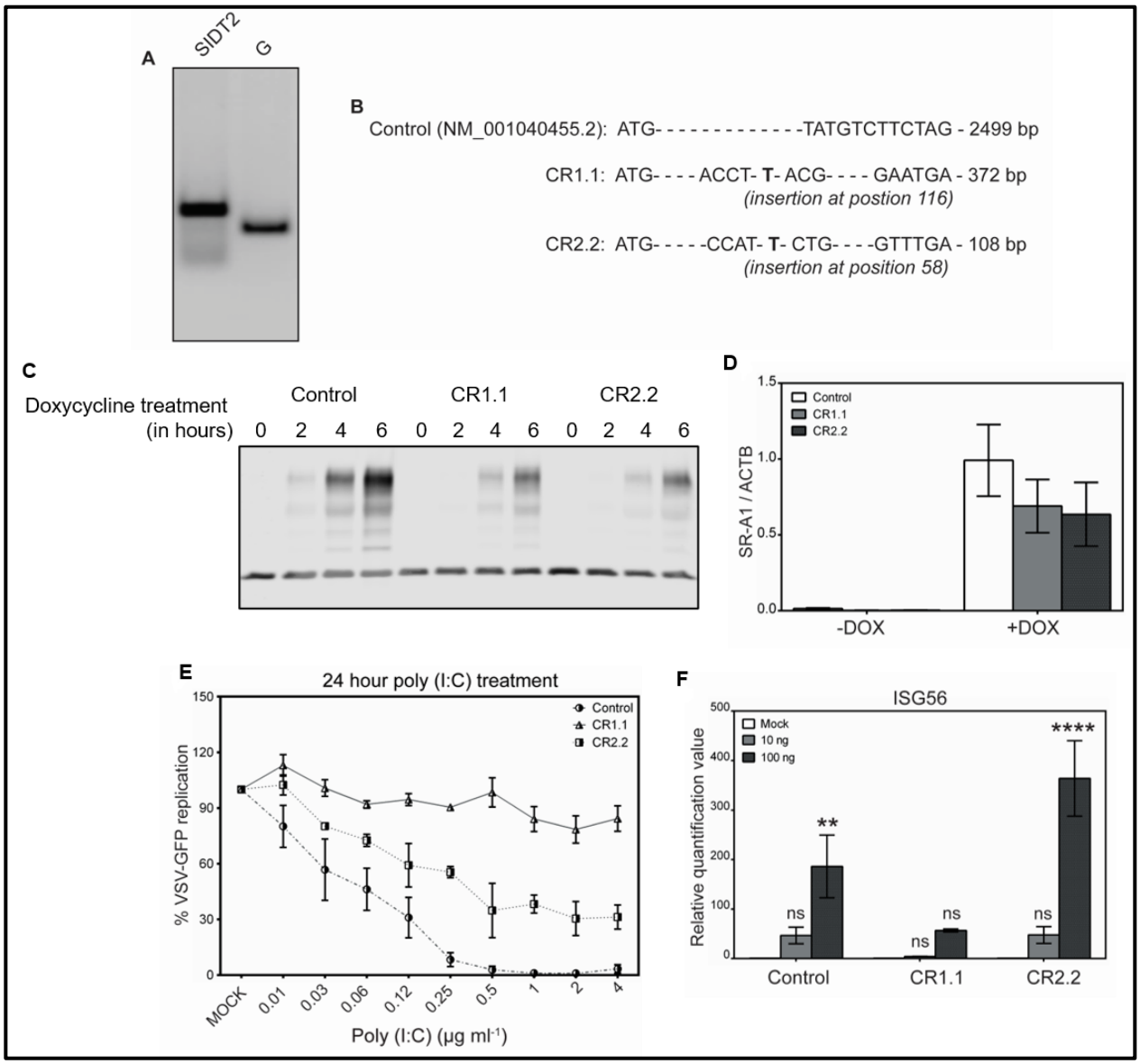

Figure 4.2.6. Human lung epithelial cells lacking SIDT2 mediate an impaired antiviral response against extracellular dsRNA.
(A) Conventional RT-PCR analysis to detect a 422 bp long portion within mRNA transcript of SIDT2 in A549 WT lung epithelial cells. GAPDH (G) was used as a control.
(B) Sanger sequencing analysis of CRISPR-Cas9 mediated indels generated at SIDT2 genomic locus in edited ΔSRA +SR-A1 cells.
(C) SR-A1 expression observed by immunoblot analysis in Control, CR1.1 and CR2.2 cells mock induced or induced with 0.1 μg ml^{-1} doxycycline for indicated lengths of time (n=3).
(D) Quantification of SR-A1 expression relative to housekeeping gene, GAPDH using immunoblot analysis in Control cells mock induced or induced with 0.1 μg ml^{-1} doxycycline for 4 h (n=3) and in CR1.1 and CR2.2 cells mock induced or induced with 0.1 μg ml^{-1} doxycycline for 6 h (n=3). No significant differences were observed.
(E) VSV-GFP replication in Control, CR1.1 and CR2.2 cells induced with 0.1 μg ml^{-1} doxycycline for 4 h (Control) and 6 h (CR1.1 and CR2.2) and treated with increasing concentrations of extracellular poly (I:C) (n=3) for 24 h.
(F) ISG56 transcript levels were measured by quantitative RT-PCR in Control, CR1.1 and CR2.2 cells mock transfected or transfected with 10 ng or 100 ng of poly (I:C) for 8 h (n=3).
Statistical significance was calculated by a two-way ANOVA with a Dunnett's post-test. Data are represented as mean ± SEM. **$p < 0.01$, ****$p < 0.0001$.
G, gapdh; bp, base pair; ns, not significant

4.3. Summary of results

Extracellular dsRNA released from virally infected cells are key inducers of bystander antiviral immunity in neighbouring cells and can also cause inappropriate inflammatory responses systemically. Therefore, it is important to gain a better understanding of the mechanisms of extracellular dsRNA signaling. In this study, we identified cellular processes that became enriched in a SR-A1-dependent manner following extracellular dsRNA binding, providing insights into extracellular dsRNA-induced signaling events (Figure 4.3). Furthermore, we illustrated that the involvement of cytoplasmic RLRs in response to extracellular dsRNA may be due to the activity of SIDT2, a mammalian ortholog of *C. elegans* SID-1 dsRNA transporter. The lack of

SIDT2 negatively impacted the ability of human lung epithelial cells to respond to extracellular dsRNA (Figure 4.3).

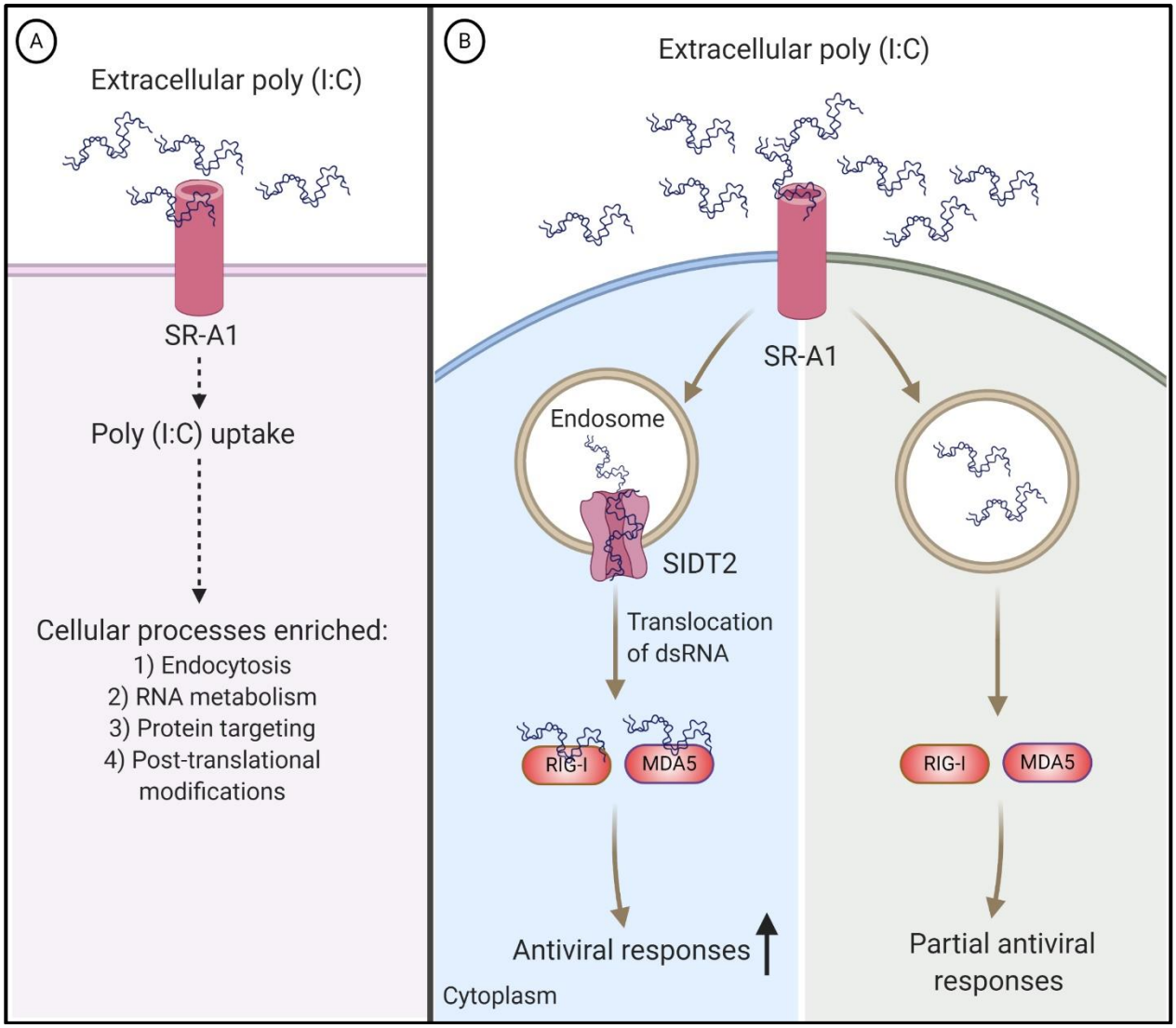

Figure 4.3. Summary of Chapter 4.
(A) Cellular processes such as endocytosis, RNA metabolism, protein targeting, and post-translational modifications are enriched in human lung epithelial cells in a SR-A1-dependent manner following extracellular dsRNA uptake.
(B) The loss of SIDT2 in human lung epithelial cells leads to an intermediate antiviral response. Image created with BioRender.com.

Chapter 5: General discussions

5.1. Overview

Nucleic acids, such as DNA and RNA, can function as pathogen- or danger-associated molecular patterns, depending on their origin, inducing innate immune responses and the development of autoimmune disorders. Scavenger receptors are a diverse group of PRRs that sense a variety of ligands (157) and SR-As are now well appreciated as important mediators of extracellular dsRNA-induced cellular responses via their ligand recognition and internalization (19, 179, 215). Despite their ability to bind and internalize extracellular, circulating dsRNA, it remains unknown whether SR-As are necessary and sufficient for this function. Furthermore, less is known about the ability of SR-As to bind and internalize DNA. Complicating these issues is the discovery of additional surface receptors that can bind and internalize nucleic acids, including DEC-205 (250), Mac-1(253) and SR-J1 (238, 251). This thesis was an effort to better understand the role of SR-A1 in the recognition, uptake and signaling mechanisms of extracellular nucleic acids including dsRNA. The discussions on the findings presented in this thesis are divided into three sections for the ease of the reader, (**a)** Ligand specificity of SR-A1, **(b)** Role of SR-A1 in extracellular dsRNA-mediated signaling, and **(c)** Intracellular fate of extracellular dsRNA.

5.1.1. Ligand specificity of SR-A1

SR-A1 exhibits a broad ligand specificity, including chemically modified or altered molecules, bacterial components, apoptotic cells and polyribonucleotides (157). SR-A1 is now well appreciated as an important mediator of cellular responses induced by extracellular dsRNA (using virus-specific sequences or poly (I:C), a viral mimetic) via its ligand recognition and internalization (19, 179, 215). While several studies suggest that SR-A1 non-specifically binds polyanionic ligands, a previous study showed that SR-A1 does not bind ligands based solely on their charge (184, 185). Indeed, while poly I and poly G demonstrate high affinity for SR-As, poly A and poly C do not, with polynucleotide binding depending on quadruplex structure as well (185). Moreover, the ability of SR-A1 to recognize and mediate uptake of DNA remains less understood. Specifically, we previously noted that poly dA:dT only partially inhibited acLDL binding to murine fibroblasts in comparison to poly (I:C) (19), suggesting a preference for specific polyanionic nucleic acids, a specificity that is poorly understood.

Based on previous studies that implicated the collagenous domain of SR-A1 as a putative ligand-binding domain (170, 181), we investigated *in vitro* the nucleic acid binding properties of purified recombinant coiled-coil collagenous domain using electrophoretic mobility shift assay. Consistent with previous studies that showed SR-A1 binds polyanionic ligands via the collagenous domain (170, 181), our findings demonstrate a strong interaction of the coiled-coil collagenous domain with all nucleic acid species tested. We noted that the purified coiled-coil collagenous domain was

sufficient to bind DNA, ssRNA and dsRNA species independent of length or sequence. To our knowledge, this is the first study that demonstrates a direct binding of different nucleic acid species to the coiled-coil collagenous domain of SR-A1. Moreover, the different nucleic acids used in this study were derived from cloned WNv envelope gene sequence instead of synthetic polynucleotides such as poly I, poly (I:C), suggesting that virus-specific sequences are also able to participate in complex conformational interactions with the collagenous domain of SR-A1. While using virus-specific sequences in nucleic acid binding assays adds novelty to our study, these observations require further validation in a cell culture system or another physiologically relevant model.

While we did not perform precise binding assays to determine whether different nucleic acid species bound the coiled-coil collagenous domain of SR-A1 with similar kinetics and affinity, we did note a similar ability to form a RNP complex when using the same ratio of nucleic acid to recombinant coiled-coil collagenous protein. Moreover, both DNA and dsRNA of similar length and sequence were able to compete for binding to the coiled-coil collagenous domain with labeled dsRNA. However, employing a technique such as surface plasmon resonance (SPR) would prove definitively whether the coiled-coil collagenous domain preferentially binds one nucleic acid species over another (296). It would also provide insights into the requirement for an overall negative charge on the molecule and the role of secondary structures of nucleic acids in mediating more robust interactions with the collagenous domain of SR-A1.

The other SR-A family members share ligand specificity with SR-A1, albeit they bind to their ligands using distinct mechanisms (256-259). We are unaware of studies that demonstrate direct binding of nucleic acids to another member of the SR-A family. The SRCR domain of SR-A6 has been postulated as a ligand binding domain due to the presence of a highly conserved, positively charged RGRAEVYY motif (171, 172). Therefore, we investigated the nucleic acid binding properties *in vitro* of purified recombinant SRCR domain as we did for coiled-coil collagenous domain. Despite the positively charged RGRAEVYY motif in the SRCR domain (171), our findings demonstrate that the SRCR domain failed to interact with all nucleic acid species tested, reiterating that the nucleic acid binding to SR-A1 is not based solely on charge and requires more complex conformational interactions (185). Interestingly, all members of the SR-A family display a high degree of conservation between their collagenous domains (161), yet their nucleic acid specificity remains unclear. In fact, trout MARCO (XM_014173984.1) fails to bind poly (I:C), a synthetic analogue of dsRNA (297). Thus, it would be interesting to determine whether the collagenous domains of other human SR-As bind different species of nucleic acids and if they do so with similar efficiency to the collagenous domain of SR-A1. Findings from these studies would enable a better understanding of the functional redundancy amongst SR-As in the context of sensing nucleic acids during virus infections and/or tissue damage.

In humans, SR-A1 and SR-A6 are important components of host defense against bacterial pathogens such as *Neisseria meningitides* (157, 298), and *S. pneumoniae* (269, 299). The SRCR domain of SR-A6 binds bacterial components or whole bacteria (171, 172) whilst the SRCR domain of SR-A1 is dispensable likely due to the lack of the RGRAEVYY motif (161, 168). Therefore, we examined the ability of recombinant coiled-coil collagenous domain of SR-A1 to bind *S. pneumoniae.* Consistent with the ability of SR-A1 to bind to the lipid A moiety of LPS (a feature of Gram-positive bacteria such as *S. pneumoniae*) (157, 299), the coiled-coil collagenous domain bound *S. pneumoniae,* albeit less efficiently relative to the SRCR domain of SR-A6. The SR-A1 has been previously shown to interact with TLR4 or TLR2 to promote phagocytosis of bacteria (157) and the lack of a co-operating PRR in our *in vitro* nucleic acid binding assays might explain the lower binding efficiency of collagenous domain to *S. pneumoniae*.

Recent studies have established that the residues outside of the ligand binding region of the SRCR domain impacts the receptor-ligand interaction (300) highlighting the importance of using a more relevant model to verify ligand binding specificities of SR-A1. Therefore, we determined whether primary human embryonic lung fibroblasts, which express SR-A1 (19), bind and internalize DNA and dsRNA. Using a quantitative nucleic acid uptake assay, we found that human fibroblasts bound and internalized dsRNA and DNA of similar sequence and length with similar efficiency. Furthermore, DNA binding and uptake was inhibited by fucoidan, a competitive ligand of SR-As, but not by the non-

competitive ligand fetuin. While these findings suggest that primary human fibroblasts bind and internalize DNA and dsRNA in a manner dependent on SR-A1, these ligands are unlikely to be specific only for SR-A1. Indeed, other cell surface receptors such as SR-J1 (238, 251) and DEC-205 (250) also recognize extracellular nucleic acids and some studies have implicated fetuin in modulating SR-J1 signaling (301), thus complicating analyses. While we found that the expression level was extremely low (SR-J1) or below the limit of detection (DEC-205) in human fibroblasts, they express other SR-As which display overlapping ligand specificities and might interfere with the analysis of SR-A1's nucleic acid specificity (19).

To investigate whether SR-A1 binds and internalizes DNA and dsRNA, we screened a diverse panel of human cell lines for SR-A family member expression using RT-PCR to identify a cell line deficient for all known SR-As. While all cell lines tested typically express several SR-A family members (19), we found that the human A549 lung epithelial cells expressed only SR-A3 and SR-A5. In addition to expression of limited SR-A members, A549 cells are also competent for IFN-I signaling and potentially express SR-J1 (265) and DEC-205 (266), thus allowing us to assess the role of SR-As in the context of additional cell surface nucleic acid binding proteins. Thus, A549 cells were used for further study; we generated SR-A deficient A549 cells using CRISPR technology and engineered them to express SR-A1 under the control of an inducible promoter. We then sought to determine the ability of cells expressing only SR-A1 to internalize DNA and dsRNA of similar length and sequence using fluorescence microscopy. Corroborating

our findings in human fibroblasts, SR-AI expressing cells internalized DNA *(unpublished work from Mossman lab)* and dsRNA efficiently, but SR-A deficient cells failed to do so. These findings strongly indicate that both DNA and dsRNA are ligands for SR-A1 and other nucleic acid receptors like SR-J1 and DEC-205 are dispensable for uptake of extracellular viral DNA or dsRNA in A549. Moreover, SR-A deficient A549 cells should serve as a suitable model to perform molecular analyses for ligand specificities of individual SR-A members which is otherwise difficult and more often inconclusive due to the presence of other SR-As.

In summary, this study shows that the coiled-coil collagenous domain of SR-A1 is sufficient to bind different nucleic acids independent of species, sequence, or length. Viral DNA and dsRNA generated *in vitro* were confirmed as ligands for SR-A1 and although other receptors like SR-J1 or DEC-205 also bind nucleic acids, there are perhaps small differences in ligand selectivity and binding mechanisms that might have evolved to increase the repertoire of innate immune recognition.

5.1.2. Role of SR-A1 in extracellular dsRNA-mediated signaling

DsRNA molecule is common to most, if not all, viral life cycles and is a potent inducer of antiviral and proinflammatory responses (15). Viral dsRNAs can also act as extracellular signaling molecules (106, 155) and prime uninfected cells for an ensuing virus challenge (90, 204), but our understanding of the mechanisms that mediate the effects of extracellular dsRNA remain unclear. SR-As have been shown to mediate cellular responses to extracellular dsRNA (19, 179, 215), but due to the presence of other cell surface receptors with similar properties, it remains unknown whether SR-As are necessary and sufficient for this function. While we previously found that siRNA-mediated knock-down of SR-A family members in MEFs resulted in lower levels of dsRNA binding and subsequent ISG induction (19), we were not able to fully deplete SR-As using this methodology. Moreover, while SR-As were initially characterized on phagocytic cells such as macrophages, we also noted that all cell types investigated express more than one SR-A, highlighting their importance and possible redundancy of function (19, 173).

To formally investigate the role of SR-A1 in extracellular dsRNA-mediated signaling, we first performed loss-of-function analyses using CRISPR-edited, SR-A deficient A549 cells (refer Section 5.1). In functional assays, we noted that parental A549 cells respond poorly to extracellular dsRNA and require the addition of DEAE-dextran to consistently observe biological responses (19, 215). DEAE-dextran is a cationic polymer that binds negatively charged nucleic acids and facilitates interactions with the negatively

charged cell membrane (302), without bypassing the requirement for cell surface receptors such as SR-As (19). Even with DEAE dextran, a relatively high concentration of poly I:C is required to induce an antiviral response in A549 cells, relative to human fibroblasts. We speculate that this observation is due to the expression of only two SR-A family members, of which SR-A3 is thought to remain intracellular (174). Notably, the ability of SR-A deficient A549 cells to respond to extracellular dsRNA was significantly reduced but not completely lost upon treatment with relatively high concentration of poly (I:C). To test if the residual activity was due to the presence of other nucleic acid binding receptors like SR-J1 or DEC-205, we assessed their expression levels by qRT-PCR and found that their expression was extremely low. It is unclear currently whether an unknown receptor or an alternative means of entry is facilitating the uptake of extracellular dsRNA when present in high concentrations along with DEAE-dextran.

To address whether SR-A1 is sufficient in mediating responses to extracellular dsRNA, we attempted to overexpress SR-A1 in SR-A deficient A549 cells. However, we were unable to generate stable clones constitutively overexpressing SR-A1 *(unpublished work from Mossman lab)*, despite validation of the expression construct in transient assays. We were successful, however, in generating an inducible cell line, where SR-A1 expression is tightly regulated by the tetracycline response element. Using this system, we can control the expression of SR-A1 and noted a concurrent increase in toxicity and cell death with increased SR-A1 expression. These data suggest that the expression level of SR-A1 needs to be tightly regulated, which is not surprising given its pleiotropic roles,

including attachment and phagocytosis (157). Controlled expression of SR-A1 restored biological function with respect to the uptake of extracellular dsRNA, and subsequent induction of an innate antiviral response. Notably, cells expressing SR-A1 were highly efficient in dsRNA uptake and did not require the addition of DEAE-dextran to the media. Since DEAE-dextran has been shown to stimulate pinocytosis and facilitate the incorporation of macromolecules into host cells in a cell-type and concentration dependent manner (302), the lack of its need in our functional assays allowed us to eliminate the potential for extracellular dsRNA entry via alternate routes.

Although it is reported that A549 cells express high levels of SR-J1 (265), we found its expression to be too low in the A549 cells used in our study. This observation is consistent with other published literature suggesting that SR-J1 levels are low during homeostatic conditions in immune cells or endothelial cells, but expression increases in pathologies in which SR-J1 ligands such as nucleic acids, S100 proteins or HMGB1 (ligands which are released during cell stress or inflammation) accumulate (303, 304). Moreover, IFN-I has been shown to induce SR-J1 expression in multiple sclerosis patients (305) suggesting that while SR-J1 may be dispensable for uptake of extracellular viral dsRNA, it is possible that it can augment the SR-A1-mediated cellular responses to dsRNA, a hypothesis that remains to be tested. Interestingly, SR-J1 activity is amplified in response to sterile tissue damage via interactions with HMGB1-DNA/RNA complexes (306-308). Given that SR-A1 and SR-J1 display overlapping ligand specificities (157, 309) it is plausible for HMGB1-DNA/RNA complexes to interact with SR-A1 and

amplify SR-A1-mediated cellular responses. However, there is currently no evidence suggesting any interplay between SR-A1, SR-J1 and/ or HMGB1.

Several studies have shown that SR-A1 can also modulate innate immune responses to bacterial and viral pathogens (173). However, the findings from these studies are inconsistent as some suggest that SR-A1 enhances immune responses (204, 211, 212) and others suggest that SR-A1 functions to limit the immune response (199, 210). Indeed, we previously noted that in response to extracellular dsRNA, SR-A1 lacks the ability to induce IFN-I production independent of the prototypic TLR3 and the RLR pathways (215) whilst others have shown it can induce proinflammatory cytokines in response to extracellular dsRNA stimulation via MAPK and PKC pathways (179, 196). The cytoplasmic tail of SR-A1 lacks any conventional signaling motifs or domains and instead, is shown to regulate cellular responses through interactions with other cellular proteins (173). Recently, a study reported that SR-A1 directly bound TRAF3 to prevent its activation and subsequent production of IFN-I was impaired in response to HBV (210). There are similar studies that suggest SR-A1 regulates the intensity of the immune response further highlighting its role in maintaining tissue homeostasis (157, 173).

Cellular functions are tightly regulated by proteins regularly forming complex and specific networks (310). Understanding the protein-protein interactions for a protein of interest (POI) helps reveal their functions and roles in different biological mechanisms (311). The traditional approaches like yeast two-hybrid and AP have been widely applied

for mapping protein-protein interactions (312, 313). In yeast two-hybrid screening, a bait (POI), and a prey protein, each fused to one half of a reporter protein (such as GFP) are overexpressed within the same cell. When the two proteins interact, the two halves of the reporter protein come together and can induce an output (such as fluorescence) (312). However, yeast two-hybrid assays can only confirm direct interactions of soluble proteins and do not work for membrane proteins (310, 312). Moreover, the overexpression of both bait and prey proteins often leads to many false positives (310).

A better alternative to yeast two-hybrid and other protein complementation assays is AP-MS to identify the interacting proteins for endogenous POI in the cell type of interest. However, AP-MS requires a good antibody against the POI (or insertion of a tag that can be pulled-down) (313). The main limitations of AP-MS are that weak or transient interactions are often lost during cell lysis and washing steps and false positives may be introduced by cellular disruption, as two proteins that normally localize in different subcellular spaces may interact with each other following cell lysis (313). Additionally, AP-MS is challenging to apply to insoluble targets including membrane proteins such as SR-A1 (310).

Proximity labeling (PL), however, was developed to provide a complimentary approach to the traditional methods for mapping protein-protein interactions in living cells. PL techniques like BioID can be applied to insoluble proteins such as SR-A1 (286). BioID typically involves the fusion of a promiscuous biotin ligase with a POI (bait),

which is then expressed in a relevant biological setting. Addition of enzyme substrate (biotin) enables covalent biotin labeling of proteins in the vicinity of the bait which can be identified using MS (286, 311). As such, the mutant biotin ligase simply releases a 'cloud' of activated biotin, which can react with nearby proteins (314). The 'cloud' of activated biotin surrounding the POI resembles a contour map, with strongest labeling occurring closest to the enzyme, and weaker labeling occurring as the distance from the POI increases (314). Importantly, in BioID, the cells remain intact when the interactome of bait is labeled and thus, the potential for false-positives due to artificial interactions caused by disruption of cells is minimized (289).

We thus sought to determine whether SR-A1 acts merely as a 'cargo delivery' protein or if it can also modulate antiviral and proinflammatory responses to extracellular dsRNA by studying its protein interaction networks. As certain tags and bulky fusions can interfere with crucial protein properties when expressed in cells (286), we first determined the optimal terminus of SR-A1 (N-terminus; N-BioID or C-terminus; C-BioID) to fuse biotin ligase to without impairing the targeting and function of the fusion protein. We noted comparable expression of both the fusion proteins and they both also localized to the cell surface but C-BioID appeared to bind acLDL less efficiently in comparison to N-BioID. It is possible that the addition of a bulky tag such as biotin ligase to the C-terminal end of SR-A1 causes steric hindrance and leads to poor ligand binding, but further experimentation is warranted to confirm that. Importantly, C-BioID and N-

BioID expression levels were much lower than SR-A1 which is consistent with the difficulties observed with overexpressing large bulky membrane proteins (315).

Next, we examined the biotinylation efficiency of C-BioID and N-BioID. Our findings demonstrate that C-BioID biotinylates lesser number of proteins than N-BioID and we hypothesize one of three reasons for such an observation. First, the steady decrease in the concentration of biotin (substrate) as it is being internalized by the cells, leaving little to no substrate for biotin ligase (enzyme) to catalyze. Second, the extracellular positioning of biotin ligase in C-BioID perhaps restricts the biotinylation events to other proximal membrane proteins and/or proteins within the endosomal lumen only (316). Third, the accessibility of biotin ligase in the context of a folded fusion protein could affect labeling as biotin ligase could be effectively 'buried' in the folded protein structure of SR-A1 (314), although this seems less likely as we still observe biotin ligase activity, albeit at lower levels. Further analyses are necessary to test whether one or more of the above theories are true in the case of C-BioID.

Alternatively, it is possible that the biotin ligase is less active in the extracellular region and within the endosomal lumen. A recent study showed that the current version of biotin ligase (TurboID) robustly biotinylated proteins in the ER lumen whereas the 1st generation biotin ligase (used in our study; conventional BioID) was only marginally active (317). While the specific mechanism(s) limiting biotinylation by conventional BioID in the ER remains unclear, we speculate that other cellular environments such as

organelles with low pH (endosomes and lysosomes) or extracellular regions may exert a similar effect on the activity of conventional BioID (316, 317). The transfection of the parental plasmid of C-BirA*, induced little to no biotinylation, which we are currently unable to explain. The concerns regarding the reduced ability of C-BioID to bind SR-A1's cognate ligand and sup-optimal biotinylation efficiency led us to exclude C-BioID from subsequent studies.

In most of the BioID studies on mammalian proteins, sequences encoding the fusion protein are stably integrated into the genome of cultured cells (318). Since we initially failed to generate a stable cell line which constitutively overexpressed N-BioID, we applied the same strategy for N-BioID as we did to generate inducible expression of SR-A1. Consistent with our earlier findings, the controlled expression of N-BioID in parental A549 cells lead to robust extracellular dsRNA-induced antiviral responses and did not require the addition of DEAE-dextran to the media. Ideally, the BioID fusion protein should be expressed in cells at a similar level to the endogenous POI or at low levels as overexpression of the fusion protein brings with it the increased possibility of mislocalization, non-specific biotinylation and identification of false positive interactions (287, 289, 318). Moreover, we had earlier noted a concurrent increase in toxicity and cell death with increased SR-A1 expression and it is highly likely that the same applies to N-BioID as well. Therefore, like SR-A1, we attempted to temporally regulate the expression levels of N-BioID and succeeded at identifying minimal concentration and duration of

doxycycline treatment required to induce N-BioID expression to levels sufficient to mediate its dsRNA-associated activity with no observable cellular toxicity.

Extended periods of biotin labeling can saturate proximal nucleophiles with biotin, enabling biotinyl-AMP intermediates to travel farther and biotinylate distal, non-specific proteins (316). At the same time, since receptor-mediated endocytosis is a relatively 'fast' biological process (290), it is critical to have generated sufficient reactive biotinyl-AMP intermediates (a cloud of activated biotin) prior to the adding the ligand (extracellular dsRNA). Thus, we sought to determine the optimal duration of biotinylation and match it with a suitable biological window to maximize biologically relevant biotinylation events for N-BioID in response to extracellular dsRNA stimulation. Our findings demonstrate that N-BioID requires at least an hour to generate some reactive biotinyl-AMP suggesting that a biotin pre-treatment is required to ensure biotinylation of proximal proteins following extracellular dsRNA treatment. Notably, the increase in biotinylated proteins in an hour following biotin treatment was subtle, likely due to the slow catalytic activity of conventional BioID (316, 317). We also noted upregulation of ISGs as early as two hours following extracellular dsRNA treatment. As dsRNA sensing precedes the production of IFN-I and ISGs (12), we identified the relevant biological window as the period prior to the induction of ISGs in response to extracellular dsRNA.

To increase the specificity of the involved biological processes in the context of receptor internalization and subsequent trafficking, we identified a time course of protein

interactions for N-BioID following extracellular dsRNA treatment. The N-BioID derived proteomes across the time course enabled us to identify pathways that were enriched at a given time following extracellular dsRNA treatment. However, based on the low catalytic efficiency of conventional BioID (310, 314, 316), we suspect that the list of candidate proteins we obtained are likely low confidence hits. Several studies using conventional BioID suggest for a protein to be considered a candidate interactor it should be enriched more than at least 3-fold (200% enrichment) in the BioID-POI samples compared to the control samples (311, 319). In contrast, we applied only a 20%-fold enrichment cut-off. Moreover, the list of candidate interactors contained several proteins like ribosomes, DNA-Dependent Protein Kinase Catalytic Subunit (PRKDC) and nuclear proteins which tend to be abundant in all BioID samples and are typically considered as background (287, 311, 314). The observation that the usual 'contaminants' are found in greater abundance suggests that the proteins biotinylated by N-BioID are underrepresented in the proteomes obtained and is likely due to the slow kinetics of conventional BioID.

The conventional BioID has mostly been applied to capture entire changes in protein complexes over a long period of time (289) and is consistent with its slow enzymatic activity which necessitates labeling with biotin for 18-24 h (sometimes much longer) to produce sufficient biotinylated material for proteomic analysis (316). This precludes the use of conventional BioID for studying dynamic processes that occur on the timescale of minutes or even a few hours like SR-A1-mediated uptake of extracellular dsRNA (316). Notably, TurboID (improved version of initial biotin ligase) can generate

as much biotinylated product in 10 mins as conventional BioID does in 18 h (316) suggesting that a 'faster' (higher catalytic efficiency) PL enzyme such as TurboID would be more suitable to study extracellular dsRNA-induced protein interactions for SR-A1. We speculate that the fast kinetics of TurboID will allow the addition of biotin and stimulus (extracellular dsRNA) simultaneously, without the need for a biotin pre-treatment step. In theory, this should decrease the non-specific biotinylation of proteins occurring during the period in which the extracellular dsRNA (stimulus) is not present. Moreover, TurboID-SR-A1 biotinylated proteins will likely be in higher abundance than the common contaminants enabling the use of stringent fold enrichment cut-off during data analysis.

Besides biotin ligases, an engineered soybean ascorbate peroxidase APEX (320) and its improved version, APEX2 (321) are also used in PL based techniques. The key advantage of APEX proteins over conventional BioID is their rapid labeling kinetics; proximal proteins can be tagged in a minute or less (320, 321). When activated by peroxide (H_2O_2), APEX and APEX2 catalyze the conversion of its substrate biotin-phenol into highly reactive biotin-phenoxyl radicals, which can covalently attach to electron-rich amino acids such as tyrosine in nearby endogenous proteins (320). However, since APEX labeling requires the use of H_2O_2 which is toxic to living cells, it cannot be used for longer periods of labeling like biotin ligases (316). Notably, the short time frame of APEX labeling (<1 min) has allowed the capture of temporally resolved snapshots of changing interactomes of proteins involved in dynamic cellular processes, such as in Wnt

(322) and GPCR signaling (290, 323) suggesting that APEX-based PL techniques might also serve as a suitable alternative to conventional BioID to dissect the extracellular dsRNA-induced signaling pathways of SR-A1.

PL approaches performed on a single POI often generates a large list of putative interactome components. However, in most cases many of these proteins are not true proximity partners for the POI. Instead, they are biotin-labeled proteins in the absence of the recombinant enzyme (e.g. endogenously biotinylated proteins such as mitochondrial carboxylases); proteins that are promiscuously biotinylated with most baits (e.g. with conventional BioID in HEK293 cells, filamin A (FLNA), PRKDC belongs to this category), nearby off-pathway proteins diffusing through the reactive biotin cloud which may not physically interact with the POI, and proteins that bind non-specifically to the affinity support (sepharose or other bead types) (314, 324). Thus, identifying relevant interactions from the PL experiments require the use of several appropriate controls and stringent downstream computational pipelines to eliminate false positive identifications (325). While using a mock treated (no extracellular dsRNA treatment) N-BioID control allowed us to subtract the endogenously biotinylated proteins efficiently, we think it is insufficient in distinguishing between true interactors for SR-A1 from background proteins that have affinity to the biotin ligase in addition to or instead of to SR-A1 (POI) and the off-pathway proteins that are in the vicinity of the N-BioID.

Controls should thus minimally include conditions that mimic endogenous biotinylation (such as no PL enzyme fused to the POI, or untransfected cells) and conditions that reproduce promiscuous biotinylation (e.g., PL enzyme alone expressed throughout the cell, and/ or fused to an irrelevant polypeptide such as the GFP) (314). To properly model promiscuous background, the control polypeptide must also be expressed to comparable levels as the fusion protein and should at least partially occupy the same intracellular locale (314). We attempted to generate a biotin ligase alone control by engineering a P2A cleavage peptide sequence in-between SR-A1 and biotin ligase (P2A-N-BioID). The P2A sequence acts as a ribosomal 'skip' site and leads to the synthesis of two individual proteins (biotin ligase and SR-A1) instead of a full-length fusion protein (326). While we succeeded in generating stable cell lines with inducible expression of P2A-N-BioID, we were unable to lower its expression to similar levels as N-BioID. Evidently, the subsequent MS data revealed that the P2A-N-BioID control 'drowned out' relevant signal from N-BioID likely due to its much higher expression than N-BioID *(unpublished data from Mossman lab)*.

Signaling proteins such as cell surface receptors often rapidly change location and protein interactions (e.g., the interactome dynamically changes from plasma membrane to early endosomes following ligand internalization) (290) and is applicable to SR-A1 as well. Thus, alongside the above controls, it may also be useful to include necessary 'compartment controls' or 'spatial' references. Spatial references serve to provide a snapshot of the composition of a structure and can be employed by expressing an

organelle marker or a sequence tag fused to the PL enzyme (310). Besides scoring of contaminants, spatial references can also be used as a secondary ‘enrichment’ strategy to help define more specific proximity interactors (314). Indeed, by identifying a time course of protein interactions via APEX-based PL and using a set of spatial references to increase specificity in the context of receptor internalization and trafficking, a recent study implicated two ubiquitin-pathway proteins as mediators of δ-opioid receptor (DOR) endosomal trafficking to the lysosome demonstrating the potential of PL based approaches to probe dynamic interactions (290).

Looking ahead, a ‘fast’ PL enzyme like TurboID or APEX proteins and appropriate controls including relevant spatial references for SR-A1 should likely eliminate the background effectively and allow the identification of high confidence interactors for SR-A1. Alternatively, a genome-wide CRISPR-Cas9 knockout screen (327) can also be applied either in parallel to PL based approaches or as an independent experiment in SR-A deficient A549 cells engineered to express SR-A1 in an inducible manner. By performing large-scale loss-of-function analyses (327), genes that are involved in mediating SR-A1 function in the context of extracellular dsRNA can be identified and thus, the question whether SR-A1 modulates innate immune responses to extracellular dsRNA or not can be addressed.

In summary, this study demonstrates that SR-A1 is sufficient to mediate extracellular dsRNA-induced cellular responses independent of other surface nucleic acid receptors. Whether SR-A1 modulates innate immune responses to extracellular dsRNA remains an open question but this study has laid the groundwork to inform future studies aimed at functional characterization of individual members of SR-A family, including SR-A1.

5.1.3. Intracellular fate of extracellular dsRNA

While our understanding about the recognition and uptake of extracellular dsRNA has grown significantly due to the discovery of surface receptors such as SR-As, Mac-1, and SR-J1, we are yet to completely dissect its intracellular trafficking which in turn would provide insights into which signaling pathways can become triggered in response to extracellular dsRNA. We previously demonstrated that besides TLR3, extracellular dsRNA also activates the RLR-sensing pathway within the cytoplasm both *in vitro* and *in vivo* (19). Other published studies also suggest that the cytoplasmic dsRNA sensing pathway plays a key role in response to systemic administration of 'naked' poly (I:C) (216). Thus, the requirement of the cytoplasmic RLR-sensing pathway in response to extracellular dsRNA is intriguing and implies the existence of a mechanism which facilitates its entry into the cytoplasm for innate immune recognition.

Recent studies have implicated SIDT1 and SIDT2, the mammalian orthologs of the *C. elegans* SID-1 dsRNA transporter (217, 218), in mediating the transport of extracellular dsRNA from endo-lysosomal vesicles into the cytoplasm in MEFs (221, 222). However, the roles of SIDT1 and SIDT2 in humans remain unclear. Since SIDT2 is more widely and abundantly expressed than SIDT1 (219), we assessed the role of SIDT2 in the intracellular trafficking of extracellular dsRNA by performing loss-of-function analyses. We generated CRISPR-edited SIDT2 knockout cells in SR-A deficient A549 cells that were engineered to express SR-A1 in an inducible manner. To address the potential problems caused by evolution and variability during single-cell clonal expansion

(328), we derived multiple independent knockout clones and randomly chose two clones for subsequent experimentation (CR1.1 and CR2.2). We noted variability in the inducible expression of SR-A1 in the two SIDT2 knockout clones in comparison to control cells and we believe it is an outcome of the single-cell clonal expansion process. Based on published literature, it is suggested that SIDT2 is localized to the endo-lysosomal compartments and mediates translocation of extracellular dsRNA following its uptake via SR-As or other surface nucleic acid receptors (221). Thus, it was imperative to have comparable uptake of extracellular dsRNA to assess the subsequent role of SIDT2 without any bias. To that end, we succeeded in inducing similar expression levels of SR-A1 in control cells and the two SIDT2 knockout clones which should result in similar or comparable levels of extracellular dsRNA uptake, although we did not directly test the efficiency of extracellular dsRNA uptake in these cells.

In functional assays, we noted significant variability in the ability of CR1.1 and CR2.2 cells to respond to extracellular dsRNA. Consistent with previous findings in MEFs (221), CR2.2 cells induce somewhat intermediate antiviral responses to extracellular dsRNA. In contrast, CR1.1 cells fail to mediate a response to extracellular dsRNA and we suspect that the functional differences between the two SIDT2 knockout clones is due to the clonal selection and expansion processes. Indeed, it has been reported that knocking out genes and subsequently generating single cell derived clonal populations can select for certain genetic alterations thus, potentially confounding downstream analysis (329). We also noted that CR1.1 cells respond poorly to transfected

dsRNA whereas, CR2.2 cells respond similarly, if not better compared to parental A549 cells. Therefore, it is possible that one or both SIDT2 knockout clones may have selected for one or more genetic changes resulting in observable functional differences between them but, it would require further investigation using multiple independent SIDT2 knockout clones to validate such a supposition.

We previously demonstrated that the MEFs lacking the RLRs also display an impairment in antiviral responses to extracellular dsRNA (19). The residual antiviral responses were shown to be dependent on TLR3 sensing of endosomal dsRNA (19) suggesting that the SIDT2 knockout cells effectively behave like RLR knockout cells by restricting the transport of extracellular dsRNA into the cytoplasm for innate immune recognition by the RLRs, a hypothesis that requires further experimentation to be tested. To address if SIDT2 transports extracellular dsRNA into the cytoplasm in human cells, gain-of-function analyses can be performed to complement the findings from studies in SIDT2 knockout cells. We believe that expression of SIDT2 in SIDT2 knockout cells would rescue the partial antiviral response and may also increase the sensitivity to the amount of extracellular dsRNA required to generate a robust antiviral response.

Looking ahead, it will be important to learn in which cells SIDT2 functions and to define its nucleic acid substrate specificity. It remains unknown what cell types respond to extracellular dsRNA to activate RLRs *in vivo* whether in the context of systemic poly(I:C) administration, or virus infection. Given the broad expression of Sidt2 (219),

many cell types could be involved. SIDT2 was recently reported as having a higher binding affinity for longer dsRNAs (~300-700 bp) (330). Indeed, we previously noted that the response to short dsRNA species (~200bp) was completely reliant on cytoplasmic RIG-I (19) in MEFs, although a direct assessment of the involvement of SIDT2 was not performed in those experiments. Moreover, the current literature about SIDT2's specificity for other nucleic acids like DNA remains contentious as some studies suggest that SIDT2 bind RNA (221, 330, 331) and others suggest that they can bind DNA as well (332-334). Our study indicated differences in intracellular trafficking pattern of extracellular dsRNA (diffuse) versus DNA (punctate) through live cell fluorescence microscopy in human embryonic lung cells. While additional biochemical analyses are required to validate and/or investigate this observation, it will also be interesting to investigate whether SIDT2 can facilitate transport of extracellular DNA into the cytoplasm. Similarly, functional investigations to determine whether SIDT2 can transport shorter substrates such as siRNAs may shed light on the development of more effective RNAi therapeutics, whose delivery continues to be hindered by poor endosomal escape (335).

We previously found that endocytosis inhibitors decreased (but did not completely block) responses to extracellular dsRNA, albeit of one length tested (19). It is possible that alternative mechanisms might facilitate the cytoplasmic entry of extracellular dsRNA. In this regard, it is notable that the proposed mechanisms of endosome escape (primarily for oligonucleotides) such as pore formation using cell-penetrating peptides,

pH-buffering effect of polycationic reagents, and lipid-based fusion of endosomal membranes require the addition of synthetic peptides or chemical agents (335-337). In contrast, transmembrane proteins can ‘flip’ within the lipid bilayer based on the changes in their surrounding phospholipid environment during processes such as trafficking following ligand binding (283, 284). Therefore, it is plausible that the SR-A1 ‘flips’ to deliver extracellular dsRNA into the cytoplasm to provide access to the RLRs but whether they possess such capacity remains to be tested.

In summary, this study provides preliminary evidence of the potential role of SIDT2 in the intracellular trafficking of extracellular dsRNA. Further validation of the current observations is necessary. This study also highlights the importance of performing studies in multiple independent clones following CRISPR-mediated editing as the clonal variability can potentially confound downstream analyses.

5.2. Concluding remarks

The continued emergence of diseases such as Ebola and COVID-19 and the occurrence of drug-resistant variants is pushing the scientific community towards development of novel strategies for more effective vaccines and antiviral therapeutics to combat viruses and mitigate human disease. Thus, it is important to better understand the dynamics of virus-host interactions at multiple levels. Bystander antiviral immunity induced via extracellular viral dsRNA is a relatively untapped strategy to benefit the host, but we require a better understanding of the signaling mechanism of extracellular dsRNA and the proteins or pathways involved to be able to identify potential targets for developing novel antiviral strategies. Further, the role of extracellular nucleic acids like dsRNA in health and disease is evident as, on one hand, nucleic acids and their sensors are being targeted to control infection and autoimmunity and on the other, dsRNA molecules such as poly (I:C) and siRNA are being developed for systemic delivery as adjuvants and targeted therapeutics, respectively. Notably, the recent approval of mRNA vaccines for COVID-19 presents an opportunity to understand the trafficking and innate immune responses to the mRNA-lipid nanoparticles which may contribute to some, if not all, adverse reactions associated with the vaccines.

I strongly believe that the findings in this thesis significantly improve our understanding about the recognition, uptake, and intracellular fate of extracellular dsRNA. Moreover, the work in this thesis has also generated some foundational knowledge about the biology of membrane receptors like SR-As which shall inform future studies aimed at functional characterization of such receptors.

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