

THE ROLE OF GAS7 IN THE INNATE IMMUNE ANTIVIRAL RESPONSE

Luana Isabel Rosinha da Silva

Mestrado Integrado em Bioengenharia

Master Thesis Dissertation

September 2021

Supervisor

Vasco Rodrigues, PhD

U932 – Immunity and Cancer, Institut Curie, Paris, France

Co-supervisor

Begoña Pérez-Cabezas, PhD

ICBAS – School of Medicine and Biomedical Sciences, Porto, Portugal

THE ROLE OF GAS7 IN THE INNATE IMMUNE ANTIVIRAL RESPONSE

Luana Isabel Rosinha da Silva

Dissertation to obtain the Degree of Master of Science in Bioengineering
(specialization in Molecular Biotechnology) at Faculdade de Engenharia da
Universidade do Porto and Instituto de Ciências Biomédicas Abel Salazar da
Universidade do Porto

September 2021

Supervisor

Vasco Rodrigues, PhD

Co-supervisor

Begoña Pérez-Cabezas, PhD

The work described in this dissertation was conducted at

Myeloid Cells and Immunity Group

U932 – Immunity and Cancer, Institut Curie, Paris, France

The work presented in this dissertation was financed through

Grants from ANRS and SIDACTION to Philippe Benaroch

ACKNOWLEDGEMENTS

First and foremost, I would like to show my appreciation for my supervisors, without which I would have never been able to finish this work. To Vasco, for being a source of motivation and admiration, for always believing in me and in my capabilities, for challenging my line of thinking and reminding me to step out of the box, for making me grow so much as a young researcher. I will be forever grateful for what you've taught me. To Begoña, for embracing the role of my co-supervisor with such dedication and enthusiasm. For always having patience for my out-of-the-norm schedules and for never failing to motivate me all the way from Portugal.

Needless to say, none of this would have been possible without Dr. Philippe Benaroch, who accepted me into his group and granted me the opportunity to be part of this project. Thank you for your words of encouragement and for being as excited over the project as I was. To everyone in the Benaroch lab (Sarah, Marion, Jovan, Flavien, François-Xavier and Anäel), I would like to thank you for the support and kindness, for having welcomed me so well in my first Parisian experience and for all of the time that we spent together, talking about the most random of things. I extend my thanks to everyone that I got to meet from the 5th floor of the *Bâtiment Burg*, for never hesitating to offer a helping hand.

Of course, I would not have been able to come this far, if not for the constant help and support from my friends, which I hold close to my heart, no matter how far apart we are or how long it takes me to reply to your messages (I'm very sorry). To everyone that University of Porto has brought into my life to make it so much brighter, I don't even think I could begin to describe how important you are to me. To my all-time companions Debas, Sabi and Vicky and the ones from Biotec and 016 that have always put a smile on my face and have been with me through this roller-coaster that is the Integrated Masters in Bioengineering. Thank you for teaching me that real friendship exists and that it's alright to be weird and, most of all, be weird together. I hope that we will be putting up with each other's antics until we turn old and are no longer able to hear each other properly or tell Debas to stop dancing to Wuthering Heights. To TVI, for always being one of my biggest sources of inspiration and having the best advice, to my M&Ms, for choosing me and making me so proud of watching how far you've come, and to the GGG and everyone else that I got to know through Praxe, truly one of the most incredible and rewarding experiences in my life. Thank you.

To all of the wonderfully crazy people I've met at the *Maison du Portugal*, especially to the Tugas in RAG, for all of the makeshift *soirées*, dinners, *promenades*, and adventures in Paris, Orléans and Antibes – *merci, mes potes*. You're the ones that pushed me through this last step. To that one random night out that started in a questionable club and ended in *Moulin Rouge* and less than 3 hours of sleep (I promised!). To Edu, to whom I'm so grateful for coming into my life, for being my giiiirl, for always accompanying me in the worst choices and best adventures, for showing me the Barcelona that you love and (almost) dying with me on a bus.

To Moutas, my Parisian companion, thank you for coming along with me twice to the city of lights, for surviving two months locked in a 35m² apartment with me and for all of the times watching anime and cartoons over chips and tea. For all of the strength and all of the laughs

from long nights staying up together to write. I hope I will visit you in a couple of years and you will be happily living your dream with small goats in the Swiss Alps.

To the friends from my hometown, that tiny city in the bottom of the Douro valley. Thank you for making life easier when we were just kids going through school and for always being up for laughs over dinner, no matter how many years pass and how much our lives have changed.

To the most important people in my life, my parents and my grandmother, my deepest thanks for giving me the wings that allowed me to fly. For always believing that I would be able to accomplish my dreams and for always caring about me more than anyone else, even when I was grumpy, tired and overworked. Thank you for always thinking of what's best for me and guiding me the best you could through all of these years. I was never an easy person, but you've nevertheless always kept me grounded and helped me through all of the hardships life threw my way. I will never be able to repay how much you've done for me.

Finally, to my grandfather and to my godfather, who would have been the proudest to see me earn this degree, thank you for never once doubting me. This thesis is for the both of you as much as it is for everyone else.

My journey has just begun.

ABSTRACT

The immune system is a multilayered structure whose main function is to protect us from invading pathogens and other dangers that compromise homeostasis. This complex network can be divided into three different major defense mechanisms: (i) anatomical and physiological barriers, (ii) innate immune response and (iii) adaptive immune response.

When an invasive pathogen is capable of breaching through the host's anatomical and physiological barriers, the innate immunity is activated, and the body responds quickly to battle the infection. Macrophages are sentinel cells from the innate immune system that mount complex immune responses once a PAMP or DAMP is sensed by its multiple PRRs, and consequently release cytokines to recruit other innate immune cell populations, along with other inflammatory components. One of such responses is led by the cGAS-STING pathway of DNA-sensing in the cytosol of host cells. Engagement of this pathway results in the production of multiple soluble mediators, including type I IFNs, that will induce an antiviral state in the cell and surrounding tissue, through the induction of ISGs. These are important defense mechanisms against DNA viruses and retroviruses that might infect the host cells.

Maintaining regulatory control of a pathway as important as cGAS-STING is of great significance, as its chronic activation leads to multiple health concerns. It is well established that STING is rapidly degraded upon stimulation, via autophagic pathways. However, in a context of chronic viral infection, cells may be continuously exposed to viral ligands. In these settings, it may be important to maintain appreciable levels of expression of STING and other antiviral factors, as to keep viral replication in check over time. However, the mechanisms that regulate the dynamics of STING expression in these contexts remain largely unknown.

Over the past few years, our laboratory has been working on the role of the F-BAR family protein GAS7 during viral infection and found that GAS7 regulates the actin cytoskeleton dynamics in macrophages, restricting the transmission and propagation of HIV-1 and HIV-2. More recently, work in the lab suggests that GAS7 may play an important role in the macrophage antiviral response: (1) the transcript and protein levels of several antiviral factors, including STING, are downregulated in the absence of GAS7, in macrophages at the steady state, (2) the ISG response to HIV is impaired in macrophages silenced for GAS7 and (3) GAS7 localizes to the nucleus in macrophages and interacts with multiple proteins involved in gene regulation and mRNA splicing and transport. Altogether, the data points to GAS7 as a factor with the ability to sustain the antiviral immunity.

In this project, the aim was to extend these initial observations and evaluate the role of GAS7 in the macrophage antiviral response, at large. We show that the absence of GAS7 does not alter the ability of macrophages to induce an ISG response to single stimulation with dsDNA or an RNA analogue delivered to the cytosol. However, when macrophages are treated with the STING ligand, 2'3'-cGAMP, the absence of GAS7 significantly impacted on the replenishment of STING after its degradation. Contrary to what was previously observed with HIV-1 and HIV-2, the absence of GAS7 does not impact the ISG response to Sendai, an RNA virus. Yet, Sendai replication was significantly augmented in macrophages depleted for GAS7; a phenotype associated with the formation of giant cells in culture. We further observed that

endogenous GAS7 accumulates in the nucleus of macrophages after stimulation with 2'3-cGAMP, further suggesting that its actions in antiviral immunity may come from its activity in this cellular compartment. Finally, by knocking down GAS7 in THP-1 cells, we observed that this cell line recapitulates certain phenotypes observed in primary macrophages, suggesting that it can be used to study the role of GAS7 during antiviral immunity, particularly to test hypotheses that require easy-to-manipulate models.

All in all, the results obtained confirm that GAS7 is a protein with a marked role in the antiviral response to viruses, particularly those that engage the cGAS-STING pathway. GAS7 can promote a faster recovery in STING levels after stimulation-induced degradation, via a mechanism that may involve an increase in its transcription or by promoting the transport, stability or splicing of its mRNA. GAS7 is also a dynamic factor able to shuttle between cellular compartments, upon stimulation, to perform certain tasks. Moreover, in the context of Sendai virus infection, we observed that GAS7 has antiviral roles that are not related to its ability to promote the ISG response, but possibly with its capacity to regulate the dynamics of the actin cytoskeleton.

INDEX

ACKNOWLEDGEMENTS	V
ABSTRACT	VIII
INDEX	XI
LIST OF ABBREVIATIONS	XV
LIST OF FIGURES	XIX
LIST OF TABLES	XX
CHAPTER I: INTRODUCTION	22
1. THE IMMUNE SYSTEM	24
2. INNATE IMMUNITY	26
2.1. MACROPHAGES: SENTINELS AND REGULATORS OF INNATE IMMUNITY.....	27
2.2. PATHOGEN RECOGNITION BY THE INNATE IMMUNE CELLS	29
3. INNATE IMMUNE SIGNALING PATHWAYS	32
3.1. CANONICAL NF- κ B PATHWAY	32
3.2. MAPK/ERK PATHWAY	33
3.3. TYPE I INTERFERON PATHWAY	34
4. RNA/DNA DETECTION: VIRAL IMMUNE SENSORS	37
4.1. NUCLEIC ACID-SENSING TLRs.....	38
4.2. RLRs – CYTOSOLIC RNA SENSORS.....	38
4.3. CYTOSOLIC DNA DETECTION	39
5. THE cGAS-STING PATHWAY OF DNA SENSING	40
5.1. MECHANISM OF cGAS ACTIVATION.....	41
5.2. ACTIVATION OF STING SIGNALING.....	42
5.3. ATTENUATION OF cGAS-STING SIGNALING	44
6. THE F-BAR PROTEIN GAS7 IN HEALTH AND DISEASE.....	46
6.1. GAS7 AS A MODULATOR OF CYTOSKELETON DYNAMICS	47
6.2. GAS7 FUNCTIONS IN THE NERVOUS SYSTEM	48
6.3. THE POTENTIAL ROLE OF GAS7 IN TUMOR SUPPRESSION.....	49
6.4. GAS7 AND ITS IMPLICATIONS IN MACROPHAGES AND INNATE IMMUNITY.....	49
CHAPTER II: PRELIMINARY DATA AND OBJECTIVES	52
PRELIMINARY DATA	54
1. GAS7 SILENCING PROMOTES HIV PROPAGATION IN MACROPHAGES.....	54
2. GAS7 SILENCING IMPAIRS ANTIVIRAL RESPONSES AGAINST HIV	56
3. GAS7 PARTIALLY LOCALIZES TO THE NUCLEUS	58

OBJECTIVES.....	60
CHAPTER III: MATERIALS AND METHODS.....	64
1. CELL CULTURE	66
1.1. 293T CELLS.....	66
1.1.1. TRANSFECTION OF 293T CELLS WITH SHRNA.....	66
1.2. MONOCYTE-DERIVED MACROPHAGES	66
1.2.1. ISOLATION OF MONOCYTES FROM HUMAN BLOOD	66
1.2.2. TRANSDUCTION AND DIFFERENTIATION TO MONOCYTE-DERIVED MACROPHAGES	68
1.3. THP-1 CELLS	68
1.3.1. DIFFERENTIATION WITH PMA	68
1.3.2. ANALYSIS OF THP-1 CELLS GROWTH KINETICS	69
1.4. SUBCELLULAR FRACTIONATION	69
1.5. VIRUS INFECTION	69
1.6. TRANSFECTION WITH 2'3'-cGAMP, HTDNA, POLY(I:C) AND TREATMENT WITH LPS.....	69
1.7. REAL-TIME QUANTITATIVE LIVE-CELL IMAGING	70
2. GENE EXPRESSION ANALYSIS USING RT-qPCR	70
2.1. RNA ISOLATION AND EVALUATION OF RNA PURITY.....	70
2.2. cDNA SYNTHESIS	70
2.3. RT-qPCR	71
2.4. GENE EXPRESSION DATA ANALYSIS AND QUANTIFICATION WITH PRISM SOFTWARE	71
3. PROTEIN EXPRESSION ANALYSIS USING WESTERN BLOT.....	72
3.1. PROTEIN QUANTIFICATION	72
3.2. GEL ELECTROPHORESIS	72
3.3. IMAGING AND QUANTIFICATION WITH IMAGELAB SOFTWARE	73
4. STATISTICAL ANALYSIS.....	73
CHAPTER IV: RESULTS AND DISCUSSION	77
GENERAL OBSERVATION: GAS7 KNOCKDOWN EFFICIENCY.....	79
1. GAS7 DOES NOT SIGNIFICANTLY IMPACT MACROPHAGE ANTIVIRAL RESPONSE TO A SINGLE STIMULATION WITH DNA OR AN RNA ANALOGUE	80
2. GAS7 IMPACTS STING DEPLETION AND REPLENISHMENT KINETICS UPON STIMULATION WITH 2'3'-cGAMP.....	84
3. SENDAI VIRUS INDUCES A STRONG ISG RESPONSE FROM MDMS UPON INFECTION, UNLIKE HSV-1	86
4. GAS7 IMPACTS STING LEVELS DURING SEV INFECTION BUT DOESN'T INTERFERE WITH THE IFN RESPONSE.....	88
5. INCREASED REPLICATION OF THE SENDAI VIRUS IN GAS7-DEPLETED CELLS IS ASSOCIATED WITH THE FORMATION OF GIANT CELLS IN CULTURE	91
6. GAS7 ACCUMULATES IN THE NUCLEUS UPON STIMULATION WITH 2'3'-cGAMP.....	94

7. THP-1 CAN POTENTIALLY BE A VALUABLE MODEL TO STUDY GAS7 FUNCTIONS IN MACROPHAGES	97
CONCLUDING REMARKS	102
FUTURE WORK	104
BIBLIOGRAPHY	108
SUPPLEMENTARY DATA	116

LIST OF ABBREVIATIONS

ABBREVIATION	EXPLANATION
ACTB	Actin Beta
AGS	Aicardi-Goutières Syndrome
AIM2	Absent in Melanoma 2
ALRs	AIM2-like Receptors
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BCA	Bicinchoninic acid
BIR	Baculoviral Inhibition of apoptosis Repeat
CARD	Caspase Recruitment Domain
CDN	Cyclic Dinucleotide
cGAMP	Cyclic Guanosine monophosphate-Adenosine monophosphate
cGAS	cGAMP synthase
CLRs	C-type lectin-like receptors
COPII	Coat Protein complex II
CpG DNA	Cytosine-phosphate-Guanine DNA
C _T	Critical Threshold
CTD	C-terminal Domain
CTT	C-terminal Tail
DAMP	Damage-Associated Molecular Pattern
DENV	Dengue Virus
DMEM	Dulbecco's Modified Eagle Medium
ER	Endoplasmic Reticulum
ERGIC	ER-Golgi intermediate compartment
ERK	Extracellular signal-regulated kinase
FBS	Fetal Bovine Serum
F-actin	Filamentous Actin
F-BAR	Fes/CIP4 homology-Bin/Amphiphysin/Rvs
GAPDH	Glyceraldehyde 3-phosphate dehydrogen
GAS7	Growth Arrest-Specific 7
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HA Units	Hemagglutination Units
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
hnRNPA2/B1	Heterogeneous nuclear ribonucleoprotein A2/B1

HPRT1	Hypoxanthine Phosphoribosyltransferase 1
HSV	Herpes Simplex Virus
htDNA	Herring testis DNA
IFI44L	Interferon induced protein 44-like
IFN	Interferon
IFNAR	Interferon- α/β Receptor
I κ B	Inhibitory κ B
IKK	I κ B Kinases
IL	Interleukin
IRAK	IL-1 receptor-associated kinase
IRF	Interferon Regulatory Factor
ISD	Interferon-Stimulatory DNA
ISG	Interferon Stimulated Gene
JAK	Janus-activated Kinase
JNK	Jun N-terminal Kinase
LBD	Ligand-Binding Domain
LC3	(Microtubule-associated proteins 1A/1B) Light Chain 3
LGP2	Laboratory of Genetics and Plpshysiology 2
LPS	Lipopolysaccharide
LRR	Leucine-rich Repeat
MAPK	Mitogen-activated Protein Kinase
MAVS	Mitochondrial Antiviral-signaling Protein
M-CSF	Macrophage Colony-Stimulating Factor
MDA5	Melanoma Differentiation-associated Protein 5
MDM	Monocyte-derived Macrophage
MOI	Multiplicity of Infection
Mx1	Myxovirus Resistance 1
MyD88	Myeloid Differentiation factor 88
NF- κ B	Nuclear Factor- κ B
NK cells	Natural Killer cells
NLRs	NOD-like Receptors
NOD	Nucleotide-binding Oligomerization Domain
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
N-WASP	Neural-Wiskott Aldrich Syndrome Protein
OAS1	2'–5' Oligoadenylate Synthase 1
PAMP	Pathogen Associated Molecular Pattern
PBMCs	Peripheral Blood Mononuclear Cells

PBS	Phosphate buffered saline
PGN	Peptidoglycan
PMA	Phorbol 12-myristate 13-acetate
Poly(I:C)	Polyinosinic:polycytidylic acid
PRR	Pattern Recognition Receptor
PYD	Pyrin Domain
RD	Repressor Domain
RIG-I	Retinoic acid-inducible Gene 1
RIPA buffer	Radioimmunoprecipitation Assay buffer
RLRs	RIG-I-like Receptors
RPMI	Roswell Park Memorial Institute medium
RPS18	Ribosomal Protein S18
RSAD2	Radical S-Adenosyl Methionine Domain Containing 2
Rt-qPCR	Real Time-quantitative Polymerase Chain Reaction
SAVI	STING-associated vasculopathy with onset in infancy
SeV	Sendai Virus
SH3	Src Homology 3
shRNA	Short-hairpin RNA
SLE	Systemic lupus Erythematosus
STAT	Signal Transducer and Activator of Transcription
STING	Stimulator of Interferon Genes
TAK1	Transforming growth factor- β -activated kinase 1
TBK1	TANK-binding Kinase 1
TGF β	Transforming Growth Factor β
TIR	Toll/interleukin 1 receptor homology
TLRs	Toll-like Receptors
TNF α	Tumor Necrosis Factor α
TRAF	Tumor necrosis factor Receptor-associated Factor
TRM	Tissue-Resident Macrophages
TYK2	Tyrosine Kinase 2
WIPI2	WD repeat domain phosphoinositide-interacting protein 2

LIST OF FIGURES

Figure I.1. Integrated human immune system	25
Figure I.2. Tissue-resident macrophages and monocyte-derived macrophages play distinct roles in tissue injury and repair.....	28
Figure I.3. Some of the cellular PRRs.....	31
Figure I.4. Canonical NF- κ B pathway.....	33
Figure I.5. Pathways of type I interferon induction and receptor signaling.....	35
Figure I.6. ISG targeting distinct steps in the viral replication cycle	36
Figure I.7. Nucleic acid sensing PRRs.....	37
Figure I.8. Cytosolic RNA-sensing pathway	39
Figure I.9. A timeline for discoveries of cGAS regulation and function.....	40
Figure I.10. Structural Basis of cGAS activation.....	41
Figure I.11. Mechanism of STING activation	43
Figure I.12. The cGAS–STING DNA sensing pathway.....	43
Figure I.13. A model of DNA-induced autophagy and signaling through the cGAS-STING pathwa	46
Figure I.14. Crystal structures of GAS7, the membrane-binding sites and protein isoforms	47
Figure II.1. GAS7 deficiency favors HIV viral propagation in macrophages	55
Figure II.2. GAS7 silencing in macrophages impairs antiviral responses against HIV	57
Figure II.3. GAS7 interacts with multiple nuclear proteins	58
Figure II.4. GAS7 partially localizes in the nucleus	59
Figure III.1. Protocol for isolation and differentiation of monocyte-derived macrophages from human blood.....	67
Figure IV.1. GAS7 does not significantly impact macrophage antiviral response to a single stimulation with cytosolic delivery of dsDNA or an RNA analogue.....	83
Figure IV.2. GAS7 impacts STING depletion and replenishment kinetics upon 2'3'-cGAMP stimulation	85
Figure IV.3. SeV induces a strong ISG response from MDMs upon infection, unlike HSV-1	87
Figure IV.4. GAS7 impacts STING levels during SeV infection but doesn't seem to significantly interfere with the IFN response	90
Figure IV.5. GAS7-depleted cells display more viral amplification and experience fusion events	93
Figure IV.6. GAS7 translocates to the nucleus upon stimulation with 2'3'-cGAMP.....	96
Figure IV.7. THP-1 can potentially be a valuable model to study GAS7 functions in macrophages	98
Figure S.1. Western Blotting data from htDNA and Poly(I:C) stimulation	118
Figure S.2. Western Blotting data from 2'3'-cGAMP stimulation	119
Figure S.3. Western Blotting data from SeV infection	120
Figure S.4. Live-cell imaging of SeV infected MDMS	120

LIST OF TABLES

Table I.1. Overview of defining features of innate and adaptive immunity	26
KEY RESOURCES TABLE.....	74

CHAPTER I: INTRODUCTION

1. THE IMMUNE SYSTEM

Throughout life we are met a multitude of foreign substances that can cause infection. Protection against pathogens is dependent on a complex network of cells, chemicals, tissues, and organs that, together, compose our immune system¹. The immune system is, consequently, a multilayered structure, and it can be divided into three different major defense mechanisms: (i) anatomical and physiological barriers, (ii) innate immune response and (iii) adaptive immune response (Figure 1.1)².

The first, whilst being the most basic form of nonspecific defense, plays a crucial role in preventing pathogens from reaching internal tissues that are susceptible to infection – external barriers such as the skin and mucosal surfaces are the first line of defense, along with destructive enzymes present in tears, saliva, and other secretions, the low pH of the stomach and the resident microbiota. Infection begins only when these barriers are breached or evaded^{1,3}.

Having penetrated the primary defenses, invading pathogens are met with an almost-instantaneous protective inflammatory response, orchestrated by the host's innate immune system. The recognition molecules that the innate system employs target conserved components shared by prominent groups of pathogens and are expressed broadly on a large number of cells, making it a fast-acting system that operates at the site of invasion^{4,5}. The cells involved in the innate response are derived from a common hematopoietic precursor that can differentiate into a quite diverse set of cells, including macrophages, dendritic cells, neutrophils, eosinophils, basophils, mast cells, natural killer (NK) cells and invariant NK T cells. These cells patrol throughout the body along with soluble recognition molecules, such as collectins, antimicrobial peptides, pentraxins (including LPS binding protein and C-reactive protein) and the proteins that make up the complement system^{3,6}.

The innate immune system is a defense mechanism already present at birth, and it does not change significantly throughout an individual's life, as it lacks the ability to learn and retain information (memory). This, nonetheless, does not change the fact that this sentinel system is vital to life, as it is remarkably conserved throughout evolution, appearing even in the simplest of animals. Moreover, innate immunity plays an essential part in initiating the subsequent adaptive immune response^{6,7}.

The adaptive immune system is precise, as it is characterized by a set of molecules and effector cells with specificity for any individual pathogen, but is relatively slow, as it might take several days or weeks to fully develop. This is due to the fact that, after encountering the foreign substance, the cells compromising the adaptive response – the T and B lymphocytes, undergo clonal selection, and thus need time to proliferate and achieve sufficient numbers as to mount a specific response that can efficiently eliminate the threat^{1,4}. In contrast to the innate immune cells' limited repertoire of receptors, these lymphocytes express highly specific antigen receptors that explicitly target the particular invader, making them virtually capable of eliminating any known pathogen². Furthermore, the adaptive immune cells are capable of establishing memory – any subsequent exposure to the same pathogen enables a faster and more robust adaptive response. Immune memory can be long lasting, and it contributes to

limiting the spread of infection in a community, as it is also possible to induce it through vaccination^{7,8}.

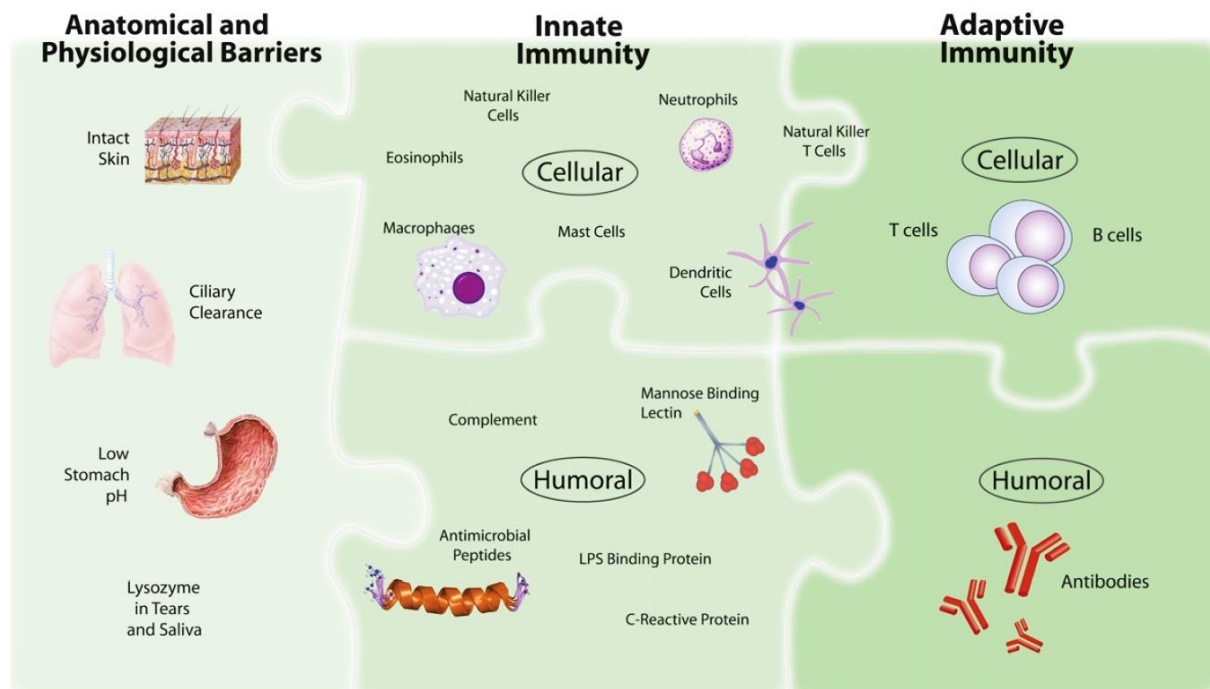


Figure I.1. Integrated human immune system (adapted)³. The human microbial defense system can be simplistically viewed as consisting of 3 levels: (1) anatomic and physiologic barriers; (2) innate immunity; and (3) adaptive immunity. In common with many classification systems, some elements are difficult to categorize. For example, NK T cells and dendritic cells could be classified as being on the cusp of innate and adaptive immunity rather than being firmly in one camp.

The defense mechanisms described are classically regarded as separate entities, but it is well established that these systems are interdependent and work together to formulate an integrated immune response. In fact, many adaptive immune responses are built with the help of innate immune cells – for instance, activation of T cells, and consecutively, of B cells, is empowered by antigen-presenting cells, such as dendritic cells and macrophages, that are able to process a protein antigen, break it into peptides, and present it appropriately to the immunoreactive lymphocytes^{3,9}.

Ultimately, an immune response can be described as a collection of different cell- and humoral-mediated mechanisms, driven by both innate and adaptive defenses, acting as one to fully eliminate a specific invading pathogen (Table I.1).

Table I.1. Overview of defining features of innate and adaptive immunity (adapted)³.

	Innate Immunity	Adaptive Immunity
Cellular elements	Hematopoietic cells: macrophages, dendritic cells, mast cells, neutrophils, eosinophils, NK cells, and NK T cells	Hematopoietic cells: T and B lymphocytes
Humoral elements	Large arsenal of components: complement proteins, LPS binding protein, C-reactive protein and other acute-phase reactants, antimicrobial peptides, and mannose-binding lectin	Immunoglobulins secreted by B cells
Receptor characteristics	Invariant, germline encoded All cells of a class express identical receptors (ie, nonclonal)	Generated by random somatic gene segment rearrangement All cells of a class express a single type of receptor with unique specificity (ie, clonal)
Ligands recognized	Conserved microbial components Common metabolic or biologic consequences of infection (eg, uric acid, K1 efflux, and MHC class I downregulation)	Specific details or epitopes of macromolecules (eg, proteins, peptides, and carbohydrates)
Types of receptors	Activating: TLR, NLR, and complement Inhibitory: killer cell immunoglobulin-like receptors	B-cell receptor and T-cell receptor
Response time	Immediate	Delayed by hours to days
Immunologic memory	None: responses are the same with each exposure. Non-anticipatory immunity	Responsiveness enhanced by repeated antigen exposure Anticipatory immunity
Risk of autoreactivity	Low: self-tolerant receptors are selected during evolution	High: random gene rearrangement generates autoreactive receptors requiring the presence of multiple tolerance mechanisms

2. INNATE IMMUNITY

When an invasive pathogen is capable of breaching through the host's anatomical and physiological barriers, some innate immune mechanisms are instantly activated to battle the infection. Soluble molecules present in extracellular fluid or blood, such as antimicrobial enzymes and peptides, create a hostile environment for microbial habitation, and some of these substances can directly induce death of the pathogens⁵. Another potent component of the early innate response is the complement system, an orchestrated mechanism made up of multiple plasma proteins that are activated upon infection and interact with each other in various pathways with the end goal of either directly causing the death of the pathogen or facilitate its phagocytosis by macrophages – a process termed opsonization².

If these processes do not succeed in eliminating the threat, the cells of the innate immune system become activated. These cells are equipped with a set of germ-line encoded pattern recognition receptors (PRRs)¹⁰, capable of recognizing a multitude of conserved molecules called pathogen-associated molecular patterns (PAMPs). PAMPs constitute vital-to-life components of pathogens and are generally representative of distinct classes of invading microbes (for instance, LPS is a PAMP characteristic of the cell walls of Gram-negative bacteria), allowing the activated innate immune cells to respond accordingly^{6, 11}.

PRRs expressed on sentinel cells of the innate immune system can also recognize danger through stress signals released by dying cells undergoing an infection event – DAMPs, or damage-associated molecular patterns. These molecules are oftentimes normal constituents of the cells, such as ATP and uric acids, that get released into the extracellular environment following injury³.

Upon recognition of pathogen- or damage-associated molecular patterns, PRRs activate a wide variety of downstream intracellular signaling pathways, culminating in the expression of specific genes and in the synthesis of a broad spectra of molecules (namely cytokines, chemokines, and cell adhesion molecules) that mediate the proinflammatory and antimicrobial response, as well as signaling the presence of infection to other cells¹².

2.1. MACROPHAGES: SENTINELS AND REGULATORS OF INNATE IMMUNITY

Macrophages are important members of the immune system and were the first innate immune cells to be described, over 130 years ago, by Ilya Metchnikoff¹³. Unlike most cell types, macrophages do not comprise a single cell population with a clearly defined phenotype. In fact, macrophages constitute a broad heterogeneous group of cells with versatile defining characteristics and functional roles in homeostatic and pathological conditions¹⁴.

For over half a century, it was believed that macrophages solely arose from the differentiation of monocytes. Monocytes are a small population of circulating blood leukocytes that represent 4-10% of mononucleated cells in the peripheral blood in humans¹⁵ and are known to have the potential to differentiate into macrophages or myeloid-lineage dendritic cells¹⁶. Monocytes and their cell progeny take part in three main functions in the immune response: (i) phagocytosis of invading pathogens, debris and dead cells, (ii) antigen presentation and (iii) cytokine production, including interleukin-1 (IL-1), IL-6 and tumor necrosis factor α (TNF α), among others¹⁷. Monocytes are usually circulating in the bloodstream, where they survive for about 1 to 3 days, patrolling for any signs of injury or infection. When needed, they rapidly move to the affected tissue and leave the blood vessels (a process called extravasation), undergoing differentiation according to the microenvironmental cues at the site of infection¹⁸.

While it is true that, in some tissues, there is a turnover of the tissue-resident macrophage population by monocyte-derived cells, that is not at all the scenario for most tissues in humans. There are multiple morphological and functional differences between the macrophages residing in tissues and the ones derived from circulating monocytes. This, along with several lineage tracing studies performed in mice over the past decade^{19, 20}, has led investigators to regard these as separated cell types. In most tissues, macrophages derive from precursors originated in the yolk sac and fetal liver during embryonic development, and are seeded before birth, retaining self-renewal capabilities that allow them to maintain a stable population independent of monocytes²¹. With this in mind, we can divide macrophages into two classes: infiltrating macrophages, which are typically found after an inciting pathology, and tissue-resident macrophages, capable of maintaining their own homeostatic population (Figure 1.2).

There are multiple populations of tissue-resident macrophages, including Kupffer cells (liver), microglia (central nervous system), histiocytes (splenic red pulp), alveolar macrophages (lungs) and osteoclasts (bone)^{22, 23}. These macrophages are of high value to the tissues they reside in, performing a variety of tasks such as apoptotic cells and debris removal, extracellular matrix remodeling and more specific functions according to the needs of the tissue, like the clearance of excess surfactant in the lungs by alveolar macrophages²⁴. These functions are

regulated by the integration of epigenetic memory and plasticity of these cells, allowing them to respond to new tissue-specific cues as the microenvironment changes²⁵.

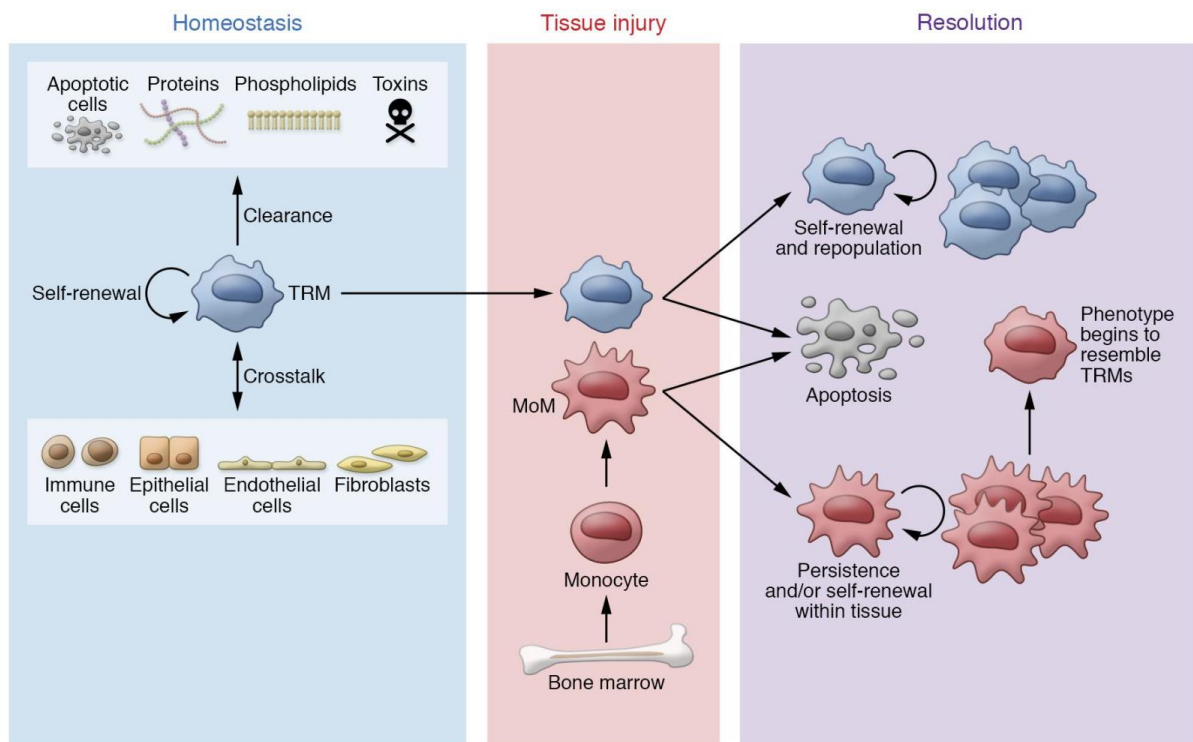


Figure 1.2. Tissue-resident macrophages and monocyte-derived macrophages play distinct roles in tissue injury and repair (adapted)²⁵. Tissue-resident macrophages (TRMs) originate from the yolk sac and fetal liver during development and persist in many tissues via self-renewal. During homeostasis (left panel), TRMs clear apoptotic cells, proteins, and phospholipids and either clear or respond to toxins, particulates, and pathogens within the local microenvironment. Many TRMs are capable of maintaining themselves by local proliferation without the contribution of monocyte-derived macrophages (MoMs). TRMs produce a variety of factors that stimulate the activation, proliferation, and differentiation of immune cells, epithelial cells, endothelial cells, fibroblasts, and stem cells that facilitate tissue homeostasis. In response to tissue injury (middle panel), bone marrow–derived monocytes are recruited to the injured tissue, where they differentiate into MoMs. During injury, TRMs and MoMs play distinct roles; usually MoMs exhibit a more robust inflammatory response. During the resolution of injury (right panel), TRMs may die or expand through self-renewal and repopulate the niche. MoMs either undergo apoptosis or persist, sometimes gaining the capacity for self-renewal. Over time, the phenotypes of TRMs and MoMs become increasingly similar. Arrows indicate interactions with other cell types.

Macrophages make up an extremely valuable component of innate cellular immunity. The remarkable plasticity of macrophages allows these cells to switch among different phenotypes, mounting a functional response to the microenvironmental stimuli and signals that they encounter in the tissue they reside in. When facing tissue injury, macrophages are confronted by different types of PAMPs or DAMPs, which induce the activation of inflammatory signaling pathways within these cells, and the consequent release of cytokines to recruit other innate immune cell populations, like monocytes and neutrophils, along with other inflammatory components. When the pathogen is eliminated and there is no longer an

imposing threat, macrophages then assume a different role as immune suppressors, mediating the consequences of the battle against the invading microbes and clearing debris, as well as producing different sets of cytokines and growth factors that allow wound healing to take place²⁶. This process of macrophage polarization driven by the local cytokine milieu, as defined by some, results in two functional subsets: the classically activated macrophages (M1) and the alternatively activated macrophages (M2)²⁷.

M1 macrophages are activated by the invasion of pathogens (through detection of different molecular patterns and inflammatory cytokines, such as interferon- γ and GM-CSF, granulocyte-macrophage colony-stimulating factor), and are able to promote the increased synthesis of pro-inflammatory cytokines (like interleukin-1 β (IL-1 β), IL-6, IL-12, IL-23, and TNF- α , among many others), along with nitric oxide or reactive oxygen species and increased complement-mediated phagocytosis, in order to efficiently eliminate the threat²⁸. M2 macrophages have anti-inflammatory and immunoregulatory healing functions and are polarized by anti-inflammatory cytokines like IL-10, IL-4 and IL-13. These macrophages then produce IL-10 and TGF β (transforming growth factor β)²⁹.

While this polarization has proven to be useful in studying macrophages *in vitro*, induced by defined mediators, we now consider the M1/M2 dichotomy far too simplistic to describe the many different phenotypes that are seen in macrophages during infection *in vivo*³⁰. This nomenclature can be particularly misleading when analyzing immune events during acute injury, when the microenvironment is inhabited by both monocyte-derived macrophages and the resident macrophages from the endangered tissue²⁵.

In recent times, it has become evident that monocytes and macrophages are key players in protecting the host against viral infection, as these cells constitute a primary source of type I interferons (IFN) upon viral infection. Type I IFN play vital roles in directly preventing viral replication, while at the same time activating/regulating the innate antiviral response^{31, 32, 33}.

2.2. PATHOGEN RECOGNITION BY THE INNATE IMMUNE CELLS

There are numerous subsets of pattern recognition receptors: (i) the Toll-like receptors (TLRs), (ii) the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), (iii) the retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs), (iv) the C-type lectin-like receptors (CLRs), (v) the absent in melanoma 2 (AIM2)-like receptors (ALRs) and (vi) other intracellular DNA sensors, such as cGAS (cyclic guanosine monophosphate (GMP)-adenosine monophosphate (AMP) synthase)^{34, 35} (Figure 1.3).

TLRs were the first PRRs identified and remain the best characterized to date³⁶. TLRs are membrane-spanning glycoproteins with an outward-facing ligand-binding domain containing leucine-rich repeat (LRR) motifs and a cytoplasmic Toll/interleukin 1 receptor homology (TIR) domain³⁷. To date, thirteen TLRs have been identified – 1-10 occur in humans and 11-13 are specific to mice. TLRs have different localizations within the cells, with most occurring in the cell surface, while others (TLR 3, 7, 8 and 9) are located intracellularly in the membranes of endosomes³⁸. They each recognize different PAMPs, such as lipopolysaccharide (LPS), flagellin, ssRNA, dsRNA and CpG DNA, characteristic of microbes like Gram-negative and Gram-positive bacteria, viruses, fungi, and protozoa. Activation of the TLRs through the TLR-PAMP

interaction results in oligomerization of the receptors, consequently triggering signaling through the nuclear factor- κ B (NF- κ B), interferon regulatory factor (IRF) and mitogen-activated protein kinase (MAPK) pathways, resulting in cytokine production^{2, 12}.

The NOD-like receptors are multidomain proteins containing a central NOD that is essential for oligomerization and activation, multiple LRR motifs at the C-terminal, responsible for ligand sensing, and one out of four known variable N-terminal domains – acidic transactivation domain, pyrin domain (PYD), baculoviral inhibition of apoptosis repeat (BIR) domain or caspase recruitment domain (CARD), all performing different effector functions and dividing the NLRs into subfamilies^{39, 40}. The NLRs can recognize a wide range of PAMPs, such as peptidoglycan (PGN, of Gram-positive bacteria), fungal hyphae, lipopeptides and flagellin, among others; DAMPs, including uric acid and ATP released by damaged cells, and some environmental factors, like alum, UV radiation, silica, etc. Recognition of these molecular patterns leads to the activation of signaling pathways (NF- κ B and MAPK) that, in turn, activate inflammatory responses⁴¹. While this is the primary function of NLRs, some also display other sets of activities, namely formation of inflammasomes with the consequent release of the proinflammatory cytokines IL-1 β and IL18, transcription activation and autophagy^{2, 42}.

The RIG-I-like receptors are RNA-sensing proteins localized in the cytosol⁵. RLRs essentially consist of a central RNA helicase-like domain, which features a DExH/D tetrapeptide motif (making it a subgroup of DEAD-box family proteins)² and a C-terminal domain (CTD), integrating a repressor domain (RD). These components are what allow RLRs to interact with viral RNAs and activate cell signaling pathways that result in the production of type I interferons (IFN I)⁴³.

The RLR family comprises three members: RIG-I, the first to be discovered, melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). Aside from the already described domains, both RIG-I and MDA5 contain an additional N-terminal region with two CARD domains, which mediate interaction with adaptor proteins and downstream signal transduction^{44, 45}. LGP2 lacks the CARD domains, and thus cannot independently induce signal transduction, however, it is thought to cooperate with RIG-I and MDA5 and regulate their viral RNA-recognition pathways⁴⁶.

C-type lectin receptors, or CRLs, have only recently been identified as a family of PRRs that is crucial to the innate immune response^{47, 48}. The CRLs comprise a wide group of proteins that share at least one carbohydrate recognition domain (also referred to as a C-type lectin-like domain), which determines the carbohydrate specificity of the CLR. Some of the most notable members of the CLR protein family include DEC-205, dectin-1, mannose receptor and langerin⁴⁹. The CLRs expressed by dendritic cells primarily recognize PAMPs such as mannose and fucose and glucan carbohydrate structures. Binding of these substances can either lead to the induction of different pathways that culminate in the expression of cytokines that can regulate T cell polarization, induce the activation of NF- κ B or modulate TLR signaling⁵⁰.

ALRs are PRRs widely accepted to be responsible for cytosolic DNA recognition and modulation of type I IFN response or inflammasome pathway induction against pathogens⁵¹. These receptor proteins are characterized by having a Pyrin signaling domain and a HIN200

DNA-binding domain; while the first is responsible for association with caspase-1 and NF- κ B activation, the latter binds to DNA. There are four different ALR genes in humans, being AIM2 and IFI16 (IFN-inducible 16) the most well-known and studied among them^{52, 53}. In fact, the function of the other ALRs is largely unclear, with a strong possibility pointing towards the activation of the ISD (interferon-stimulatory DNA) pathway⁵⁴.

Cyclic GMP-AMP synthase, or cGAS, is a newly identified cytosolic enzyme whose discovery was able to clarify the previously poorly understood mechanism by which DNA led to the immune stimulation of the downstream signaling pathway that was responsible for the induction of type I IFN and NF- κ B activation⁵⁵. It is now firmly acknowledged that cGAS, upon double-stranded DNA recognition, generates the second messenger 2'3'-cGAMP (cyclic-GMP-AMP), which, in turn, binds to and activates STING (stimulator of interferon genes), establishing the cGAS-STING pathway of DNA sensing, especially important in the detection and elimination of several viruses^{2, 56}.

A more thorough and extensive description of PRRs that are not involved in nucleic acids detection and their signaling transduction pathways can be found elsewhere^{36, 57, 58, 59, 60}.

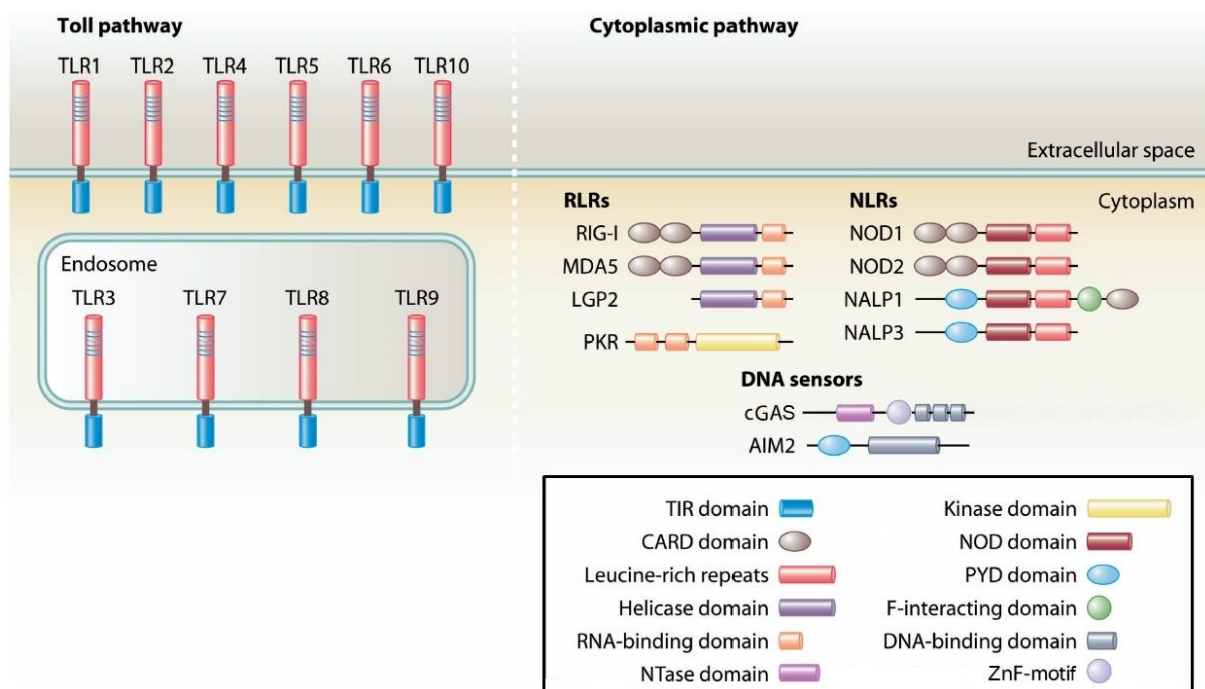


Figure I.3. Some of the cellular PRRs (adapted and modified to include cGAS, not yet discovered at the time of the publishing of this paper)¹². TLRs are membrane-bound receptors localized at the cellular or endosomal membranes, recognizing PAMPs via the LRR domain and transducing signals to the intracellular environment through the TIR domain. RLRs with a C-terminal helicase domain bind RNA and become activated to transduce CARD-dependent signaling. The dsRNA-activated kinase PKR is an intracellular PRR that senses RNA through binding to two N-terminal dsRNA-binding domains. NLRs are a class of intracellular proteins characterized by a central NOD domain and a C-terminal LRR domain, the latter of which serves as a pattern recognition domain. Signals are transduced through N-terminal domains, including CARD and pyrin (PYD) domains. cGAS and AIM2 are intracellular DNA sensors.

3. INNATE IMMUNE SIGNALING PATHWAYS

The different subsets of PRRs described in the previous section diverge in both structural properties and in which PAMPs and DAMPs they are able to recognize. This leads to the activation of different downstream cascades that culminate in the expression of a plethora of proinflammatory molecules, which, in turn, orchestrate the early response to infection and subsequently activate the adaptive immune system, effectively eliminating the threat⁶¹. These signaling pathways share many similarities among the different PRRs and are responsible for controlling a vast number of cellular processes – such as inflammation, apoptosis, and autophagy, among others, determining the fate of the affected cell, as well as the fate of the cells in its immediate surroundings. As a matter of fact, the integrated regulation of the many recognition receptors is dependent on a rather small number of downstream signaling pathways, the major ones being the NF- κ B pathway, the MAPK/ERK pathway and the Type I IFN or IRF3/IRF7 pathway^{12, 62}.

3.1. CANONICAL NF- κ B PATHWAY

In mammalian cells, the NF- κ B family of inducible transcription factors consists of five members – p105/p50 (NF- κ B1), p100/52 (NF- κ B2), RELA (or p65), RELB and c-REL, which share a conserved amino-terminal Rel homology domain (RHD). This 300 amino-acid long domain is responsible for dimerization, interaction with other molecules, binding to DNA and nuclear translocation⁶³.

There are two major signaling pathways that give way to NF- κ B activation: the canonical and the noncanonical pathways⁶⁴. The canonical NF- κ B pathway constitutes one of the most common downstream signaling events following stimuli detection by the PRRs (Figure 1.4). This signaling cascade is primarily mediated by a complex of Inhibitory κ B (I κ B) kinases (IKKs), consisting of three core elements: the IKK α and IKK β kinases and the regulatory subunit IKK γ (also known as NEMO, or NF- κ B essential modifier)⁶⁵. The I κ Bs are inhibitors of NF- κ B activity, constitutively bound to and retaining the transcription factor in the cytoplasm.² The IKK complex, when activated, is responsible for the site-specific phosphorylation of I κ B at two serine residues through its IKK β subunit. Phosphorylation targets I κ B for ubiquitin-dependent degradation through the proteasome pathway, which ultimately frees NF- κ B, allowing its activation in driving the transcription of multiple genes for pro-inflammatory cytokines including TNF α , IL-6 and pro-IL-1 β ^{66, 67}.

All TLRs are known to induce NF- κ B network activation in host cells. Most TLRs use MyD88 (Myeloid differentiation factor 88) as a signal transduction adapter, binding to it through the TIR domain in its cytoplasmatic tail and MyD88's own TIR domain, present at its C-terminus⁶⁸. MyD88 uses its death domain to recruit the serine–threonine members of the IRAK (IL-1 receptor-associated kinase) family, IRAK-1 and 4, in association with the ubiquitin E3 ligase TRAF-6 (tumor necrosis factor receptor-associated factor 6)⁶⁹. TRAF-6 is capable of promoting IKK γ ubiquitination and, together, they recruit a signaling component shared by different PRRs networks for the NF- κ B pathway, the transforming growth factor- β -activated kinase 1 (TAK1)⁷⁰. Upon binding to poly-ubiquitin chains, TAK1 is activated and is then capable of phosphorylating and thus, activating, the IKK complex^{2, 67}.

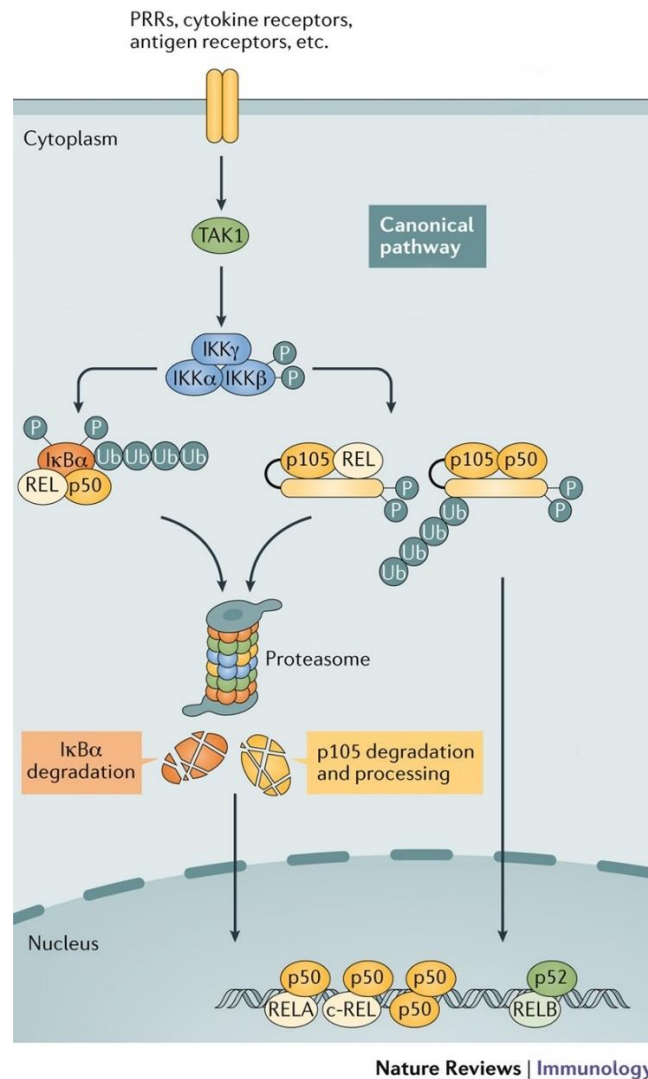


Figure I.4. Canonical NF-κB pathway (adapted)⁷¹. The canonical NF-κB cascade is triggered by signals from a large variety of immune receptors, which activate the kinase TAK1. TAK1 then activates a trimeric IκB kinase (IKK) complex, composed of catalytic (IKKα and IKKβ) and regulatory (IKKγ) subunits, via phosphorylation of IKKβ. Upon stimulation, the IKK complex, largely through IKKβ, phosphorylates members of the IκB family, IκBα and the IκB-like molecule p105, which sequester NF-κB members in the cytoplasm. IκBα and p105 associate with p50 and members of the REL family (RELA or c-REL). Upon phosphorylation by IKK, IκBα and p105 are targeted for ubiquitin-dependent degradation in the proteasome, resulting in the nuclear translocation of canonical NF-κB family members, which bind to specific DNA elements.

3.2. MAPK/ERK PATHWAY

The MAPK family consists of fourteen members in mammals, the most thoroughly investigated being extracellular signal-regulated kinase 1 (ERK1), ERK2, p38α, Jun N-terminal kinase 1 (JNK1) and JNK2⁶². MAPK activation in cells of the innate immune response has been mostly studied in regard to the TLRs, with a vast set of similarities and shared elements regarding the NF-κB pathway.

Much like in the NF-κB pathway, detection of PRRs by most TLRs will result in the activation of MyD88, which then recruits members of the IRAK family to form a complex with TRAF-6 and

the E2 ubiquitin-conjugating enzyme 13, UBC13. TRAF-6 and UBC13 will lead to the activation of the MAPK pathway through TAK1, as, aside from phosphorylating the IKK complex, it is also capable of phosphorylating members of the MAPK kinase (MKK) family. Ultimately, these signaling cascades result in the activation of activator protein (AP-1), a transcription factor that will transcribe cytokine genes¹².

3.3. TYPE I INTERFERON PATHWAY

Interferons are an extensive class of pro-inflammatory molecules essential for mobilizing the host immune response, especially against viral infections, and are thus of great importance. There are three different classes of interferons: type I IFNs (IFN α , IFN β and other less characterized elements), type II IFN (IFN γ) and type III IFN (IFN λ -1, 2 and 3)^{5, 72}.

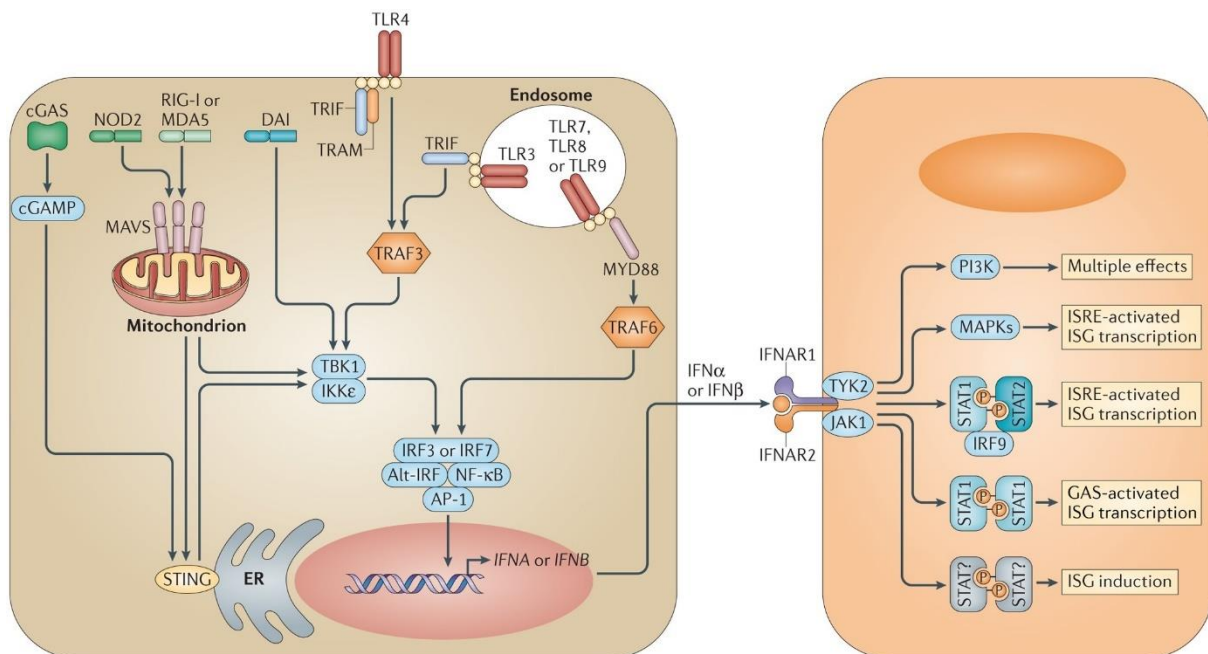
The Type I IFN were initially discovered as soluble factors that interfered with viral infection⁷³. It is now known that these cytokines have powerful antiviral activity that is able to shape both innate and adaptive immunity and are produced by many different cells, mainly monocytes, macrophages, and fibroblasts. These interferons can be detected by most, if not all, cells in humans, due to the broad expression of the IFN receptor⁷⁴. The human genome encodes for thirteen partially homologous IFN α subtypes, a single IFN β and multiple poorly established single gene products (IFN ϵ , IFN τ , IFN κ , IFN ω , IFN δ and IFN ζ)⁷⁵.

Under homeostatic conditions, type I interferons are expressed at very low and oftentimes even undetectable levels. In case of infection, their expression in host cells is promptly induced through several signaling pathways⁷⁶. These signaling cascades are usually initiated by nucleic acid sensors – PRRs such as some TLRs (TLR3, TLR7-TLR9), the RLRs, AIM2 and cGAS, and usually converge on the IRF family of transcription factors. Consequently, the genes encoding for IFN α and IFN β are transcribed. For the most part, IRF3 and IRF7 are the fundamental IRFs partaking in this function, although others can also induce IFN α/β gene transcription^{77, 78}. It is disputed whether NF- κ B is required as a cofactor to type I IFN production and the explicit role of the NF- κ B cascade in this pathway⁷⁸.

When cells secrete type I IFN, they can communicate with their neighboring cells through either an autocrine or a paracrine process. In these cells, type I IFN binds to its widespread receptor, IFNAR (interferon- α/β receptor), eliciting a new cascade of antiviral gene expression.

IFNAR is a heterodimeric transmembrane receptor consisting of two functional subunits, IFNAR1 and IFNAR2. IFNAR1 is constitutively associated to tyrosine kinase 2 (TYK2) and IFNAR2 is constitutively associated to Janus-activated kinase 1 (JAK1); following the engagement of the heterodimer, these kinases undergo rapid autophosphorylation and the Janus kinase–signal transducer and activator of transcription (JAK–STAT) pathway is initiated^{79, 80}. Activation of the TYK2 and JAK1 kinases results in phosphorylation of members of the STAT family, especially STAT1 and STAT2, which then combine with interferon regulatory factor 9 (IRF9) to form the interferon-stimulated gene factor 3 (ISGF3) complex, capable of translocating to the nucleus and inducing the transcription of a plethora of type I IFN-stimulated genes, ISGs (Figure I.5)⁸¹.

The JAK–STAT pathway of immune signaling is the most thoroughly studied downstream pathway upon IFN I secretion; nevertheless, it has now become apparent that several other cascades are required for the generation of many of the responses to type I interferons, some operating independently of the JAK-STAT pathway and others cooperating with STATs to optimize transcription of ISGs⁸⁰.



Nature Reviews | Immunology

Figure I.5. Pathways of type I interferon induction and receptor signaling (adapted)⁷⁴. Recognition of microbial products by a range of cell-surface and intracellular PRRs, including TLRs and RIG-I, can lead to induction of the genes encoding type I interferons, which is mediated by several distinct signaling pathways. On the binding of type I IFNs to their receptor (IFNAR), multiple downstream signaling pathways can be induced, leading to a diverse range of biological effects. The canonical STAT1–STAT2–IRF9 signaling complex (also known as the IFN-stimulated gene factor 3 (ISGF3) complex) binds to IFN-stimulated response elements (ISREs) in gene promoters, leading to induction of a large number of IFN-stimulated genes. Type I IFNs can also signal through STAT1 homodimers, and other STAT heterodimers and homodimers may also be activated downstream, including STAT3, STAT4 and STAT5. Other signaling pathways that do not rely on JAK and/or STAT activity may also be activated, thereby leading to diverse effects on the cell.

Interferon-stimulated genes (ISGs), the products of the IFN signaling pathway, were discovered in the 1980s and have since then been an ever-growing field, with hundreds of ISGs identified in many cell types^{82, 83}. The ISG family even includes genes that encode some of the previously mentioned PRRs, IRFs and several transducing proteins, including STAT1/2 and RIG-I, which are already present in cells at basal levels, but are also induced by type I IFN to more efficiently reinforce the immune response, from pathogen detection to its elimination⁷². Some ISGs especially target viruses that have invaded host cells, being that every stage of the virus life cycle, from its entry to the replication of its genome, can become an ideal moment for ISG intervention (Figure I.6). The majority of the more widely known ISGs are subject of recent

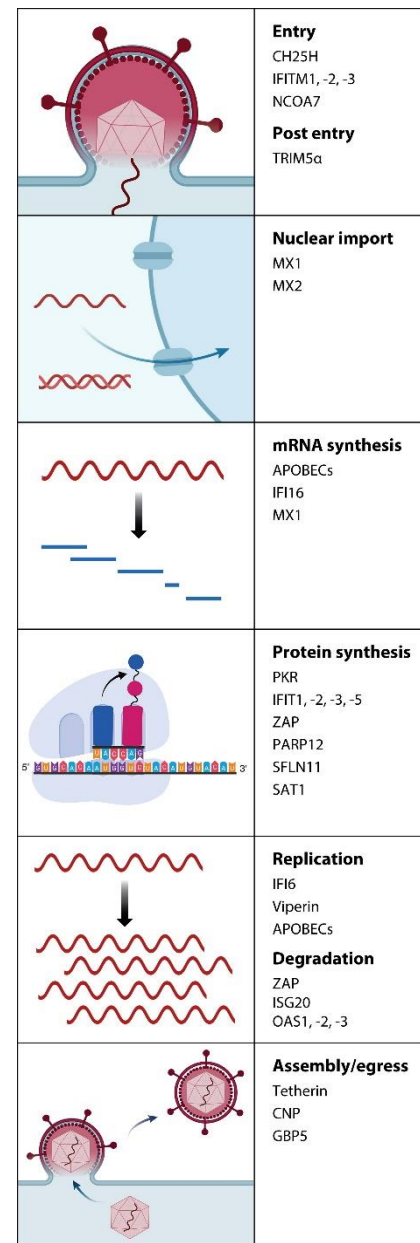
reviews^{72, 84, 85, 86}, and will not be discussed in great detail here. Thus, only a summary of antiviral ISGs relevant to this dissertation is provided.

A popular way to categorize the antiviral ISGs by their mechanism of action is by assessing which part of the viral life cycle they target. There are three main stages from the point where the virus invades the organism: (i) attachment, entry, and trafficking, (ii) translation and replication, and (iii) particle assembly and egress^{72, 86}.

One of the first identified ISGs responsible for inhibition of viral entry was murine myxovirus resistance 1 (Mx1), which encodes for the MxA protein in humans. Evidence suggests that MxA is a dynamin-like GTPase that targets viral nucleocapsids by forming highly ordered oligomer rings around these structures, preventing them from reaching their desired cellular destination, and hindering the viral cycle prior to the establishment of replication^{87, 88}.

Interferon induced protein 44-like, IFI44L, is an ISG belonging to the IFI44 family, which reportedly acts on viral replication, possibly limiting resources that the viruses need to make more copies of themselves, although the mechanism is still vastly uncertain. IFI44L is markedly antiproliferative, which might be related to how it is able to reduce viral replication and thus, have potent antiviral activity towards viruses such as HCV (Hepatitis C virus) and RSV (Respiratory Syncytial Virus)^{89, 90}.

RSAD2 (radical S-adenosyl methionine domain containing 2) is one of the most well-studied and highly induced antiviral ISGs, and it encodes the Viperin (virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible) effector protein. Viperin is anchored to the endoplasmic reticulum (ER) and also localizes to ER-derived lipid droplets, organelles of importance when it comes to the replication cycle of many viruses⁹¹. Viperin can act against many clinically important viruses, and the mechanism of action seems to vary according to their different life cycles – for the influenza A virus and HIV-1 (human immunodeficiency virus 1), Viperin has been described to inhibit budding at the host cell membrane^{92, 93} and for HCV and DENV-2 (dengue virus type 2) it interacts with components of the viral replication complex, inhibiting RNA replication^{94, 95}.



 Schoggins JW. 2019.
Annu. Rev. Virol. 6:567–84.

Figure I.6. ISG targeting distinct steps in the viral replication cycle (adapted)⁷⁹. Viral replication steps are shown in boxes. Examples of some of the currently identified ISG effectors targeting viral entry, viral genome nuclear import, viral gene or protein synthesis, viral genome replication, or virion assembly/egress are indicated next to each box.

4. RNA/DNA DETECTION: VIRAL IMMUNE SENSORS

Many pathogens depend on nucleic acids for replication and propagation within the host. It is, thus, indispensable that mechanisms capable of detecting and targeting abnormal RNA and DNA molecules exist and evolve within innate immunity to eliminate this threat.

The heterogeneous group of pathogen recognition receptors responsible for nucleic acid sensing are localized inside of the cells, either in endosomes or the cytosol. These PRRs include the endosomal TLRs (TLR3, TLR7–TLR9) and the cytosolic RNA sensors RIG-I and MDA5, along with the cytosolic DNA sensors AIM2 and cGAS (Figure I.7).

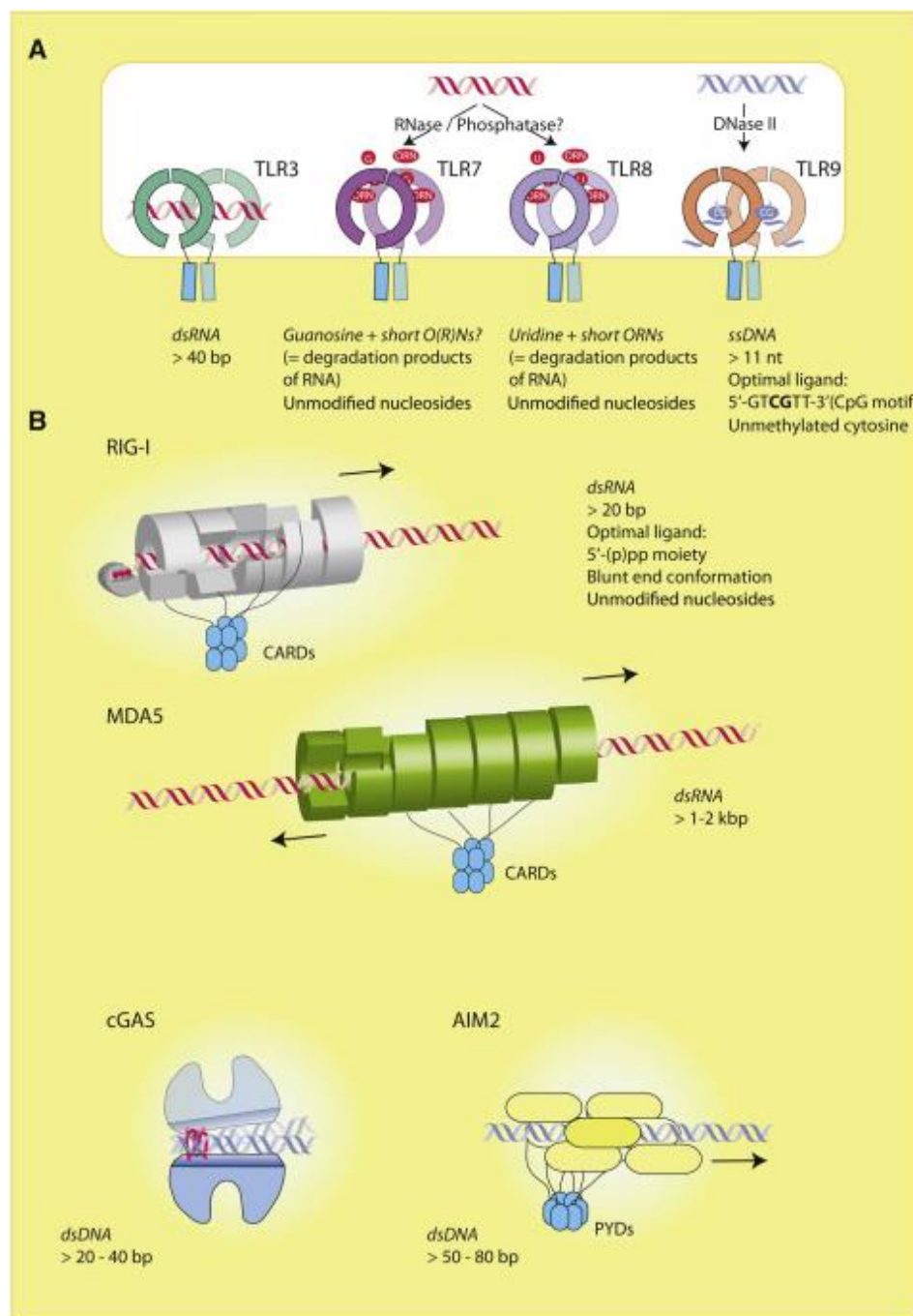


Figure I.7. Nucleic acid sensing PRRs (adapted)⁹⁶. A number of PRRs scan the cell for the presence of nucleic

acids. This includes **(A)** TLR3 and TLR7–TLR9, whose nucleic acid-binding domains face the lumen of the endosomal compartment, and **(B)** the sensors RIG-I, MDA5, cGAS, and AIM2 in the cytoplasm. A simplified schematic view of their structures and cognate ligands is shown.

4.1. NUCLEIC ACID-SENSING TLRs

The endosomal Toll-like receptors detect extracellular RNA and DNA that enter the host cells through endocytosis⁹⁷. In humans, the first identified PRR capable of sensing nucleic acids was TLR9. This Toll-like receptor recognizes unmethylated CpG (cytosine-phosphate-guanine) dinucleotides, a common feature of bacterial and viral DNA^{98, 99}. Binding of CpG DNA to TLR9 is thought to result in the recruitment of MyD88, activating the NF- κ B and MAPK signaling pathways¹⁰⁰.

The other TLRs in the endosomal compartment are RNA sensors: TLR3 recognizes dsRNA and induces production of both IFN- β and proinflammatory cytokines through IRF3 and NF- κ B activation¹⁰¹, while TLR7 and TLR8 are activated by ssRNA (single-stranded RNA), inducing MAPK cascades that lead to AP-1 activation and NF- κ B-dependent type I cytokines, respectively¹⁰².

4.2. RLRs – CYTOSOLIC RNA SENSORS

Invading RNA viruses deliver their genome into the cytoplasm of host cells and take advantage of the host machinery, forcing the cells to produce viral proteins and replicate the foreign RNA multiple times to produce progeny¹⁰³. In the cytosol, the RLRs RIG-I and MDA5 recognize different pathogen-derived dsRNA species. These receptors are encoded by ISGs (DDX58, DExD/H-Box Helicase 58, for RIG-I, and IFIH1, Interferon Induced With Helicase C Domain 1, for MDA5), and thus, their expression levels are heavily increased with IFN exposure observed during viral infection⁴². It is now a known fact that infections with almost all the most well studied RNA virus families are recognized by RLRs⁴⁵.

RIG-I recognizes blunt-end short dsRNA bearing 5'-triphosphate or 5'-diphosphate (although with lower affinity), along with ssRNA bearing 5'-triphosphate¹⁰⁴, whilst MDA5 is known to preferentially bind to long dsRNA (> 1-2 kbps)¹⁰⁵.

Signaling through RIG-I and MDA5 is mediated by a common adaptor, MAVS (mitochondrial antiviral-signaling protein), a CARD-containing protein which locates to the outer mitochondrial membrane. RNA detection prompts the RLRs to associate with MAVS and establish CARD-CARD interactions, eventually leading to the formation of a signalosome of helically assembled MAVS proteins, which ultimately results in the induction of IRF3/IRF7 (through preceding activation of TBK1, TANK-binding kinase 1) and NF- κ B signaling^{77, 106} (Figure I.8). The resulting antiviral response is mediated by IFN β and a multitude of ISGs and proinflammatory cytokines.

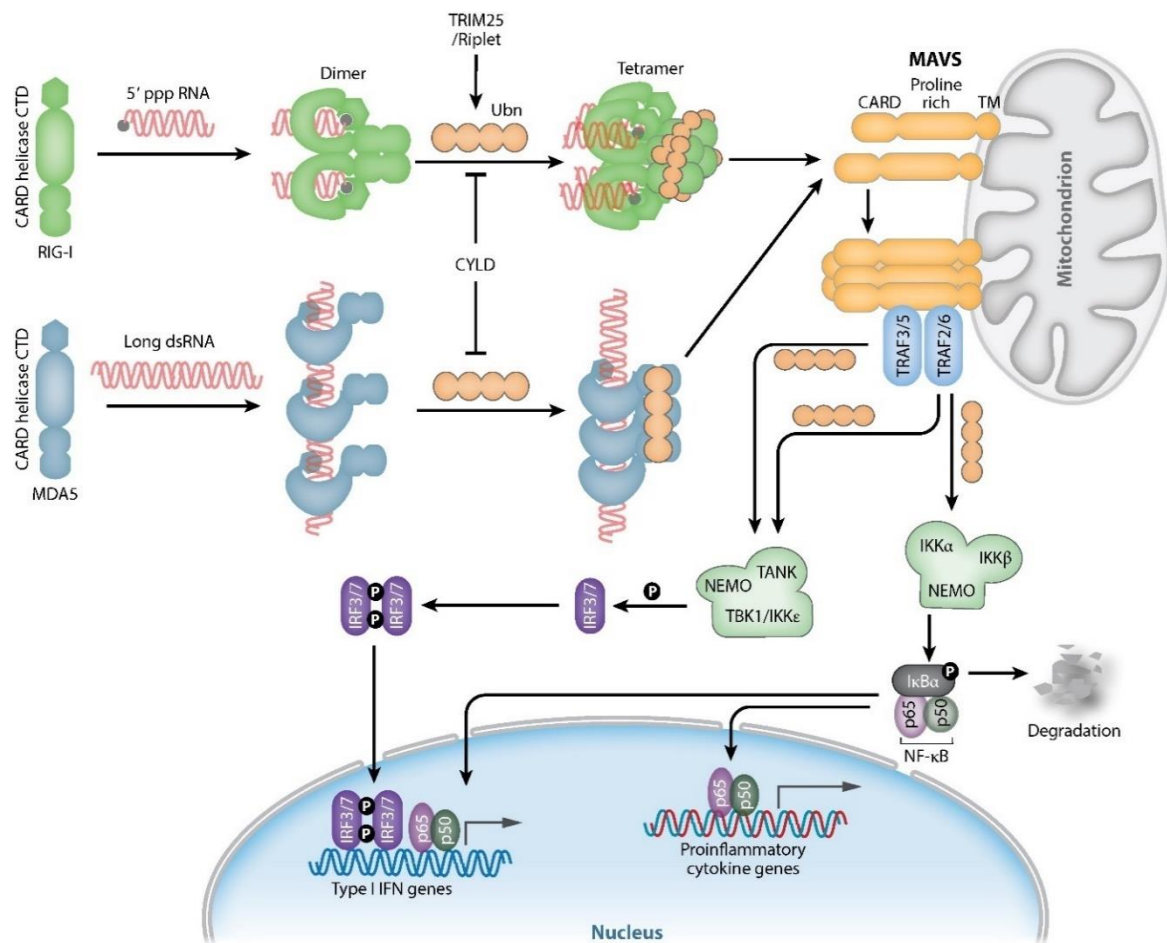


Figure I.8. Cytosolic RNA-sensing pathway (adapted)⁴⁴. Viral RNA in the cytosol is sensed by RLRs, which include RIG-I and MDA5. RIG-I specifically recognizes dsRNA bearing 5'-triphosphate, or 5'-diphosphate. RNA binding induces a conformational change of RIG-I that exposes its tandem CARDs. MDA5 recognizes long dsRNA, and multiple MDA5 molecules can be assembled along RNA, with the CARDs exposed. Binding of K63-linked polyubiquitin chains to CARDs of either RIG-I or MDA5 triggers their oligomerization, which provides oligomer templates to initiate a helical assembly of the adaptor protein MAVS on the outer membrane of mitochondria. Through recruitment of TRAF proteins, MAVS activates TBK1 and IKK through a K63 polyubiquitin-dependent mechanism. TBK1 and IKK complexes activate transcription factors IRF3 and NF-κB, respectively, and turn on type I IFN and proinflammatory cytokines. Bar-ended lines indicate inhibition. Abbreviations: P, phosphoryl group; TM, transmembrane domain; Ubn, polyubiquitin chain.

4.3. CYTOSOLIC DNA DETECTION

Every microbial pathogen, with the exception of RNA viruses, depends on DNA for their replication. In homeostatic conditions, DNA is usually confined within the nuclear and mitochondrial compartments of eukaryotic cells. If DNA is found by sensors in abnormal cell sites, such as the cytosol, powerful immune cascades resulting in inflammasome activation and type I IFN induction are triggered¹⁰⁷.

To date, numerous proteins have been proposed as cytosolic DNA sensors, among which AIM2 and cGAS represents essential ones, both detecting dsDNA in a length-dependent manner,

originated not only from viruses, retroviruses, bacteria and protozoa, but also self-DNA such as mitochondrial and nuclear DNA that gain access to the cytosol^{108, 109}.

AIM2 is a cytosolic DNA sensor, that, upon binding to dsDNA (preferably at a length of around 200bps or more), prompts a signaling cascade that culminates in the formation of a multiprotein inflammasome complex. The inflammasome, together with ASC (apoptosis-associated speck-like protein containing a CARD), activates caspase-1. This allows for the maturation of the IL-1 β and IL-18 cytokines and activation of the pore-forming protein gasdermin D, ultimately resulting in programmed pyroptotic cell death as an indicator for the cell's danger status^{110, 111}.

cGAS represents the most widely accepted dsDNA sensor, playing a core role in recognizing dsDNA in the cytosol, independently of its sequence and inducing both the production of type I IFNs and proinflammatory cytokines. This pattern recognition receptor, along with its downstream signaling pathway, is discussed in more detail in the next section.

5. THE cGAS-STING PATHWAY OF DNA SENSING

It has been known that DNA is capable of enabling immune responses since as early as the beginning of the 20th century¹¹². However, cyclic GMP–AMP synthase, cGAS, the dsDNA sensor responsible for activating the signaling cascade inside the host cells was identified only less than 10 years ago, in 2013, through the use of biochemical fractionation and quantitative mass spectrometry⁵⁵. Since its discovery, our understanding of cGAS has increased to the point where we now know about its structure, regulation and function, as well as how it impacts the immune response and its role in some human diseases (Figure I.9)¹¹³.

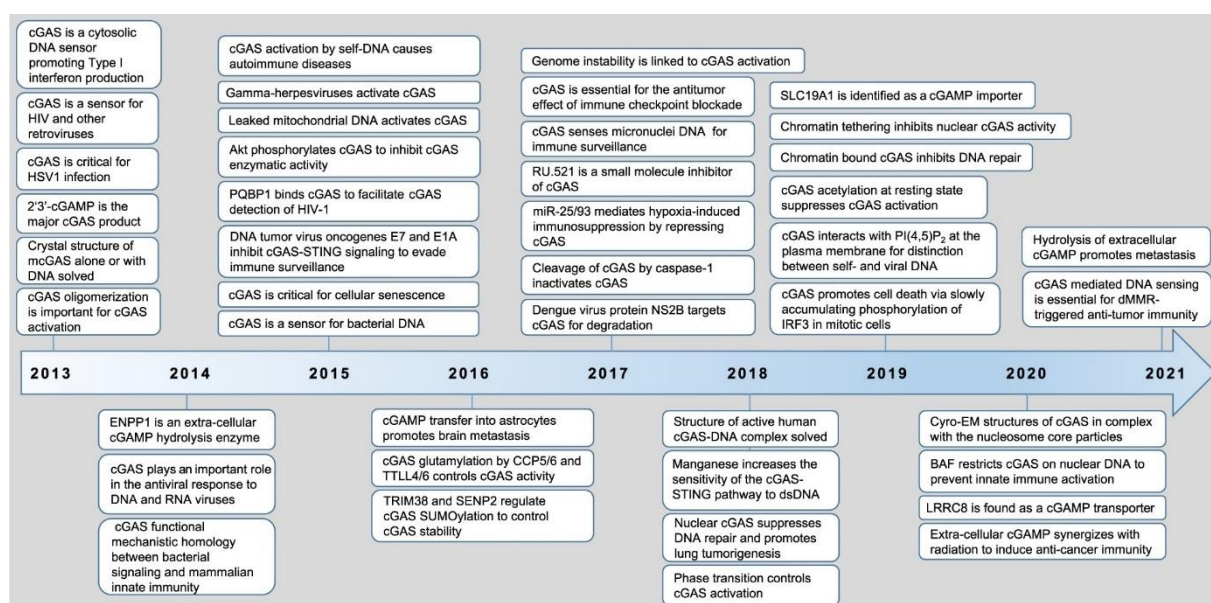


Figure I.9. A timeline for discoveries of cGAS regulation and function (adapted)¹¹³. Within less than a decade, our understanding of cGAS structure, regulation, and function in human diseases has been significantly advanced due to contributions from many intriguing studies.

5.1. MECHANISM OF cGAS ACTIVATION

Under homeostatic conditions, cGAS resides as an inactive protein in the cytosol of host cells. Recent studies have reported that cGAS can also localize to the cell's nucleus, which raises questions regarding how its activation is prevented when confronted with normal self-DNA present in this cellular compartment¹¹⁴.

Upon dsDNA binding, cGAS is activated and synthesizes the cyclic dinucleotide cGAMP. The binding of DNA to cGAS is mediated through mostly electrostatic and hydrogen bonding interactions. cGAS is comprised of two functional lobes, the N-lobe and the C-lobe, which are separated by a deep groove harboring the active site of the protein. The N-lobe is much similar to the ones characteristic of most template-independent nucleotidyltransferase (NTase) enzymes, such as OAS1 (2'-5' oligoadenylate synthase 1)^{115, 116}, while the C-lobe is made up of a tight five α -helix bundle, including a unique zinc-binding motif, which is also appended to the NTase core scaffold, essential for dsDNA recognition¹¹⁵. In its unbound state, cGAS is monomeric, yet, instead of forming a 1:1 complex with DNA, two active cGAS molecules bind to two dsDNA molecules, constituting a 2:2 cGAS:DNA complex¹¹⁷ (Figure I.10). Short dsDNA is unable to activate cGAS since it's incapable of inducing cGAS dimerization. In this manner, longer dsDNA, ideally with a length of over 45bps, is required for efficient dimerization and activation of cGAS¹¹⁸.

The binding of DNA to cGAS triggers conformational changes in cGAS that induce its catalytic activity – its cleft becomes permissive to binding of adenosine triphosphate (ATP) and guanosine triphosphate (GTP), the substrates necessary for subsequent production of 2'3'-cGAMP¹¹⁷. cGAS generates a cGAMP with two unique phosphodiester bonds: one between the 2'-hydroxyl group (OH) of GMP and the 5' phosphate of AMP, and the other between the 3' OH of AMP and 5' phosphate of GMP^{119, 120}. DNA binding to cGAS additionally induces an event of liquid-liquid phase separation, with the formation of liquid-like droplets, where the reactants for cGAMP production are at increased concentrations, allowing for heightened cGAS activity^{121, 122}.

The second messenger 2'3'-cGAMP binds to and activates the ER-resident adaptor STING.

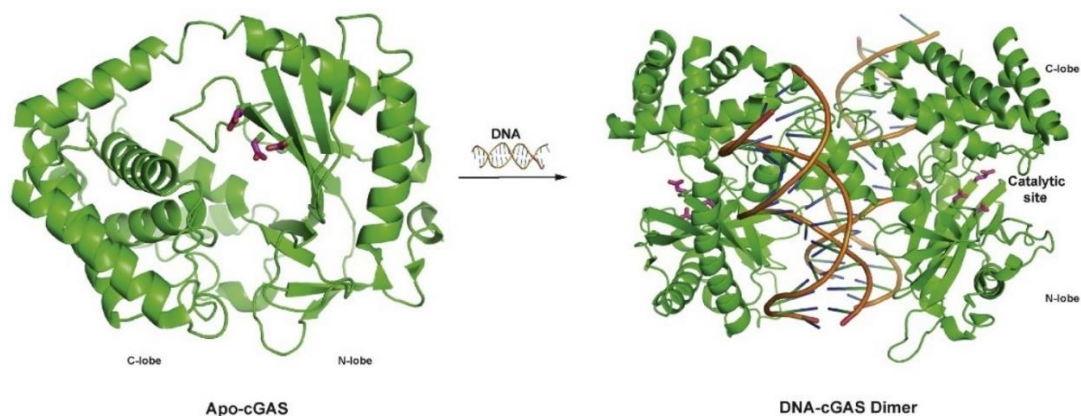


Figure I.10. Structural Basis of cGAS activation (adapted)¹²³. Inactive cGAS is a monomer. Binding to the sugar-phosphate backbone of DNA leads to dimerization of the cGAS-DNA complex and structural rearrangements in cGAS that expose its catalytic site (highlighted) for nucleotide binding and catalysis.

5.2. ACTIVATION OF STING SIGNALING

STING, encoded by TMEM173, is a dimeric protein anchored in the endoplasmic reticulum. Crystallization studies have shown that STING is composed of an N-terminal segment with four transmembrane helices, a connector region and a cytosolic C-terminal portion comprising a ligand-binding domain (LBD) and a C-terminal tail (CTT)¹²⁴ (Figure I.11a).

STING is a known cyclic dinucleotide (CDN) sensor, including cyclic di-GMP and cyclic di-AMP, second messengers in gram-negative and gram-positive bacteria, respectively^{125, 126}, along with the newly found 3'3'-cGAMP (also known as bacterial cGAMP)¹²⁷ and the product of cGAS enzymatic activity, 2'3'-cGAMP.

Upon binding of CDNs to both subunits of the STING dimer, STING is activated and undergoes extensive conformational rearrangements, even more so with 2'3'-cGAMP when compared to the other CDNs, which, aside from the standard 180° clockwise rotation (or untwisting) of the LBD, induces a ~20Å inward rotation, originating a deeper groove for cGAMP binding, along with the closing of a β -sheet lid on top of it^{128, 129} (Figure I.11b). The untwisting of the LBD is suggested to alter the surface geometry of the STING homodimer in such a way that lateral oligomerization of several STING dimeric molecules, in a side-by-side configuration, is facilitated. Upon oligomerization, STING exits the ER and traffics through the ERGIC (ER-Golgi intermediate compartment) towards the Golgi, with the help of iRhom2 (inactive rhomboid protein 2)¹³⁰, COPII (coat protein complex II) and ARF (ADP-ribosylation factor) GTPases^{131, 132}. During this process, STING interacts with TBK1, inducing its trans-autophosphorylation. TBK1 phosphorylates other TBK1 molecules in close proximity, ultimately exerting catalytic activity towards the Ser residue within the pLxIS motif present in the CTT of STING. Following this, the CTT of STING serves as a docking site for IRF3, which is then phosphorylated and activated by TBK1, forming a homodimer to enter the nucleus and induce the transcription of type I IFN¹³³ (Figure I.11c).

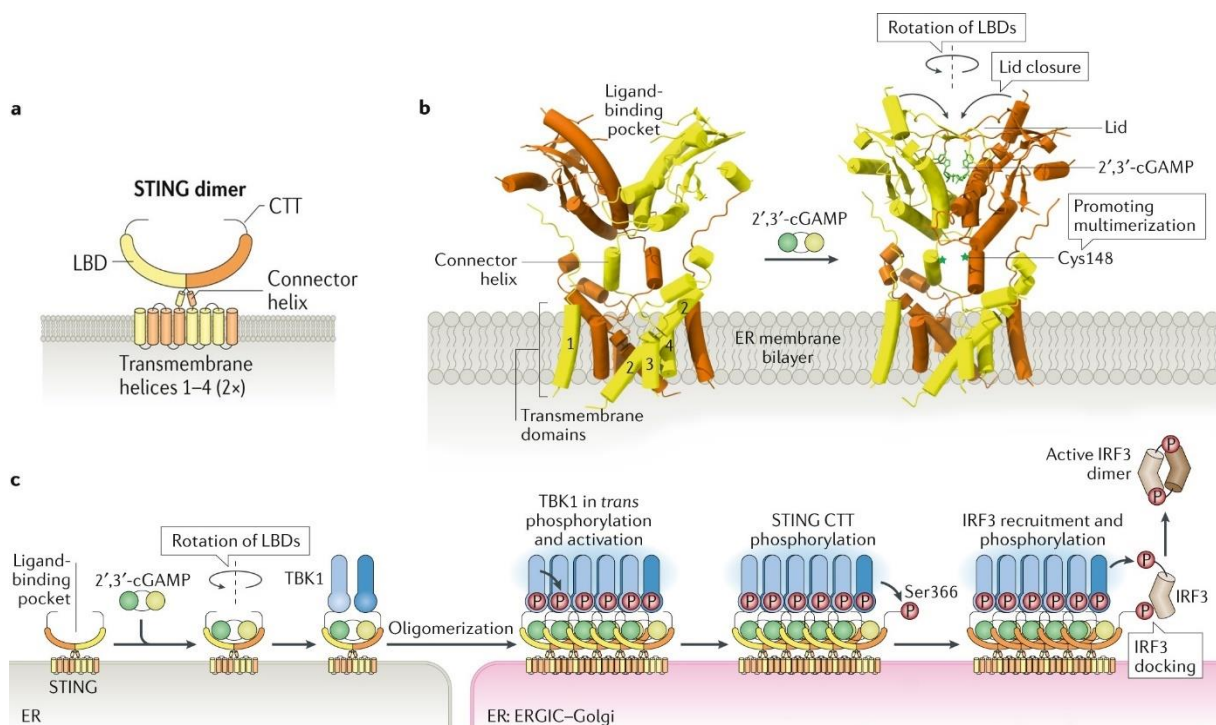


Figure I.11. Mechanism of STING activation (adapted)¹⁰⁸. (a) Membrane-bound STING dimer with annotated structural domains and elements. (b) Cryo-electron microscopy structure of the ligand-free chicken STING dimer (PDB ID 6NT6) and the 2'3'-cGAMP-bound STING dimer (PDB ID 6NT7). 2'3'-cGAMP binding results in closing of the lid and a rotation of the ligand-binding domains (LBDs) with respect to the transmembrane domains, generating a surface geometry for oligomerization. Cys148 of human STING (green stars) is implicated in disulfide bond formation that stabilizes oligomers. (c) The events leading to IRF3 activation. 2'3'-cGAMP binds to the ligand-binding pocket formed by the LBDs of the STING dimer. This is associated with a conformation change, suggested to generate a surface geometry that promotes lateral STING oligomerization. STING dimers associate — via their CTTs — with TBK1, promoting in *trans* phosphorylation of TBK1. Active TBK1 then phosphorylates the STING CTT, creating a docking site for IRF3, which is subsequently phosphorylated by TBK1. IRF3 then dimerizes, gaining transcriptional activity.

STING translocation through the ERGIC and TBK1 are redundantly responsible for the activation of the IKK complex¹³⁴, leading to signaling through the NF- κ B pathway and culminating in the transcription of proinflammatory cytokines and chemokines, as previously described¹³⁵. Following this complex cascade of events, and upon continued stimulation, STING activity is suppressed and rapid degradation of this adaptor is issued, so as to avoid continued cytokine production, which could lead to autoinflammatory pathologies¹³⁶.

A full view of the cGAS-STING pathway of DNA sensing is shown in Figure I.12.

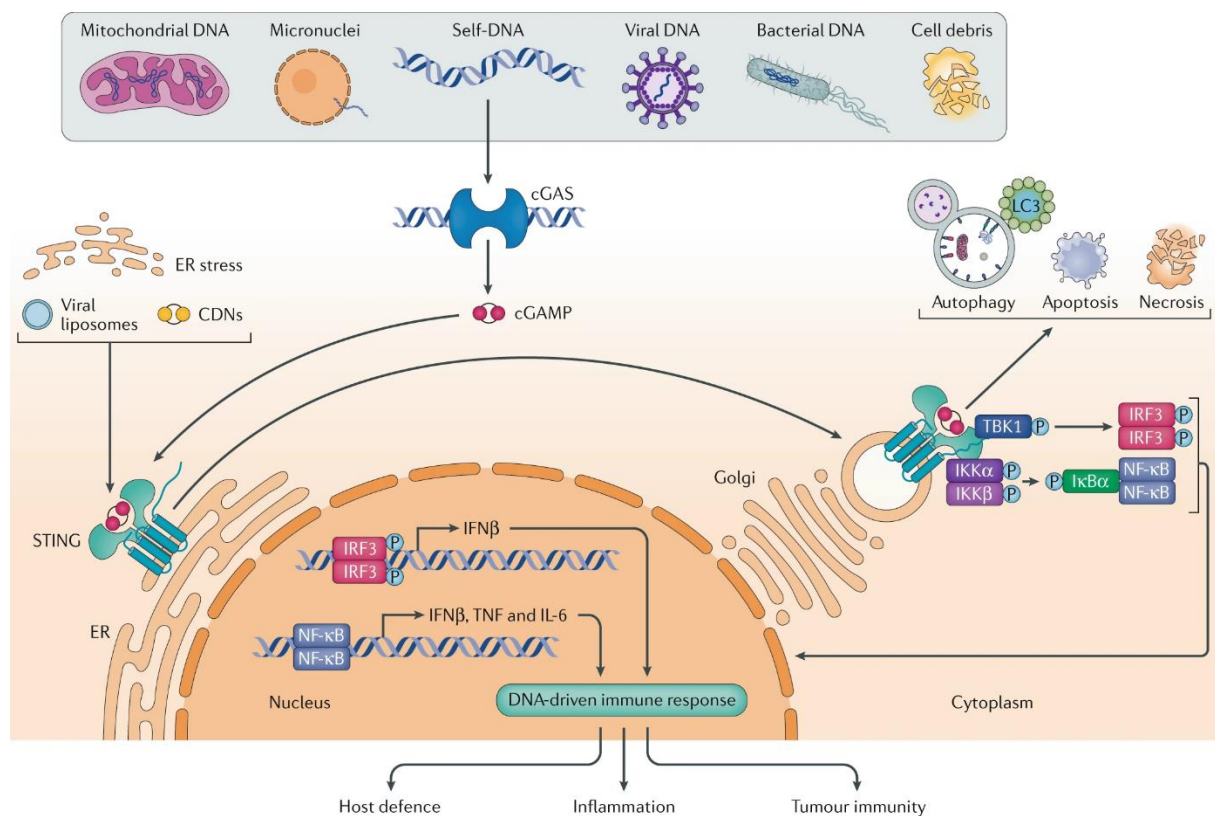


Figure I.12. The cGAS–STING DNA sensing pathway (adapted)¹³⁷. DNA from various sources can enter the cytosol and engage the cGAS–STING pathway. DNA binding activates cGAS to generate the second messenger cGAMP, which binds to STING. Stimuli other than cGAMP, including ER stress, viral liposomes, and cyclic dinucleotides, can also activate STING. Upon activation, STING translocates from the ER to the

Golgi, where it recruits TBK1 and IKK, which then phosphorylate IRF3 and I κ B α , respectively. Phosphorylated IRF3 dimerizes and translocates to the nucleus to activate transcription of genes encoding type I IFN. I κ B α phosphorylation results in translocation of NF- κ B to the nucleus, where it activates transcription of genes encoding proinflammatory cytokines. STING signaling also results in activation of other cellular process such as autophagy, apoptosis, and necrosis.

5.3. ATTENUATION OF cGAS-STING SIGNALING

Maintaining regulatory control of a pathway as important as cGAS-STING is of great significance, as its chronic activation leads to multiple health concerns. In fact, the abnormal activation of this pathway leads to excessive cytokine activation, originating autoimmune diseases such systemic lupus erythematosus (SLE), Aicardi-Goutières syndrome (AGS) and STING-associated vasculopathy with onset in infancy (SAVI)¹³⁸. A tight regulation of cGAS-STING activators and inhibitors has to continuously take place, however, how this modulation is achieved is still not fully understood, and there is much yet to investigate.

To ensure that cGAS does not bind to self-dsDNA, multiple DNase enzymes that digest dsDNA are present in the cytosol, controlling its availability as a ligand for cGAS¹³⁸. Compartmentalization is also a major mechanism of cGAS-STING regulation, as self-DNA is usually contained within the nucleus and mitochondrial compartments of the cell. However, it has recently been proven that cGAS is also present in the nucleus¹¹⁴. Recent studies, based largely on cryoelectronic microscopy, unveiled the structural basis through which nuclear cGAS is maintained inactive despite being surrounded by chromatin^{139, 140, 141, 142, 143, 144}. In the nucleus, cGAS is tightly tethered to the nucleosome, via interactions with the histones H2A and H2B. These interactions appear to have higher affinity than the cGAS-dsDNA interaction and serve to hinder the binding of cGAS to chromosomal DNA and its inappropriate activation in the nucleus.

There are numerous negative regulators limiting cGAS signaling. Some examples are the induction of autophagy¹⁴⁵, and post-translational modifications, among which acetylation, mono- and poly-glutamylation and phosphorylation^{146, 147, 148}. Aside from this, cGAS can also suffer cleavage through the action of apoptotic caspases¹⁴⁹ and its activity can be restricted by AIM2 pathway-induced gasdermin D¹⁵⁰.

Negative regulation at the level of 2'3'-cGAMP occurs primarily through the action of ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (ENPP1)¹⁵¹.

STING levels can also be regulated at various stages. STING trafficking is a potent inducer of autophagy, which, in fact, constitutes a primordial function of the cGAS-STING pathway and ends in the degradation of STING in the lysosome^{132, 152}. There is evidence pointing towards the fact that STING degradation via autophagy can occur via two distinct mechanisms: (i) basal autophagy (independent of TBK1 activation)¹³² and (ii) DNA-stimulated autophagy (mediated by TBK1 after IRF3 activation)¹⁵³.

Type (i) autophagy is a robust output of STING signaling, and it operates independently of the CTT domain of STING and in the absence of TBK1 and IRF3 activation (Figure I.13)¹³². Upon

exiting the ER and translocating to the ERGIC in COPII vesicles, WIPI2 (WD repeat domain phosphoinositide-interacting protein 2) is recruited to the site and LC3 (microtubule-associated proteins 1A/1B light chain 3) lipidation ensues, leading to the formation of autophagosomes that target microbial pathogens for degradation. While, for the most part, STING is also degraded in the lysosome, in a Ras-related protein Rab-7a (RAB7)-dependent manner, a portion of STING is able to traffic to the Golgi, and the remaining steps in the cGAS-STING pathway take place.

On the other hand, type (ii) autophagy occurs after activation of the cGAS-STING pathway to the level of IRF3¹⁵³, when TBK1 is capable of recruiting and phosphorylating p62/SQSTM1, a ubiquitin-binding selective autophagy receptor, at the S403 site. This, in turn, increases its affinity for K63 ubiquitin chains and targets STING for trafficking to autophagosomes and ubiquitin-linked degradation. This type of autophagy selectively impairs the levels of actively signaling STING.

STING can also be negatively regulated by inflammasome components from the NLR family, which sequester STING before it is able to recruit and activate TBK1^{154, 155}.

Additionally, the Nrf2 (nuclear factor (erythroid-derived 2)-like 2) transcription factor, is able to repress STING levels post-transcriptionally. Nrf2 is also linked to the autophagy machinery, as this factor regulates the expression of p62/SQSTM1, involved in the degradation of STING as per already described above.

Positive regulation of STING expression has also been described. The heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2/B1) selectively exports the mRNAs encoding for STING and cGAS from the nucleus to the cytoplasm to promote their transduction, during viral infection¹⁴⁴. Also, TMEM173, the gene encoding STING, also behaves as an ISG, as its expression is induced by type I IFN, in a STAT-1-dependent manner¹⁵⁶.

Despite all the described regulatory mechanisms, it is appropriate to believe that our understanding of the dynamic regulation of cGAS-STING during infection is still at its infancy.

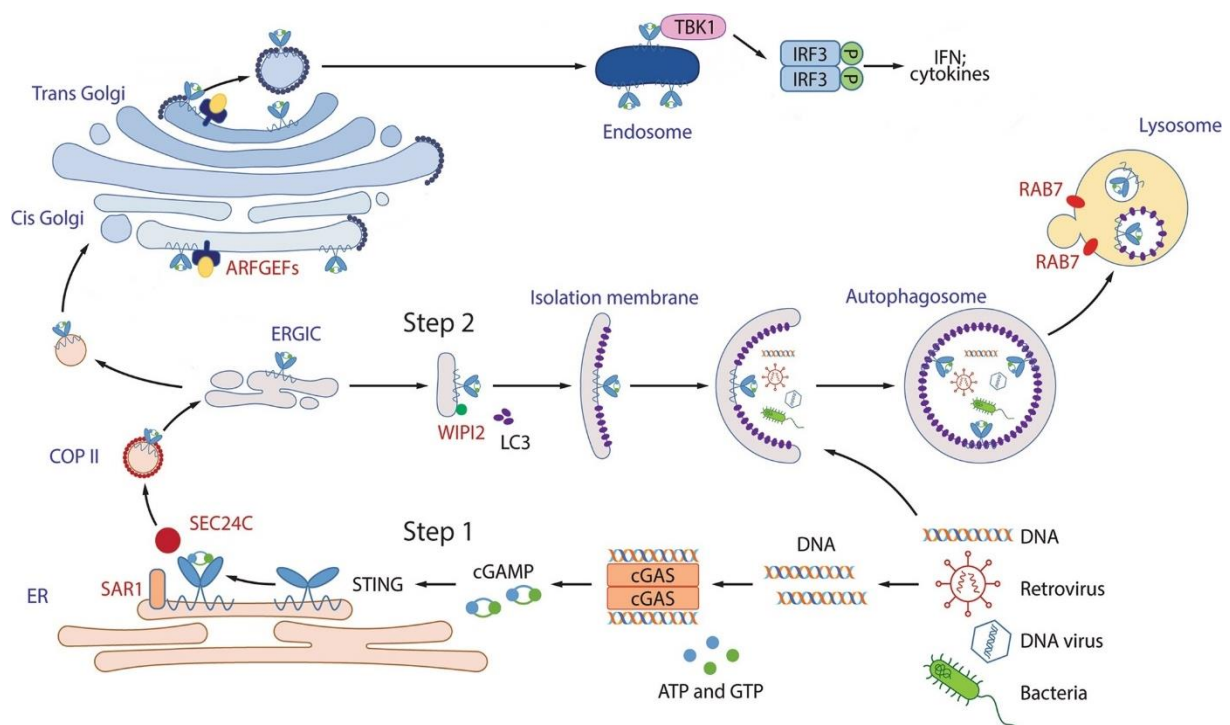


Figure I.13. A model of DNA-induced autophagy and signaling through the cGAS-STING pathway (adapted)¹³². Step 1, DNA activates cGAS to synthesize cGAMP, which binds to STING and triggers STING translocation from the ER to the ERGIC and Golgi. Step 2, the ERGIC, which contains cGAMP-bound STING, serves as a membrane source for the LC3 recruitment and lipidation through a WIPI2-dependent mechanism. LC3-positive membranes target DNA and pathogens to autophagosomes, which are subsequently fused with lysosomes in a process that requires RAB7 GTPase.

6. THE F-BAR PROTEIN GAS7 IN HEALTH AND DISEASE

Growth arrest-specific (Gas) genes are preferentially expressed in cells in the G0 phase of the cell cycle, whose quiescent state is a result of serum deprivation and growth factor-induced confluence¹⁵⁷. GAS7 (growth arrest-specific gene 7), was isolated in 1989 through the usage of a retroviral-based gene trapping strategy in serum-starved mouse embryonic fibroblasts (NIH3T3 cells)¹⁵⁸. In humans, the GAS7 gene is located on chromosome 17p13.1, which is rich in protein-coding genes and known for its remarkably high gene density, comprising a considerable amount of disease genes^{159, 160}.

The individual GAS7 gene generally encodes for more than one protein isoform, across all species in which GAS7 has been identified^{161, 162}. In humans, alternative RNA splicing leads to differential expression of the GAS7 gene into three GAS7 protein isoforms: hGAS7a, hGAS7b and hGAS7c¹⁶³ (Figure I.14). GAS7 isoform c is composed of three defined functional domains, an N-terminal SH3 (Src Homology 3) domain, followed by a WW domain and a central F-BAR (Fes/CIP4 homology-Bin/Amphiphysin/Rvs) domain. Among these, the SH3 domain is exclusive of hGAS7c, and possesses a characteristic peptide binding mode when compared to canonical SH3 domains¹⁶⁴. The human GAS7 isoform b possesses the F-BAR and WW domains, while hGAS7a contains only the F-BAR domain¹⁶⁵. The F-BAR domain, the only domain

common to all three isoforms, mediates GAS7 oligomerization and is responsible for its interaction with actin and the establishment of connections between microtubules and actin filaments by GAS7^{166, 167}.

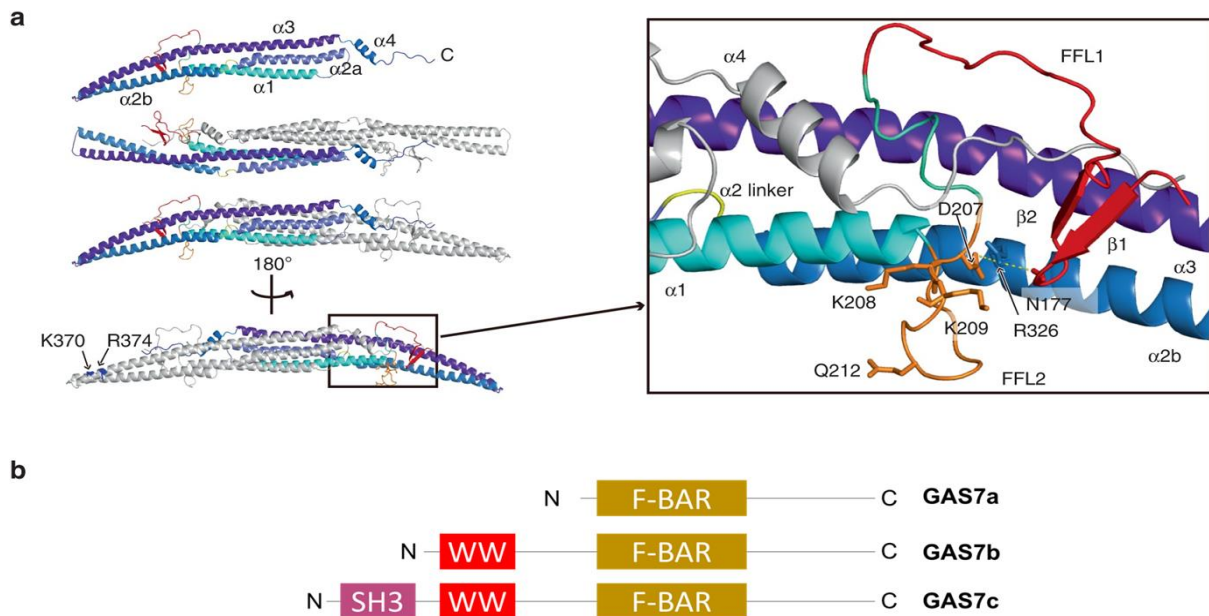


Figure I.14. Crystal structures of GAS7, the membrane-binding sites and protein isoforms (partially adapted)¹⁶³. (a) Ribbon diagrams of the crystal structure of the isolated GAS7 F-BAR domain and the GAS7-specific N-terminal region including filament forming loops, FFL1 and FFL2. Secondary structural elements are indicated. (b) GAS7 protein isoforms in humans. The F-BAR domain is the only common denominator among the isoforms, constituting the only domain present in hGAS7a. hGAS7b possesses an additional WW domain, while hGAS7c contains the previously mentioned domains, along with a unique SH3 domain.

GAS7 is predominantly expressed in brain tissues, including the cerebral cortex, hippocampus, and cerebellum¹⁶⁸, but is also present in the spleen, lung, testis and immune cells, including macrophages¹⁶³. At a subcellular level, GAS7 is mostly distributed in the cytoplasm with a preference for the submembrane region. In addition, it can also be colocalized with actin filaments and focal adhesion sites^{160, 169}.

6.1. GAS7 AS A MODULATOR OF CYTOSKELETON DYNAMICS

The F-BAR domain of GAS7 is a common feature among all of the known protein isoforms and it is thought to be present in every organism that expresses GAS7, making it the most consequential domain of the GAS7 protein. It is through the F-BAR domain of GAS7 that this protein can interact with actin, while also serving as the connecting bridge between actin filaments and microtubules^{166, 167}.

A report from the laboratory of Sue Lin-Chao was the first to describe the association of GAS7 with actin¹⁶⁶. In their work, the authors propose a model that membrane-localized GAS7 is responsible for promoting actin assembly and the crosslinking of actin filaments near the plasma membrane to induce cell protrusions. Subsequent studies revealed the colocalization and interaction between GAS7 and Neural-Wiskott Aldrich syndrome protein (N-WASP), a

crucial regulator of actin dynamics¹⁷⁰. Through its WW domain, mouse GAS7 can interact directly with proline-rich sequences from N-WASP, prompting the structural change necessary for N-WASP activation, ultimately directing the reorganization of the cortical cytoskeleton, and thus initiating the formation of membrane protrusions in neuroblastoma cells.

It was later found that overexpression of GAS7c in lung cancer cell models results in a drastic reduction of cell migration, while its silencing correlates with increased cell motility¹⁷¹. Similar to the lung cancer cell model, it has been reported that GAS7 is able to block actin polymerization in human breast cancer cells, and that this negative regulation of actin suppresses migration of these cells¹⁷². These findings suggest that GAS7 can have a negative impact on actin dynamics and doesn't solely act as a positive regulator of this component.

6.2. GAS7 FUNCTIONS IN THE NERVOUS SYSTEM

Microtubules and actin filaments (F-actin) are key molecular factors that, together, control neuronal morphology and neurodevelopment^{173, 174}. Neurite initiation, outgrowth and branching are heavily dependent on these dynamics, as the association of actin filaments with microtubules prompts the filopodia forward in the axon growth cones of neurons^{175, 176}.

The ability of GAS7 to mediate actin polymerization and establish a connection between microtubules and actin filaments makes it a vital protein during neuronal maturation and differentiation. The specific interactions established by the GAS7 protein with N-WASP and F-actin, along with its role in maintaining microtubule stability, are responsible for the reorganization of the cortical cytoskeleton and thus, for the neurite outgrowth and overall differentiation of hippocampal neurons¹⁷⁰. Furthermore, the GAS7 isoform b protein, when ectopically expressed in Neuro 2A cells, promotes crosslinking between microtubules and actin filaments, prompting alterations in cell morphology with the growth of dendrite-like branched processes¹⁶⁷. This is the case for immature neurons, in which a deficiency in GAS7 levels suppresses the maturation process of these cells, reaffirming the important role of GAS7 during neuronal maturation/differentiation.

These findings fueled further research on the possible associations between GAS7 and diverse pathogeneses of the nervous system: (1) GAS7 can be a schizophrenia risk gene, as its overexpression in mice is shown to increase branching of leading processes and consequently arrest neuronal migration, while suppression of GAS7 leads to neuronal migration inhibition¹⁷⁷; (2) another neuropathology possibly associated with GAS7 is primary open angle glaucoma (POAG), as two population studies have identified single nucleotide polymorphisms in the GAS7 gene for patients^{178, 179}; (3) GAS7 could be implicated in Alzheimer's disease and, possibly, in other tauopathies – hGAS7b shares an indispensable bond with phosphorylated tau and is a stabilizing agent for sheet intermediates¹⁶⁵, and a drastic reduction of the GAS7b levels was observed for neurons in brains from patients diagnosed with Alzheimer's disease, suggesting that a disturbance of GAS7 baseline levels might impair microtubular maintenance in this pathology.

6.3. THE POTENTIAL ROLE OF GAS7 IN TUMOR SUPPRESSION

The first association of GAS7 and its potential role as a tumor-suppressor gene dates back to 2015, when Tseng and coworkers found that GAS7 transcription was deficient in lung cells from cancer patients¹⁷¹. Both loss of function and reduced gene expression of tumor suppressor genes promote tumor cell development, dissemination, and migration, contributing to the tumor's malignancy. In this particular case, downregulation of hGAS7c significantly increases the motile behavior and metastasis of lung tumor cells.

Oxaliplatin is an antineoplastic agent used in systemic chemotherapy and approved in China for the treatment of advanced Hepatocellular Carcinoma (HCC)¹⁸⁰. A comparison between HCC cell lines and healthy liver cells revealed that GAS7c mRNA and protein expression levels are low in HCC cells. However, treatment with oxaliplatin could rescue GAS7c expression¹⁸¹. Ectopic expression of GAS7c resulted in inhibition of cell proliferation and migration abilities of HCC cells, as well as induction of apoptosis of said cells, whereas GAS7c downregulation reversed the anticancer effects of oxaliplatin, revealing that GAS7c plays a major direct role in the antineoplastic action of this agent.

A study by Chang et al. proposed yet another tumor suppressor role for GAS7 in breast tumorigenesis: hGAS7b can induce blocking of actin polymerization and dynamics, resulting in the suppression of breast cancer cell migration, invasion, and metastasis¹⁷².

Finally, in zebrafish and mammalian models of neuroblastoma, the absence of GAS7 led to accelerated metastasis possibly due to increased migratory capacity of tumor cells¹⁸².

6.4. GAS7 AND ITS IMPLICATIONS IN MACROPHAGES AND INNATE IMMUNITY

GAS7 is expressed at significant levels in some immune cell types, such as macrophages and other myeloid cells¹⁶³. Yet, apart from a few recent studies detailed below, little is known about its actual roles in immune cell function.

Macrophages are formidable phagocytic cells that internalize large, micron-sized particles unable to be internalized by other endocytic pathways¹⁸³. Phagocytosis is heavily dependent on the extension of the plasma membrane to be able to engulf particles. A recent study on the potential role of GAS7 in mediating phagocytosis found that mouse GAS7b plays an essential role in phagocytic cup formation in macrophages¹⁶³. GAS7b binds to the plasma membrane through the side surface of its F-BAR domain and is able to induce sheet formation on the flat membranes of the phagocytic cup, which is of the essence in membrane deformation associated with phagocytosis. The WASP family of proteins might cooperate with GAS7 on phagocytic cups formation through Actin related proteins (Arp)2/3 complex mediated actin polymerization and overall dynamics¹⁶³, however, further research is needed to clarify exactly how sheet formation and actin polymerization concertedly participate in membrane deformation during phagocytosis.

It is possible that GAS7 can have a significant impact in the innate immune response of tissue-resident alveolar macrophages. GAS7 is upregulated in an LPS/IFN γ environment, via NF- κ B activation, as observed during *Klebsiella pneumoniae* infection. GAS7 is also seen to impact

the cytokines secreted by alveolar macrophages in the context of infection, as its knockdown leads to the reduction of IL-6 and IL-12 expression¹⁸⁴.

A lot of questions remain when it comes to the regulation and specific functions of GAS7, especially in the vast field of immunology, although there is a pattern showing that this could be an important adaptor for future studies.

CHAPTER II: PRELIMINARY DATA AND OBJECTIVES

PRELIMINARY DATA

1. GAS7 SILENCING PROMOTES HIV PROPAGATION IN MACROPHAGES

CD4+ T cells are undoubtedly the major target of human immunodeficiency virus (HIV). However, there are other CD4+ immune cells that serve as viral targets, among which are cells of macrophage lineage. Although there are significantly less macrophages infected with HIV in the host when compared to CD4+ lymphocytes, there are numerous circumstances that contribute to the fact that macrophages are an ideal reservoir for HIV, helping to spread the viral particles and contributing to the overall pathogenesis of this HIV infection^{185, 186}.

While GAS7 is highly expressed in macrophages, it is absent from CD4+ T cells. Due to their ability to induce membrane curvature and impact on cytoskeletal dynamics, F-BAR family members can potentially act as viral cofactors, or instead restrict viral replication. Such is the case, for instance, for the F-BAR protein PACSIN2, which serves as a host factor that assists HIV-1 transmission across host cells¹⁸⁷. Over the past few years our lab, at Institut Curie, has been studying the role of GAS7 during HIV infection in macrophages. It was found that GAS7 restricts HIV viral particle production and that silencing of this protein promotes HIV-1 and HIV-2 propagation in macrophages (Figure II. 1A and B). Strikingly, when infected with HIV-2, GAS7 deficient macrophages undergo significantly higher fusion events with other macrophages, as compared with control macrophages, forming highly virus-productive multinucleated giant cells usually characteristic of chronic infection¹⁸⁸ (Figure II. 1C).

It is well established that actin dynamics are critical for HIV transmission and propagation from myeloid cells¹⁸⁹, while not having a significant impact in other cell types¹⁹⁰. For instance, less than a year ago, it was reported that PSGL-1, a leukocyte-specific single-pass transmembrane protein, is able to stabilize cellular F-actin (filamentous actin) dynamics to restrict HIV infection¹⁹¹. GAS7 is an F-BAR protein that is a strong F-actin binding partner, and has complex roles in regulating actin dynamics^{166, 167, 181}. Our current line of research in the laboratory points to a mechanism in which GAS7 impairs the remodeling of the actin network that promotes HIV spread and the formation of multinucleated cells. Yet, the specific molecular players underlying such effect are still under investigation.

While investigating the role of GAS7 in macrophages, our team also made additional intriguing observations. Silencing GAS7 led to decreased expression of genes encoding for antiviral molecules within the cells. In GAS7-deficient macrophages, at the steady state, the mRNAs encoding for selected ISGs (such as RSAD2 and Mx1), are significantly downregulated, including the ER-based effector protein STING (Figure II. 1D-E).

It is intriguing to observe the depletion of STING levels in GAS7 deficient cells at the steady state, as STING is a key member of the cGAS-STING pathway of DNA-sensing. This could have an impact in the innate response of macrophages to a multitude of viruses that trigger the cGAS-STING pathway of DNA-sensing, including HIV, whose reverse transcribed DNA is recognized by the cGAS DNA sensor¹⁹².

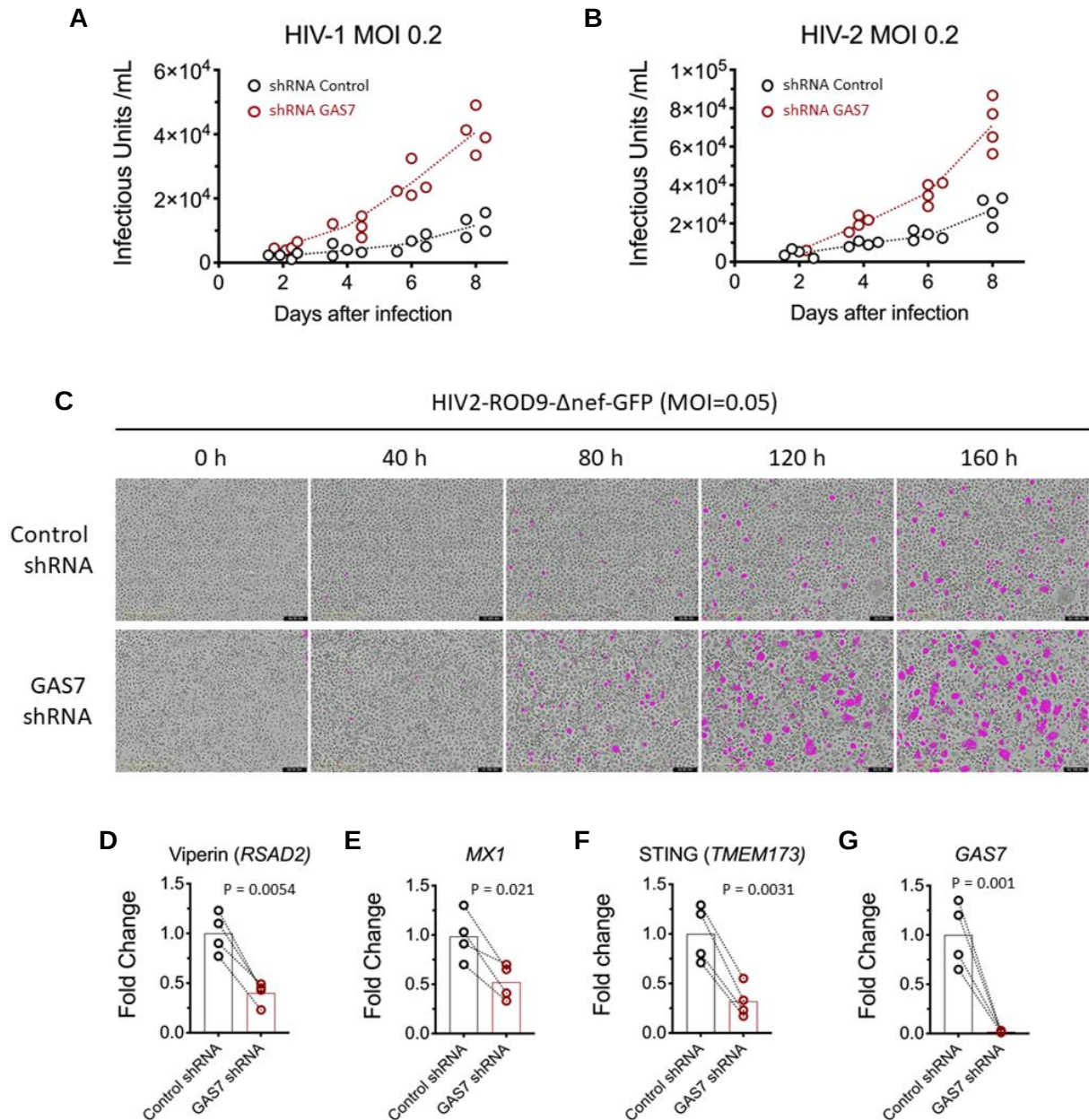


Figure II.1. GAS7 deficiency favors HIV viral propagation in macrophages. (A-B) MDMs transduced with a control or GAS7 shRNA were infected with HIV-1 (NL-AD8-VSVG) or HIV-2 (ROD9-VSVG) at MOI=0.2. Culture supernatants were harvested at the indicated time-points and the concentration of infectious viral particles were quantified using the GHOST reporter cell line. (C) MDMs transduced with a control or GAS7 shRNA were infected with HIV-2 (ROD9-GFP-VSVG) at MOI=0.05. Cells were placed in the Incucyte live cell imaging system and pictures were acquired at the indicated time-points. The GFP signal that measures viral spread is highlighted in pink. (D-G) MDMs transduced with a control or GAS7 shRNA were lysed in RNA lysis buffer. Quantitative PCR was performed for the indicated genes.

2. GAS7 SILENCING IMPAIRS ANTIVIRAL RESPONSES AGAINST HIV

After establishing that GAS7 is important to maintain favorable levels of antiviral effectors, such as some ISGs, including STING, at the steady state, it was important to verify the importance of GAS7 in the antiviral response to HIV infection in macrophages.

Monocyte-derived macrophages transduced with a control or GAS7 shRNA were infected with HIV-1 (NL-AD8-VSVG) with increasing MOIs and assessed for their levels of IFN β , as well as ISGs (RSAD2 and Mx1, as done previously, and IFI44L) and STING. The results match what was observed at the steady state, as mRNA levels encoding for the aforementioned immune effectors are severely downregulated in HIV-1 infected macrophages silenced for GAS7 (Figure II. 2A-F)

These results were further confirmed by Western Blot analysis of MDMs, silenced or not for GAS7, that were infected with HIV-1 (NL-AD8-VSVG) and HIV-2 (ROD9) (Figure II. 2G). It is especially clear in infection with HIV-2 that STING is progressively depleted with increasing MOI in macrophages silenced for GAS7.

This seems to allude at a possible association between GAS7 and STING levels and has led to a hypothesis regarding whether GAS7 can be an important factor to promote the renewal of STING in macrophages that are under chronic infection and thus require mechanisms to promote steady levels of anti-viral molecules to sustain the response.

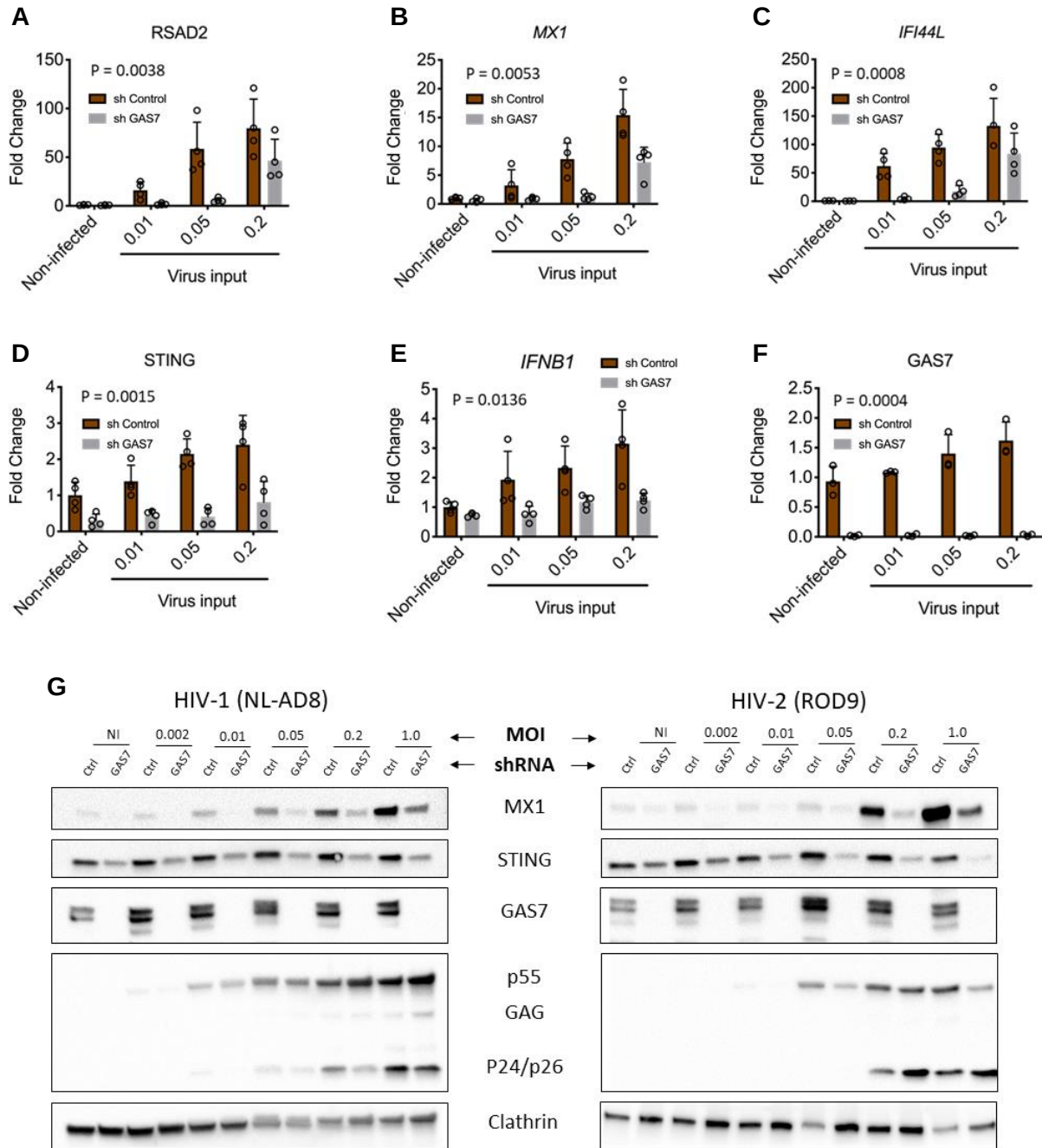


Figure II.2. GAS7 silencing in macrophages impairs antiviral responses against HIV. (A-F) MDMs transduced with a control or GAS7 shRNA were infected with HIV-1 (NL-AD8-VSVG) at the indicated MOIs for 8 days. Cells were lysed for quantification of the indicated mRNAs by qPCR. (G) MDMs transduced with a control or GAS7 shRNA were infected with HIV-1 (NL-AD8-VSVG) and HIV-2 (ROD9) at the indicated MOIs for 8 days. Cells were lysed for quantification of the indicated proteins by western blot. NI, non-infected.

3. GAS7 PARTIALLY LOCALIZES TO THE NUCLEUS

Aiming to characterize the role of GAS7 during HIV infection in macrophages, our laboratory has recently performed a proteomic analysis, by mass spectrometry, aiming to identify the binding partners of GAS7 in primary human macrophages. It was found that, while GAS7 binds several previously described cytoskeleton factors, most of its binding partners in macrophages are, surprisingly, nuclear proteins (Figure II.3A). These binding partners are enriched for biological processes ranging from mRNA metabolic processing to mRNA splicing and regulation of gene expression (Figure II. 3B).

Previous research already suggested that GAS7 partly localizes to the cell nucleus in some cell types, and it seemingly enhances the gene expression of osteoblastogenesis transcription factors, such as Runx2. Indeed, the data shows that that GAS7 functionally influences the transcriptional activity of Runx2, potentially triggering the differentiation of MSCs into osteoblasts^{159, 193}.

These studies unveiled the presence of GAS7 in the nucleus. However, little is known about its nuclear functions, and it would be of interest to explore whether nuclear GAS7 is able to regulate gene expression or mRNA transport in cell types that express high levels of this protein, such as macrophages.

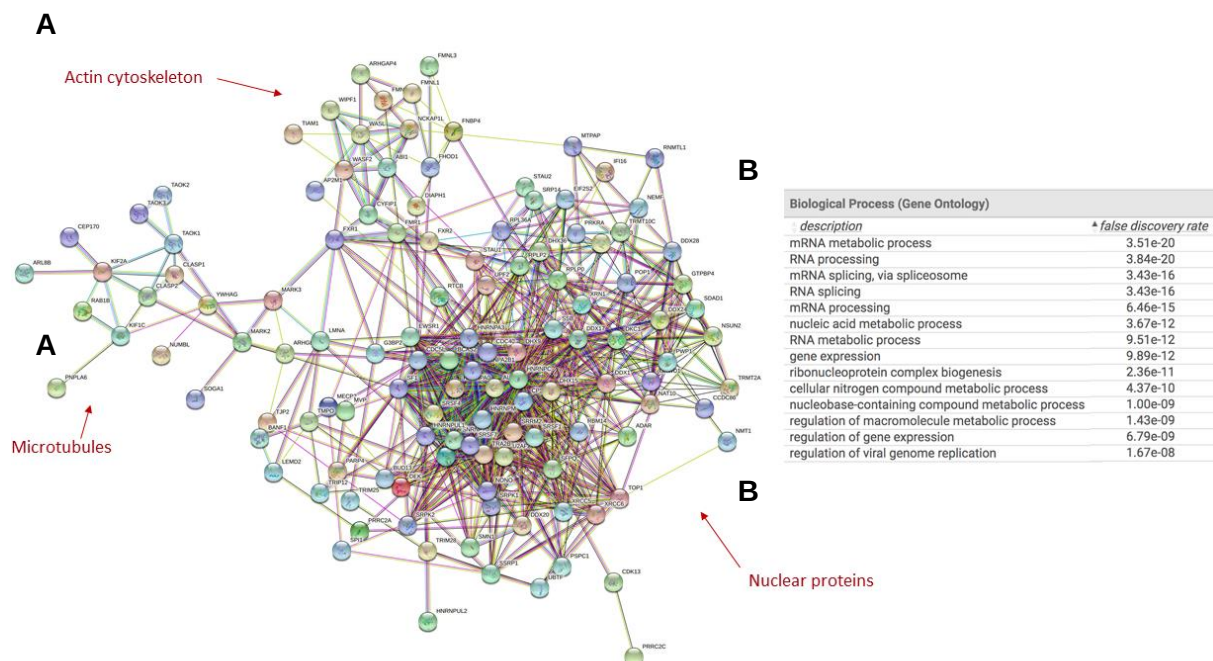


Figure II.3. GAS7 interacts with multiple nuclear proteins. (A) MDMs transduced with GAS7C-FLAG were immunoprecipitated using anti-FLAG-coated agarose beads. The GAS7 binding partners were obtained by mass spectrometry. The protein-protein interaction network was obtained using STRING (<https://string-db.org/>). **(B)** Gene Ontology terms most enriched among the GAS7-binding partners. Obtained using <http://geneontology.org/>.

To further confirm the presence of GAS7 in the nucleus of macrophages, our laboratory performed a cell fractionation assay on wild-type MDMs, and the collected cellular cytosolic, membrane and nuclear fractions were analyzed by Western Blotting for the presence of GAS7 (Figure II. 4A). In parallel, wild-type MDMs were also fixed and stained for GAS7, for analysis of the GAS7 subcellular distribution by confocal microscopy imaging (Figure II. 4B).

Both analyses demonstrate that GAS7 is present in the nucleus of macrophages, and thus can presumably impact in gene expression or mRNA splicing or transport. This offers a promising hypothesis as to how GAS7 could be influencing the mRNA levels of STING in macrophages, and thus, should be further investigated.

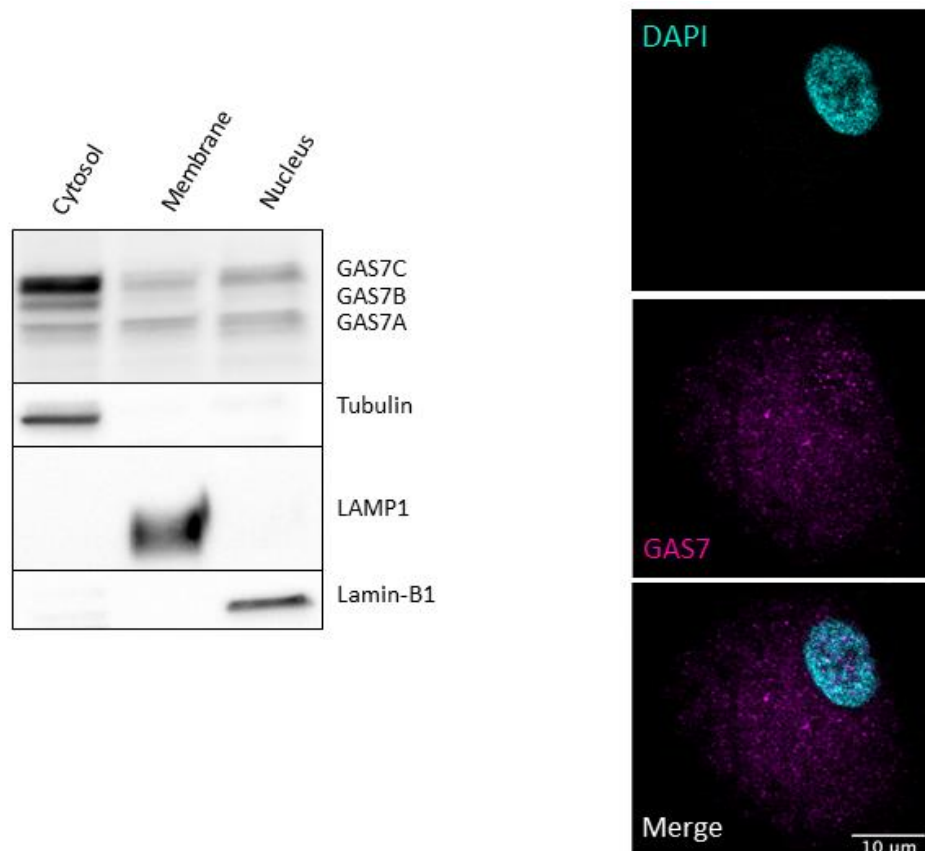


Figure II.4. GAS7 partially localizes in the nucleus. (A) WT MDMs were fractionated into cytosol, membrane, and nuclear fractions, using a commercially available kit. Fractions were probed by western blot for the indicated proteins. (B) WT MDMs were fixed with PFA and stained for GAS7. Images were acquired using confocal microscopy.

OBJECTIVES

Our preliminary data, detailed above, suggests that GAS7 plays an important role in the macrophage antiviral response. First, we found that the levels of several antiviral factors, including STING, are downregulated in the absence of GAS7, in macrophages at the steady state. Second, the ISG response to HIV is severely impaired in macrophages silenced for GAS7. Third, GAS7 localizes to the nucleus in macrophages and interacts with multiple proteins involved in gene regulation and mRNA splicing and transport.

In the current project, the aim was to extend these initial observations and evaluate the role of GAS7 in the macrophage antiviral response, at large. To achieve this, we will expose macrophages silenced or not for GAS7 to different classes of viruses, or viral PAMPs and evaluate the impact of GAS7 absence on the capacity of the cell to respond to these foreign insults. Next, we aim to explore whether the subcellular distribution of GAS7 is impacted during stimulation of the macrophage with a STING activator. Finally, in the last task we want to characterize the impact of depleting GAS7 from a commonly used myeloid human cell line. The 3 distinct tasks, and encompassed subtasks, of the project, are detailed below.

Task 1: Defining the importance of GAS7 in the antiviral response of primary human macrophages

This first task will constitute the core of this project and aims to elucidate the importance of GAS7 during the macrophage response to different classes of viruses, DNA and RNA analogues, or STING activators. By using a panoply of diverse stimuli, while varying different other parameters, we aim to evaluate how the presence or absence of GAS7 impacts the response to stimuli of distinct nature, their doses and the timing of analysis after stimulation.

1.1: Determining whether GAS7 impacts the macrophage ISG response to DNA and an RNA analog

In this subtask, we will stimulate macrophages, silenced or not for GAS7, with DNA and an RNA analog delivered to the cytoplasm – htDNA or Poly(I:C), respectively. The macrophage ISG response will be analyzed at the mRNA and protein levels. This first task should allow us to dissect whether GAS7 impacts on the response to any or both viral mimetics and elucidate dose-response effects.

1.2: Evaluating the kinetics of STING degradation and replenishment in MDMs upon stimulation with its ligand, 2'3'-cGAMP

STING is rapidly degraded upon stimulation with cytosolic DNA or its specific ligand 2,3-cGAMP, through two independent mechanisms of autophagy. Yet, how fast STING levels are brought back to normal, and which factors impact on STING replenishment are topics that remain severely unexplored. This may, in part, be because most studies are performed in cell lines that cannot be maintained in culture for extended periods of time and thus do not allow examining the replenishment of STING levels, which appears to be a relatively slow event.

Previous work in our lab demonstrated that silencing GAS7 leads to reduced STING transcript and protein levels at the steady state. Most strikingly, when macrophages are infected with HIV-2, STING levels are severely depleted in macrophages silenced for GAS7, particularly at higher viral MOIs (Figure II.2). Thus, we hypothesize that GAS7 is important to maintain steady levels of STING, that may be critical to maintain immunity in the context of a chronic viral infection.

To study the importance of GAS7 in promoting the replenishment of STING in macrophages, we will deliver 2'3'-cGAMP, a direct ligand of STING, to the cytoplasm of human macrophages. After stimulation we will follow the kinetics of STING degradation and replenishment overtime aiming to examine the impact of GAS7 absence.

1.3: Assessing whether GAS7 influences the innate macrophage response to DNA and RNA viruses

Our lab has established the importance of GAS7 in the immune response to HIV, a retrovirus whose antiviral response depends on both the DNA and RNA detection systems. In this subtask, we aim to extend these observations to other RNA and DNA viruses. Based on previous findings, it is expected that GAS7 has an impact on the response to DNA viruses, mostly governed by the cGAS-STING pathway. Less predictable is how GAS7 may impact the macrophage response to RNA viruses.

We will, therefore, expose MDMs, depleted or not for GAS7, to HSV-1 (herpes simplex virus type 1), a DNA virus, and SeV (Sendai virus, also known as murine respirovirus), an RNA virus, and analyze the magnitude of the antiviral response at both the mRNA and protein levels.

We will also evaluate if the absence of GAS7 impacts on the replication of these viruses, by quantifying the levels of viral transcripts in MDMs depleted or not for GAS7 and using live imaging to observe the formation of giant cells.

Task 2: Evaluating whether GAS7 translocates to the nucleus upon stimulation of the cGAS-STING pathway

Preliminary data shows that GAS7 is present at the macrophage nucleus where it binds multiple proteins implicated in diverse cellular processes. We can thus speculate that the impact of GAS7 on the antiviral response may be mediated by its actions on the nucleus, where it may regulate gene expression, mRNA transports, among other processes. An aspect requiring clarification is whether the distribution of GAS7 across the different cellular compartments is stable or it accumulates in the nucleus during viral infection, to promote the expression of antiviral molecules.

In this task we will test whether GAS7 translocates to the nucleus upon stimulation with the STING ligand 2'3'-cGAMP. We have chosen cGAMP stimulation given our observations that GAS7 is critical to maintain steady STING transcript levels. Upon stimulation and at defined time-points we will separate the cellular compartments to observe GAS7 distribution in stimulated and non-stimulated cells.

Task 3: Generation and validation of a GAS7 KD monocytic cell line

While working with primary cells is preferable to ascertain the physiological significance of experimental observations, there are instances where cell lines can be advantageous as they are easier to genetically manipulate and expand. In the context of this project, it would be useful for instance to generate a KO cell line for GAS7 that could subsequently be complemented with defined GAS7 constructs to give insight into the GAS7 domains that are important for the antiviral response. The THP-1 monocytic cell lines are routinely used as models of human macrophages in studies involving HIV or the immune response – these cells are known to respond to the virus in a manner that resembles primary cells.

To determine whether THP-1 can be a good model to study the antiviral roles of GAS7, we will generate a GAS7 KD cell line by shRNA transduction and assess the impact of GAS7 silencing of ISG expression at the transcript and protein levels. Growth assessment of the cells and fractionation studies will also be performed.

CHAPTER III: MATERIALS AND METHODS

1. CELL CULTURE

1.1. 293T CELLS

This 293T cell line is a stable clone derivative of the HEK-293 cell line expressing the large T antigen of simian virus 40 (SV40), as described elsewhere^{194, 195}. These cells are broadly used in scientific research for lentiviral particle production.

The 293T cell line was seeded on T-150 cm² TPP flasks and maintained in Gibco® DMEM complete medium, high glucose, GlutaMAX™ (Thermo Fisher Scientific, Massachusetts, United States) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin/streptomycin (P/S), 1% (v/v) sodium pyruvate and 1% (v/v) Gibco® MEM non-essential amino acids solution at 37 °C with 5% CO₂. Cells were routinely maintained by seeding at 2 x10⁶ cells and sub-passaged for 2-3 times per week to avoid confluency.

1.1.1. TRANSFECTION OF 293T CELLS WITH SHRNA

For each lentivector, the transfection mix was prepared with NaCl 150mM, PEI 7.5mM (diluted from PEI MAX®, Polysciences, Pennsylvania, United States), Gibco® Opti-MEM™ I Reduced Serum Medium (Thermo Fisher Scientific), DMEM complete medium and DNA (detailed sequences in Key Resources Table, at the end of the present chapter). 293T cells were transfected with the pMD2.G plasmid (encoding VSV-G) and the psPAX2 plasmid (encoding Gag, Pol, Rev, and Tat) whereas SIV (HIV-2 or Simian Immunodeficiency Virus) DNA was transfected with the pSIV3+ plasmid. SIV DNA contains the Vpx protein, an antagonist of SAMHD1, which is a viral restriction factor expressed in myeloid cells responsible for inhibiting infection by HIV-1 particles. By inducing the degradation of SAMHD1, Vpx is crucial for efficient transfection, as it allows more efficient lentiviral transduction rates^{196, 197}. All plasmids are further described in Key Resources Table.

14x10⁶ 293T cells were transfected with each transfecting mix. After 12h of incubation, the medium was replaced by Gibco® RPMI 1640 complete medium, GlutaMAX™ (Thermo Fisher Scientific) supplemented with 5% (v/v) fetal bovine serum (FBS), 5% (v/v), Human AB serum (Sigma-Aldrich, Missouri, United States), 1% (v/v) penicillin/streptomycin (P/S). Lentivector-containing supernatants were collected 24h after medium change, clarified by 5 minutes of centrifugation at 300 g, filtered through a 0.45µm-pore-size-filter and stored at 4 °C until further usage.

1.2. MONOCYTE-DERIVED MACROPHAGES

1.2.1. ISOLATION OF MONOCYTES FROM HUMAN BLOOD

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque Plus™ (GE-Healthcare, Illinois, United States) gradient centrifugation. All apheresis rings used were obtained with blood from healthy human voluntary donors provided by “Établissement Français du Sang”.

CD14+ cells were isolated from human PBMCs through column-based magnetic cell separation. As such, cells were subjected to positive selection using CD14 MicroBeads human,

LS Columns and QuadroMACS™ Separator, following manufacturer's instructions (Miltenyi Biotec, Germany). CD14⁺ PBMCs were considered human primary monocytes.

A schematic description of the protocol can be found in Figure III.1.

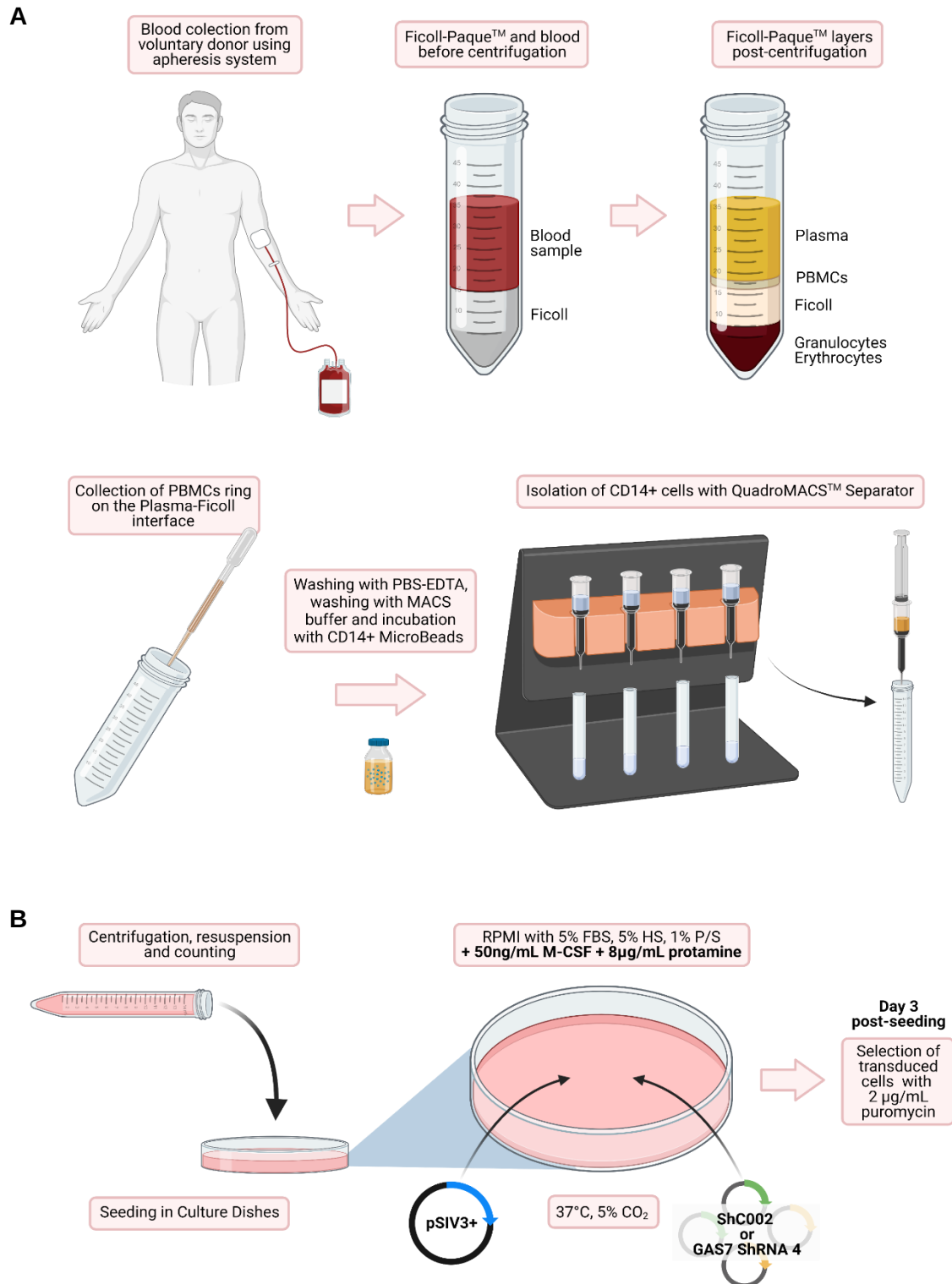


Figure III.1. Protocol for isolation and differentiation of monocyte-derived macrophages from human blood. (A) Cells present in a blood sample obtained from a donation apheresis system underwent Ficoll-Paque Plus™ gradient centrifugation. The resulting ring of PBMCs was washed multiple times using PBS-

EDTA and MACS buffer, and isolation of CD14⁺ cells was achieved through positive-selection column based magnetic cells separation. **(B)** The monocytes were seeded in adequate macrophage-inducing media and co-transduced with SIV viral particles and lentivectors encoding the conditions of interest (control shRNA, termed ShC002 or GAS7 shRNA). All steps were performed under sterile conditions in a laminar flow chamber of a Class II security laboratory.

1.2.2. TRANSDUCTION AND DIFFERENTIATION TO MONOCYTE-DERIVED MACROPHAGES

Differentiation of monocytes to macrophages was achieved by culturing CD14⁺ cells for 6-10 days in RPMI complete medium supplemented with 5% (v/v) fetal bovine serum (FBS), 5% (v/v) Human AB serum (Sigma-Aldrich), 1% (v/v) penicillin/streptomycin (P/S). Macrophage differentiation was promoted by adding 50ng.mL⁻¹ M-CSF (Miltenyi Biotec) to the cultures. Finally, 8µg.mL⁻¹ of protamine (Sigma-Aldrich) were added to promote the transduction efficiency of the lentiviral particles. Cells were incubated Corning® 100 mm Not TC-treated Culture Dishes (Sigma-Aldrich).

The primary monocytes were co-transduced with equal volumes of SIV viral particles and lentivectors encoding the conditions of interest. ShC002 MISSION® pLKO.1-puro (Sigma-Aldrich) was used as the control shRNA, and GAS7 shRNA 4 pLKO.1-puro (Sigma-Aldrich) to induce the knockdown of GAS7 in the corresponding cells (for more information, consult Key Resources Table). Transduced macrophages were selected with 2µg.mL⁻¹ puromycin (InvivoGen, California, United States), at day 3 (Figure III.1B).

Cells were used for experiments after 10 days of differentiation.

1.3. THP-1 CELLS

THP-1 is a human monocytic cell line, derived from the peripheral blood of a child patient with acute monocytic leukemia. THP-1 cells and their genetically engineered derivatives, represent valuable tools for investigating monocyte biology in multiple environments and conditions¹⁹⁸.

THP-1 cells were seeded on T-75 cm² TPP flasks and maintained in Gibco® RPMI 1640 complete medium (GlutaMAX™) supplemented with 5% (v/v) fetal bovine serum (FBS), 5% (v/v) human serum (HS), 1% (v/v) penicillin/streptomycin (P/S) at 37 °C with 5% CO₂. Cells were passed every two days, at a 1:10 of the total concentration, equaling 2,5x10⁶ cells.mL⁻¹.

Genome-edited THP-1-derived cells were generated with shRNA technology to achieve Control shRNA THP-1 cells and GAS7 deficient THP-1 cells, as described in previous sections.

1.3.1. DIFFERENTIATION WITH PMA

Control shRNA and GAS7 deficient THP-1 cells were washed with PBS and cultured (1,5 x 10⁵ cells/well) in 24-well plates in RPMI complete medium, 12 wells per condition. Differentiation was induced for 48h with PMA (Sigma-Aldrich) at 30ng.mL⁻¹, after which the medium was changed.

1.3.2. ANALYSIS OF THP-1 CELL-GROWTH KINETICS

Control shRNA THP-1 cells and GAS7-deficient THP-1 cells were washed with PBS and cultured (5×10^4 cells/well) in 6-well plates in RPMI complete medium, 3 wells per condition. Cell quantification was assessed every 24 hours using the LUNA-FL™ Automated Fluorescence Cell Counter (Logos BioSystems, South Korea), at consecutive 24h timepoints, over 10 days.

1.4. SUBCELLULAR FRACTIONATION

Subcellular fractionation of cells was performed according to the Mem-PER™ Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific), with some modifications to the manufacturer's protocol, as subsequently described. Briefly, cells were permeabilized in 60μL of ice-cold Permeabilization Buffer (Thermo Fisher Scientific), supplemented with 1% phosphatase inhibitor cocktail (Cell Signaling Technology, Massachusetts, United States) and 2% complete protease inhibitor cocktail (Roche, Switzerland), and incubated on ice for 30 minutes. The permeabilized cells were centrifuged at 16000 g for 15 minutes at 4°C, after which the supernatant containing the cytosolic proteins was transferred to new tubes. The pellet was resuspended in 30μL of Solubilization Buffer (Thermo Fisher Scientific), supplemented with 1% phosphatase inhibitor cocktail (Cell Signaling Technology) and 2% complete protease inhibitor cocktail (Roche) and incubated on ice for 30 minutes. Tubes were centrifuged at 16000g for 15 minutes at 4°C, after which the supernatant containing the solubilized membrane and membrane-associated proteins was transferred to new tubes. The pellet containing the nuclei was resuspended in 30μL of PBS, to which 6μL of 6X Laemmli SDS sample buffer (Alfa Aesar, Thermo Fisher Scientific) was added. All samples were stored at -20°C until further use.

1.5. VIRUS INFECTION

For *in vitro* virus infection, monocyte-derived macrophages (1.5×10^5 cells/well) were cultured in 24-well plates (12 wells per condition) in RPMI for 12 h and thereafter infected with HSV-1 VP26-GFP (MOI = 10) (previously prepared by transfection of Vero cells with the plasmid encoding HSV-1 VP26-GFP, kindly gifted by Nicolas Manel, from Institut Curie) or SeV (3 hemagglutination units [HA Units].mL⁻¹) (originally from ATCC, Virginia, United States, kindly gifted by Nicolas Manel) with four consecutive 3-fold dilutions.

1.6. TRANSFECTION WITH 2'3'-cGAMP, htDNA, POLY(I:C) AND TREATMENT WITH LPS

Monocyte-derived macrophages (1.5×10^5 cells/well) were incubated overnight in a 24-well plate, 12 wells per condition. For transfection with 2'3'-cGAMP (InvivoGen), a transfection mix with Lipofectamine 3000 reagent (Thermo Fisher Scientific) and 1mg.mL⁻¹ of 2'3'-cGAMP was prepared as per standard protocol. Cells were stimulated with two different doses of 2'3'-cGAMP (4μg.mL⁻¹ and 1μg.mL⁻¹) at different timepoints in a reverse kinetics manner, incubated with the stimuli overnight and subjected to media change the following day. At the end of day 6, all cells were lysed and subjected to western blotting. For assessment of the response of monocyte derived macrophages to models for DNA and RNA stimuli, htDNA (Sigma-Aldrich) and Poly(I:C) (InvivoGen) were used, respectively. A transfection mix with

Lipofectamine 3000 reagent (Thermo Fisher Scientific) and $0,5\mu\text{g.mL}^{-1}$ of htDNA or $0,5\mu\text{g.mL}^{-1}$ of Poly(I:C) was prepared as per standard protocol, with four 3-fold dilutions. Cells were incubated with the nucleic acids stimuli overnight. Supernatants were collected after 24h for further assays and cells were lysed for RT-qPCR.

For the assessment of GAS7 subcellular location, stimulation of MDMs was performed, using a transfection mix of Lipofectamine 3000 (Thermo Fisher Scientific) and $4\mu\text{g.mL}^{-1}$ of 2'3'-cGAMP (InvivoGen) or 100ng.mL^{-1} of LPS-EB Ultrapure (InvivoGen), as per standard protocol. Cells were incubated with the stimuli overnight and after 24h, the cells were lysed, and subcellular fractionation of cells was performed as previously described. Cellular fractions were further analyzed by Western Blot.

1.7. REAL-TIME QUANTITATIVE LIVE-CELL IMAGING

For some experiments, upon media change at 24-hours post-infection, MDMs were placed in a real time live-cell analysis system (Incucyte® S3, Sartorius, Germany) and contrast phase pictures were obtained at 3h intervals for 6 to 8 days. Individual pictures were acquired, and videos and figures were further mounted using the ImageJ software.

2. GENE EXPRESSION ANALYSIS USING RT-QPCR

2.1. RNA ISOLATION AND EVALUATION OF RNA PURITY

Cells were lysed by adding lysis reagent: $350\mu\text{L}$ RA1 + 2% TCEP Reducing Agent (MACHEREY-NAGEL, Germany). Samples were then stored at -20°C until further usage. Then, NucleoSpin® RNA MiniPrep kit (MACHEREY-NAGEL) was used for RNA extraction and purification according to the manufacturer's protocol. RNA samples were eluted with $20\mu\text{L}$ RNase-free H_2O .

RNA final concentration and purity ($\text{OD}_{260/280}$) were assessed using a NanoDrop 2000 Instrument (NanoDrop Technologies, Thermo Fisher Scientific) coupled to a computer with the NanoDrop software. Only samples that satisfied the acceptable range defined for the assessed parameter ($\text{OD}_{260/280}$ – ratio of 2,0-2,2, according to the technical specifications provided by the instrument's manufacturer) were used for cDNA synthesis.

2.2. cDNA SYNTHESIS

To reverse transcribe the RNA into cDNA, the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used, according to manufacturer's protocol. In $20\mu\text{L}$ PCR tubes, $14,2\mu\text{L}$ RNA from each sample was incubated with $2\mu\text{L}$ 10X RT Buffer, $2\mu\text{L}$ 10X RT Random Primers, $0,8\mu\text{L}$ 25X dNTP Mix (100mM) and $1\mu\text{L}$ MultiScribe Reverse Transcriptase ($50\text{U}/\mu\text{L}$), at 25°C for 10 minutes and then at 37°C for 2 hours. The reaction then was inactivated by heating at 85°C for 5 seconds and posterior chilling at 12°C , until sample retrieval. The cDNA products were stored at -20°C until use.

2.3. RT-qPCR

RT-qPCR experiments were run on the LightCycler® 480 System (Roche Diagnostics), making use of the LightCycler® 480 version 1.5.0 SP4 software for the run setup and for the analysis.

To quantify the ISG response by qPCR, we employed TaqMan™ assays using sets of primers/probes already optimized and available commercially (Thermo Fisher Scientific), for each gene of interest. These sets include a pair of Forward and Reverse primers and a probe labeled on its 5' with a fluorescent reporter dye (FAM) and a non-fluorescent quencher (NFQ) at the 3'. Consult Key Resources Table for full details concerning used primers/probes sets.

The mix used for the TaqMan RT-qPCR contained 5µL of TaqMan™ Universal Master Mix II (Applied Biosystems, Thermo Fisher Scientific), 0,5µL of each primer/probe, 3,5µL of nuclease free-water and 1µL of cDNA product of each sample. A master mix containing the required total amount of TaqMan™ Universal Master Mix II and Primer/Probe was prepared and dispensed into the wells of a LightCycler® 480 Multiwell Clear Plate 384 (Roche). The second mix, including the total amount of nuclease free-water and cDNA product, was prepared thereafter, and dispensed into the wells of the same plate, using a E1-ClipTip™ Bluetooth™ Electronic Multichannel Pipette (Thermo Fisher Scientific). The thermal cycling protocol involved a pre-incubation step of 95°C for 5 minutes, followed by 45 amplification cycles (denaturation step at 95°C for 20 seconds; annealing step at 60°C for 15 seconds and final extension at 72 °C for 15 seconds), and a cooling step at 40°C for 30 seconds.

To quantify the relative viral loads of Sendai virus in infected MDMs we employed qPCR using the SYBR Green method. All SYBR Green gene-specific primers (Eurogentec, Belgium) against Sendai genes, as well as the housekeeping genes, were designed using the Primer3 program. Primers' sequences, as well as the length of the respective amplification product, can be found in Key Resources Table.

For the SYBR Green RT-qPCR, the final mix was made up of 5µL of FastStart Universal SYBR® Green Master (ROX) (Roche), 0,25µL of each diluted primer (forward and reverse primers at 10µM, full list of primers used in Key Resources Table) 3,5µL of LightCycler® 480 SYBR Green I Master H₂O, PCR grade (Roche) and 1µL of cDNA product of each sample. A master mix containing the required total amount of FastStart Universal SYBR® Green Master and Primers was prepared and dispensed into the wells of a LightCycler® 480 Multiwell Clear Plate 384 (Roche). The second mix was prepared and loaded into the qPCR plate as described in the section above. The thermal cycling protocol involved a pre-incubation step of 95°C for 5 minutes, followed by 45 amplification cycles (denaturation step at 95°C for 20 seconds; annealing step at 60°C for 15 seconds and final extension at 72°C for 15 seconds), followed by melting curve acquisition (starting at 70°C for 1 minute, with the temperature increasing incrementally until reaching 95°C), and, at last, a cooling step at 40°C for 30 seconds.

2.4. GENE EXPRESSION DATA ANALYSIS AND QUANTIFICATION WITH PRISM SOFTWARE

Relative quantification of target gene expression levels was performed making use of the critical threshold (C_T) values obtained for each condition according to Equations 1-3.

This methodology requires a similar efficiency (approximately 100%) in the amplification of target and reference genes. For the TaqMan qPCR protocol, HPRT1, ACT B and RPS18 were used as housekeeping genes for the normalization of the C_T values (Equation 1), while for the SYBR Green protocol, GAPDH was used instead of HPRT1.

Equations:

- (1) $\Delta C_{T \text{ Sample}} = C_{T \text{ Gene of interest}} - \text{Geomean} (C_{T \text{ Housekeeping genes}})$
- (2) $\Delta(\Delta C_T) = \Delta C_{T \text{ Sample}} - \Delta C_{T \text{ Reference Sample}}$
- (3) $\text{Fold change} = 2^{(-\Delta\Delta C_T)}$

3. PROTEIN EXPRESSION ANALYSIS USING WESTERN BLOT

3.1. PROTEIN QUANTIFICATION

For the different experiments, cells were lysed in 30 μ L of ice-cold RIPA Buffer (Sigma-Aldrich), supplemented with 1% phosphatase inhibitor cocktail (Cell Signaling Technology) and 2% complete protease inhibitor cocktail (Roche).

Protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Whole cell lysates were normalized for protein quantity, mixed with 6X Laemmli SDS sample buffer (Alfa Aesar, Thermo Fisher Scientific) and denatured for 20 minutes at 95°C and 1000rpm, followed by centrifugation at RT for 5 minutes, at max speed, to remove debris.

3.2. GEL ELECTROPHORESIS

A total of 10-20 μ g of reduced samples was separated by SDS-PAGE on 4-20% Criterion® TGX™ Precast Midi Protein gels or 4-15% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, California, United States). During the first 10-15 minutes each gel ran at 60V, and after, the voltage was altered to 120V until completion.

To transfer the proteins to a blotting membrane, Trans-Blot Turbo™ Transfer System® (Bio-Rad) was used. Membranes were blocked in 5% skim-milk supplemented with 10X TBS and 0,1% Tween-20 for 30 minutes at RT and were left incubating overnight at 4°C with the appropriate primary antibody diluted in TBST.

After being washed thrice in PBS-Tween (10% PBS in deionized H₂O supplemented with 0,1% Tween-20), membranes were incubated with secondary antibodies diluted in blocking buffer at RT for 1h.

All antibodies used for blotting are described in Key Resources Table.

3.3. IMAGING AND QUANTIFICATION WITH IMAGELAB SOFTWARE

After three washes in PBS-Tween, all the membranes were exposed using 2mL of a Clarity™ Western ECL Substrate with equal parts Peroxide Solution and Luminol/Enhancer Solution (Thermo Fisher Scientific), along with a ChemiDoc XRS+ Imaging System (Bio-Rad).

Quantification of band densitometry of the levels of each protein was performed using the Image Lab 6.0.1 software (Bio-Rad). The quantification was normalized according to band density of the loading controls used in each experiment, namely: Clathrin, Tubulin, Histone H3, Laminβ, LAMP-I, GAPDH and MAVS (consult Key Resources Table for full disclosure of items used).

4. STATISTICAL ANALYSIS

Experiments were repeated two to four times. Data analysis was performed using GraphPad Prism 9.0 for Mac (GraphPad Software). Statistical differences between groups were calculated using two-way analysis of variance (ANOVA). In column statistics, to identify statistical differences when the control shRNA and shGAS7 groups were being compared, Multiple paired t test was performed.

Differences between groups were considered statistically significant when: $0.01 < p < 0.05$ (*), $0.001 < p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).

KEY RESOURCES TABLE

PRIMARY ANTIBODIES USED FOR WESTERN BLOTTING

Target Antigen	Species	Dilution	Source	Identifier
Clathrin Heavy Chain	Mouse	1:1000	BD Biosciences	610499
RIG-I	Rabbit	1:1000	Cell Signaling	37435
GAPDH	Goat	1:1000	Abcam	ab9483
GAS7	Rabbit	1:1000	Abcam	ab168370
Histone H3	Rabbit	1:1000	Abcam	ab1791
Lamin β	Rabbit	1:1000	Abcam	ab16048
LAMP-I	Rabbit	1:1000	Abcam	ab24170
MAVS	Rabbit	1:1000	Cell Signaling	830005
MxA	Rabbit	1:1000	Abcam	ab95926
STING	Rabbit	1:1000	Cell Signaling	136475
α -Tubulin	Rat	1:2000	Bio-Rad	MCA78G

SECONDARY ANTIBODIES USED FOR WESTERN BLOTTING

Target Species	Species	Dilution	Source	Identifier
Goat	Bovine	1:3000	Jackson Immuno Research Europe	AB_2340872
Mouse	Horse	1:3000	Cell Signaling	70765
Rabbit	Goat	1:3000	Cell Signaling	70745
Rat	Goat	1:3000	Cell Signaling	70775

RT-QPCR TAQMAN PRIMERS AND PROBES

Target gene	Source	Identifier
ACTB	Thermo Fisher Scientific	Hs01060665_g1
CXCL8	Thermo Fisher Scientific	Hs00174103_m1
DDX58	Thermo Fisher Scientific	Hs01061433_m1
GAS7	Thermo Fisher Scientific	Hs00243470_m1
HPRT1	Thermo Fisher Scientific	Hs02800695_m1
IFI44L	Thermo Fisher Scientific	Hs05580845_s1
IFNB1	Thermo Fisher Scientific	Hs01077958_s1
CXCL10	Thermo Fisher Scientific	Hs00171051_m1
MAVS	Thermo Fisher Scientific	Hs00920075_m1
Mx1	Thermo Fisher Scientific	Hs00895608_m1
RPS18	Thermo Fisher Scientific	Hs02387368_g1
RSAD2	Thermo Fisher Scientific	Hs00369813_m1
SDHA	Thermo Fisher Scientific	Hs07291714_mH
TMEM173	Thermo Fisher Scientific	Hs00736955_g1
TNF	Thermo Fisher Scientific	Hs00174128_m1

RT-QPCR SYBR GREEN GENE-SPECIFIC PRIMERS

Target gene	Sequence	Source	Identifier
Actin B (s)	5'-GCACAGAGCCTCGCCTT-3'	Eurogentec	#5702379
Actin B (as)	5'-GTTGTCGACGACGAGCG-3'	Eurogentec	#5702378
GAPDH (s)	5'-CAACTACATGGTTTACATGTTCC-3'	Eurogentec	#6050400
GAPDH (as)	5'-GAAGATGGTGATGGGATTTC-3'	Eurogentec	#6050401
RPS18 (s)	5'-CTGCCATTAAGGGTGTGG-3'	Eurogentec	#6050402
RPS18 (as)	5'-TCAATGTCTGCTTCTCTCAAC -3'	Eurogentec	#6050403
Large, SeV (s)	5'-ACTTCATCTGGGTTGCCGTA-3'	Eurogentec	#7352843
Large, SeV (as)	5'-TGGTGTATACGTGGGCCTAC-3'	Eurogentec	#7352844
Matrix, SeV (s)	5'-CTCACTTGGGTAAATCGGCG-3'	Eurogentec	#7352841
Matrix, SeV (as)	5'-CTACAGATGCCGCCAAATC-3'	Eurogentec	#7352842
Nucleocapsid, SeV (s)	5'-GGTTCCTCGTCTCTCTGCTT-3'	Eurogentec	#7352837
Nucleocapsid, SeV (as)	5'-ACCATAGGTCCAACAGCCA-3'	Eurogentec	#7352838
Phosphoprotein, SeV (s)	5'-AAGAGGGGAGAAGACCAAGC-3'	Eurogentec	#7352839
Phosphoprotein, SeV (as)	5'-GAGTTCGGGGCTAGGAATCA-3'	Eurogentec	#7352840

PLASMIDS

Plasmid	Source	Identifier
psPAX2	Addgene	#12260
pMD2.G	Addgene	#12259

pSIV3+	199	Non-applicable
ShC002 MISSION® pLKO.1-puro	Sigma-Aldrich	Catalog N. SHC002
GAS7 shRNA 4 pLKO.1-puro	Sigma-Aldrich	TRCN0000013226

OTHER REAGENTS

Reagent	Source	Identifier
10x Tris/Glycine/SDS buffer	Bio-Rad	#1610772
2'3'-cGAMP	Invivogen	#tlrl-nacga23-1
Acridine orange	Logos Biosystems	F23001
CD14 Microbeads human	Miltenyi Biotec	5210610232
Clarity™ Western ECL Substrate Luminol/Enhancer Solution	Bio-Rad	102031615
Clarity™ Western ECL Substrate Peroxide Solution	Bio-Rad	102031618
Complete protease inhibitor cocktail	Roche	11836153001
EDTA	Sigma-Aldrich	20-158
FastStart Universal SYBR® Green Master (ROX)	Roche	04913914001
FBS	Eurobio	CVFSVF00-01
Ficoll-Paque™ PLUS	GE Healthcare	10294423
HSV-1 VP26-GFP	200	Non-applicable
htDNA	Sigma-Aldrich	D6898
Human Serum	Thermo Fisher Scientific	35-060-CI
Laemmli SDS sample buffer	Thermo Fisher Scientific	R24F521
Lipofectamine 3000	Thermo Fisher Scientific	L3000015
LPS-EB Ultrapure	InvivoGen	#tlrl-3pelps
MEM non-essential amino acids	Thermo Fisher Scientific	11140050
M-CSF	Miltenyi Biotec	130-096-491
P/S	Thermo Fisher Scientific	15140122
PEI MAX®	Polysciences	24765-100
PBS	Thermo Fisher Scientific	10010023
Phosphatase inhibitor cocktail	Cell Signaling	5870S
PMA	Sigma-Aldrich	P1585
Poly(I :C)	InvivoGen	#tlrl-picw
Protamine	Sigma-Aldrich	1.10123
Puromycin	InvivoGen	ant-pr-5
RIPA Buffer	Sigma-Aldrich	#SLBZ0792
SeV	ATCC	VR-907
Sodium pyruvate	Thermo Fisher Scientific	11360070
TaqMan™ Universal Master Mix II	Applied Biosystems	#01067609
Tween-20	Thermo Fisher Scientific	9005-64-5

CRITICAL COMMERCIAL KITS

Kit	Source	Identifier
BCA protein assay kit	Thermo Fisher Scientific	23225
High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific	4368814
Mem-PER™ Plus Membrane Protein Extraction Kit	Thermo Fisher Scientific	89842
NucleoSpin® RNA MiniPrep kit	MACHERY-NAGEL	740955.50

CHAPTER IV: RESULTS AND DISCUSSION

GENERAL OBSERVATION: GAS7 KNOCKDOWN EFFICIENCY

The monocyte-derived macrophages were stably transduced with pLKO.1 lentiviral particles either containing short-hairpin RNA targeting GAS7 (GAS7 KD) or with no shRNA sequence (shRNA Control). After positive selection with puromycin for transduced cells, the levels of GAS7 were assessed by quantitative PCR and Western Blot analysis. The shRNA targeting GAS7 showed efficient knockdown of GAS7 levels of over 90% for all experiments (Figure IV.1A, L and R, Figure IV.2A and E and Figure IV.4 A and F).

1. GAS7 DOES NOT SIGNIFICANTLY IMPACT MACROPHAGE ANTIVIRAL RESPONSE TO A SINGLE STIMULATION WITH DNA OR AN RNA ANALOGUE

In order to assess the potential impact that GAS7 could have in the macrophage response, we first tested macrophages with reduced GAS7 expression for their capacity to respond to viral mimetics delivered to the cytosol. The magnitude of the response was evaluated by measuring the protein and mRNA levels of several ISGs. The GAS7 knockdown and control shRNA MDMs were treated with htDNA or Poly(I:C), complexed with a liposomal transfection reagent, to efficiently deliver them to the cytosol. These stimuli should induce type I IFN response, and consequently, of ISGs, through DNA-sensing and RNA-sensing pathways, respectively.

The ISG response to cytosolic htDNA or Poly(I:C) was evaluated by both western blot (Figure IV. 1A-G) and qPCR (Figure IV. 1H-S). We observed increased expression of RSAD2, Mx1, IFI44L and DDX58 with increasing doses of both htDNA and Poly(I:C), at both mRNA and protein levels. We also observed, and in agreement with our preliminary data, that in unstimulated MDMs, or in MDMs treated with the lipofectamine vehicle alone, the levels of most ISGs appear reduced in MDMs depleted for GAS7 (see, for instance Figure IV.1A and I, for Mx1 levels). However, upon exposure to either stimulus, the levels of these ISGs were not statistically different when comparing control and GAS7 KD MDMs (Figure IV. 1A-B, D-E, G-J, M-P and S).

Collectively, the data from these experiments indicates that, while the absence of GAS7 impacts on the steady-state levels of certain antiviral molecules, GAS7-silenced macrophages are still able to mount an ISG response to cytosolic dsDNA and dsRNA that is undistinguishable from normal macrophages. This is in clear contrast with what was previously observed with HIV infection, where the absence of GAS7 clearly impaired the ability of the cell to sense and respond to the infection. We can speculate that the importance of GAS7 to the antiviral response becomes clearer in the context of a chronic infection, where the cell may be continuously stimulated over a period of time. In this setting, GAS7 may be critical by promoting the replenishment of antiviral molecules overtime that help fight the infection. Thus, a single stimulation with viral mimetics, as performed in this task, might not be the optimal strategy to examine the role of GAS7, and so in the next sections we will explore how GAS7 impacts the antiviral response over more extended periods.

When there is an ongoing infection that activates the cGAS-STING pathway, STING levels decrease via autophagy-mediated degradation, as seen previously, to avoid chronic stimulation of the pathway^{132, 153}. In accordance with this, STING protein levels have previously been shown to decrease upon htDNA delivery to the cytosol, in a dose-dependent fashion¹³². However, we did not observe significant STING degradation after htDNA delivery to the cytosol (Figure IV.1A, C and K).

One could argue that htDNA could be detected by other DNA-sensing pathways such as TLR9. Using Lipofectamine as a transfection reagent, htDNA is directly delivered to the cytosol, and so htDNA is compulsorily detected by cytosolic DNA sensors. It is possible that our protocol failed to efficiently deliver htDNA to the cytosol. However, previous work in the laboratory showed that “naked” htDNA fails to induce any ISG response in MDMs (data not shown),

clearly indicating that our experimental approach was able to deliver htDNA to the cytosol. Other cytosolic DNA sensors, such as AIM2, could detect htDNA. However, AIM2 is more associated with inflammasome activation rather than ISG induction. Therefore, it is reasonable to assume that htDNA is detected by the cGAS-STING pathway, and it has been shown to do so in multiple studies^{132, 201}.

The reason why STING levels did not decline post-stimulation can be related with the doses used for transfection. The highest dose of htDNA used for this experiment was $0,5\mu\text{g.mL}^{-1}$, and while it induced strong ISG responses, it might not have been high enough to trigger autophagy and thus, insufficient to induce a marked decline in STING levels. htDNA-induced STING degradation was shown to happen in previous studies for a dose 4 times higher ($2\mu\text{g.mL}^{-1}$) than the highest dose used for these experiments¹³². For this reason, in the next section, where our main goal is to observe variation in STING levels and examine whether GAS7 could have an impact in these levels, we switched our approach and used the STING agonist, 2'3'-cGAMP, that more efficiently induces STING degradation after stimulation.

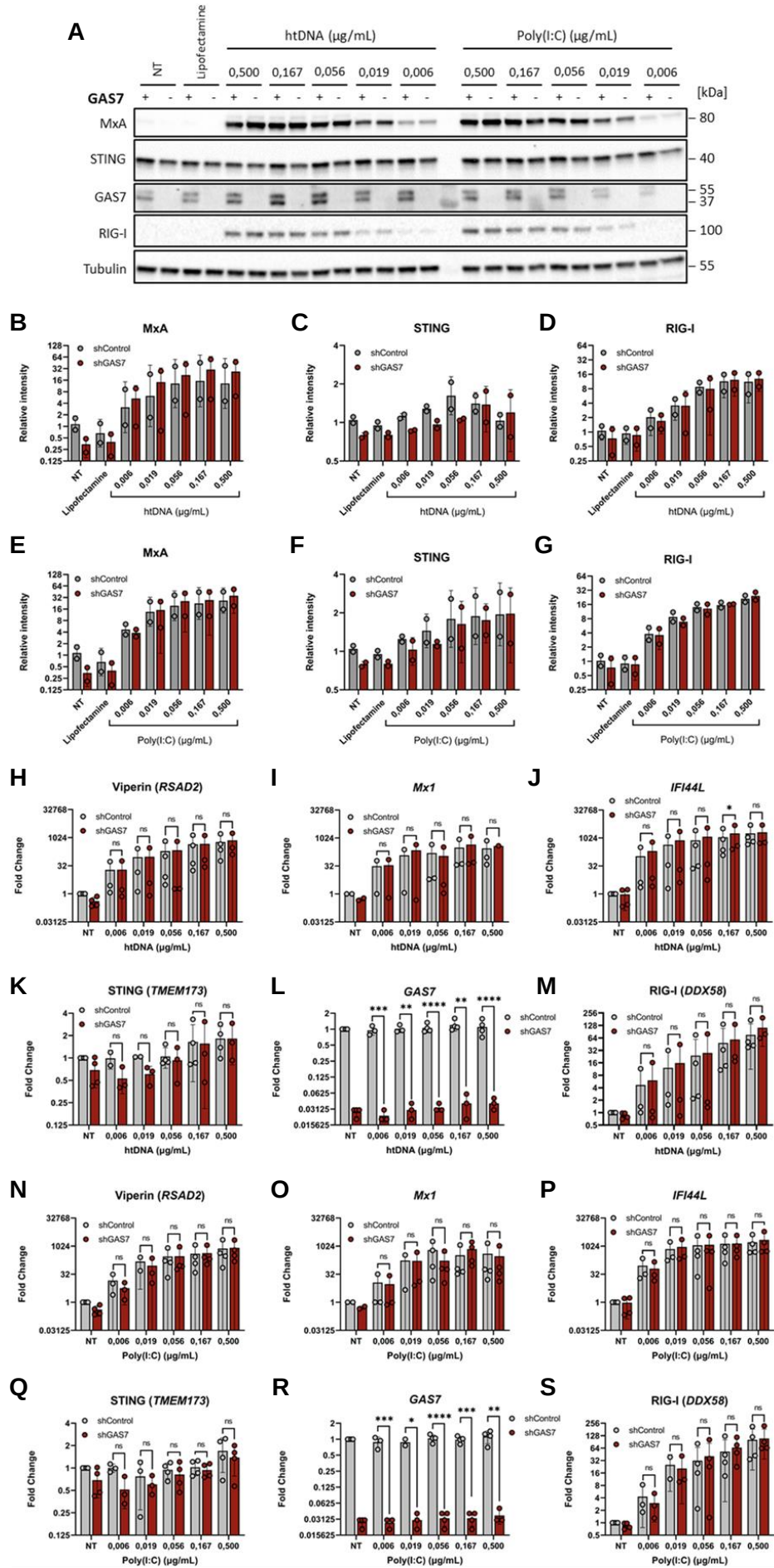


Figure IV.1. GAS7 does not significantly impact macrophage antiviral response to a single stimulation with cytosolic delivery of dsDNA or an RNA analogue. (A) MDMs transduced with a control or GAS7 shRNA were treated with htDNA or Poly(I:C), complexed with lipofectamine at the indicated concentrations. Cells were lysed after 24h for quantification of the indicated proteins by western blot. One representative donor is shown. (B-D) Graphical summary of the indicated proteins in MDMs treated with htDNA, corresponding to quantification of western blot analysis. (E-G) Graphical summary of the indicated proteins in MDMs treated with Poly(I:C), corresponding to quantification of western blot analysis. (H-S) MDMs transduced with a control or GAS7 shRNA were treated with htDNA and Poly(I:C) at the indicated concentrations. Cells were lysed after 24h for quantification of the indicated mRNAs by qPCR. NT, non-treated.

Data information: The data in panels (B-G) are presented as means of two replicates, including (A), $\pm$ s.d. The data in panels (H-S) are presented as means of four replicates $\pm$ s.d. Data from a total of five independent donors, with each dot representing an individual donor. Statistical analysis was performed using Multiple paired t test for multiple comparisons. Differences between groups were considered statistically significant when: $0.01 < p < 0.05$ (*), $0.001 < p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).

2. GAS7 IMPACTS STING DEPLETION AND REPLENISHMENT KINETICS UPON STIMULATION WITH 2'3'-cGAMP

Our preliminary data indicates that the STING mRNA and protein levels appear decreased at the steady state in MDMs silenced for GAS7. Also, when infected with HIV-2 for 8 days, a virus known to activate the cGAS-STING pathway, we observed a severe depletion of STING levels in GAS7-silenced MDMs, as compared with normal macrophages, which could still maintain a sizeable pool of cellular STING, despite chronic infection. This data suggested that GAS7 could be important to maintain STING levels during chronic infection, via mechanisms that we still do not understand. To test more directly if the absence of GAS7 impacts on the replenishment of STING levels after its stimulation-induced degradation, we decided to deliver 2'3'-cGAMP to the cytosol of MDMs, as this is arguably the STING agonist that activates this protein the most.

We decided to employ 2 doses of lipofectamine-complexed 2'3'-cGAMP to examine dose-effects on the possible role of GAS7 in the replenishment of STING levels: a higher dose of $4\mu\text{g.mL}^{-1}$ (Figure IV. 2A-D) and a lower dose of $1\mu\text{g.mL}^{-1}$ (Figure IV. 2E-H). Cells were stimulated and 24h later the media was renewed to remove the stimulus and the cells were followed overtime to examine STING depletion and replenishment. Treatment with cGAMP elicited efficient STING degradation in both shControl and shGAS7-transduced MDMs, with, as expected, the higher dose inducing a more severe depletion of the protein. STING levels appear to reach a minimum at around 48h post-stimulation, after which the levels of the protein start to rise. Interestingly, in shControl-transduced macrophages, STING levels recovered to a greater extent as compared with GAS7-silenced macrophages (Figure IV. 2A, C-E and G-H). This was more evident after treatment with a low dose of cGAMP, where the levels of STING recovered almost completely at 6 days after stimulation in shControl MDMs but not in shGAS7 cells. We did not observe significant differences in the Mx1 response overtime, between shControl and shGAS7 macrophages.

These experiments provide, to our knowledge, the first instance in which the replenishment of STING levels was examined overtime, after stimulation-induced degradation. We observed that this process is slower than autophagic degradation after stimulation, as the cell takes about one week to completely recover the levels of STING expression, probably longer for higher doses of cGAMP. We observed that, in the absence of GAS7, the protein levels of STING appear to take longer to recover, which could help explain the previous observations in the laboratory with HIV-2 infection. There was, however, no apparent reduction in the Mx1 response after cGAMP stimulation of GAS7-depleted macrophages, further confirming that, in the context of a single stimulation, the absence of GAS7 does not seem to significantly impact the ISG response. In this sense, it would be interesting to expose macrophages to multiple or consecutive rounds of cGAMP stimulation. Such context would perhaps better replicate a chronic viral infection and would reveal in a clearer manner the impact of GAS7 on the antiviral response. We anticipate that in the context of repeated exposure to cGAMP, the absence of GAS7 would result in poor replenishment of STING levels and progressively decreased response to consecutive stimulation.

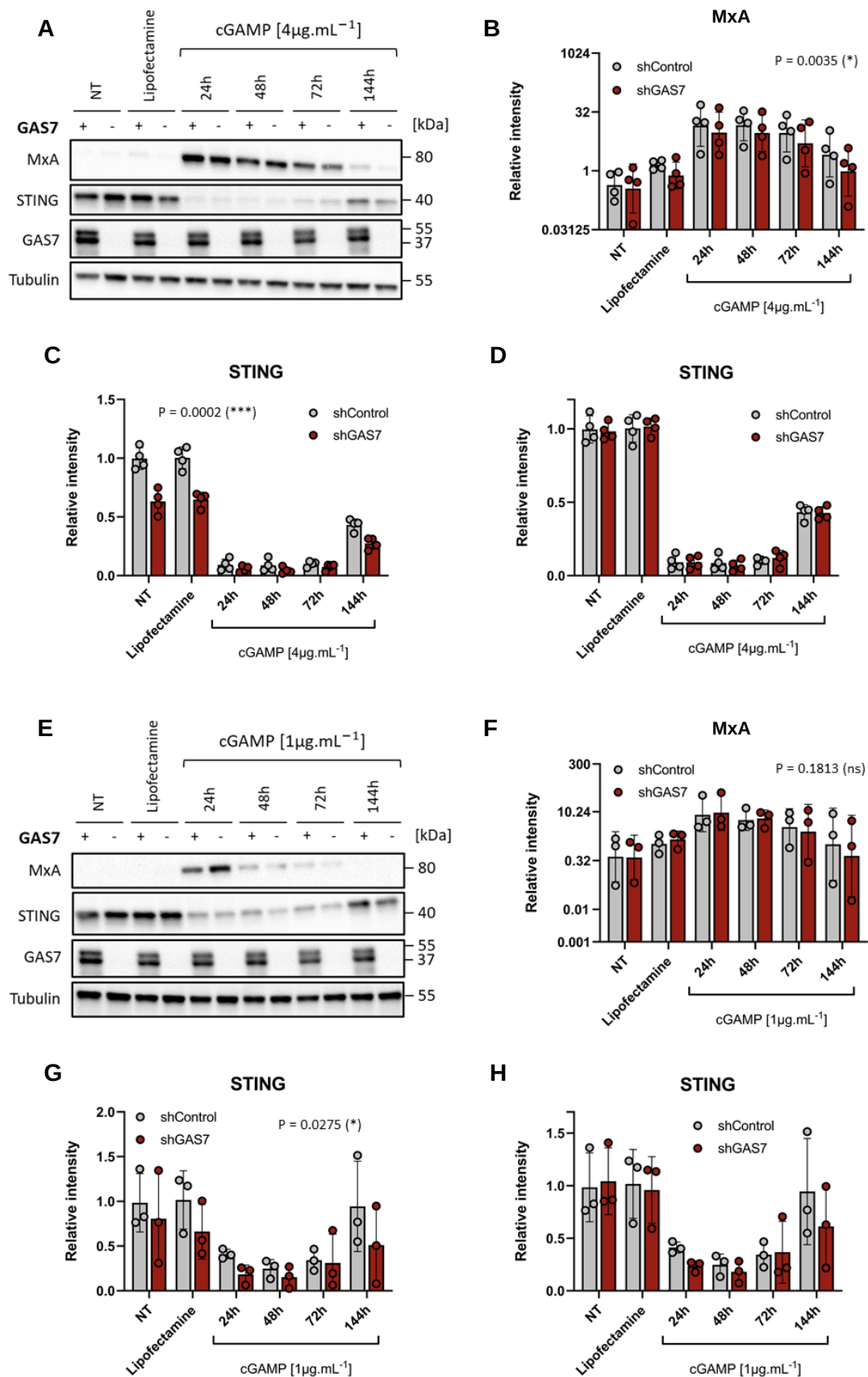


Figure IV.2. GAS7 impacts STING depletion and replenishment kinetics upon 2'3'-cGAMP stimulation. (A) MDMs transduced with a control or GAS7 shRNA were stimulated with 2'3'-cGAMP ($4\mu\text{g.mL}^{-1}$) at the indicated times. Cells were lysed for quantification of the indicated proteins by western blot. One

representative donor is shown. **(B-C)** Graphical summary corresponding to quantification of western blot analysis. **(D)** Another approach at a graphical summary corresponding to quantification of STING in western blot analysis. shControl values are normalized relative to the values for NT and Lipofectamine in the control cells, while shGAS7 values are normalized relative to the values for NT and Lipofectamine in the GAS7-depleted cells. **(E)** MDMs transduced with a control or GAS7 shRNA were stimulated with 2'3'-cGAMP ($1\mu\text{g.mL}^{-1}$) at the indicated times. Cells were lysed for quantification of the indicated proteins by western blot. One representative donor is shown. **(F-G)** Graphical summary corresponding to quantification of western blot analysis. **(H)** Another approach at a graphical summary corresponding to quantification of STING in western blot analysis. shControl values are normalized relative to the values for NT and Lipofectamine in the control cells, while shGAS7 values are normalized relative to the values for NT and Lipofectamine in the GAS7-depleted cells. NT, non-treated.

Data information: The data in panels (B-D) are presented as means of four replicates, including (A), $\pm$ s.d. The data in panels (F-H) are presented as means of three replicates, including (D), $\pm$ s.d. Data from a total of four independent donors, with each dot representing an individual donor. Statistical analysis was performed using two-way ANOVA. Differences between groups were considered statistically significant when: $0.01 < p < 0.05$ (*), $0.001 < p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).

3. SENDAI VIRUS INDUCES A STRONG ISG RESPONSE FROM MDMS UPON INFECTION, UNLIKE HSV-1

Next, we aimed to study the importance of GAS7 in the innate immune response to additional RNA and DNA viruses and extend our preliminary observations with HIV. We started by performing a pilot experiment where WT MDMs were exposed to the DNA virus HSV-1, and the RNA virus SeV, at multiple doses, to ascertain their ability to induce an antiviral response in our experimental system (Figure IV.3).

SeV induced a potent, dose dependent ISG response (Figure IV. 3A-C and F), and a rise in STING levels was also observed for the MDMs infected with this RNA virus (Figure 3.D). HSV-1, on the other hand, failed to induce an ISG response in our system (Figure IV. 3G-L).

The lack of response to HSV-1 was surprising, as it is a DNA virus known for activating the cGAS-STING pathway, especially in microglia from the central nervous system²⁰². However, HSV-1 is a master of immune evasion, and it has recently been documented that one of its viral proteins, UL41, is able to target and selectively degrade cGAS mRNA in HEK293T cells. This leads to deficient cGAMP production and allows HSV-1 to successfully evade the cGAS-STING pathway of cytosolic DNA-sensing^{203, 204}. The same mechanism of pathway evasion might be happening in MDMs, hence the lack of a response to the infection with HSV-1.

Based on these observations, we decided not to proceed our investigation on the role of GAS7 during the antiviral response with HSV-1. Due to time constraints, it was not possible to acquire and test another DNA virus, and we proceeded the experiments with SeV only.

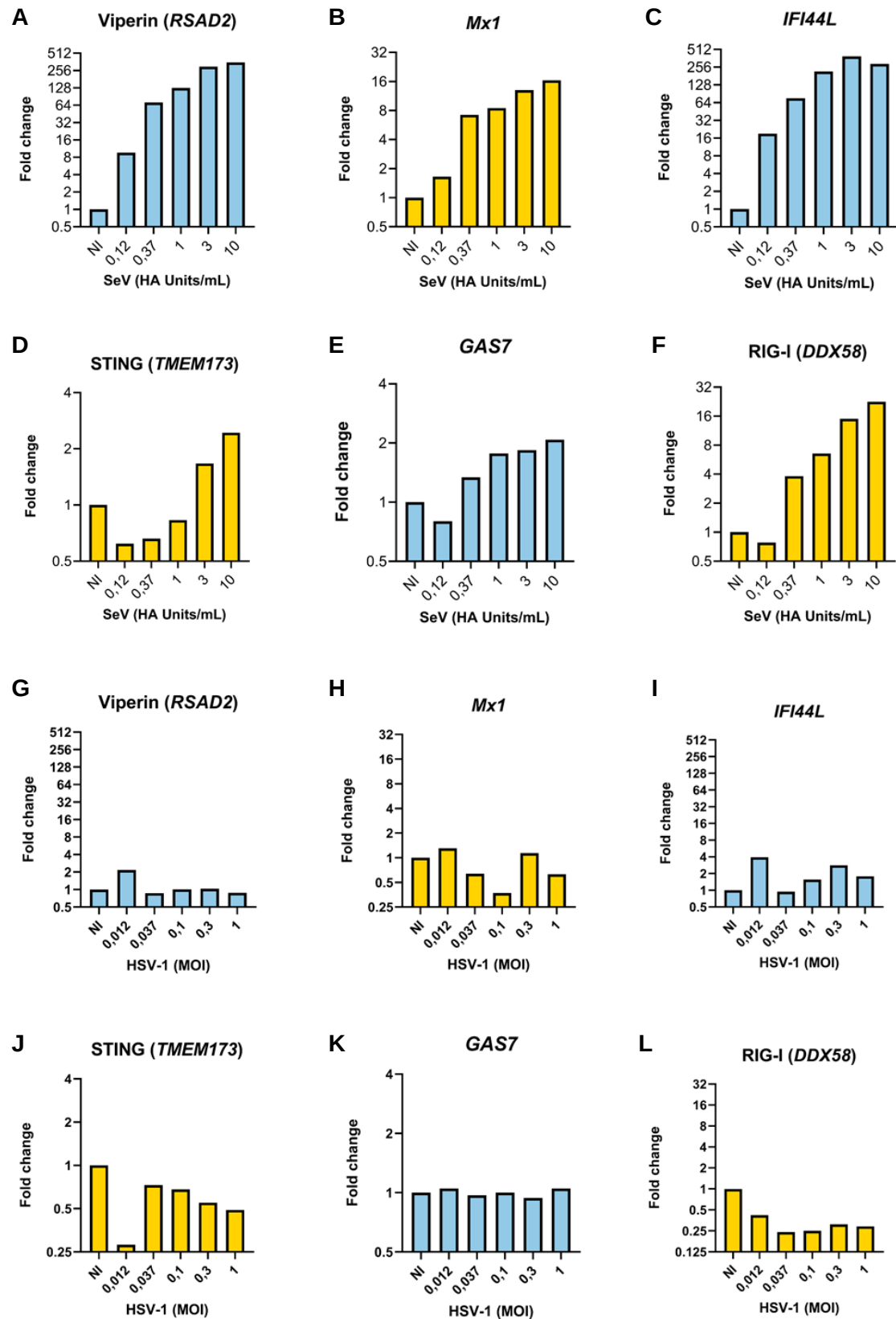


Figure IV.3. SeV induces a strong ISG response from MDMs upon infection, unlike HSV-1. (A-F) WT MDMs were infected with SeV at indicated HA Units. At 48 hours post-infection, cells were lysed in RNA lysis buffer. qPCR was performed for the indicated genes. **(G-L)** WT were infected with HSV at indicated MOI. At 48 hours post-infection, cells were lysed in RNA lysis buffer. qPCR was performed for the indicated genes. NI, non-infected.

4. GAS7 IMPACTS STING LEVELS DURING SEV INFECTION BUT DOESN'T INTERFERE WITH THE IFN RESPONSE

To assess whether GAS7 impacts the antiviral response to an RNA virus, MDMs, depleted or not for GAS7, were infected with SeV at several (HA Units).mL⁻¹ and lysed for analysis after 7 days. The protein levels of several ISGs were analyzed by western blot (Figure IV. 4A-D), while their transcript levels were examined by qPCR (Figure IV. 4E-J). As observed in our previous experiments, most ISGs have their expression reduced in shGAS7 macrophages in non-infected cells (see for instance for RSAD2 – Figure IV.4E). However, this is no longer apparent after SeV infection, as the magnitude of the ISG response was essentially similar between control and shGAS7 macrophages at the different viral doses. There was nevertheless a consistent trend observed for several ISGs in which the response appeared stronger for shGAS7 cells at lower viral doses (0,04 and 0,12 HA Units.mL⁻¹) and it becomes undistinguishable at higher doses (0,37, 1,0 and 3,0 HA Units.mL⁻¹). This was observed for example for Mx1 (Figure IV. 4A, B and G) and DDX58 (Figure IV.4D and J) at both protein and mRNA levels. These trends, however, did not reach statistical significance. STING protein and transcript levels were reduced in shGAS7 MDMs after infection with SeV, at certain doses (Figure IV. 4A, C and H).

These experiments demonstrate that, unlike our previous data with HIV-1 and HIV-2, the immune response to SeV is not impaired in the absence of GAS7. SeV is a virus detected by the RIG-I-MAVS pathway of RNA sensing. Unlike STING, we did not observe any decrease in MAVS mRNA levels upon GAS7 silencing (Figure IV.4A and data not shown). This suggests that GAS7 may be more important in the antiviral response to viruses that engage the cGAS-STING pathway, such DNA viruses and retroviruses.

Nevertheless, we decided to test the role of GAS7 in the response to SeV, because recent studies show that STING might also play a role in the response to RNA viruses. In fact, STING has been reported to interact with RIG-I and MAVS, in a complex that is stabilized upon virus infection at the mitochondrial-associated endoplasmic reticulum membranes (MAM)^{133, 205, 206}. Studies have shown that for some RNA viruses, including SeV, vesicular stomatitis virus (VSV), Newcastle disease virus (NDV), and Japanese encephalitis virus (JEV), that are recognized by RIG-I, STING can function as a co-factor in the IFN response^{133, 205, 207, 208, 209}.

Regarding SeV, it was found that a loss of STING resulted in less IFN β induction and that binding of RIG-I to STING occurred in cells infected with SeV. STING may, thus, link the intracellular RNA and DNA sensing pathways and, hypothetically, be required for efficient functioning of the RIG-I-MAVS pathway for certain RNA viruses^{207, 210}.

In our experiments, while the levels of STING appear decreased after GAS7 silencing, this did not result in impaired responses to SeV. GAS7-depleted cells do not have a complete loss of STING levels (dropping about 25 to 40%), and this adaptor protein works as a co-factor of RIG-I-MAVS in this context. Because of this, it is presumable that the cells are still able to mount a satisfying IFN type I response and induce the expression of ISGs based solely on RIG-I-MAVS signaling.

In fact, our data suggests that the response to SeV may be even amplified in GAS7-depleted cells, at least for lower viral input doses (0,04 and 0,12 HA Units.mL⁻¹). Although our data did not reach statistical significance, it was consistent across multiple ISGs and for both mRNA and protein levels. While the reasons for such increase remain obscure and mostly speculative at this stage, ongoing work in our lab is aiming to shed light in this and other questions by performing genome-wide transcriptomic analyses that will elucidate in a global manner the impact that GAS7 has on the antiviral response.

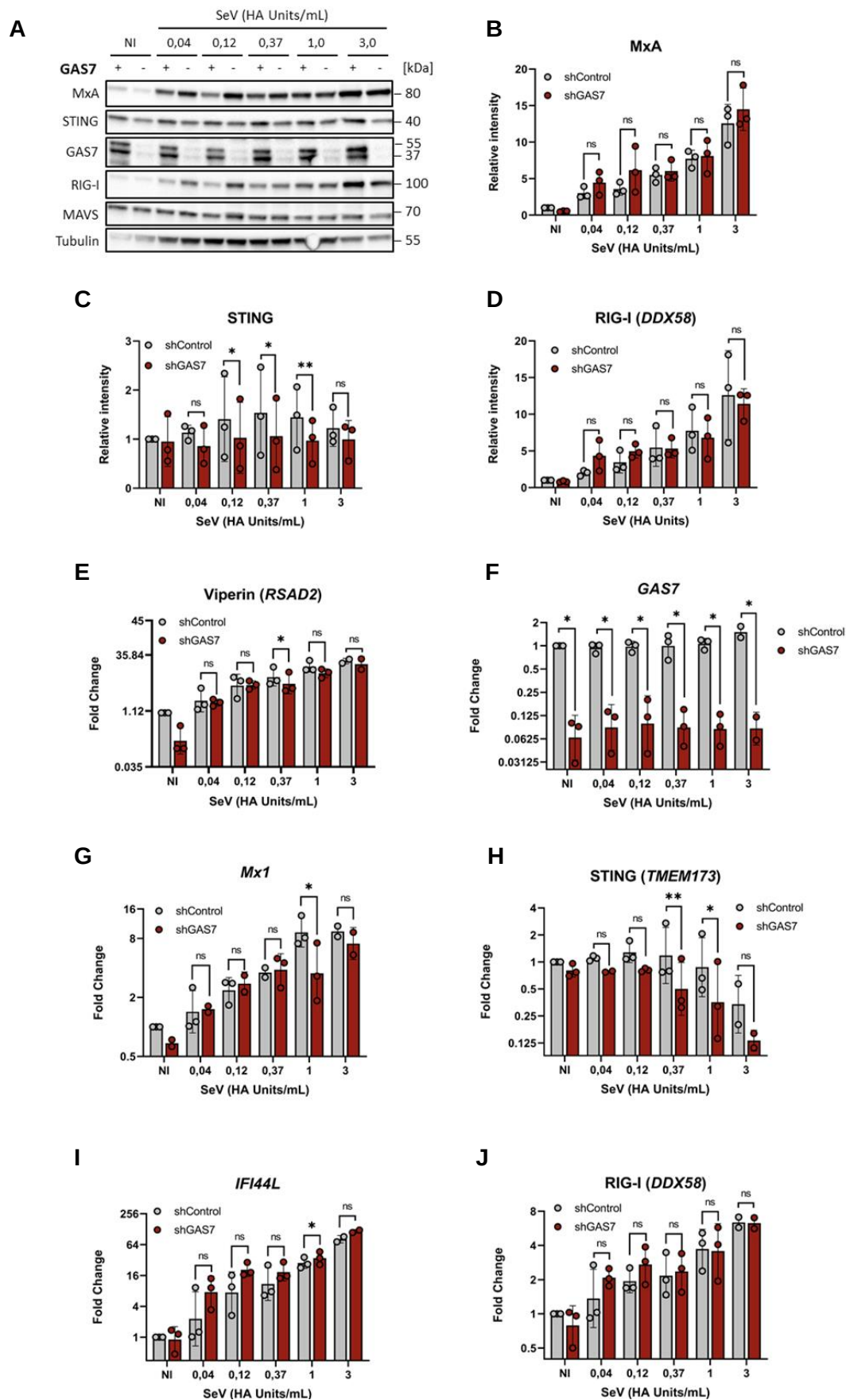


Figure IV.4. GAS7 impacts STING levels during SeV infection but doesn't seem to significantly interfere with the IFN response. (A) MDMs transduced with a control or GAS7 shRNA were infected with SeV at indicated HA Units. Cells were lysed for quantification of the indicated proteins by western blot. **(B-D)**

Graphical summary corresponding to quantification of western blot analysis illustrated in (A). **(E-J)** MDMs transduced with a control or GAS7 shRNA were infected with SeV at indicated HA Units. Cells were lysed after 24h for quantification of the indicated mRNAs by qPCR. NI, non-infected.

Data information: The data in panels (B-D) are presented as means of three replicates, including (A), and the data in panels (E-J) are presented as means of three replicates $\pm$ s.d. Data from a total of three independent donors, with each dot representing an individual donor. Statistical analysis was performed using Multiple paired t test for multiple comparisons. Differences between groups were considered statistically significant when: $0.01 < p < 0.05$ (*), $0.001 < p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).

5. INCREASED REPLICATION OF THE SENDAI VIRUS IN GAS7-DEPLETED CELLS IS ASSOCIATED WITH THE FORMATION OF GIANT CELLS IN CULTURE

While GAS7 has a clear impact on the antiviral response to HIV in MDMs, parallel work in our lab has described that it also restricts the propagation of HIV via mechanisms that appear to rely on inhibiting the dynamics of the actin cytoskeleton. Thus, in the case of HIV-1 and HIV-2, the antiviral activities of GAS7 appear to rely on two completely distinct mechanisms. On one side, GAS7 is required for an adequate ISG response against the virus, and, on the other side, it restricts the propagation of the virus by impacting on the actin dynamics required for viral transmission from cell to cell. In the case of HIV-2, such increased transmission in the absence of GAS7 is revealed by the drastic increase in the number of giant multinucleated cells in culture (Figure II.1C).

The macrophage antiviral response to SeV infection is not negatively impacted by the absence of GAS7, however, it is well described that this virus, as HIV-2, induces the formation of large multinucleated cells, in a process that involves the host cell actin cytoskeleton²¹¹. Thus, we asked whether the absence of GAS7 would impact the replication of SeV in MDMs and the formation of giant cells

To that end, MDMs transduced with a control or GAS7 shRNA were infected with SeV for 7 days and lysed, as before. To quantify the relative viral loads in the infected MDMs, we designed primers for qPCR targeting 4 SeV genes that encode important proteins – the nucleocapsid, large, matrix and phosphoprotein (Figure IV. 5A-D).

Silencing of GAS7 resulted in a significant increase in the viral load in infected macrophages, especially at the lower doses (0,12 and 0,37 HA Units.mL⁻¹), where we could see a 5 to 10 fold increase of the viral load in GAS7 silenced macrophages, as compared with shControl transduced cells, across the 4 different viral genes employed. The effect is still observed for higher doses (1,0 and 3,0 HA Units.mL⁻¹), despite being less significant (Figure IV. 5A-D). This most likely happens due to the fact that there is a toxic effect of the virus when the cells exert a lot of cellular response – typically, viruses try to amplify within the host cells in a way that does not over activate them so as to not generate an overly intense response and lead to apoptosis²¹². However, when infecting MDMs directly with greater viral concentrations, that highly activated state is reached, which results in immense cell death over the 7 days before collecting the cells for analysis.

Given these results, we also decided to place MDMs transduced with control or GAS7 shRNAs and infected with SeV, in a real time live-cell analysis system to observe the formation of giant cells as the infection progresses (Figure IV. 5E). Non-infected MDMs, either shControl or shGAS7-transduced, do not appear to significantly change their morphology after 96 h in culture (Figure IV. 5E-top two panels).

While SeV induces syncytia in different types of eukaryotic cells, we did not observe formation of giant cells in infected shControl MDMs (Figure IV. 5E – third panel from top). We cannot exclude the formation of smaller syncytia, because we did not stain the nucleus to ascertain the exact number of nuclei per cell, but their size and morphology over-time seems stable and consistent with mononucleated cells. In contrast, in SeV-infected cells silenced for GAS7, we frequently observed the formation of giant cells that could measure more than 500 μ m (Figure IV. 5E – bottom panel).

These results show increased replication of SeV in the absence of GAS7, even though this protein does not impact on the antiviral response to this virus. Such increased replication is reflected by the formation of giant cells in culture that are most likely multinucleated syncytia. While we did not explore further this phenotype, because the goals of this project were more focused on the role of GAS7 in the antiviral immune response, it seems clear that GAS7 has other important antiviral roles, not related to its ability to promote the ISG response. In the case of HIV-2, our current data indicates that GAS7 regulates the actin cytoskeleton, which impairs the ability of the virus to induce multinucleated syncytia. A similar mechanism may be operational during SeV infection, suggesting that GAS7 may be a broad antiviral factor. The data from these experiments can serve as a starting ground for future studies aiming to explore mechanistically how GAS7 impairs SeV replication.

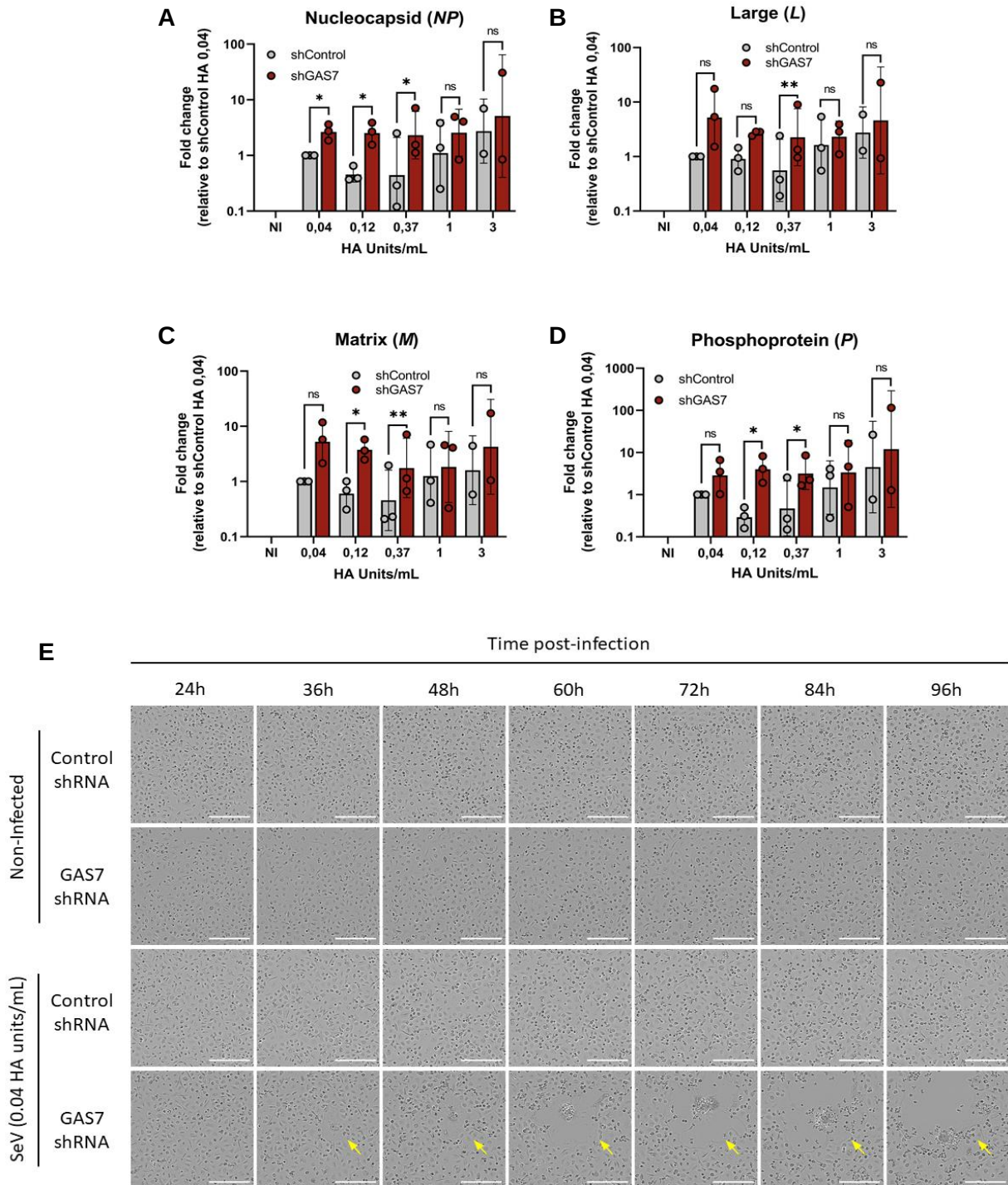


Figure IV.5. GAS7-depleted cells display more viral amplification and experience fusion events. (A-D) MDMs transduced with a control or GAS7 shRNA were infected with SeV at indicated HA Units. Cells were lysed at 7 days after infection, and the quantification of infectious viral load was achieved through qPCR for the indicated genes. **(E)** MDMs transduced with a control or GAS7 shRNA were infected with SeV at 0,04 HA Units.mL⁻¹. Cells were placed in the Incucyte live cell imaging system and pictures were acquired at the indicated time-points. Scale bar = 200 μ m. NI, non-infected.

Data information: The data in panels (A-D) are presented as means of three replicates $\pm$ s.d. Statistical analysis was performed using Multiple paired t test for multiple comparisons. Data from a total of three independent donors, with each dot representing an individual donor. Differences between groups were considered statistically significant when: 0.01 < p < 0.05 (*), 0.001 < p < 0.01 (**), p < 0.001 (***) and p < 0.0001 (****).

6. GAS7 ACCUMULATES IN THE NUCLEUS UPON STIMULATION WITH 2'3'-cGAMP

Our lab has previously shown that GAS7 is partially in the nucleus at the steady state in macrophages, and it interacts with multiple nuclear proteins involved in diverse processes such as mRNA splicing and export, regulation of gene expression, etc (Figure II.3 and Figure II.4). Thus, while we still do not understand how GAS7 influences the antiviral response, it is possible that its effects come from actions of this protein in the nucleus.

GAS7 impacts the expression of STING and other antiviral genes at the mRNA level, suggesting that it may regulate their expression, splicing, stability, or transport of their mRNAs. For this task, we asked whether the subcellular distribution of GAS7 changes upon stimulation with 2'3'-cGAMP. We hypothesize that if GAS7 further accumulates in the nucleus after a stimulus that induces STING degradation, it would further favor the hypothesis that it may regulate STING mRNA levels from its actions in the nucleus. As a control, the subcellular location of GAS7 was also evaluated for cells stimulated with LPS, which primes the cells via TLR signaling, allowing transcriptional induction of several proinflammatory genes.

WT MDMs were thus stimulated with lipofectamine-complexed 2'3'-cGAMP or LPS and a cell fractionation protocol was employed at defined time-points to separate the cytosolic, membrane-enriched and nuclear fractions (Figure IV.6). Strikingly, at the 24h timepoint, in the MDMs stimulated with cGAMP, cytosolic GAS7 appears severely depleted and GAS7 accumulates in the nucleus. In non-treated and LPS-treated cells, GAS7 levels remain elevated in the cytosol. GAS7 is also present at relatively elevated levels in the nucleus of WT cells, confirming our preliminary studies (Figure II.4). In cGAMP stimulated MDMs, GAS7 levels in the cytosol start to come back to pre-stimulation levels by 48 hours and become undistinguishable from non-treated by 96 hours. Intriguingly, in LPS-stimulated cells we observed a selective decrease in the levels of nuclear GAS7, which does not appear to accumulate in any other compartment suggesting induction of degradation of the protein.

Mx1 expression was induced by stimulation with both cGAMP and LPS and localized mostly to cytosolic and membrane fractions. STING degradation was observed, as expected, only in the cGAMP treated MDMs, and does not recover even after 96h, probably due to the high dose of cGAMP employed for this experiment (4 μ g.mL⁻¹).

Interestingly, in non-treated and LPS treated cells STING is present in the membrane fraction, as expected for an ER-resident protein, but also in the nucleus. The presence of STING in the nucleus remains a controversial topic, however, and this could be happening due to a fault in the fractionation protocol, where a part of the ER could be included in the nuclear fraction, instead of in the membrane fraction, as the compartments are very closely packed. On the other hand, our fractionation protocol indicates that the reagent used to dissolve the membranes is capable of efficiently dissolving the plasma, mitochondrial and endoplasmic reticulum-Golgi membranes, while not solubilizing the nuclear membranes, and thus fully separating these compartments. Moreover, recent studies are corroborating the observation of STING in the nucleus, stating that a less investigated pool of STING might partly reside at the inner membrane of the nuclear envelope^{213, 214}.

These experiments suggest that GAS7 is a dynamic factor able to shuttle between distinct cellular compartments after certain stimulations to perform a role in a particular location. Because of its action in promoting STING mRNA and protein expression, we can speculate that GAS7 accumulation in the nucleus after a stimulus that induces STING degradation may work to promote a faster recovery in the levels of this antiviral factor, via a mechanism that may involve an increase in its transcription or by promoting the transport, stability or splicing of its mRNA. These alternative mechanisms are currently being studied in our lab. The fact that LPS appears to induce the degradation of GAS7 specifically in the nucleus is very intriguing and may suggest that this protein is impacted in different ways by different classes of PAMPs, further emphasizing the need to perform additional studies on how GAS7 expression is itself regulated.

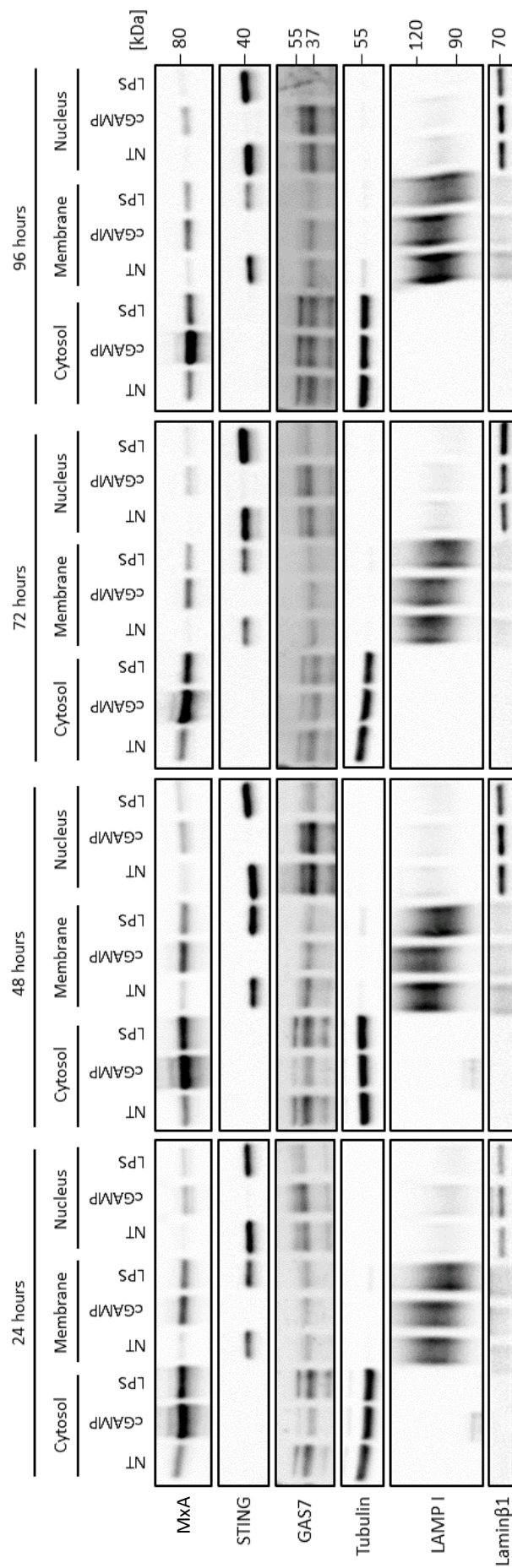


Figure IV.6. GAS7 translocates to the nucleus upon stimulation with 2'3'-cGAMP. WT MDMs were stimulated with 2'3'-cGAMP (4μg.mL⁻¹) or LPS (100ng.mL⁻¹). After 24 hours, the cells were fractionated into cytosol, membrane, and nuclear fractions, using a commercially available kit. Fractions were probed by western blot for the indicated proteins. Tubulin was used as a cytosolic marker, LAMP1 to mark the membrane-enriched fraction and Laminβ1 as a nuclear marker. NT, non-treated.

7. THP-1 CAN POTENTIALLY BE A VALUABLE MODEL TO STUDY GAS7 FUNCTIONS IN MACROPHAGES

In the last task of this project, we wanted to test whether THP-1 cells could constitute a valuable model to study the antiviral roles of GAS7, in a way where the experiments would not be dependent on blood donations and laborious primary cell differentiation.

We silenced the expression of GAS7 in THP-1 cells, using the same shRNA strategy employed in primary macrophages, and found that the silencing with the use of shRNA is sufficient to deplete GAS7 levels with great efficiency (Figure IV. 7A and D). To further characterize the THP-1 cells as an appropriate model for MDMs, we evaluated whether the control shRNA and the GAS7 shRNA cells behaved similarly in terms of growth kinetics over the course of 10 days (Figure IV. 7C). It is reasonable to conclude that both cell types grow similarly over time and at a fast rate, achieving 2×10^6 cells.mL⁻¹ over the indicated period, when only 1.5×10^4 cells.mL⁻¹ were initially plated in each well of a 6-well plate.

THP-1 cells were then stimulated with PMA to achieve differentiation into adherent macrophage-like cells – once these cells are plated and induced with PMA, they enter growth arrest and differentiate into cells that adhere to the bottom of the wells and look morphologically like primary macrophages. Western blot analyses of THP-1 cells depleted for GAS7 showed no significant differences in terms of STING or MXA expression, unlike primary MDMs, where those molecules were often downregulated at the steady-state (Figure IV. 7A). Using qPCR, we nevertheless observed significant decrease in the transcript levels of both RSAD2 (Figure IV. 7E) and STING (TMEM173) (Figure IV. 7G).

Finally, subcellular fractionation of WT THP-1 cells also showed the presence of endogenous GAS7 in the nucleus of those cells, as observed in primary macrophages.

Altogether, this last part of the work supports the use of THP-1 cells as a potential model to study GAS7 in macrophages, with the advantages and facility to manipulate associated with cell lines.

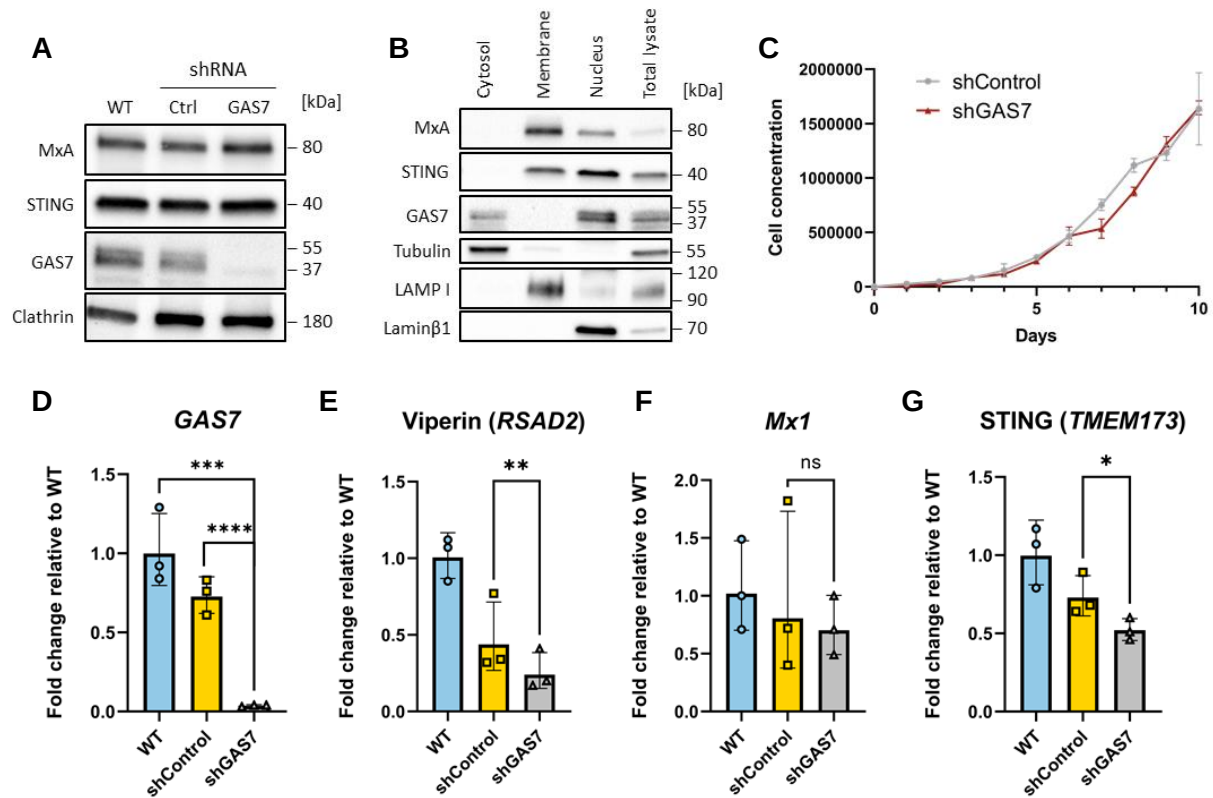


Figure IV.7. THP-1 can potentially be a valuable model to study GAS7 functions in macrophages. (A) Protein levels of GAS7, STING and Mx1 were elucidated by western blot in PMA stimulated (30ng.mL^{-1}) WT, shRNA Control and shGAS7 THP-1 cells. Cells were lysed upon 48 hours of differentiation. (B) THP-1 cells were stimulated with PMA (30ng.mL^{-1}). After 48 hours, the cells were fractionated into cytosol, membrane, and nuclear fractions, using a commercially available kit. Fractions were probed by western blot for the indicated proteins. (C) THP-1 cell quantification was assessed using an automated fluorescence cell counter, at consecutive 24h intervals, over 10 days. (D-E) Levels of GAS7, RSAD2, Mx1 and STING mRNA were detected by qPCR in WT, shRNA Control and shGAS7 THP-1 cells.

Data information: The data in panel C are presented as means of two replicates $\pm$ s.d. The data in panels (D-G) are presented as means of three replicates $\pm$ s.d. Statistical analysis was performed using ratio paired t test. Differences between groups were considered statistically significant when: $0.01 < p < 0.05$ (*), $0.001 < p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).

CHAPTER V: CONCLUDING REMARKS AND FUTURE WORK

CONCLUDING REMARKS

The work presented in this thesis extends initial observations made in our laboratory and confirms that GAS7 is a protein with important roles in the innate immune response to viruses, particularly those that engage the cGAS-STING pathway. With its ability to shuttle between cellular compartments, GAS7 may selectively accumulate in sites where its action is required at a given point in time. We speculate that nuclear GAS7 can promote the turnover of the mRNA encoding for STING, and other antiviral molecules, via a mechanism that may involve an increase in their transcription or by promoting the transport, stability or splicing of their mRNA. Additionally, data from this thesis, along with past and ongoing work in the laboratory, also suggests that GAS7 might have additional antiviral roles that are not related to its ability to promote the ISG response. These seem to result from the ability of GAS7 to regulate the dynamics of the actin cytoskeleton, including the formation of cell protrusions and filopodia, which are structures known to be involved in the propagation of multiple viruses. Interestingly, these later functions are most likely mediated by GAS7 actions in the cytosol, further highlighting the importance of compartmentalization in the different antiviral roles of GAS7.

Our initial experiments aimed at evaluating the impact that GAS7 could have in the macrophage response to a single stimulation by viral mimetics. While the absence of GAS7 impacts on the steady-state levels of certain antiviral molecules, the ISG response to cytosolic dsDNA and dsRNA was undistinguishable between normal and GAS7-depleted macrophages.

This was in contrast with the previous observations from the lab concerning HIV infection, where GAS7 clearly impacted the ISG response, and led us to conclude that perhaps a context of a single stimulation is not suited to appreciate the importance of GAS7 in the antiviral response. While certain antiviral factors appear to be reduced in GAS7-silenced macrophages at the steady state, the cell is still capable of mounting a response comparable to normal macrophages. However, our previous work with HIV-2 demonstrated severe depletion of STING in GAS7-silenced macrophages after infection, suggesting an important role for GAS7 in maintaining the levels of this antiviral effector in the context of a lasting infection. Thus, we tested directly if the absence of GAS7 impacts on the replenishment of STING levels after its stimulation-induced degradation, by delivering 2'3'-cGAMP to the cytosol of MDMs. Using primary cells, we observed the degradation and replenishment of STING levels after cGAMP stimulation, for the first time, to our knowledge. We further show that STING levels recovered to a greater extent in shControl-transduced macrophages as compared with GAS7-silenced macrophages. Because the levels of STING are depleted with similar efficiency in both normal and GAS7-silenced macrophages (even if slightly delayed in GAS7-silenced cells), we speculate that the role of GAS7 in promoting the recovery of STING levels comes from *de novo* synthesis of the protein or its transcript, rather than GAS7 inhibiting STING degradation via autophagy.

In the present work, we also evaluated the importance of GAS7 in the innate immune response to additional RNA and DNA viruses. SeV, an RNA virus, induced a potent, dose dependent ISG response and a rise in STING levels for the MDMs infected, while HSV-1 failed to induce an ISG response in our system, which was surprising for a DNA virus. We assume that HSV-1 can evade the cGAS-STING pathway, as it has been proven to be able to do so in HEK293T cells.

Due to time constraints, we were not able to evaluate the role of GAS7 in the ISG response to another DNA virus; yet this remains an important task for the future.

By comparing the magnitude of the ISG response to SeV between normal and GAS7-silenced macrophages, we observed that they were not significantly different. This suggests that GAS7 does not play a role in the antiviral ISG response to RNA viruses. Indeed, past data from the lab revealed that the levels of MAVS, an adaptor molecule that plays critical roles in the antiviral response to RNA viruses through RIG-I and MDA5, are not impacted by GAS7 silencing. Possibly, the distinct impact that GAS7 has on RNA viruses versus retroviruses comes from its ability to promote STING expression (whose actions parallel those of MAVS, but in a context of infection with DNA viruses or retroviruses.), but not MAVS.

While the macrophage response to SeV is not negatively impacted by the absence of GAS7, this absence still induces a significant increase in the viral load in infected cells, associated with the formation of giant cells, that are most likely multinucleated syncytia. Even though it wasn't the aim of the project, we established that GAS7 has other important antiviral roles, not related to its ability to promote the ISG response. In the case of HIV-2, GAS7 regulates the actin cytoskeleton, which impairs the ability of the virus to induce multinucleated syncytia – a similar mechanism may be operational during SeV infection, suggesting that GAS7 may be a broad antiviral factor.

Combining the knowledge gathered from this thesis and other work in the lab, we hypothesized that GAS7 impacts the expression of STING and other antiviral genes at the mRNA level, by regulating their expression, or the splicing, stability, or transport of their mRNAs, from its actions in the nucleus. It was thus important to know whether the subcellular distribution of GAS7 changed upon stimulation with 2'3'-cGAMP. At 24h post-stimulation with cGAMP, cytosolic GAS7 appears severely depleted and accumulates in the nucleus, coming back to pre-stimulation levels by 48h and becoming undistinguishable from WT by 96h. These experiments suggest that GAS7 is a dynamic factor able to shuttle between distinct cellular compartments after certain stimulations and we can speculate that GAS7 accumulation in the nucleus after a stimulus that induces STING degradation may work to promote a faster recovery in the levels of this antiviral factor.

Finally, we established the use of THP-1 cells as a potential model to study GAS7 in macrophages, with the advantages and facility to manipulate associated with cell lines.

FUTURE WORK

This work establishes GAS7 as a novel regulator of the antiviral innate immune response. However, the mechanisms behind such actions remain obscure to date. Thus, future experiments should focus on elucidating those mechanisms. Because of the presence of GAS7 in the nucleus, its multiple interactions with nuclear proteins involved in RNA-related processes and the fact that the transcripts for many antiviral molecules are reduced in the absence of GAS7, we hypothesize that nuclear GAS7 is responsible for the actions of this protein in the antiviral immunity. It is thus critical to evaluate the transcriptomics changes induced by GAS7 silencing in macrophages, at the genome scale. An RNA-seq analysis comparing macrophages depleted or not for GAS7 is ongoing in the laboratory and should elucidate the full breadth of transcriptomic changes induced by GAS7. Such RNA-seq analysis can also evaluate whether RNA splicing is progressing normally in the absence of GAS7 and give clues about a role for GAS7 in this process. GAS7 may also impact the expression of antiviral factors by epigenetic mechanisms. To evaluate this hypothesis, techniques such as ATAC-seq could be employed to evaluate and compare chromatin accessibility in macrophages in the absence of GAS7. GAS7 may also impact on the transport of the mRNAs of STING and other molecules from the nucleus to the cytoplasm, as recently reported for hnRNPA2/B1¹⁴⁴. To evaluate this, one could quantify and compare the partition between nucleus and cytoplasm of selected mRNAs between normal and GAS7-silenced macrophages, by qPCR after cell fractionation.

Related to the question of whether GAS7 regulates antiviral immunity from the nucleus is how it shuttles between different compartments, as we observed after cGAMP stimulation. So, which motifs of GAS7 could be involved in nuclear transport or mediating gene transcription? In human MSCs, GAS7 has been shown to regulate Runx2-dependent gene expression, however, it is unknown if this regulation is achieved through posttranslational modifications or epigenetic activation of the Runx2 promoter region¹⁵⁹. The functions of the F-BAR domain are already well established – aside from mediating GAS7 oligomerization, it is also responsible for its interaction with actin, and the connection of microtubules with actin filaments. However, to date, the function of both the WW and the SH3 domains of hGas7 in term of ligand interaction remains unclear¹⁶⁴. It seems that tyrosine phosphorylation might be of significant importance in the regulation of many SH3 domains and it may interfere with the binding of SH3 domain to interacting partners²¹⁵. Thus, one may speculate that post-transcriptional modifications, such as phosphorylation or others, may drive the shuttling of GAS7 between different subcellular compartments. To date nothing is known about the post-transcriptional modifications of GAS7, although *in silico* tools suggest that it may be phosphorylated in several tyrosines. It would thus be interesting to combine site-directed mutagenesis of such residues and evaluate the impact on the translocation of GAS7 to the nucleus. This could be followed by identification of the putative kinases that phosphorylate GAS7. Our proteomic analysis already provided certain candidates that interact with GAS7 in macrophages. Thus, while it is very preliminary to conclude that phosphorylation is driving GAS7 to shuttle to the nucleus, it is nevertheless an interesting point of view that remains to be addressed.

We observed that GAS7 impacts the innate antiviral response to retroviruses such as HIV-1 and HIV-2, but not to an RNA virus, SeV. Thus, GAS7 may be more important in the antiviral response to viruses that directly engage the cGAS-STING pathway, such as DNA viruses and retroviruses. We couldn't evaluate the role of GAS7 in the immune response to a DNA virus, but this remains a critical task for the future. HSV-1 did not prove to be a good model in our studies possibly due to its capacity to evade the cGAS-STING pathway as it imposes a strong selective pressure to pathogens. As such, in future studies, DNA viruses that are not known to evade the cGAS-STING pathway should be used, such as poxviruses, like the modified vaccinia Ankara (MVA) or adenoviruses (Ad), for instance.

Moreover, other non-viral pathogens can also activate STING, and it would be interesting to evaluate the role of GAS7 in the innate responses they induce. For instance, certain bacteria produce CDNs (cyclic di-GMP, cyclic di-AMP and 3'3'-cGAMP), known to directly bind to and activate STING. *Listeria monocytogenes* is an interesting pathogen for these studies, for example. This pathogen is a Gram-positive bacterium replicating in myeloid cells that secretes c-di-AMP and it thus able to activate STING. *Listeria monocytogenes* does this for its own benefit, as activation of STING actually prevents the induction of specific cell-mediated immunity by induction of type I interferon²¹⁶. Aside from direct STING activation, *Listeria* DNA present in the cytosol of monocyte-derived macrophages has also been proven to activate cGAS, heightening STING induction even further²¹⁷.

Our experiments with SeV confirmed our previous data with HIV-1 and HIV-2 in that GAS7 possesses antiviral functions that are independent of its capacity to modulate the antiviral innate response. We defend that a similar mechanism as to what happens with HIV-2, where GAS7 seems to regulate the actin cytoskeleton and consecutively impairs the ability of the virus to induce multinucleated syncytia, might be operational during SeV infection. SeV is known to propagate by inducing the formation of multinucleated syncytia that depend in the host actin cytoskeleton for formation. Thus, GAS7 may impair the spread of SeV via mechanisms that block cell fusion. This data can serve as a starting ground for future mechanistic studies as to how GAS7 impairs SeV replication.

Another interesting topic to develop in further studies would be whether GAS7 impacts STING levels in cancer cells, and if that could have any impact in cancer progression. Cancer cells are replete with cytosolic dsDNA (from genomic, mitochondrial, and exogenous origins) and thus, constitutively induce the activation of the cGAS-STING pathway. The effects of this activation of the pathway are still poorly understood, and can result in protumorigenesis or antitumorigenesis, depending on a lot of different factors^{218, 219}. Constitutive activation of the pathway can lead to other diseases, especially severe autoimmune disorders such as systemic lupus erythematosus (SLE), Aicardi-Goutières syndrome (AGS) and STING-associated vasculopathy with onset in infancy (SAVI)¹³⁸. If future studies focus on whether GAS7 is able to affect STING levels in the pathologies mentioned, GAS7 could be an important factor for future therapies concerning some of these diseases.

Finally, the classical limitations associated with the use of human blood in laboratory research will continue to exist and must be kept in mind. For this reason, it is important to develop further studies in an alternative model that can thoroughly mimic monocyte-derived

macrophages. In this study, we have started to evaluate whether the THP-1 monocytic cell line could be a valuable candidate for this position. These studies should be continued and extended to assess whether these cells react the same way as primary macrophages to infection with different stimuli and viruses.

BIBLIOGRAPHY

1. Moser, M. & Leo, O.J.V. Key concepts in immunology. **28**, C2-C13 (2010).
2. Murphy, K. & Weaver, C. *Janeway's immunobiology*. Garland science, 2016.
3. Turvey, S.E., Broide, D.H.J.J.o.A. & Immunology, C. Innate immunity. **125**, S24-S32 (2010).
4. Chaplin, D.D.J.J.o.A. & Immunology, C. Overview of the immune response. **125**, S3-S23 (2010).
5. Lydyard, P., Whelan, A. & Fanger, M. *BIOS Instant notes in immunology*. Taylor & Francis, 2011.
6. Hato, T. & Dagher, P.C.J.C.J.o.t.A.S.o.N. How the innate immune system senses trouble and causes trouble. **10**, 1459-1469 (2015).
7. Parkin, J. & Cohen, B.J.T.L. An overview of the immune system. **357**, 1777-1789 (2001).
8. Nicholson, L.B.J.E.i.b. The immune system. **60**, 275-301 (2016).
9. Cruse, J., Lewis, R. & Wang, H. *Immunology guidebook*. Elsevier, 2004.
10. Ahn, J., Barber, G.N.J.E. & medicine, m. STING signaling and host defense against microbial infection. **51**, 1-10 (2019).
11. Akira, S., Uematsu, S. & Takeuchi, O.J.C. Pathogen recognition and innate immunity. **124**, 783-801 (2006).
12. Mogensen, T.H.J.C.m.r. Pathogen recognition and inflammatory signaling in innate immune defenses. **22**, 240-273 (2009).
13. Mass, E. & Lachmann, N.J.G.T. From macrophage biology to macrophage-based cellular immunotherapies. 1-4 (2021).
14. Stefater III, J.A., Ren, S., Lang, R.A. & Duffield, J.S.J.T.i.m.m. Metchnikoff's policemen: macrophages in development, homeostasis and regeneration. **17**, 743-752 (2011).
15. Auffray, C., Sieweke, M.H. & Geissmann, F.J.A.r.o.i. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. **27**, 669-692 (2009).
16. Germic, N., Frangez, Z., Yousefi, S., Simon, H.-U.J.C.D. & Differentiation. Regulation of the innate immune system by autophagy: monocytes, macrophages, dendritic cells and antigen presentation. **26**, 715-727 (2019).
17. Wynn, T.A., Chawla, A. & Pollard, J.W.J.N. Macrophage biology in development, homeostasis and disease. **496**, 445-455 (2013).
18. Gerhardt, T. & Ley, K.J.C.r. Monocyte trafficking across the vessel wall. **107**, 321-330 (2015).
19. Yona, S. *et al.* Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. **38**, 79-91 (2013).
20. Guillelliams, M. *et al.* Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. **210**, 1977-1992 (2013).
21. Hashimoto, D. *et al.* Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. **38**, 792-804 (2013).
22. Murray, P.J. & Wynn, T.A.J.N.r.i. Protective and pathogenic functions of macrophage subsets. **11**, 723-737 (2011).
23. Epelman, S., Lavine, K.J. & Randolph, G.J.J.I. Origin and functions of tissue macrophages. **41**, 21-35 (2014).
24. Rodrigues, V., Ruffin, N., San-Roman, M. & Benaroch, P. Myeloid cell interaction with HIV: a complex relationship. *Front Immunol* **8**: 1698. 2017.
25. Watanabe, S., Alexander, M., Misharin, A.V. & Budinger, G.S.J.T.J.o.c.i. The role of macrophages in the resolution of inflammation. **129**, 2619-2628 (2019).
26. Vannella, K.M. & Wynn, T.A.J.A.r.o.p. Mechanisms of organ injury and repair by macrophages. **79**, 593-617 (2017).
27. Sica, A. & Mantovani, A.J.T.J.o.c.i. Macrophage plasticity and polarization: in vivo veritas. **122**, 787-795 (2012).
28. Martinez, F.O., Helming, L. & Gordon, S.J.A.r.o.i. Alternative activation of macrophages: an immunologic functional perspective. **27**, 451-483 (2009).
29. Murray, P.J. *et al.* Macrophage activation and polarization: nomenclature and experimental guidelines. **41**, 14-20 (2014).
30. Ley, K.J.T.J.o.I. M1 means kill; M2 means heal. **199**, 2191-2193 (2017).
31. Eloranta, M. & Alm, G.J.S.j.o.i. Splenic marginal metallophilic macrophages and marginal zone macrophages are the major interferon-alpha/beta producers in mice upon intravenous challenge with herpes simplex virus. **49**, 391-394 (1999).
32. Kumagai, Y. *et al.* Alveolar macrophages are the primary interferon- α producer in pulmonary infection with RNA viruses. **27**, 240-252 (2007).
33. Goritzka, M. *et al.* Alveolar macrophage-derived type I interferons orchestrate innate

- immunity to RSV through recruitment of antiviral monocytes. **212**, 699-714 (2015).
34. Amarante-Mendes, G.P. *et al.* Pattern recognition receptors and the host cell death molecular machinery. **9**, 2379 (2018).
 35. Chiffolleau, E.J.F.i.i. C-type lectin-like receptors as emerging orchestrators of sterile inflammation represent potential therapeutic targets. **9**, 227 (2018).
 36. Kawasaki, T. & Kawai, T.J.F.i.i. Toll-like receptor signaling pathways. **5**, 461 (2014).
 37. O'Neill, L.A. & Bowie, A.G.J.N.R.I. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. **7**, 353-364 (2007).
 38. Zheng, W. *et al.* Toll-like receptor-mediated innate immunity against herpesviridae infection: a current perspective on viral infection signaling pathways. **17**, 1-15 (2020).
 39. Franchi, L., Warner, N., Viani, K. & Nuñez, G.J.I.r. Function of Nod-like receptors in microbial recognition and host defense. **227**, 106-128 (2009).
 40. Saxena, M. & Yeretssian, G.J.F.i.i. NOD-like receptors: master regulators of inflammation and cancer. **5**, 327 (2014).
 41. Kim, Y.K., Shin, J.-S. & Nahm, M.H.J.Y.m.j. NOD-like receptors in infection, immunity, and diseases. **57**, 5-14 (2016).
 42. Zhang, L., Wang, C.-C.J.H. & International, P.D. Inflammatory response of macrophages in infection. **13**, 138-152 (2014).
 43. Loo, Y.-M. & Gale Jr, M.J.I. Immune signaling by RIG-I-like receptors. **34**, 680-692 (2011).
 44. Tan, X., Sun, L., Chen, J. & Chen, Z.J.J.A.r.o.m. Detection of microbial infections through innate immune sensing of nucleic acids. **72**, 447-478 (2018).
 45. Rehwinkel, J. & Gack, M.U.J.N.R.I. RIG-I-like receptors: their regulation and roles in RNA sensing. **20**, 537-551 (2020).
 46. Rodriguez, K.R., Bruns, A.M. & Horvath, C.M.J.J.o.v. MDA5 and LGP2: accomplices and antagonists of antiviral signal transduction. **88**, 8194-8200 (2014).
 47. Gringhuis, S.I. *et al.* C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF- κ B. **26**, 605-616 (2007).
 48. Rogers, N.C. *et al.* Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. **22**, 507-517 (2005).
 49. Osorio, F. & e Sousa, C.R.J.I. Myeloid C-type lectin receptors in pathogen recognition and host defense. **34**, 651-664 (2011).
 50. Geijtenbeek, T.B. & Gringhuis, S.I.J.N.R.I. Signalling through C-type lectin receptors: shaping immune responses. **9**, 465-479 (2009).
 51. Nakaya, Y., Lilue, J., Stavrou, S., Moran, E.A. & Ross, S.R.J.M. AIM2-like receptors positively and negatively regulate the interferon response induced by cytosolic DNA. **8**, e00944-00917 (2017).
 52. Briard, B., Place, D.E. & Kanneganti, T.-D.J.P. DNA sensing in the innate immune response. **35**, 112-124 (2020).
 53. Hornung, V. *et al.* AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. **458**, 514-518 (2009).
 54. Gray, E.E. *et al.* The AIM2-like receptors are dispensable for the interferon response to intracellular DNA. **45**, 255-266 (2016).
 55. Sun, L., Wu, J., Du, F., Chen, X. & Chen, Z.J.J.S. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. **339**, 786-791 (2013).
 56. Xiao, T.S. & Fitzgerald, K.A.J.M.c. The cGAS-STING pathway for DNA sensing. **51**, 135-139 (2013).
 57. Hayashi, T., Nakamura, T. & Takaoka, A.J.N.R.M.e.G.k.J.j.o.c.i. Pattern recognition receptors. **34**, 329-345 (2011).
 58. Chen, G., Shaw, M.H., Kim, Y.-G. & Nuñez, G.J.A.R.o.P.M.o.D. NOD-like receptors: role in innate immunity and inflammatory disease. **4**, 365-398 (2009).
 59. Dixit, E. & Kagan, J.C.J.A.i.i. Intracellular pathogen detection by RIG-I-like receptors. **117**, 99-125 (2013).
 60. Cambi, A. & Figdor, C.G.J.C.o.i.c.b. Dual function of C-type lectin-like receptors in the immune system. **15**, 539-546 (2003).
 61. Liu, T., Zhang, L., Joo, D., Sun, S.-C.J.S.t. & therapy, t. NF- κ B signaling in inflammation. **2**, 1-9 (2017).
 62. Arthur, J.S.C. & Ley, S.C.J.N.R.I. Mitogen-activated protein kinases in innate immunity. **13**, 679-692 (2013).
 63. Baldwin Jr, A.S.J.A.r.o.i. The NF- κ B and I κ B proteins: new discoveries and insights. **14**, 649-681 (1996).
 64. Wang, S., Liu, Z., Wang, L., Zhang, X.J.C. & immunology, m. NF- κ B signaling pathway, inflammation and colorectal cancer. **6**, 327-334 (2009).
 65. Napetschnig, J. & Wu, H.J.A.r.o.b. Molecular basis of NF- κ B signaling. **42**, 443-468 (2013).
 66. Israël, A.J.C.S.H.p.i.b. The IKK complex, a central regulator of NF- κ B activation. **2**, a000158 (2010).
 67. Karin, M. & Greten, F.R.J.N.r.i. NF- κ B: linking inflammation and immunity to cancer

- development and progression. **5**, 749-759 (2005).
68. Kawai, T. & Akira, S.J.T.i.m.m. Signaling to NF- κ B by Toll-like receptors. **13**, 460-469 (2007).
 69. Akira, S. & Takeda, K.J.N.r.i. Toll-like receptor signalling. **4**, 499-511 (2004).
 70. Adhikari, A., Xu, M. & Chen, Z.J.O. Ubiquitin-mediated activation of TAK1 and IKK. **26**, 3214-3226 (2007).
 71. Sun, S.-C.J.N.r.i. The non-canonical NF- κ B pathway in immunity and inflammation. **17**, 545-558 (2017).
 72. Schneider, W.M., Chevillotte, M.D. & Rice, C.M.J.A.r.o.i. Interferon-stimulated genes: a complex web of host defenses. **32**, 513-545 (2014).
 73. Isaacs, A.J.A.i.v.r. Interferon. **10**, 1-38 (1964).
 74. McNab, F., Mayer-Barber, K., Sher, A., Wack, A. & O'garra, A.J.N.R.I. Type I interferons in infectious disease. **15**, 87-103 (2015).
 75. Pestka, S., Krause, C.D. & Walter, M.R.J.I.r. Interferons, interferon-like cytokines, and their receptors. **202**, 8-32 (2004).
 76. Ivashkiv, L.B. & Donlin, L.T.J.N.R.I. Regulation of type I interferon responses. **14**, 36-49 (2014).
 77. Goubau, D., Deddouche, S. & e Sousa, C.R.J.I. Cytosolic sensing of viruses. **38**, 855-869 (2013).
 78. Honda, K., Takaoka, A. & Taniguchi, T.J.I. Type I inteferon gene induction by the interferon regulatory factor family of transcription factors. **25**, 349-360 (2006).
 79. Darnell, J.E., Kerr, I.M. & Stark, G.R.J.S. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. **264**, 1415-1421 (1994).
 80. Platanias, L.C.J.N.R.I. Mechanisms of type-I-and type-II-interferon-mediated signalling. **5**, 375-386 (2005).
 81. Stark, G.R. & Darnell Jr, J.E.J.I. The JAK-STAT pathway at twenty. **36**, 503-514 (2012).
 82. Larner, A. *et al.* Transcriptional induction of two genes in human cells by beta interferon. **81**, 6733-6737 (1984).
 83. De Veer, M.J. *et al.* Functional classification of interferon-stimulated genes identified using microarrays. **69**, 912-920 (2001).
 84. Schoggins, J.W. & Rice, C.M.J.C.o.i.v. Interferon-stimulated genes and their antiviral effector functions. **1**, 519-525 (2011).
 85. Schoggins, J.W.J.C.o.i.v. Interferon-stimulated genes: roles in viral pathogenesis. **6**, 40-46 (2014).
 86. Schoggins, J.W.J.A.r.o.v. Interferon-stimulated genes: what do they all do? **6**, 567-584 (2019).
 87. Haller, O., Kochs, G.J.J.o.l. & Research, C. Human MxA protein: an interferon-induced dynamin-like GTPase with broad antiviral activity. **31**, 79-87 (2011).
 88. Haller, O., Staeheli, P., Schwemmle, M. & Kochs, G.J.T.i.m. Mx GTPases: dynamin-like antiviral machines of innate immunity. **23**, 154-163 (2015).
 89. Huang, W.-C., Tung, S.-L., Chen, Y.-L., Chen, P.-M. & Chu, P.-Y.J.B.c. IFI44L is a novel tumor suppressor in human hepatocellular carcinoma affecting cancer stemness, metastasis, and drug resistance via regulating met/Src signaling pathway. **18**, 1-10 (2018).
 90. Busse, D. *et al.* Interferon-induced protein 44 and interferon-induced protein 44-like restrict replication of respiratory syncytial virus. **94**, e00297-00220 (2020).
 91. Hinson, E.R. & Cresswell, P.J.J.o.B.C. The N-terminal amphipathic α -helix of viperin mediates localization to the cytosolic face of the endoplasmic reticulum and inhibits protein secretion. **284**, 4705-4712 (2009).
 92. Wang, X., Hinson, E.R., Cresswell, P.J.C.h. & microbe. The interferon-inducible protein viperin inhibits influenza virus release by perturbing lipid rafts. **2**, 96-105 (2007).
 93. Nasr, N. *et al.* HIV-1 infection of human macrophages directly induces viperin which inhibits viral production. **120**, 778-788 (2012).
 94. Moradpour, D., Penin, F. & Rice, C.M.J.N.r.m. Replication of hepatitis C virus. **5**, 453-463 (2007).
 95. Helbig, K.J. *et al.* Viperin is induced following dengue virus type-2 (DENV-2) infection and has anti-viral actions requiring the C-terminal end of viperin. **7**, e2178 (2013).
 96. Roers, A., Hiller, B. & Hornung, V.J.I. Recognition of endogenous nucleic acids by the innate immune system. **44**, 739-754 (2016).
 97. Lester, S.N. & Li, K.J.J.o.m.b. Toll-like receptors in antiviral innate immunity. **426**, 1246-1264 (2014).
 98. Hemmi, H. *et al.* A Toll-like receptor recognizes bacterial DNA. **408**, 740-745 (2000).
 99. Krieg, A.M. *et al.* CpG motifs in bacterial DNA trigger direct B-cell activation. **374**, 546-549 (1995).
 100. Latz, E. *et al.* Ligand-induced conformational changes allosterically activate Toll-like receptor 9. **8**, 772-779 (2007).
 101. Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T. & Seya, T.J.N.i. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon- β induction. **4**, 161-167 (2003).
 102. Heil, F. *et al.* Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. **303**, 1526-1529 (2004).

103. Barr, J. & Fearn, R. Genetic instability of RNA viruses. *Genome Stability*. Elsevier, 2016, pp 21-35.
104. Pichlmair, A. *et al.* RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. **314**, 997-1001 (2006).
105. Kato, H. *et al.* Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. **205**, 1601-1610 (2008).
106. Okude, H., Ori, D. & Kawai, T.J.F.i.l. Signaling through nucleic acid sensors and their roles in inflammatory diseases. **11**, 3657 (2020).
107. Schlee, M. & Hartmann, G.J.N.R.I. Discriminating self from non-self in nucleic acid sensing. **16**, 566-580 (2016).
108. Hopfner, K.-P. & Hornung, V.J.N.r.M.c.b. Molecular mechanisms and cellular functions of cGAS–STING signalling. **21**, 501-521 (2020).
109. Kumar, V.J.F.i.l. The trinity of cGAS, TLR9, and ALRs guardians of the cellular galaxy against host-derived self-DNA. **11**, 3902 (2021).
110. Sagulenko, V. *et al.* AIM2 and NLRP3 inflammasomes activate both apoptotic and pyroptotic death pathways via ASC. **20**, 1149-1160 (2013).
111. Broz, P. & Dixit, V.M.J.N.R.I. Inflammasomes: mechanism of assembly, regulation and signalling. **16**, 407-420 (2016).
112. O'Neill, L.A.J.C. DNA makes RNA makes innate immunity. **138**, 428-430 (2009).
113. Yu, L., Liu, P.J.S.T. & Therapy, T. Cytosolic DNA sensing by cGAS: regulation, function, and human diseases. **6**, 1-15 (2021).
114. Gentili, M. *et al.* The N-terminal domain of cGAS determines preferential association with centromeric DNA and innate immune activation in the nucleus. **26**, 2377-2393. e2313 (2019).
115. Kranzusch, P.J., Lee, A.S.-Y., Berger, J.M. & Doudna, J.A.J.C.r. Structure of human cGAS reveals a conserved family of second-messenger enzymes in innate immunity. **3**, 1362-1368 (2013).
116. Li, X. *et al.* Cyclic GMP-AMP synthase is activated by double-stranded DNA-induced oligomerization. **39**, 1019-1031 (2013).
117. Zhang, X. *et al.* The cytosolic DNA sensor cGAS forms an oligomeric complex with DNA and undergoes switch-like conformational changes in the activation loop. **6**, 421-430 (2014).
118. Andreeva, L. *et al.* cGAS senses long and HMGB/TFAM-bound U-turn DNA by forming protein–DNA ladders. **549**, 394-398 (2017).
119. Ablasser, A. *et al.* cGAS produces a 2'-5'-linked cyclic dinucleotide second messenger that activates STING. **498**, 380-384 (2013).
120. Diner, E.J. *et al.* The innate immune DNA sensor cGAS produces a noncanonical cyclic dinucleotide that activates human STING. **3**, 1355-1361 (2013).
121. Du, M. & Chen, Z.J.J.S. DNA-induced liquid phase condensation of cGAS activates innate immune signaling. **361**, 704-709 (2018).
122. Xie, W. *et al.* Human cGAS catalytic domain has an additional DNA-binding interface that enhances enzymatic activity and liquid-phase condensation. **116**, 11946-11955 (2019).
123. Cai, X., Chiu, Y.-H. & Chen, Z.J.J.M.c. The cGAS–cGAMP–STING pathway of cytosolic DNA sensing and signaling. **54**, 289-296 (2014).
124. Su, Y.-C. *et al.* Crystallization studies of the murine c-di-GMP sensor protein STING. **68**, 906-910 (2012).
125. Burdette, D.L. *et al.* STING is a direct innate immune sensor of cyclic di-GMP. **478**, 515-518 (2011).
126. Burdette, D.L. & Vance, R.E.J.N.i. STING and the innate immune response to nucleic acids in the cytosol. **14**, 19-26 (2013).
127. Tao, J., Zhou, X. & Jiang, Z.J.I.l. cGAS–cGAMP–STING: The three musketeers of cytosolic DNA sensing and signaling. **68**, 858-870 (2016).
128. Zhang, X. *et al.* Cyclic GMP-AMP containing mixed phosphodiester linkages is an endogenous high-affinity ligand for STING. **51**, 226-235 (2013).
129. Shang, G. *et al.* Crystal structures of STING protein reveal basis for recognition of cyclic di-GMP. **19**, 725-727 (2012).
130. Luo, W.-W. *et al.* iRhom2 is essential for innate immunity to DNA viruses by mediating trafficking and stability of the adaptor STING. **17**, 1057-1066 (2016).
131. Dobbs, N. *et al.* STING activation by translocation from the ER is associated with infection and autoinflammatory disease. **18**, 157-168 (2015).
132. Gui, X. *et al.* Autophagy induction via STING trafficking is a primordial function of the cGAS pathway. **567**, 262-266 (2019).
133. Ishikawa, H., Ma, Z. & Barber, G.N.J.N. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. **461**, 788-792 (2009).
134. Balka, K.R. *et al.* TBK1 and IKKε act redundantly to mediate STING-induced NF-κB responses in myeloid cells. **31**, 107492 (2020).
135. Abe, T. & Barber, G.N.J.J.o.v. Cytosolic-DNA-mediated, STING-dependent proinflammatory gene induction necessitates canonical NF-κB

- activation through TBK1. **88**, 5328-5341 (2014).
136. Konno, H., Konno, K. & Barber, G.N.J.C. Cyclic dinucleotides trigger ULK1 (ATG1) phosphorylation of STING to prevent sustained innate immune signaling. **155**, 688-698 (2013).
 137. Motwani, M., Pesiridis, S. & Fitzgerald, K.A.J.N.R.G. DNA sensing by the cGAS–STING pathway in health and disease. **20**, 657-674 (2019).
 138. Ahn, J. & Barber, G.N.J.C.o.i.i. Self-DNA, STING-dependent signaling and the origins of autoinflammatory disease. **31**, 121-126 (2014).
 139. Boyer, J.A. *et al.* Structural basis of nucleosome-dependent cGAS inhibition. **370**, 450-454 (2020).
 140. Cao, D., Han, X., Fan, X., Xu, R.-M. & Zhang, X.J.C.R. Structural basis for nucleosome-mediated inhibition of cGAS activity. **30**, 1088-1097 (2020).
 141. Kujirai, T. *et al.* Structural basis for the inhibition of cGAS by nucleosomes. **370**, 455-458 (2020).
 142. Michalski, S. *et al.* Structural basis for sequestration and autoinhibition of cGAS by chromatin. **587**, 678-682 (2020).
 143. Pathare, G.R. *et al.* Structural mechanism of cGAS inhibition by the nucleosome. **587**, 668-672 (2020).
 144. Zhao, B. *et al.* The molecular basis of tight nuclear tethering and inactivation of cGAS. **587**, 673-677 (2020).
 145. Chen, M. *et al.* TRIM14 inhibits cGAS degradation mediated by selective autophagy receptor p62 to promote innate immune responses. **64**, 105-119 (2016).
 146. Dai, J. *et al.* Acetylation blocks cGAS activity and inhibits self-DNA-induced autoimmunity. **176**, 1447-1460. e1414 (2019).
 147. Seo, G.J. *et al.* Akt kinase-mediated checkpoint of cGAS DNA sensing pathway. **13**, 440-449 (2015).
 148. Xia, P. *et al.* Glutamylation of the DNA sensor cGAS regulates its binding and synthase activity in antiviral immunity. **17**, 369-378 (2016).
 149. Rongvaux, A. *et al.* Apoptotic caspases prevent the induction of type I interferons by mitochondrial DNA. **159**, 1563-1577 (2014).
 150. Banerjee, I. *et al.* Gasdermin D restrains type I interferon response to cytosolic DNA by disrupting ionic homeostasis. **49**, 413-426. e415 (2018).
 151. Kato, K. *et al.* Structural insights into cGAMP degradation by Ecto-nucleotide pyrophosphatase phosphodiesterase 1. **9**, 1-8 (2018).
 152. Gonugunta, V.K. *et al.* Trafficking-mediated STING degradation requires sorting to acidified endolysosomes and can be targeted to enhance anti-tumor response. **21**, 3234-3242 (2017).
 153. Prabakaran, T. *et al.* Attenuation of cGAS-STING signaling is mediated by a p62/SQSTM1-dependent autophagy pathway activated by TBK1. **37**, e97858 (2018).
 154. Zhang, L. *et al.* NLRC3, a member of the NLR family of proteins, is a negative regulator of innate immune signaling induced by the DNA sensor STING. **40**, 329-341 (2014).
 155. Guo, H. *et al.* NLRX1 sequesters STING to negatively regulate the interferon response, thereby facilitating the replication of HIV-1 and DNA viruses. **19**, 515-528 (2016).
 156. Ma, F. *et al.* Positive feedback regulation of type I interferon by the interferon-stimulated gene STING. **16**, 202-212 (2015).
 157. Schneider, C., King, R.M. & Philipson, L.J.C. Genes specifically expressed at growth arrest of mammalian cells. **54**, 787-793 (1988).
 158. Brenner, D.G., Lin-Chao, S. & Cohen, S.N.J.P.o.t.N.A.o.S. Analysis of mammalian cell genetic regulation in situ by using retrovirus-derived "portable exons" carrying the *Escherichia coli lacZ* gene. **86**, 5517-5521 (1989).
 159. Chao, C.C.-K., Hung, F.-C. & Chao, J.J.J.S.c.i. Gas7 is required for mesenchymal stem cell-derived bone development. **2013** (2013).
 160. Su, W.B. *et al.* Gas7. *Madame Curie Bioscience Database [Internet]*. Landes Bioscience, 2013.
 161. Chao, C.C.K., Chang, P.Y. & Lu, H.H.P.J.J.o.n.r. Human Gas7 isoforms homologous to mouse transcripts differentially induce neurite outgrowth. **81**, 153-162 (2005).
 162. Chang, P.Y., Kuo, J.T., Lin-Chao, S. & Chao, C.C.K.J.J.o.n.r. Identification of rat Gas7 isoforms differentially expressed in brain and regulated following kainate-induced neuronal injury. **79**, 788-797 (2005).
 163. Hanawa-Suetsugu, K. *et al.* Phagocytosis is mediated by two-dimensional assemblies of the F-BAR protein GAS7. **10**, 1-13 (2019).
 164. Nie, Y. *et al.* Solution NMR structure and ligand identification of human Gas7 SH3 domain reveal a typical SH3 fold but a non-canonical ligand-binding mode. **516**, 1190-1195 (2019).
 165. Akiyama, H. *et al.* A Novel Role for hGas7b in Microtubular Maintenance. **284**, 32695-32699 (2009).
 166. She, B.-R., Liou, G.-G. & Lin-Chao, S.J.E.c.r. Association of the growth-arrest-specific protein Gas7 with F-actin induces reorganization of microfilaments and

- promotes membrane outgrowth. **273**, 34-44 (2002).
167. Gotoh, A., Hidaka, M., Hirose, K. & Uchida, T.J.J.o.B.C. Gas7b (growth arrest specific protein 7b) regulates neuronal cell morphology by enhancing microtubule and actin filament assembly. **288**, 34699-34706 (2013).
 168. Hidaka, M. *et al.* Alzheimer's disease-related protein hGas7b interferes with kinesin motility. **151**, 593-598 (2012).
 169. Thul, P.J. *et al.* A subcellular map of the human proteome. **356** (2017).
 170. You, J.-J. & Lin-Chao, S.J.J.o.B.C. Gas7 functions with N-WASP to regulate the neurite outgrowth of hippocampal neurons. **285**, 11652-11666 (2010).
 171. Tseng, R.-C. *et al.* Growth-arrest-specific 7C protein inhibits tumor metastasis via the N-WASP/FAK/F-actin and hnRNP U/ β -TrCP/ β -catenin pathways in lung cancer. **6**, 44207 (2015).
 172. Chang, J.-W. *et al.* Wild-type p53 upregulates an early onset breast cancer-associated gene GAS7 to suppress metastasis via GAS7–CYFIP1-mediated signaling pathway. **37**, 4137-4150 (2018).
 173. Nanda, S., Bhattacharjee, S., Cox, D.N. & Ascoli, G.A.J.A.a.S. Distinct roles of microtubules and actin filaments in defining dendritic architecture. (2019).
 174. Alberts, B. *et al.* The self-assembly and dynamic structure of cytoskeletal filaments. *Molecular Biology of the Cell. 4th edition.* Garland Science, 2002.
 175. Fawcett, J. Spinal Cord Regeneration and Functional Recovery: Strategies. (2009).
 176. Hung, F.C. & Chao, C.C.K.J.J.o.n.r. Knockdown of growth-arrest-specific gene 7b (gas7b) using short-hairpin RNA desensitizes neuroblastoma cells to cisplatin: Implications for preventing apoptosis of neurons. **88**, 3578-3587 (2010).
 177. Zhang, Z. *et al.* Growth arrest specific gene 7 is associated with schizophrenia and regulates neuronal migration and morphogenesis. **9**, 1-13 (2016).
 178. Shiga, Y. *et al.* Genetic analysis of Japanese primary open-angle glaucoma patients and clinical characterization of risk alleles near CDKN2B-AS1, SIX6 and GAS7. **12**, e0186678 (2017).
 179. Xu, J. *et al.* Association of polymorphism rs11656696 in GAS7 with primary open-Angle Glaucoma in a Chinese Population. **40**, 237-241 (2019).
 180. Qin, S. & Wu, Q. Systemic chemotherapy with oxaliplatin is a good option for advanced hepatocellular carcinoma. **2**, 203-207 (2015).
 181. Li, D., Zhang, B. & Hu, C.J.A.b.e.b.S. Oxaliplatin inhibits proliferation and migration of human hepatocellular carcinoma cells via GAS7C and the N-WASP/FAK/F-actin pathway. **49**, 581-587 (2017).
 182. Dong, Z. *et al.* GAS7 deficiency promotes metastasis in MYCN-driven neuroblastoma. **81**, 2995-3007 (2021).
 183. Mosser, D.M. & Edwards, J.P.J.N.r.i. Exploring the full spectrum of macrophage activation. **8**, 958-969 (2008).
 184. Xu, Q. *et al.* Growth arrest-specific protein 7 regulates the murine M1 alveolar macrophage polarization. **65**, 1065-1073 (2017).
 185. Aquaro, S. *et al.* Macrophages and HIV infection: therapeutical approaches toward this strategic virus reservoir. **55**, 209-225 (2002).
 186. Koppensteiner, H., Brack-Werner, R. & Schindler, M.J.R. Macrophages and their relevance in Human Immunodeficiency Virus Type I infection. **9**, 1-11 (2012).
 187. Popov, S., Popova, E., Inoue, M., Wu, Y. & Göttinger, H.J.P.o.t.N.A.o.S. HIV-1 gag recruits PACSIN2 to promote virus spreading. **115**, 7093-7098 (2018).
 188. McNally, A.K., Anderson, J.M.J.C.F.i.H. & Disease. Macrophage fusion and multinucleated giant cells of inflammation. 97-111 (2011).
 189. Ménager, M.M. & Littman, D.R.J.C. Actin dynamics regulates dendritic cell-mediated transfer of HIV-1 to T cells. **164**, 695-709 (2016).
 190. Waki, K. & Freed, E.O.J.V. Macrophages and cell-cell spread of HIV-1. **2**, 1603-1620 (2010).
 191. Liu, Y. *et al.* PSGL-1 inhibits HIV-1 infection by restricting actin dynamics and sequestering HIV envelope proteins. **6**, 1-15 (2020).
 192. Gao, D. *et al.* Cyclic GMP-AMP synthase is an innate immune sensor of HIV and other retroviruses. **341**, 903-906 (2013).
 193. Hung, F.C., Chang, Y., Lin-Chao, S. & Chao, C.C.K.J.J.o.O.R. Gas7 mediates the differentiation of human bone marrow-derived mesenchymal stem cells into functional osteoblasts by enhancing Runx2-dependent gene expression. **29**, 1528-1535 (2011).
 194. Lin, Y.-C. *et al.* Genome dynamics of the human embryonic kidney 293 lineage in response to cell biology manipulations. **5**, 1-12 (2014).
 195. Aldrich, M.K.-S. 293T cell line human. 2021 [cited 2021 06/08/2021] Available from: https://www.sigmaaldrich.com/FR/fr/product/sigma/cb_12022001
 196. Puigdomènech, I., Casartelli, N., Porrot, F. & Schwartz, O.J.J.o.v. SAMHD1 restricts HIV-1

- cell-to-cell transmission and limits immune detection in monocyte-derived dendritic cells. **87**, 2846-2856 (2013).
197. Li, D. *et al.* Vpx mediated degradation of SAMHD1 has only a very limited effect on lentiviral transduction rate in ex vivo cultured HSPCs. **15**, 271-280 (2015).
 198. Bosshart, H. & Heinzelmann, M.J.A.o.t.m. THP-1 cells as a model for human monocytes. **4** (2016).
 199. Goujon, C. *et al.* With a little help from a friend: increasing HIV transduction of monocyte-derived dendritic cells with virion-like particles of SIV MAC. **13**, 991-994 (2006).
 200. Desai, P. & Person, S.J.J.o.v. Incorporation of the green fluorescent protein into the herpes simplex virus type 1 capsid. **72**, 7563-7568 (1998).
 201. Dunphy, G. *et al.* Non-canonical activation of the DNA sensing adaptor STING by ATM and IFI16 mediates NF- κ B signaling after nuclear DNA damage. **71**, 745-760. e745 (2018).
 202. Reinert, L.S. *et al.* Sensing of HSV-1 by the cGAS-STING pathway in microglia orchestrates antiviral defence in the CNS. **7**, 1-12 (2016).
 203. Christensen, M.H. *et al.* HSV-1 ICP 27 targets the TBK 1-activated STING signalsome to inhibit virus-induced type I IFN expression. **35**, 1385-1399 (2016).
 204. Su, C. & Zheng, C.J.J.o.v. Herpes simplex virus 1 abrogates the cGAS/STING-mediated cytosolic DNA-sensing pathway via its virion host shutoff protein, UL41. **91**, e02414-02416 (2017).
 205. Zhong, B. *et al.* The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. **29**, 538-550 (2008).
 206. Castanier, C., Garcin, D., Vazquez, A. & Arnoult, D.J.E.r. Mitochondrial dynamics regulate the RIG-I-like receptor antiviral pathway. **11**, 133-138 (2010).
 207. Ishikawa, H. & Barber, G.N.J.N. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. **455**, 674-678 (2008).
 208. Sun, W. *et al.* ERIS, an endoplasmic reticulum IFN stimulator, activates innate immune signaling through dimerization. **106**, 8653-8658 (2009).
 209. Nazmi, A., Mukhopadhyay, R., Dutta, K. & Basu, A.J.S.r. STING mediates neuronal innate immune response following Japanese encephalitis virus infection. **2**, 1-10 (2012).
 210. Ni, G., Ma, Z. & Damania, B.J.P.p. cGAS and STING: at the intersection of DNA and RNA virus-sensing networks. **14**, e1007148 (2018).
 211. Chandra, S., Kalaivani, R., Kumar, M., Srinivasan, N. & Sarkar, D.P.J.M.b.o.t.c. Sendai virus recruits cellular villin to remodel actin cytoskeleton during fusion with hepatocytes. **28**, 3801-3814 (2017).
 212. Hilleman, M.R.J.P.o.t.N.A.o.S. Strategies and mechanisms for host and pathogen survival in acute and persistent viral infections. **101**, 14560-14566 (2004).
 213. Cheradame, L. *et al.* STING Promotes Breast Cancer Cell Survival by an Inflammatory-Independent Nuclear Pathway Enhancing the DNA Damage Response. (2020).
 214. Dixon, C.R. *et al.* STING Nuclear Partners Contribute to Innate Immune Signalling Responses. (2020).
 215. Tatarova, Z., Brabek, J., Rösel, D. & Novotný, M.J.P.o. SH3 domain tyrosine phosphorylation-sites, role and evolution. **7**, e36310 (2012).
 216. Archer, K.A., Durack, J. & Portnoy, D.A.J.P.p. STING-dependent type I IFN production inhibits cell-mediated immunity to *Listeria monocytogenes*. **10**, e1003861 (2014).
 217. Hansen, K. *et al.* *Listeria monocytogenes* induces IFN β expression through an IFI16-, cGAS-and STING-dependent pathway. **33**, 1654-1666 (2014).
 218. Kwon, J. & Bakhoum, S.F.J.C.d. The cytosolic DNA-sensing cGAS-STING pathway in cancer. **10**, 26-39 (2020).
 219. Cheng, Z. *et al.* The interactions between cGAS-STING pathway and pathogens. **5**, 1-15 (2020).

SUPPLEMENTARY DATA

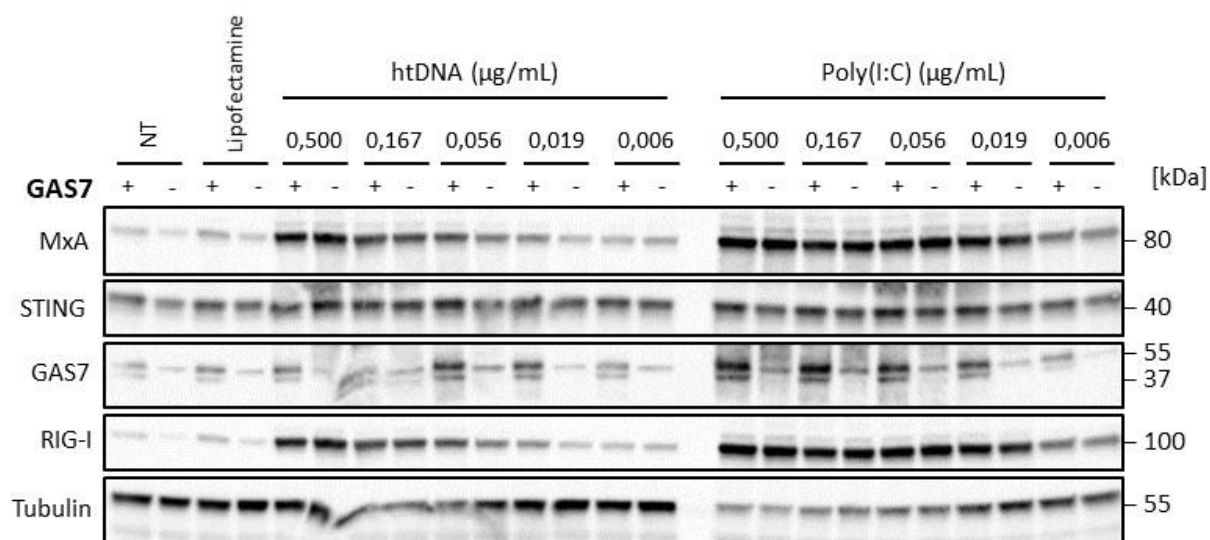


Figure S.1. Western Blotting data from htDNA and Poly(I:C) stimulation. MDMs transduced with a control or GAS7 shRNA were treated with htDNA and Poly(I:C) at the indicated concentrations. Cells were lysed after 24h for quantification of the indicated proteins by western blot. This figure represents the other replicate this experiment, aside from the one shown in Figure IV. 1A. NT, non-treated.

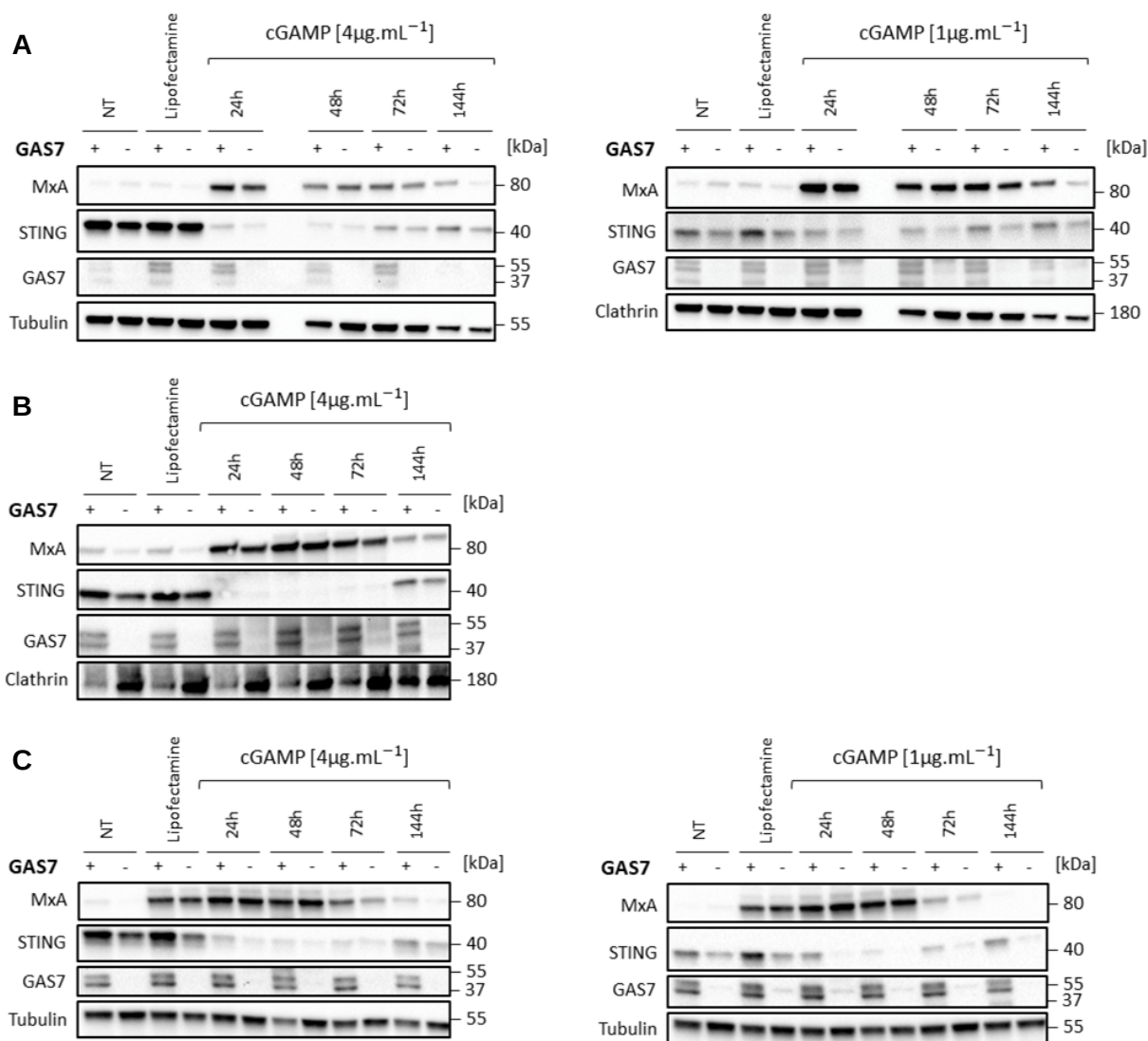


Figure S.2. Western Blotting data from 2'3'-cGAMP stimulation. MDMs transduced with a control or GAS7 shRNA were stimulated with 2'3'-cGAMP (4 $\mu\text{g.mL}^{-1}$ or 1 $\mu\text{g.mL}^{-1}$) at the indicated times. Cells were lysed for quantification of the indicated proteins by western blot. A, B and C represent the three replicates in 2'3'-cGAMP stimulation experiments, aside from the one shown in Figure IV. 2A and D. NT, non-treated.

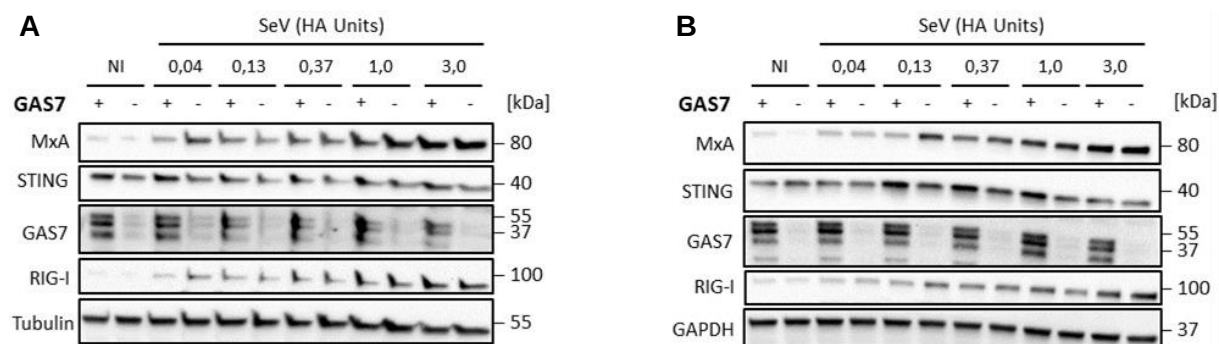


Figure S.3. Western Blotting data from SeV infection. MDMs transduced with a control or GAS7 shRNA were infected with SeV at indicated HA Units. Cells were lysed for quantification of the indicated proteins by western blot. A and B represent the two replicates in SeV infection experiments, aside from the one shown in Figure IV. 3A. NI, non-infected.

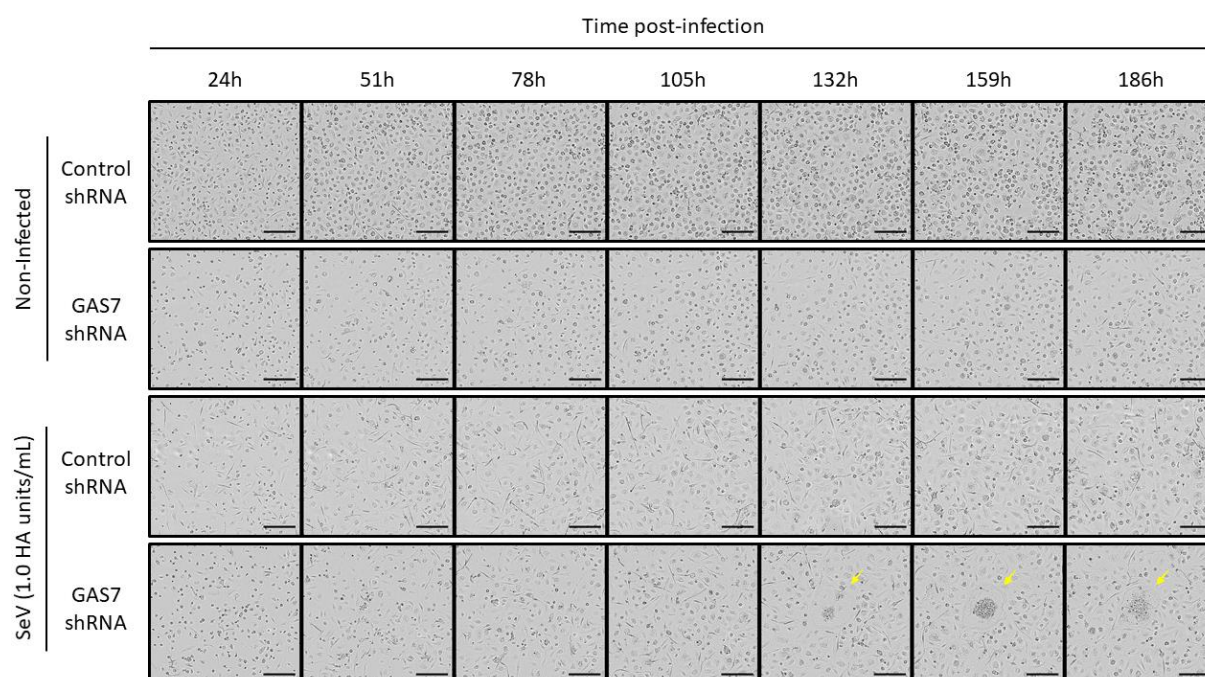


Figure S.4. Live-cell imaging of SeV infected MDMS. MDMs transduced with a control or GAS7 shRNA were infected with SeV at 1,0 HA Units.mL⁻¹. Cells were placed in the Incucyte live cell imaging system and pictures were acquired at the indicated time-points. Scale bar = 200 μ m. This figure represents the replicate in live-cell imaging experiments, aside from the one shown in Figure IV. 5E. NI, non-infected.