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Mechanisms of gasdermin pore formation in response to viral sensing in human respiratory epithelial cells

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the degree of Doctor of Philosophy

Coralie GUY

Supervisor: Professor Andrew G. Bowie

School of Biochemistry and Immunology
Trinity College Dublin
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Abstract

A key component of the innate immune response to infection or cellular stress is the activation of inflammasomes which leads to gasdermin (GSDM) pore formation, interleukin-1 β (IL-1 β) release and lytic cell death. GSDMs are a family of pore-forming proteins with six members namely GSDMA, GSDMD, GSDMC, GSDME and Pejvakin. Most inflammasome and GSDM studies have been performed in myeloid cells where the NLRP3 inflammasome is often activated after viral infection leading to formation of GSDMD pores. Respiratory epithelial cells are an essential line of defence to initiate a robust immune response against viral pathogens but the mechanism of GSDM activation in epithelial cells upon sensing of double-stranded RNA (dsRNA), a key viral pathogen-associated molecular pattern, and of viral infection, remains unclear. Therefore the aim of this study was to investigate the mechanism of inflammasome activation and GSDM pore formation in response to viruses and dsRNA in epithelial cells. Our results demonstrated that undifferentiated primary normal human bronchial epithelial (NHBE) cells are a better model to study inflammasome and GSDM activation compared to commonly used immortalised and cancerous cell lines such as BEAS-2B and A549 cells. Transfection of poly(I:C), a mimic of viral dsRNA, both triggered caspase-1-dependent GSDMD cleavage into a pore-forming fragment but also led to caspase-3, -8, -9-mediated GSDMD inactivation and GSDME pore formation. Further investigation using pharmacological inhibitors and siRNA technology showed that NLRP1 inflammasome activation, but not NLRP3, resulted in GSDMD pore formation, whereas PKR activation led to GSDMD inactivation and GSDME activation. Additionally, TRIF, the adaptor for the dsRNA receptor TLR3, contributed to early GSDME cleavage, lytic cell death and cytokine secretion following dsRNA delivery. In contrast MAVS, the adaptor for the viral RNA sensors RIG-I and MDA5, wasn't involved in GSDM processing. While GSDME triggered lytic cell death, both GSDME and GSDMD pores were required for robust IL-1 β secretion. Preliminary results indicated that dsRNA sensing also triggered GSDMA pore formation at the plasma membrane. Importantly, NLRP1 and PKR were also involved in upregulation of pro-IL-1 β , RIG-I and MDA5 expression in dsRNA-stimulated NHBE cells. Infection of NHBE cells with influenza A virus (IAV) mainly led to GSDME pore formation and GSDMD inactivation in a PKR-caspase-3-dependent but inflammasome- and MAVS-independent manner. Interestingly, caspase-3 and caspase-8, but not caspase-1 were required for IAV-mediated lytic cell death and IL-1 β secretion. Suppression of GSDMD and GSDME expression using siRNA led to enhanced IAV replication suggesting that GSDM pore formation in response

to IAV is intrinsically antiviral in basal respiratory epithelial cells. Preliminary results with respiratory syncytial virus (RSV) suggested that PKR might also contribute to caspase-3-mediated GSDME pore formation during RSV infection. Overall different pathways lead to GSDM cleavage during viral sensing in NHBE cells. While intracellular dsRNA triggers NLRP1 inflammasome-dependent GSDMD pore formation, both dsRNA sensing and IAV infection activate PKR that mediates caspase-3-dependent GSDMD inactivation and GSDME activation.

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Table of abbreviations

AIM2:	Absent in melanoma 2
AP-1:	Activator protein 1
APS:	Amonium persulfate
ASC:	Apoptosis-associated speck-like protein containing a CARD
BEBM:	Bronchial epithelial cell growth basal medium
BIR:	Baculovirus inhibitor of apoptosis protein repeat
BMDC:	Bone marrow-derived dendritic cell
BMDM:	Bone marrow-derived macrophage
BSA:	Bovine albumin fraction
CARD:	Caspase activation and recruitment domain
cGAMP:	Cyclic guanosine monophosphate-adenosine monophosphate
cGAS:	Cyclic GMP-AMP synthase
DAI:	DNA-dependent activator of IFN-regulatory factors
DAMP:	Damage-associated molecular pattern
DDX41:	DEAD-Box Helicase 41
DFNA5:	Deafness autosomal dominant 5
DMEM:	Dulbecco's Modified Eagle's Medium
DMSO:	Dimethyl sulfoxide
DPP8/9:	Dipeptidyl peptidase 8 and 9
dsDNA:	Double-stranded DNA
dsRNA:	Double-stranded RNA
DSS:	Disuccinimidyl suberate
dTGN:	Dispersed <i>trans</i> -Golgi network
dTHP-1:	Differentiated THP-1 cell
DTT:	Dithiothreitol
ELISA:	Enzyme-linked immunosorbent assay
ESCRT-III:	Endosomal sorting complexes required for transport-III
FADD:	Fas-associated protein with death domain

FCS: Fetal calf serum

FIIND: Function to find domain

GSDM: Gasdermin

GSDMA-E: Gasdermin A-E

HIN: Hematopoietic interferon-inducible nuclear

HMW: High molecular weight

hNLRP1: Human NLRP1

hpi: h post infection

HpIC: High molecular weight poly(I:C)

hpRNA: 5'-triphosphate hairpin RNA

IAV: Influenza A virus

IFI16: IFN inducible protein 16

IFN: Interferon

IFNAR: Interferon- α/β receptor

IKK: Inhibitor of nuclear factor- κ B (I κ B) kinase

IL: Interleukin

IL-1R: IL-1 receptor

IMDM: Iscove's Modified Dulbecco's Medium

IP-10: Interferon gamma-induced protein 10

iPSC: induced pluripotent stem cells

IRF: Interferon regulatory factor

ISG: Interferon stimulated genes

ISGF3: IFN-stimulated factor 3

ISRE: IFN-stimulated response element

JAK1: Janus kinase 1

KSHV: Kaposi's sarcoma-associated herpesvirus

LDH: Lactate dehydrogenase

LGP2: Laboratory of Genetics and Physiology 2

LpIC: Low molecular weight poly(I:C)

LPS: Lipopolysaccharide

LRR: Leucine rich repeat
 LTA: Lipoteichoic acid
 LMW: Low molecular weight
 MAL: MyD88-adaptor-like
 MAVS: Mitochondrial antiviral-signaling protein
 MD-2: Myeloid differentiation factor 2
 MDA5: Melanoma differentiation associated gene 5
 MDCK: Madin-Darby canine kidney
 MEM: Minimum Essential Medium
 MERS-CoV: Middle East respiratory syndrome coronavirus
 mNLRP1B: Mouse NLRP1
 MOI: Multiplicity of infection
 mtDNA: Mitochondrial DNA
 MyD88: Myeloid differentiation primary response protein 88
 NACHT: NAIP, CIITA, HET-E, and TP-1
 NEK7: Never in mitosis gene a-related kinase 7
 NF- κ B: Nuclear factor kappa B
 NHBE: Normal human bronchial epithelial cell
 NINJ1: Ninjurin 1
 NLR: NOD-like receptor
 NLRA: NLR family, acidic domain containing
 NLRB: NLR family, BIR domain containing
 NLRC: NLR family, CARD domain containing
 NLRP: NLR family, PYD domain containing
 NLRX: NLR family, mitochondrial-addressing sequence containing
 NOD: Nucleotide-binding oligomerisation domain
 NP: Nucleoprotein
 NS: Nonstructural protein
 ORF45: Open reading frame 45
 PACT: PKR activating protein

PAMP: Pathogen-associated molecular patterns

PBS: Phosphate buffered saline

PKR: Protein kinase R

PMA: Phorbol 12-myristate 13-acetate

PMSF: Phenylmethanesulfonyl fluoride

Poly(dA:dT): Poly(deoxyadenylic-deoxythymidylic) acid sodium salt

Poly(I:C): Polyinosine-polycytidylic acid

PRR: Pattern recognition receptor

PYD: Pyrin

qPCR: Real-time quantitative PCR

qRT-PCR: Quantitative real-time polymerase chain reaction

RIG-I: Retinoic acid-inducible gene I

RIP1: Receptor-interacting protein 1

RLR: RIG-I-Like Receptor

RPMI: Roswell Park Memorial Institute

ROS: Reactive oxygen species

RSV: Respiratory syncytial virus

SARM: Sterile α - and armadillo-motif-containing protein

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2

SD: Standard deviation

SDS: Sodium dodecylsulfate

SDS-PAGE: SDS-polyacrilamide gel electrophoresis

SEM: Standard error of the mean

SFM: Serum free medium

ssRNA: Single stranded RNA

STAT: Signal transducer and activator of transcription

STING: Stimulator of interferon genes

TAK1: Transforming growth factor beta-activated kinase 1

TBK1: TANK-binding Kinase 1

TBS: Tris-buffered saline

TCID₅₀: 50 % tissue culture infective dose

TEMED: N,N,N',N'-Tetramethylethylenediamine

TGN: *Trans*-Golgi network

Th: T helper

TIR: Toll/interleukin-1 receptor

TLR: Toll like receptor

TNF: Tumor necrosis factor

TRAF: TNF receptor-associated factor

TRAM: TRIF-related adaptor molecule

TRIF: TIR-domain containing adaptor inducing interferon (IFN)- β

TRIM25: Tripartite motif containing 25

TYK2: Tyrosine kinase 2

VBP: Val-boroPro

vRNP: viral ribonucleoprotein

Chapter 1. Introduction

1.1 Biology of respiratory virus infection

Respiratory virus infections are a major burden to human health and the economy. Before the COVID-19 pandemic, influenza virus and respiratory syncytial virus (RSV) were the main cause of hospitalisations and deaths during seasonal epidemics [1]. Nevertheless many respiratory viruses contribute to annual infections such as rhinovirus, adenovirus, parainfluenza virus and coronavirus. Respiratory viruses that have animal reservoirs, including influenza virus and coronavirus, are a constant threat to human health since they can be the origin of pandemics. The recent and ongoing COVID-19 pandemic illustrates the need to understand how respiratory viruses trigger lung inflammation in infected individuals. In this study, we focus on influenza A virus (IAV) and RSV.

1.1.1 Influenza A virus

1.1.1.1 Epidemiology, transmission and pathogenesis

Influenza virus infection causes mild respiratory disease of the upper respiratory tract in most of the human population but leads to severe symptoms in very young, elderly and immunocompromised individuals. According to World Health Organisation estimation, influenza epidemics cause one billion infections, three to five million cases of severe illness and 290,000 to 650,000 deaths each year. Four types of influenza virus have been isolated, namely A, B, C and D. While influenza A and B viruses cause annual seasonal epidemics, influenza C virus only induces asymptomatic or very mild infections in human and influenza D virus infects cattle. IAV is the only influenza virus that can provoke flu pandemics because, unlike influenza B virus that only circulates in humans, IAV is also detected in wild birds, domestic ducks, poultry and pigs. The appearance of swine or avian IAV viruses in human population had indeed engendered four pandemics since 1918 [2].

Influenza viruses are transmitted by the respiratory route in the human population through aerosols, droplets or self-inoculation after touching a contaminated surface. Low humidity and low temperatures favour transmission, explaining why epidemics usually happen during winter months. IAV can also be transmitted from infected animal to human through aerosols, contaminated water or fomites. Influenza virus preferentially targets respiratory epithelial cells of the upper respiratory tract. Severe influenza cases are associated with viral replication in the lower respiratory tract which can cause viral pneumonia, severe inflammation due to a cytokine storm, or secondary bacterial infection [2, 3].

1.1.1.2 Genome structure and life cycle

IAV belongs to the *Orthomyxoviridae* family and to the *Influenza A* genus. IAV is an enveloped virus containing eight single-stranded, negative-sense RNAs segments [Figure 1.1]. IAV segmented genome encodes for hemagglutinin (HA) and neuraminidase (NA) glycoproteins and membrane protein (M2) that are present on the viral envelope, but also for viral RNA-dependent RNA polymerase, composed of three subunits PB1, PB2 and PA; viral nucleoprotein (NP), matrix protein (M1), nuclear export protein, nonstructural (NS) protein NS1, PB1-F2 and PA-X. RNA genome segments are coated with viral nucleoprotein and associate with viral polymerase to form viral ribonucleoproteins (vRNPs) [3, 4].

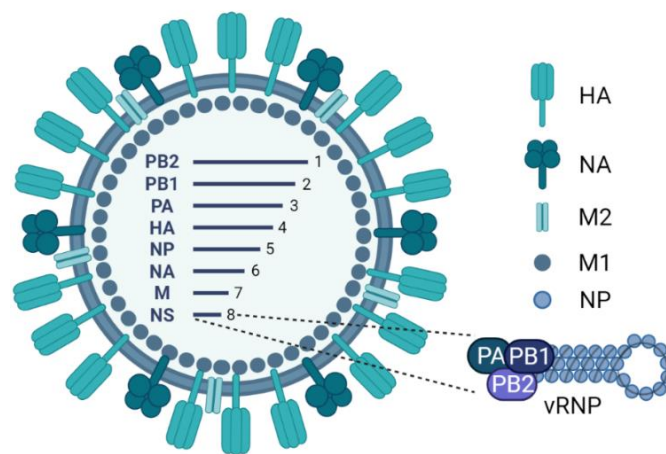


Figure 1.1: Influenza A virion and genome.

IAV virion contains eight segmented RNAs per genome. Segments 1, 2 and 3 encode for PB2, PB1 and PA which are the three subunits of the viral RNA-dependent RNA polymerase. Segments 2 and 3 also encode for PB1-F2 and PA-X respectively. Segments 4 and 6 encode for hemagglutinin (HA) and neuraminidase (NA) glycoproteins. Segment 5 encodes the nucleoprotein (NP). Segment 7 encodes for the matrix protein (M1) and membrane protein (M2) and segment 8 encodes for the nonstructural protein NS1 and nuclear export protein. Figure created with BioRender.

Human IAV targets epithelial cells of the human respiratory tract to replicate. Viral hemagglutinin protein preferentially binds to α 2,6-linked sialic acids, a plasma membrane glycan, which leads to endosomal uptake. Acidification of the endosome induces a conformational change in the hemagglutinin that triggers the release of the eight viral vRNPs of the segmented genome into the cytoplasm, through the fusion between the viral envelope and the endosomal membrane. vRNPs are then imported into the nucleus in order to be

transcribed and replicated by the viral RNA-dependent RNA polymerase. Newly transcribed viral mRNAs are exported into the cytoplasm to be translated into viral proteins. Structural proteins traffic to the plasma membrane and insert into the host membrane to create virion assembly sites. To be fully functional, hemagglutinin requires to be cleaved by cellular proteases present in respiratory epithelial cells. In addition, PB2, PB1, PA and nucleoprotein are imported into the nucleus to associate with the viral segmented genome to form vRNP. Then interaction between vRNPs, viral M1 and nuclear export protein promotes their export into the cytoplasm where they traffic to the plasma membrane to be incorporated into virions. Neuraminidase facilitates virion release and viral spread from infected cells by cleaving sialic acids bound to hemagglutinin. During IAV life cycle, NS1, PB1-F2 and PA-X prevent antiviral responses that would limit viral replication [3, 4] [Figure 1.2].

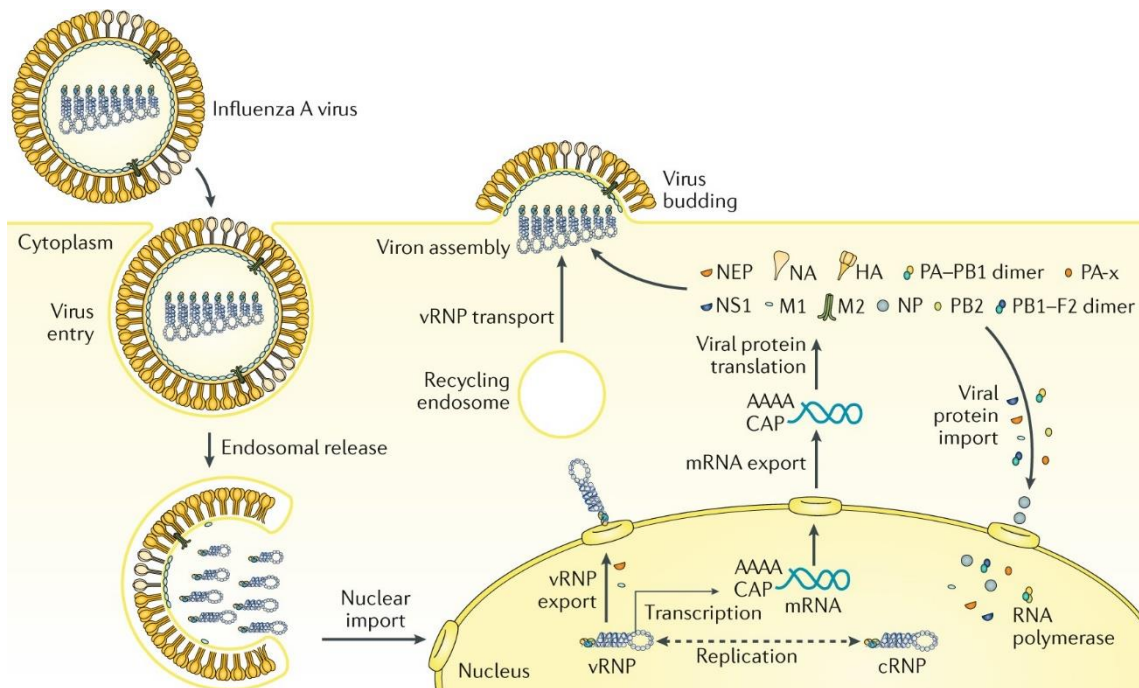


Figure 1.2: Influenza A virus life cycle.

IAV binds to the surface of targeted cells through interaction between viral glycoproteins and host receptors. This leads to endosomal uptake and membrane fusion which facilitates the release of vRNPs into the cytoplasm. vRNPs are then imported into the nucleus where transcription and replication of the viral genome occur. Then viral mRNAs are exported and translated in the cytoplasm. Finally newly synthesised protein and vRNPs are assembled into virions which are released into the extracellular space. cRNP: complementary RNP; NEP: nuclear export protein. Taken from [3].

1.1.1.3 Antigenic changes in influenza hemagglutinin protein

IAV causes seasonal epidemics, and pandemics, through two distinct mechanisms, namely antigenic drift and antigenic shift respectively. Prior infections and vaccinations generate antibodies that can recognise the next IAV infection. However, hemagglutinin accumulates mutations to prevent recognition by neutralizing antibodies leading to escape from the antiviral response and seasonal epidemics. This mechanism is called antigenic drift. In the course of infection, co-infections between human IAV and animal IAV strains can result in exchange of viral RNA segments where hemagglutinin derived from animal IAV strains is incorporated into a human IAV virion. This type of reassortment, known as antigenic shift, is the origin of pandemics since the human population don't have pre-existing immunity against this particular hemagglutinin [5].

1.1.2 Respiratory syncytial virus

1.1.2.1 Epidemiology, transmission and pathogenesis

RSV was first isolated in 1955 from chimpanzee [6] and then isolated from infants with severe lower respiratory tract illness [7]. RSV circulates throughout the world and mainly causes seasonal outbreaks especially during winter months. RSV is classified in two subtypes, namely RSV type A and type B, and both subgroups co-circulate with one usually predominant. Nearly all children by two years of age have been exposed to this virus. It is the major cause of acute respiratory tract illness such as pneumonia and bronchiolitis in infants and young children. RSV infection can also be severe in the elderly, immunocompromised persons, and individuals with comorbidities. RSV is mainly transmitted by close contact but can also be spread in droplets [8, 9].

RSV induces less cytopathic effects, such as sloughing of cells, than other respiratory viruses because it only replicates in apical ciliated cells in the stratified airway epithelium. Thereby the pathology observed during infection is thought to be caused by the host antiviral response rather than viral replication. Nevertheless, RSV replication can cause inclusion bodies and multinucleated syncytial giant cells in epithelial cells which could potentially correlate with severe cases. Most of the damage from RSV infection is observed in bronchi and bronchioles. Neutrophils are the main immune cells recruited to the site of infection but the recruitment of cytotoxic and helper T cells is essential for viral clearance. RSV infection also induces eosinophilic infiltration which was thought to contribute to pathogenesis but its direct implication is still debated. In more severe cases, RSV induces mucus production and

inflammation leading to the obstruction of the lower airway, and exacerbates preexisting airway diseases in patients suffering from asthma and chronic obstructive pulmonary disease. The damages induced by viral infection increase the probability of having a bacterial coinfection which could further enhance RSV replication [8].

1.1.2.2 Genome structure and life cycle

RSV belongs to the *Pneumoviridae* family and *Orthopneumovirus* genus. RSV is an enveloped, negative-sense, single-stranded RNA virus [Figure 1.3A]. The RSV genome, composed of ten genes encoding eleven proteins [Figure 1.3B]. The glycoprotein (G), the fusion glycoprotein (F) and the small hydrophobic (SH) protein, a viroporin, constitute the viral envelope. The polyprotein also contains five structural proteins, namely the large RNA-dependent RNA polymerase (L), the nucleoprotein (N), the phosphoprotein (P), the matrix (M) and M2; and two nonstructural proteins, NS1 and NS2 that inhibit interferon response and apoptosis. The two open reading frames present in M2 mRNA encode two proteins, namely the transcription processivity factor M2-1 and the replication regulator M2-2 [9].

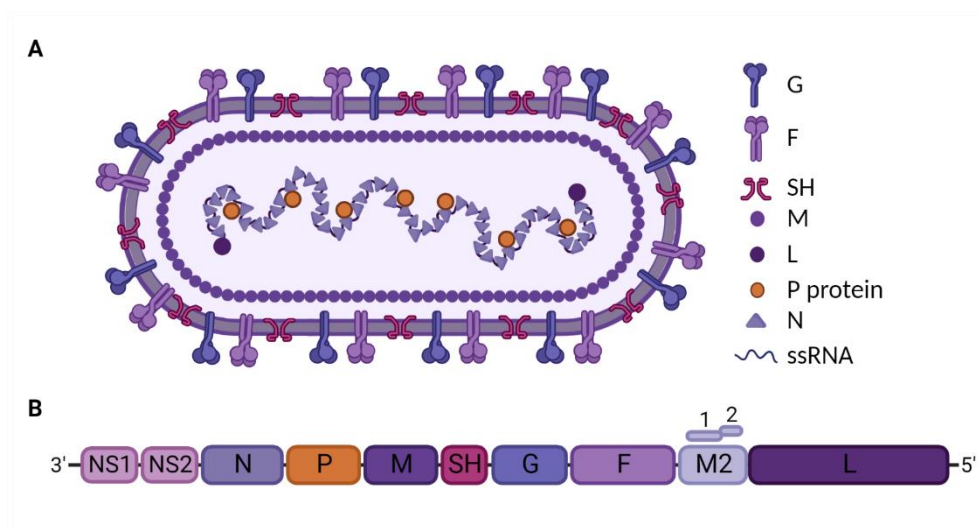


Figure 1.3: Respiratory syncytial virus virion and genome.

A. Morphology of RSV virion. The viral envelope is composed of the glycoprotein (G), the fusion (F) protein and the small hydrophobic (SH) protein. The matrix (M) and M2 proteins are present below the viral membrane. The nucleoprotein (N) binds to the single-stranded RNA (ssRNA) and is associated with the large RNA-dependent RNA polymerase (L) and phosphoprotein (P). **B.** The RSV genome is composed of 10 genes encoding 11 proteins. The M2 gene contains two open reading frames leading to the translation of two proteins. Figure created with BioRender.

Many cellular receptors have been described for RSV cell entry, such as heparan sulfate proteoglycans, CX₃ chemokine receptor 1 (CX₃CR1) and nucleolin [8, 9]. The binding of the viral glycoproteins to the host surface factors brings RSV closer to the plasma membrane. The fusion protein then drives fusion of the viral and host cell membranes leading to the release of the vRNP into the cytoplasm. Virus entry can also be performed by macropinocytosis where the fusion protein induces fusion of the viral and host endosomal membranes thereby releasing the viral genome in the cytoplasm. Next, viral RNA-dependent RNA polymerase transcribes viral mRNA and replicates the genome by producing positive-sense anti-genome intermediates in viral inclusion bodies. A characteristic of linear negative-sense RNA is that the genome is transcribed in a polar gradient with the genes present in the 3' end more amplified than the genes present in the 5' end. The viral nucleic acids generated during replication can be sensed by the host RNA sensors and trigger an antiviral response. The host cell machinery translates proteins from viral mRNA transcripts. Finally, the virion is assembled at the plasma membrane and released from the infected cells [8, 9] [Figure 1.4].

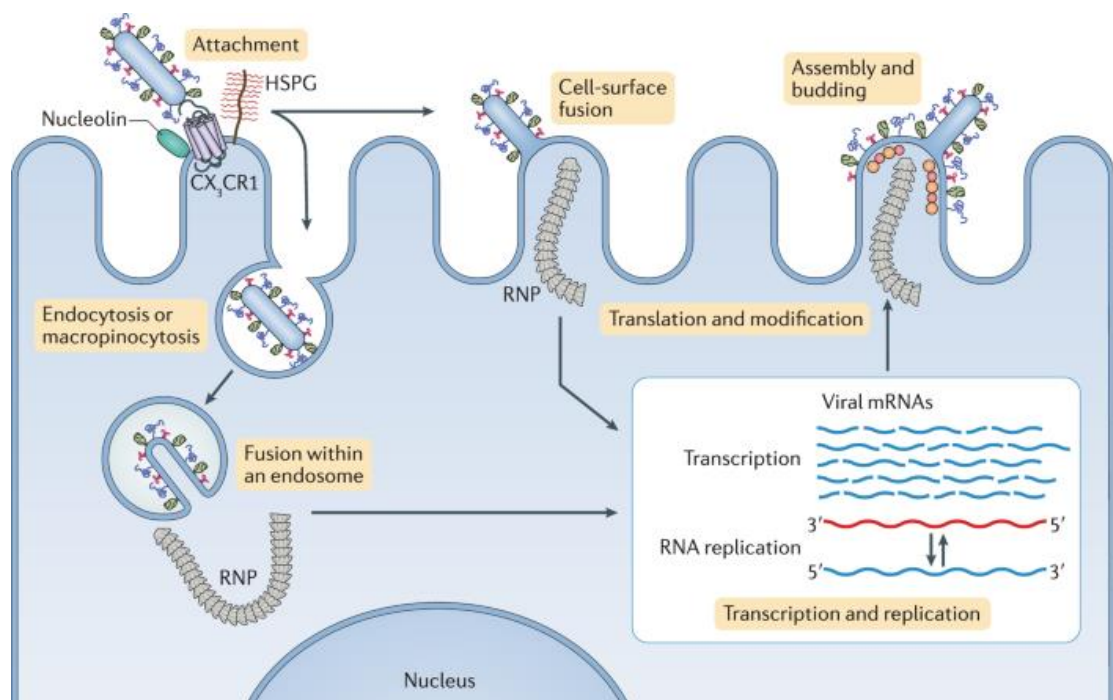


Figure 1.4: Respiratory syncytial virus life cycle.

Viral glycoproteins mediate attachment of RSV to the surface of the targeted cells. Then, viral entry is facilitated either by direct fusion of the viral and host plasma membranes or by macropinocytosis, followed by fusion of the viral and host endosomal membranes. The transcription and replication of the viral genome and translation occur in the cytoplasm. Lastly, the virion is assembled and released from the cells. HSPG: heparan sulfate proteoglycans; RNP: ribonucleoprotein complex. Taken from [9].

1.1.2.3 Vaccine development

Although there are currently no licensed vaccines for RSV, more than 30 candidates are in development [9, 10]. Several vaccine approaches have been developed for three distinct populations, namely infants before exposure to RSV, pregnant women and elderly people. For instance, particle-based and subunit-based vaccines are generated for high-risk adults but won't be used to vaccinate RSV-naïve infants as it could cause vaccine-enhancement disease after natural infection. Live-attenuated and chimeric RSV vaccines are tested for young children instead [9, 10].

1.2 The innate immune response against respiratory RNA viruses

The immune system is composed of three barriers, namely the anatomical and physiological barriers, the innate immune system and the adaptive immune system, which are all important to protect the host against microbial infections. The anatomical and physiological barriers which include the skin, mucociliary clearance mechanisms, low pH in the stomach and bacteriolytic lysozyme in tears and saliva, are the first line of defence against pathogens. Once the pathogen bypasses these barriers, the innate immune response is rapidly elicited, generally within minutes. This response is generated by hematopoietic and non-hematopoietic cells such as neutrophils, monocytes, macrophages, dendritic cells and respiratory or gastrointestinal epithelial cells. These cells can detect the conserved molecular structures present in most pathogens, the cellular stress caused by infection or injury, and the absence of molecules on infected cells which are normally expressed on non-infected cells. In addition, innate immunity has a humoral system composed of complement proteins, collectins and anti-microbial peptides. Thereby innate immunity is essential for cellular homeostasis, infection clearance and activation of the adaptive immune system. Adaptive immunity has cellular and humoral responses which takes several days to be generated. This specific response is based on T and B lymphocytes [11].

1.2.1 Airway epithelial cells are the first line of defence against respiratory viruses

The respiratory tract is composed of the upper and the lower respiratory tract. The upper airway includes the nasal cavity, paranasal sinuses, the pharynx and the part of the larynx above the vocal cords while the lower airway comprises the part of the larynx below the vocal cords, trachea, bronchi, bronchioles and alveoli [12]. Since the alveoli are primordial

for the exchange of oxygen and carbon dioxide, the respiratory tract can also be divided into the conducting airways, namely upper respiratory tract, trachea, bronchi and bronchioles; and the respiratory part which mainly comprises alveoli [12]. The conducting airways are lined with pseudostratified epithelium which is composed of different cell types such as basal cells, club cells, goblet cells and ciliated cells, whereas the respiratory part is formed of type I and type II alveolar cells [12, 13]. Basal cells are progenitor cells that are important for the regeneration of epithelial cells following injury [13].

Airway epithelial cells establish the first barrier against the environment and can detect non-self components, such as pathogen molecules, and initiate an immune response. Similar functions are found in alveolar epithelial cells, besides their fundamental role in gas exchange. In steady state, the innate and adaptive immune responses must be regulated in order to maintain homeostasis and avoid chronic diseases [13]. To achieve this, airway and alveolar epithelial cells have two main intrinsic properties. Firstly, they act as a physical barrier against the outside environment to prevent infection and thus avoid disruption of homeostasis. Indeed goblet cells secrete mucins in the conducting airways that protect the pseudostratified epithelium from inhaled particles which are removed from the lung by mucociliary clearance mechanism [13]. This process is facilitated by ciliated cells. Similarly alveolar epithelial cells produce alveolar lining fluid to protect the lung [14]. Mucins, club cells and alveolar epithelial cells have antimicrobial, antiprotease and antioxidant functions [12, 13]. In addition, transcellular junctions between the epithelial cells are essential to separate a potential stimulus from professional immune cells [12, 13]. Secondly, during homeostasis airway and alveolar epithelial cells are hyporesponsive towards agonists of Toll-like receptor 2 (TLR2) and TLR4 receptors under a certain threshold [12]. It is worth noting that these receptors are mainly expressed on the basolateral side of the airway epithelial cells to avoid uncontrolled activation [12, 15]. Besides their intrinsic characteristics, airway and alveolar epithelial cells regulate immune cells such as alveolar macrophages, lung-resident dendritic cells and lymphocytes during homeostasis by direct cell-cell contact, secretion of anti-inflammatory cytokines and lipophilic factors [12].

For a long time, respiratory epithelial cells have been described as a passive barrier in the defence against viral infection. Nevertheless both airway and alveolar epithelial cells express pattern recognition receptors (PRRs) that recognise viral infection such as IAV and RSV leading to the production of cytokines and chemokines [16, 17] (further described in **section 1.2.3**). For instance, epithelial cells predominantly secrete type I and type III interferon (IFN), tumor necrosis factor α (TNF- α), interleukin 6 (IL-6), CXCL8 also known

as IL-8, CXCL10 also known as interferon gamma-induced protein 10 (IP-10), IL-1 α and IL-1 β [15, 17–19]. Usually these cells first produce TNF- α , IL-1 α and IL-1 β that further enhance the secretion of IL-6 and IL-8 [17, 18]. However airway epithelial cells elicit antiviral responses that are virus-specific. For instance IAV infection triggers a more robust and diverse response than RSV infection [15]. The release of cytokines and chemokines have both autocrine and paracrine effects resulting in the amplification of mediators secreted by respiratory epithelial cells and in the activation and recruitment of other immune cells, such as monocytes, neutrophils, natural killer cells and CD4⁺ T cells, to the lung [16, 17]. In the context of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, lung epithelial cells activate pro-inflammatory macrophages that further enhance inflammatory state [20]. Thereby, the hyporesponsive state of respiratory epithelial cells is overcome during viral infection. Nevertheless apoptotic cells and cellular debris are cleared by alveolar macrophages and other phagocytes to re-establish homeostasis in the respiratory tract following viral infection [16].

1.2.2 Detection of respiratory RNA viruses by pattern recognition receptors

In order to elicit an innate immune response against viral infections, immune and non-immune cells directly recognise pathogen-associated molecular patterns (PAMPs) *via* their PRRs. PAMPs are conserved motifs found in pathogens, for example nucleic acids, bacterial lipoproteins and viral glycoproteins. PRRs can also detect the infection in an indirect manner. Indeed, infection causes many changes in the cells and damage-associated molecular patterns (DAMPs) are released from dying cells in the extracellular space but also from damaged organelles in the cytoplasm. For example, the viroporin M2 of IAV induces the release of mitochondrial DNA (mtDNA) into the cytosol during infection causing the activation of specific PRRs [21] **[Figure 1.5]**.

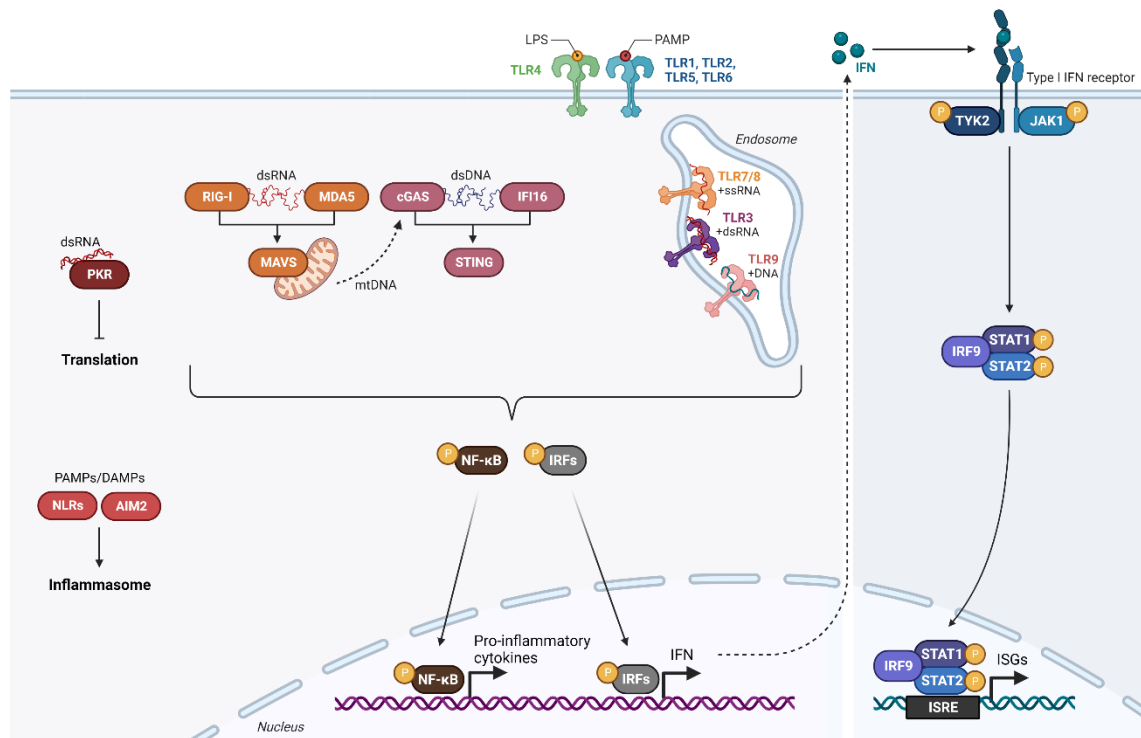


Figure 1.5: Pattern recognition receptor signaling pathways induce innate immune responses during infection.

PRRs recognise PAMPs or DAMPs during infection. TLRs, RLRs and cytosolic DNA receptors are the main sensors of viral infections. In endosomes, TLR7 and TLR8 detect ssRNA, TLR3 senses dsRNA and TLR9 recognises unmethylated CpG DNA. In the cytosol, RIG-I and MDA5 bind to viral dsRNA and interact with MAVS to induce an antiviral response. The DNA sensors cGAS and IFI16 recognise dsDNA and interact with STING to trigger the signaling pathway. During viral infection mtDNA can be released in the cytosol and thus be detected by cGAS. Viral coat proteins can also be recognised by surface TLRs. All these pathways lead to the phosphorylation of the transcription factors NF- κ B and IRFs which translocate to the nucleus and induce the transcription of pro-inflammatory cytokines and IFN respectively. Once secreted, IFN binds to its receptor which activates TYK2 and JAK1. This leads to the phosphorylation of the transcription factors STAT1 and STAT2 which associate with IRF9 and translocate to the nucleus to induce the transcription of ISGs. In addition, PKR interacts with dsRNA in the cytosol and inhibits protein translation. NLRs and AIM2 sense PAMPs and DAMPs which trigger inflammasome activation. Figure created with BioRender.

1.2.2.1 Toll-like receptors

The first innate immune receptor was discovered in 1996 and was named Toll. Toll produced antimicrobial peptides to fight fungal infection in *Drosophila melanogaster* [22]. Shortly afterwards a group identified a homologous protein that induces an inflammatory and adaptive immune response in human [23]. Since then, nine additional human Toll-like receptors (TLR) have been identified. These TLRs are type I transmembrane domain proteins that are localised either on the plasma membrane (TLR-1,-2,-4,-5,-6) to recognise microbial proteins, or on the endosomal membrane (TLR-3,-7,-8,-9) to interact with microbial nucleic acids [24] **[Figure 1.5]**.

TLR2 forms a heterodimer with either TLR1 or TLR6. While a TLR1-TLR2 complex binds triacylated lipoproteins [25], a TLR2-TLR6 heterodimer recognises diacylated lipoproteins [26] and Gram-positive bacterial cell wall components such as lipoteichoic acid (LTA) [27]. TLR4 senses lipopolysaccharide (LPS) from Gram-negative bacteria when the receptor interacts with myeloid differentiation factor 2 (MD-2) [28]. The flagellin of both Gram-positive and Gram-negative bacteria is recognised by TLR5 [29]. In the endosome, viral and synthetic double-stranded RNA (dsRNA) such as polyinosine-polycytidylic acid (poly(I:C)) triggers TLR3 [30] whereas viral and synthetic single-stranded RNA (ssRNA) activates TLR7 and TLR8 [31]. In addition TLR9 senses unmethylated CpG-containing DNA motifs [32] found in both bacterial and viral DNA.

RNA viruses are mainly detected by TLR3 and TLR7 in the intracellular compartment. Indeed in IAV-infected plasmacytoid dendritic cells, TLR7 recognises viral genomes in the endosome leading to the production of IFN- α [33]. In human bronchial epithelial cells infected with IAV, TLR3 triggers the secretion of inflammatory cytokines and type I IFN [34]. Nevertheless IAV-infected TLR3-deficient mice have reduced lung injuries and reduced lethality, but higher viral loads in the lungs, compared to wild-type mice [35], showing that despite its essential role in viral clearance, TLR3 signaling can also lead to pathogenesis. Moreover TLR2 and TLR4 receptors, that are important to trigger immune response against bacterial infection, can also detect viral protein present at the surface of the virus at early stages of infection. For instance, the recognition of RSV fusion protein by the complex TLR4-CD14 induces the production of pro-inflammatory cytokines in human monocytes [36] and TLR2-TLR6-deficient mouse macrophages infected with RSV secrete less pro-inflammatory cytokines than wild-type cells [37]. *In vivo* experiments demonstrated that TLR4, TLR2 and TLR6 are important for RSV clearance in the lung [36, 37].

TLRs are composed of an amino (N)-terminal ectodomain which contains extracellular leucine rich repeats (LRRs), a carboxyl (C)-terminal cytoplasmic Toll/interleukin-1 receptor (TIR) signaling domain and a transmembrane region which links the domains together. While the ectodomain has a horseshoe-like form and interacts with PAMPs ligands, the TIR domain interacts with adaptor proteins to induce signal transduction [24]. The interaction between the ectodomain and its ligand results in receptor conformational changes which bring TIR domains closer together. These domains can then dimerise and recruit adaptor proteins to induce downstream signaling [31, 32]. Five adaptor proteins are involved in the TLRs-mediated signaling, namely myeloid differentiation primary response protein 88 (MyD88), MyD88-adaptor-like (MAL), TIR-domain-containing adaptor protein inducing IFN- β (TRIF), TRIF-related adaptor molecule (TRAM) and sterile α - and armadillo-motif-containing protein (SARM) [38]. The adaptor MyD88 is recruited by all TLRs, except for TLR3 which uses TRIF. TLR2 and TLR4 signaling are dependent on the recruitment of MyD88 by MAL. Endocytosed TLR4 can also signal through a MAL/MyD88-independent but TRAM/TRIF-dependent signaling [38]. Unlike the other adaptors, SARM is a negative regulator of the TRIF-dependent signaling pathway and thus inhibits TLR3 and TLR4 signaling [39]. Except for SARM, adaptor proteins then couple to downstream kinases to phosphorylate and activate the transcription factors, namely nuclear factor- κ B (NF- κ B), activator protein 1 (AP-1) and members of the IFN-regulatory factor (IRF) family such as IRF1, IRF5, IRF3 and IRF7. These transcription factors induce the production of type I and type III IFNs, inflammatory cytokines, chemokines and IFN stimulated genes (ISGs) [Figure 1.5]. Due to the induction of strong immunostimulatory signals, TLR activation must be tightly regulated to prevent inflammatory diseases [24].

1.2.2.2 RIG-I-like receptors

The retinoic acid-inducible gene-I (RIG-I)-like receptor (RLR) family contains melanoma differentiation-associated gene 5 (MDA5), RIG-I and laboratory of genetics and physiology 2 (LGP2) proteins. Even though both RIG-I and MDA5 bind to viral dsRNA replicative intermediates and poly(I:C) [40, 41], these receptors don't recognise the same ligands. While short dsRNA activates the RIG-I pathway, long dsRNA is sensed by MDA5 [41]. Contrary to cellular mRNA, viral RNA replicative intermediates don't usually have a cap structure at their 5' ends allowing RIG-I, but not MDA5, to detect and binds to this uncapped unmodified 5'-triphosphate group on the blunt-end of a dsRNA panhandle structure [42, 43]. Based on these characteristics, RIG-I and MDA5 recognise distinct RNA

viruses. For instance RIG-I detects IAV, Sendai virus and Japanese encephalitis virus infection whereas MDA5 induces IFN response during picornavirus infection [44, 45].

Three domains are present in RLRs, namely a caspase activation and recruitment domain (CARD) in the N-terminal position, a central DExD/H-Box RNA helicase domain and a C-terminal domain [43]. Both C-terminal domain and helicase domain are important for RNA binding. Indeed the C-terminal domain of RIG-I recognises the 5'-triphosphate on an RNA ligand directly, which then allows the dsRNA portions to bind to the helicase domain. This interaction triggers conformational changes which expose the CARD domain for downstream signaling [43]. Indeed the CARD domains of RLRs oligomerise and interact with the CARD domains of mitochondrial antiviral signaling protein (MAVS) resulting in the activation of the kinases TANK-binding Kinase 1 (TBK1) and inhibitor of nuclear factor- κ B (I κ B) kinase β (IKK β) which phosphorylate and activate the transcription factors IRF3 and IRF7 [46, 47]. In addition, MAVS interacts with Fas-associated protein with death domain (FADD) and the kinase receptor-interacting protein 1 (RIP1) *via* its C-terminal effector domain and activates NF- κ B [46]. These two pathways lead to the secretion of type I IFN and pro-inflammatory cytokines [46, 47] [Figure 1.5]. To counteract RLRs-mediated antiviral response, RSV NS2 diminishes IFN response by binding to RIG-I CARD which prevents its association with MAVS [48]. In addition IAV NS1 interacts the E3 ubiquitin-protein ligase tripartite motif containing 25 (TRIM25) which prevents RIG-I activation and thereby limits downstream signaling [49].

Interestingly, RIG-I can also activate a multiprotein complex called the inflammasome which triggers a pro-inflammatory response during RNA virus infection [50]. This outcome will be further discussed in the **section 1.3.2.4**. Besides eliciting an IFN response, RIG-I can also directly restrict SARS-CoV-2 replication in an IFN-independent manner [51]. Mechanistically, RIG-I binds to the 3' untranslated region of the viral genome which prevents viral RNA-dependent RNA polymerase recruitment and thus viral replication. In addition, interaction between RIG-I and SARS-CoV-2 genome doesn't involve RIG-I C-terminal domain, but its helicase domain, preventing its conformational change and further interaction with MAVS to trigger IFN response [51].

LGP2 acts as a negative regulator of the RIG-I and MDA5 signaling pathway. For instance, LGP2 also has the ability to bind to dsRNA but unlike RIG-I and MDA5, LGP2 lacks the CARD domain which prevents the activation of the downstream signal pathway [52]. Also, by interacting with dsRNA, LGP2 limits the recognition of dsRNA by RIG-I and

MDA5 and their subsequent activation [53]. Moreover, LGP2 can block RIG-I oligomerisation which restrains its interaction with MAVS [54] but can also directly binds to MAVS preventing its interaction with IKKi and subsequently the antiviral response [55]. Yet, LGP2 can also act as a positive regulator by increasing the recognition of viral RNA by RLRs [56].

1.2.2.3 Protein kinase R

Protein kinase R (PKR) is a serine-threonine kinase that is found in an inactive form in mammalian cells. Since PKR promoter contains an IFN-stimulated response element (ISRE), PKR transcription is enhanced by IFN during viral infection [57]. PKR is composed of N-terminal dsRNA binding domain and a C-terminal kinase domain bound together by linker region [58]. The dsRNA binding domain consist of two dsRNA binding motifs and binds to viral dsRNA leading to PKR homodimerisation and autophosphorylation [59]. Activated PKR phosphorylates the initiation factor of the translation eIF2 α which triggers inhibition of host protein translation initiation, and consequently causes arrest of viral translation [59] **[Figure 1.5]**. In addition, PKR can mediate FADD-dependent caspase-8 activation, which further activates caspase-3 and the apoptosome, leading to apoptotic cell death [60, 61]. Additionally, PKR is involved in control of transcription through NF- κ B activation, and cell proliferation [59]. PKR can also recognise self-dsRNA in absence of viral infection, such as mitochondrial RNAs (mtRNA) released from the mitochondria or nuclear noncoding RNA present in the cytoplasm, and trigger translation shut down or apoptotic cell death [62]. Besides dsRNA recognition, PKR can be activated through the interaction with the cellular PKR activating protein (PACT) during cellular stress [59].

RNA viruses produce dsRNA replicative intermediates which can trigger PKR-dependent antiviral response that is important to contain viral replication. For instance, in mice infected with IAV, the viral titre is higher in PKR-deficient mice than in wild-type mice [63]. Many viruses target PKR to escape this antiviral mechanism. For example, the dsRNA-binding domain of Middle East respiratory syndrome coronavirus (MERS-CoV) p4a protein binds to viral dsRNA to prevent their recognition by PKR resulting in the increase of translation initiation [64]. In addition, IAV NS1 interacts with PKR which limits its activation *in vitro* and *in vivo* [65]. This inhibition is however independent of the RNA-binding activity of NS1 [65] illustrating different viral strategies to counteract PKR activation.

1.2.2.4 NOD-like receptors

Nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) are cytosolic receptors that recognise PAMPs and DAMPs during infection or cellular stress. The human genome encodes 22 NLR proteins [66]. These proteins have several common domains: a N-terminal domain, a central NAIP, CIITA, HET-E, and TP-1 (NACHT) domain, also known as NOD, and a C-terminal LRR [67]. The N-terminal domain is either composed of one of the following domains: CARD, pyrin (PYD), baculovirus inhibitor of apoptosis repeat (BIR) domain, acidic transactivation domain or mitochondrial-addressing sequence [66, 67]. Based on the domain present at the N-terminal position, the NLR family is classified into five subfamilies, namely the acidic transactivation domain (NLRA), BIR domain (NLRB), CARD domain (NLRC), PYD domain (NLRP) and mitochondrial-addressing sequence (NLRX) [67].

This N-terminal domain enables interaction with downstream signaling molecules whereas the LRR domain is important for autoregulation and ligand binding. The NACHT domain is required for receptor oligomerisation which triggers recruitment of other proteins to form a multiprotein signaling platform that activates caspase-1 named the inflammasome [66]. NLRs have roles in signal transduction, regulation of transcription, autophagy and inflammasome formation [66]. Owing to their implication in immune responses, mutations and polymorphisms in genes encoding the NLR family are associated with many chronic inflammatory, autoinflammatory, neurodegenerative and metabolic diseases such as asthma, Crohn's disease, vitiligo, Alzheimer's disease and diabetes [66, 67].

Several members of this family are involved in antiviral responses to fight infection. NOD2 recognises RSV ssRNA genome and triggers MAVS and IRF3-dependent type I IFN response [68]. Additionally NLRC5 acts in concert with NLRP3 inflammasome to induce the secretion of IL-1 β and IL-18 cytokines during rhinovirus infection [69]. Moreover NLRX1 diminishes IAV-mediated macrophages apoptosis by interacting with viral PB1-F2 protein which results in enhanced macrophage survival and IFN response, thereby limiting viral replication [70]. Nevertheless to prevent excessive antiviral responses that are deleterious for the host, NLRX1 interferes with RIG-I and MAVS interaction and associates with TNF receptor-associated factor 6 (TRAF6) to decrease IRF and NF- κ B signaling pathway activation respectively [71]. Finally other NLRs receptors, such as NLRP3 and NLRP1, have been shown to induce the formation of the inflammasome during viral infections [Figure 1.5]. The mechanism will be further described in section 1.3.2.

1.2.2.5 Cytosolic DNA receptors

In eukaryotic cells, DNA is only present in the nucleus and in the mitochondria. Thereby the presence of self or foreign DNA in the cytoplasm during infections, cellular damages or autoimmune diseases, is recognised as a danger signal by DNA sensors in mammalian cells. This leads to the activation of a host immune response such as type I IFN production. In the last decade many cytosolic DNA sensors have been discovered. DNA-dependent activator of IFN-regulatory factors (DAI) was the first receptor to be identified, followed by the discovery, among others, of interferon-inducible protein (AIM2), interferon gamma inducible protein 16 (IFI16), RNA polymerase III, DEAD-Box Helicase 41 (DDX41) and cyclic GMP-AMP synthase (cGAS) [72]. Interestingly, DNA sensors such as AIM2, IFI16 and cGAS are also important to initiate an immune response against RNA virus infection. AIM2 inflammasome formation [Figure 1.5] will be described in section 1.3.2.3.

1.2.2.5.1 cGAS/STING pathway

Upon DNA binding cGAS catalyses the production of 2'3'-cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) from ATP and GTP molecules [73]. This second messenger binds to and activates the endoplasmic reticulum-resident stimulator of interferon genes (STING) protein, resulting in the production of type I IFN and other cytokines [74] [Figure 1.5]. Additionally, cGAMP produced in infected cells can be transferred through gap junctions to naive neighbouring cells where it can interact with STING and activate the antiviral pathway [75]. Almost all DNA sensors signal through STING which then activates TBK1 resulting in the phosphorylation and translocation of transcription factors NF- κ B and IRF3 [76–78]. However a recent study challenged this model as they showed that TBK1 was dispensable for NF- κ B activation whereas the kinase activity of TBK1 was essential for the activation of IRF3 [79]. TBK1 and IKK ϵ have redundant functions to induce STING and NF- κ B-dependent responses [79].

As well as mediating antiviral responses against DNA viruses, cytosolic DNA sensors have also been implicated in targeting RNA viruses. Several reports support this hypothesis. For instance RNA virus replication is increased in mice or cells deficient for cGAS or STING respectively [80, 81] and STING interacts with known components of the RNA sensing pathway, namely RIG-I and MAVS, to promote type I IFN response [82, 83]. Several mechanisms have been proposed to explain how STING limits RNA virus replication. Firstly membrane fusion, a necessary step for enveloped RNA viruses to enter the host cell, activates

a STING-dependent, but cGAS-independent, IFN response [84]. Secondly RNA virus infection causes mtDNA release in the cytoplasm that is sensed by cGAS which subsequently triggers STING-dependent IFN response [21] [**Figure 1.5**]. Indeed IAV M2 viroporin localises at the mitochondria and induces the translocation of mtDNA into the cytoplasm [21]. Finally STING limits viral infection independently of IFN signaling through inhibition of translation which decreases the level of viral and host proteins [81]. Importantly, this mechanism is independent of membrane fusion since transfection of RNA genome of enveloped virus in STING-deficient cells leads to increase viral replication compared to control cells, suggesting that STING-mediated translation shut down prevents both naked and enveloped RNA virus infection [81].

Another line of evidence that the cGAS/STING pathway is required for the innate immune response against RNA viruses is that this signaling pathway is antagonised by RNA viruses. For instance, IAV NS1 interacts with mtDNA released in the cytoplasm during viral infection to evade cGAS activation [21]. In addition, IAV hemagglutinin binds to STING and blocks its dimerisation which prevents STING activation [84]. Similarly the papain-like protease of coronavirus disrupts STING assembly and dimerisation and thus inhibits the induction of the IFN response [85].

1.2.2.5.2 IFI16 receptor

IFI16 belongs to the same PYHIN family as AIM2 and recognises DNA viruses in the cytoplasm and nucleus of infected cells. IFI16 directly restricts herpesvirus and papillomavirus replication by preventing the binding of host transcription factors to viral promoters, and through the recruitment of markers for heterochromatin on viral promoters [86–88]. In addition, IFI16 cooperates with cGAS to trigger cGAMP production and subsequent STING-dependent IFN response [89, 90] [**Figure 1.5**]. IFI16 is another example of the crosstalk between DNA and RNA sensing pathways, since IFI16 enhances RIG-I-mediated type I IFN response during IAV infection by increasing RNA polymerase II recruitment to RIG-I promoter, which amplifies RIG-I transcription [91]. In addition, IFI16 promotes RIG-I recognition of IAV RNA in the cytoplasm by interacting with both viral RNA and RIG-I [91]. Importantly IFI16 can also inhibit IAV replication by directly binding to IAV genome which triggers a STING-dependent antiviral response leading to the release of pro-inflammatory cytokines and type I IFN, and to lytic cell death [92].

1.2.3 Mediators of immune responses

The interaction between PAMPs or DAMPs and PRRs activates a signal cascade leading to the phosphorylation and the translocation of the two major families of transcription factors, NF- κ B and IRFs [Figure 1.5]. These factors induce the transcription of pro-inflammatory and antiviral genes, namely type I and type III IFNs, ISGs and pro-inflammatory cytokines [Figure 1.5]. Those cytokines enable the attraction and activation of immune cells, allowing the establishment of the adaptive immune response.

1.2.3.1 Interleukins

ILs are a vast family of immunomodulatory cytokines that mediate communication between cells. They have a role in cell proliferation, maturation, adhesion, differentiation, migration and activation. ILs can either have pro- or anti-inflammatory responses.

The IL-1 family comprises ten receptors and eleven soluble molecules including IL-1 α , IL-1 β and IL-18. IL-1 α and IL-1 β signal through the same receptor, named IL-1 receptor (IL-1R), whereas IL-18 binds to IL-18R receptor. Phylogenetic analysis shows that IL-1 α probably arose from an ancestral gene duplication of IL-1 β between 320 and 160 million years ago [93]. Moreover IL-1 α , IL-1 β and IL-18 possess a pro-domain that can be cleaved by proteases. Pro-IL-1 β and pro-IL-18 are inactive and need to be cleaved by caspase-1, which is part of the inflammasome complex, to be active, whereas pro-IL-1 α is directly biologically active after translation [94]. IL-1 is secreted by various cell types such as macrophages, dendritic cells, neutrophils and epithelial cells, whereas IL-18 is principally produced by macrophages and dendritic cells [95]. However, unlike IL-1 β and IL-18, IL-1 α can also act as a membrane protein that binds to its receptor on neighbouring cells [96]. Additionally, IL-1 α can shuttle into the nucleus *via* its nuclear localisation sequence and somehow up-regulate the transcription of genes [96]. Finally these ILs can stimulate various cell types and have different roles in innate and adaptive immunity and immunopathology. For instance, IL-1 regulates hematopoiesis and induces pro-inflammatory cytokines production such as TNF- α and IL-6, and T helper 17 (Th17) cell differentiation. As for IL-18, this cytokine promotes IFN- γ production, natural killer cell cytotoxicity, innate lymphoid 1 cell activation and Th1 responses [94, 95]. Nevertheless, uncontrolled release of IL-1 or IL-18 contributes to inflammatory or autoimmune diseases [94, 95].

IL-6 is a pleiotropic cytokine which belongs to the IL-6 family. IL-6 is only present in small amounts in the cell, but its expression is greatly induced after stimulation or infection

[97]. IL-6 is secreted by immune and non-immune cells and can either bind to its transmembrane receptor IL-6R or to its soluble receptor sIL-6R leading to a classic or trans-signaling pathway respectively. The classic signaling mainly triggers anti-inflammatory responses whereas the trans-signaling pathway mostly elicits pro-inflammatory responses. IL-6 regulates immune cells and as such contributes to the initiation of the hepatic acute phase response, the differentiation of B cells into plasma cells that produce antibodies, and the proliferation and differentiation of T cells [97].

1.2.3.2 Chemokines

Chemokines are a group of around fifty chemotactic cytokines classified into four subfamilies according to the position of the cysteine residues at their N-terminal: XC, CC, CXC and CX₃C. Chemokines are generally secreted, and they bind to two types of receptors that are expressed on leukocytes, namely G-protein-coupled chemokine receptors and atypical chemokine receptors. Receptors are named after the chemokines they bind to, i.e., XCR, CCR, CXCR and CX₃CR. Chemokines were first described to attract the innate immune cells to the site of infection or inflammation to induce an acute inflammatory response. Henceforth several roles have been described such as coordination of interactions between the innate and adaptive immune responses and regulation of homeostasis [98].

CXCL10, also known as IP-10, is part of the CXC chemokine group and is a ligand for the CXCR3 receptor. IP-10 is produced by several cell types including leukocytes, monocytes, epithelial cells and keratinocytes in response to type II IFN [99]. Epithelial cells enhance IP-10 secretion during rhinovirus-induced asthma, severe cases of IAV infection and chronic obstructive pulmonary disease exacerbations [100–102]. Also, IP-10 contributes to airway hyperactivity and inflammation in an asthma murine model [103]. Additionally IP-10 functions as a chemoattractant for macrophages, dendritic cells, natural killer cells and activated T cells, and has a role in apoptosis, angiogenesis and regulation of proliferation and cell growth [99].

1.2.3.3 Interferons

Isaacs and Lindenmann described for the first time in 1957 the antiviral role of IFN against IAV virus [104]. IFNs are cytokines divided into three families according to the cell surface receptors they bind. The type I IFN family is composed of thirteen IFN- α subtypes

and a single IFN- β , IFN- ϵ , IFN- κ , and IFN- ω , whereas type II IFN is only composed of a single IFN- γ . The type III IFN family includes IFN- λ 1 (IL-29), IFN- λ 2 (IL-28A), IFN- λ 3 (IL-28B) and more recently IFN- λ 4 [105]. All these cytokines have different functions depending on their receptor location and expression and the transcription factors they activate. Indeed, the type I IFN receptor is ubiquitously expressed whereas the type III IFN receptor is mainly present on mucosal epithelial tissues and on hepatocytes, explaining why not all cells can respond to IFN- λ compared to IFN- α/β . The type II receptor is also broadly expressed on cell membranes but IFN- γ is only produced by immune cells and enhances both innate and adaptive responses [105].

In response to respiratory viruses, epithelial cells mainly secrete IFN- λ whereas peripheral blood mononuclear cells produce more IFN- α although both induce IFN- α , - β , - λ [106]. Type I and type III IFNs have different receptors but similar signaling pathways. Both receptors are heterodimers composed of interferon- α/β receptor (IFNAR)1 and IFNAR2 for type I IFN and IFNLR, also called IL28R α , and IL-10R2 for type III IFN. Mice lacking both IFNAR1 and IL28R α receptors are more susceptible to SARS-CoV, RSV and IAV infection than mice lacking only one of the receptors, showing that the pathways are non-redundant [107]. Once secreted, type I and type III IFNs have an autocrine and paracrine action and bind to their respective receptors which induce their dimerisation. This activates tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1) kinases which phosphorylate signal transducer and activator of transcription (STAT)1 and STAT2. Phosphorylated STAT1-STAT2 then associates with IRF9 to form a heterotrimeric complex called IFN-stimulated factor 3 (ISGF3). ISGF3 translocates to the nucleus, binds to ISREs within promoters of ISGs and induces their transcription [105] **[Figure 1.5]**. Nevertheless antiviral response and antiproliferative effects induced by IFN- λ are generally less potent than type I IFNs [108].

1.3 Inflammasome activation during RNA virus infection

The inflammasome is a cytosolic multiprotein complex formed during cellular stress or infection that activates the inflammatory caspase-1, leading to the secretion of pro-inflammatory IL-1 β and IL18 cytokines, and/or pyroptosis which is an inflammatory type of lytic programmed cell death. Dysregulation of the activation of this innate immune pathway is implicated in many inflammatory diseases [109]. For instance, NLRP3

inflammasome is implicated in atherosclerosis, Alzheimer's disease and type II diabetes [110] and NLRP1 is associated with vitiligo, inflammatory bowel disease, celiac disease and systemic lupus erythematosus [111].

1.3.1 Role of IL-1 α / β in respiratory virus infections

IL-1 α / β have a broad range of roles on immune and non-immune cells during viral infection. In human epithelial cells infected with rhinovirus, blockade of IL-1R1 decreased the release of pro-inflammatory cytokines such as IL-6 and IL-8 and neutrophil chemoattractants but didn't affect the secretion of T-cell chemoattractants, production of IFNs and ISGs, or viral replication. This indicates that for rhinovirus, IL-1 has a role in induction of pro-inflammatory signals and the recruitment and activation of immune cells to the site of infection but has no direct antiviral activity that blocks viral replication [112]. In line with this, infection of IL-1R1^{-/-} mice with IAV showed a reduced lung inflammatory pathology and an enhanced mortality induced by the virus compared to wild-type mice [113]. Also, IAV-mediated IL-1 α / β release induced an effective viral clearance by the recruitment of neutrophil and CD4⁺ T cells and the enhancement of the antiviral IgM antibody response by B cells [113]. It is worth noting that by blocking IL-1R1 receptor, these studies don't distinguish the role of IL-1 α *versus* IL-1 β . Importantly, IAV-infected mice release IL-1 α following cell death that is essential for optimal IL-1 β secretion and immune responses [114].

1.3.2 Activation of inflammasome complexes after PAMPs and DAMPs recognition

To date multiple inflammasomes have been described. Many of them are formed in response to bacterial infections, including NLRP6 inflammasome [115] and NAIP-NLRC4 inflammasome, pyrin inflammasome [109], or after viral infections such as NLRP3, NLRP1 and AIM2 inflammasomes. Inflammasomes either directly recognise the pathogen or indirectly sense the infection through pathogen-inflicted cellular damage [Figure 1.5].

1.3.2.1 NLRP3 inflammasome

The NLRP3 protein is composed of three domains, namely a N-terminal PYD domain, a NACHT domain and a C-terminal LRR domain [116] [Figure 1.6].

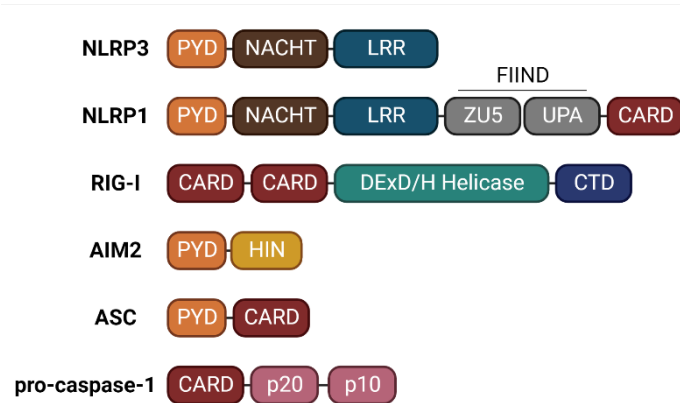


Figure 1.6: Protein domains of inflammasome components.

NLRP3 has three domains: PYD, NACHT and LRR domains. NLRP1 is composed of PYD, NACHT, LRR, FIIND which consist of ZU5 and UPA and CARD domains. RIG-I contains CARD, DExD/H helicase and C-terminal domain (CTD). AIM2 is composed of PYD and HIN domains. ASC contains PYD and CARD domains. Pro-caspase-1 is composed of CARD p20 and p10 domains. Figure created with BioRender.

Besides some exceptions, activation of the NLRP3 inflammasome requires two steps: priming and activation. Firstly, priming is necessary to upregulate the transcription of the components of the inflammasome. Indeed during infection or cellular damage, PRRs induce signal transduction pathway that activates the transcription factor NF- κ B. This upregulates the transcription of genes such as NLRP3 and pro-IL-1 β [116] [Figure 1.7]. Additionally the priming step induces post-translational modifications of NLRP3 such as ubiquitylation and phosphorylation [116] [Figure 1.7]. These modifications stabilise NLRP3 which is then in a competent state for an activation signal. Finally, an activation step is necessary to fully activate the inflammasome. Contrary to specific PRRs, NLRP3 can be activated by a wide range of unrelated stimuli that induce cellular stress. These signals could be potassium (K⁺) or chloride efflux, binding of extracellular ATP on P2X7 receptor, modification of intracellular calcium concentration, lysosomal disruption, mitochondrial dysfunction, release of mitochondrial DNA, production of reactive oxygen species (ROS), metabolic changes and *trans*-Golgi disassembly [116] [Figure 1.7]. It has also been shown that never in mitosis gene a-related kinase 7 (NEK7) protein is essential for NLRP3 inflammasome activation in mouse

macrophage cells [Figure 1.7]. Indeed in mouse bone marrow-derived macrophages (BMDMs) lacking NEK7, activation of caspase-1 and secretion of IL-1 β are diminished in the presence of activators of the NLRP3 inflammasome compared to wild-type BMDMs [117]. K⁺ efflux is necessary for interaction between NEK7 and NLRP3 in the cytosol, which leads to NLRP3 oligomerisation and apoptosis-associated speck-like protein containing a CARD (ASC) speck formation [117]. Thereby many activation mechanisms have been proposed since the discovery of NLRP3 inflammasome but no consensus model has emerged [116]. Many activators of NLRP3 induce K⁺ efflux to trigger NLRP3 inflammasome activation [118]. Nevertheless Chen and colleagues have recently proposed that many different activators of NLRP3 induce the disassembly of the *trans*-Golgi network (TGN) into dispersed TGN (dTGN). NLRP3 is then recruited *via* ionic bonding with phosphatidylinositol-4-phosphate on the dTGN. This interaction promotes the aggregation and activation of NLRP3 that can then form the inflammasome complex. It is worth noting that this mechanism operates for NLRP3 activators that are known to be both dependent and independent of K⁺ efflux [119].

Nevertheless most of these studies have been done in mouse macrophages and studies in human myeloid cells have pointed out differences in the priming and activation mechanisms. For instance, the activation of NLRP3 inflammasome is not dependent on NEK7 in human monocyte cell lines and human induced pluripotent stem cells (iPSC)-derived macrophages even though NEK7 and NLRP3 can interact together [120]. Instead NLRP3 is recruited to phosphatidylinositol-4-phosphate on the TGN by the kinase IKK β . The kinase activity of IKK β is needed for this post-translational priming of NLRP3 [120]. However the activation of NLRP3 is enhanced by NEK7 at early times in LPS-primed human iPSC-derived macrophages until IKK β is fully active [120]. Thereby the authors suggest that NEK7 acts more as a primer than an activator of the NLRP3 inflammasome. Interestingly NEK7 bypass and IKK β -dependent priming have also been found in mouse macrophage cells with certain stimuli, although NEK7-dependent NLRP3 inflammasome activation is the predominant pathway [120].

Besides this canonical inflammasome activation, a non-canonical inflammasome pathway has been described during bacterial infection. Caspase-11 in mice or caspase-4 and caspase-5 in human can directly bind to LPS in the cytosol which induces caspase oligomerisation and activation [121]. Caspase-11 then cleaves gasdermin D (GSDMD) to form pores which then facilitate K⁺ efflux leading to NLRP3 inflammasome activation and

thereby caspase-1-dependent IL-1 β maturation [109, 122]. Thus caspase-11 can directly cause pyroptosis, but indirectly trigger IL-1 β release.

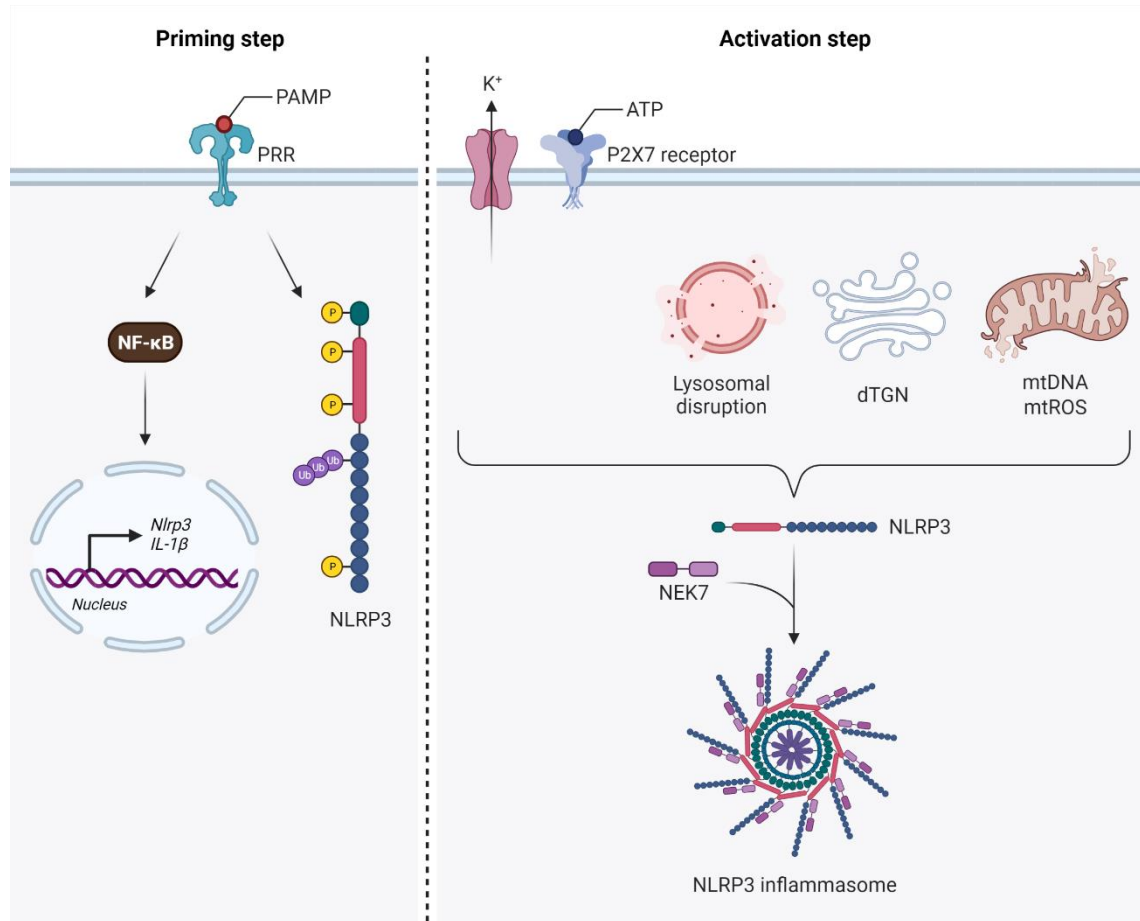


Figure 1.7: Mechanisms of NLRP3 inflammasome activation.

Activation of PRRs by PAMPs leads to the priming step, also called signal 1, which induces the transcription of pro-IL-1 β and NLRP3 through NF- κ B. The priming step can also mediate post-translational modifications of NLRP3 such as phosphorylation and ubiquitylation. The activation step, also called signal 2, is triggered by extracellular ATP, release of mitochondrial ROS (mtROS) or mtDNA, lysosomal disruption and TGN disassembly into dTGN. Many of these activating events cause K⁺ efflux, which is often required for NLRP3 activation. Then interaction between NLRP3 and NEK7 leads to NLRP3 inflammasome assembly and activation. Figure created with BioRender.

NLRP3 inflammasome is also activated following RNA virus infection. Several studies showed that IAV PB1-F2 in the presence of viral RNA and ion channel M2 can trigger NLRP3 inflammasome in mouse macrophage cells [123–125]. In addition SARS-CoV

viroporin 3A protein induces mitochondrial ROS release and K⁺ efflux that trigger NLRP3 inflammasome in BMDMs [126]. However, only a few papers have claimed that NLRP3 is activated in airway epithelial cells after respiratory virus infection. Two papers from the same group showed that in RSV-infected lung epithelial cells, viral SH protein forms pores into the cell and organelle membranes that induces their permeabilisation and NLRP3 inflammasome activation [127]. That group also showed that in primary bronchial cells infected with rhinovirus, viral ion channel protein 2B induces efflux of calcium from endoplasmic reticulum and Golgi that activates NLRP3 and NLRC5 inflammasomes [69]. NLRP3 inflammasome triggers a crucial inflammatory response at the early stage of IAV infection but causes harmful inflammation at later stages that drives pathogenesis [128]. This shows that despite having a protective role in viral infection, a sustained or uncontrolled NLRP3-dependent inflammatory response can be detrimental for the host. In addition, NLRP3 inflammasome formation has a protective role against enterovirus 71 infection *in vivo* [129]. To overcome this immune response, enterovirus 71 proteases cleave NLRP3 preventing inflammasome activation and IL-1 β secretion [129]. Several viral proteins interact with NLRP3 and counteract NLRP3-dependent response following different mechanisms. For instance, bound human parainfluenza virus type 3 C protein induces NLRP3 proteasomal degradation [130], bound IAV NS1 prevents NLRP3 interaction with the adaptor protein ASC [131] and bound Sendai virus V protein and human parainfluenza virus type 2 V protein limit NLRP3 oligomerisation and subsequently ASC recruitment [132]. Nevertheless, instead of evading an NLRP3-mediated response, Zika virus infection triggers NLRP3 inflammasome and viral NS1 prevents proteasomal degradation of caspase-1 to counteract host immune response [133]. Indeed caspase-1 cleaves cGAS which limits the recognition of mtDNA release during Zika infection and subsequently STING-dependent signaling [133]. Thereby Zika virus-mediated NLRP3 inflammasome activation diminishes the type I IFN response which favours viral infection [133].

1.3.2.2 NLRP1 inflammasome

In 2002 the NLRP1 inflammasome was the first NLR inflammasome to be described [134] but its activation mechanism in human cells has remain elusive until recently. Human NLRP1 (hNLRP1) has five domains, namely PYD, NACHT, LRRs, function to find domain (FIIND) that can be divided into ZU5 and UPA subdomains, and CARD in C-terminal position [Figure 1.6]. The PYD and NACHT domains are separated by a disordered linker. Mouse NLRP1B (mNLRP1B) is composed of the same domains except for PYD which is

absent. Constitutive autocleavage between the ZU5 and UPA subdomains of the FIIND domain is indispensable for NLRP1 inflammasome activity. The resulting N- and C-terminal fragments remain noncovalently bound to avoid constitutive inflammasome activation [135–137]. Indeed the PYD and LRR domains act as repressor domains to prevent the release of the C-terminal inflammatory CARD domain [138] and further interaction with the adaptor protein ASC.

Several mechanisms of NLRP1 inflammasome activation have been described that varies between species. Studies first focused on mNLRP1B to reveal its activation mechanism. Anthrax lethal factor (LF), a metalloprotease, directly cleaves mNLRP1B or rat NLRP1 leading to the activation of NLRP1 inflammasome [139, 140] **[Figure 1.8A]**. This cleavage induces the degradation of the N-terminal domain by the N-end rule proteasome pathway and the release of the C-terminal fragment. The latter is not degraded due to the break caused by the autoproteolysis within the FIIND domain. The C-terminal fragment then self-oligomerises and activates caspase-1 to form an inflammasome [141, 142]. This activation mechanism called “functional degradation” suggests that the proteasomal degradation is the key event for NLRP1 inflammasome activation and not the protease sensing *per se*. In line with this model, the E3 ubiquitin ligase IpaH7.8 of *Shigella flexneri* ubiquitylates mNLRP1B N-terminal fragment which leads to its proteasomal degradation and subsequent inflammasome activation [142]. Based on the mechanism described for mNLRP1B, several studies hypothesised that viral proteases could cleave and activate hNLRP1. In airway epithelial cells, human rhinovirus 3C protease indeed cleaves hNLRP1 in the disordered linker inducing the proteasomal degradation of the autoinhibitory N-terminal fragment and inflammasome activation [143] **[Figure 1.8A]**. Similar conclusions with the 3C protease from diverse picornaviruses and 3C-like protease from SARS-CoV-2 have been highlighted in keratinocytes and lung epithelial cells respectively [144, 145]. Interestingly picornavirus protease cleaves hNLRP1 at residue sites that mimic the highly conserved natural sites used by viral protease to cleave picornavirus polyprotein [144]. In addition, picornavirus 3C protease can also cleave and activate mNLRP1B [144].

A second mechanism common to both human and mouse has been discovered following the observation that inhibition of dipeptidyl peptidase 8 and 9 (DPP8/9) by Val-boroPro (VBP) triggered NLRP1 inflammasome in both mouse and human cells [146–148] **[Figure 1.8B]**. Further investigation showed that DPP9 binds to the hNLRP1 FIIND domain, and that both this interaction and DPP9 catalytic function are required to inhibit hNLRP1 inflammasome [148]. Cryo-electron microscopy analysis revealed that DPP9

interacts with two NLRP1 molecules and forms a ternary DPP9-NLRP1^A-NLRP1^B complex where NLRP1^A is the full-length NLRP1 and NLRP1^B is composed of the UPA-CARD C-terminal domains [149, 150]. Thereby DPP8/9 inhibition by VBP triggers inflammasome activation by displacing NLRP1^B from the repressor DPP9 which can then assemble into an inflammasome [150]. However, it is still unclear how the NLRP1^B molecule is produced in cells. Interestingly a similar mechanism might be involved during parasite infection. *Toxoplasma gondii* infection triggers NLRP1 inflammasome in human and rodent cells but doesn't proteolytic cleave the N-terminal fragment of NLRP1 [151] suggesting that a mechanism other than functional degradation is involved. A report indeed indicated that *Nlrp1* alleles had similar sensitivities to *Toxoplasma gondii* infection and inhibition of DPP8/9 in rodent macrophages [152].

Three additional activation mechanisms for hNLRP1 have been revealed that are not found in mouse, exemplifying the difference in signaling pathway between species. Firstly, hNLRP1 can sense both synthetic dsRNA, and viral dsRNA produced during Semliki Forest virus infection, in primary keratinocytes [153] **[Figure 1.8C]**. Even though hNLRP1 binds to short dsRNA and dsDNA, only long dsRNA triggers ATP hydrolysis by the NACHT domain. This probably results in a change in hNLRP1 conformation which then enables inflammasome formation. In addition, the LRRs were sufficient and necessary for nucleic acid binding but the NACHT domain enhances binding affinity [153]. Secondly, hNLRP1 can also respond to DNA viruses such as Kaposi's sarcoma-associated herpesvirus (KSHV). KSHV open reading frame 45 (ORF45) interacts with the disordered linker of hNLRP1 leading to N-terminal fragment displacement from C-terminal domain [154] **[Figure 1.8D]**. Thereby activation of the hNLRP1 inflammasome is not dependent on functional degradation of its N-terminal fragment [154] which further illustrates that hNLRP1 can sense viral infection in a non-protease-dependent manner. Interestingly this function was also found in primates but not in rodents [154]. Finally, ribotoxic stress and ultraviolet B radiation are detected by hNLRP1 as danger signals in keratinocytes [155, 156]. Ribotoxic stress response triggered by ultraviolet B or by specific toxins activated ZAK α kinase and p38 that then hyperphosphorylate the disordered linker region of hNLRP1 leading to inflammasome activation [156] **[Figure 1.8E]**. This activation is thought to be independent of proteasomal degradation and N-terminal fragment degradation [156].

Overall, all these recent studies demonstrate that hNLRP1 has an important role in sensing different types of cellular stress in barrier epithelial cells.

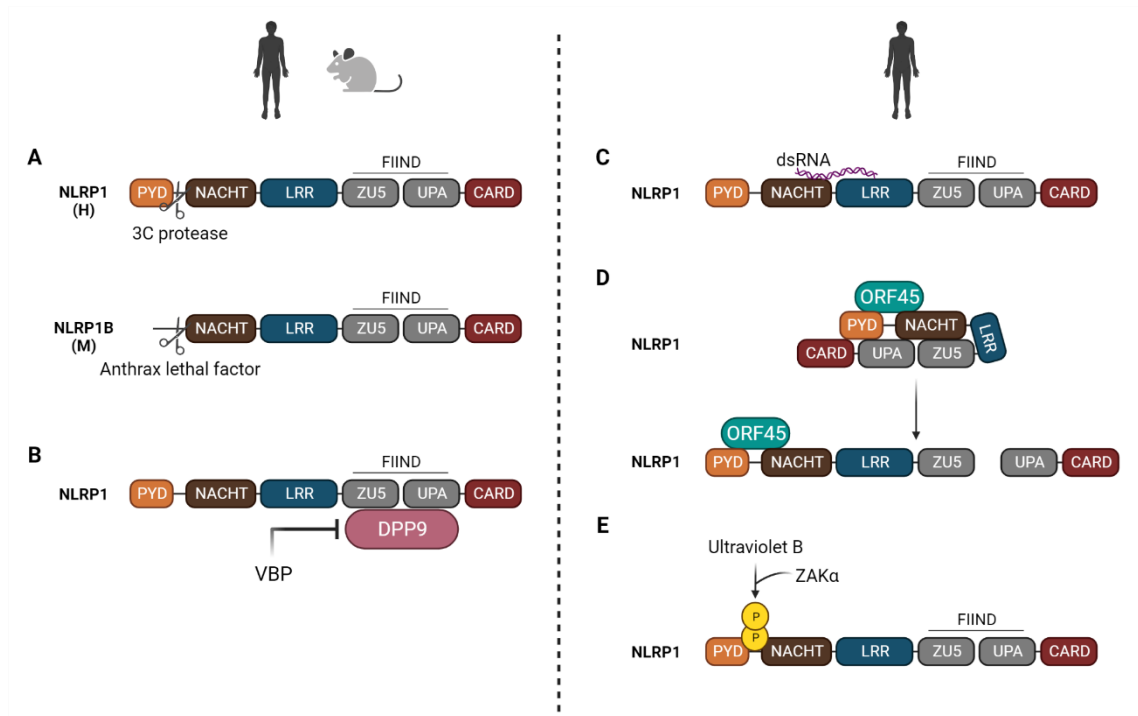


Figure 1.8: Mechanisms of NLRP1 inflammasome activation in human and mouse cells.

hNLRP1 and mNLRP1B can be activated through two similar mechanisms. (A) Picornavirus 3C protease and anthrax lethal factor cleave hNLRP1 and mNLRP1B respectively in their N-terminal domain leading to its proteasomal degradation. The release of the C-terminal fragment induces the inflammasome formation. (B) Inhibition of DPP9 by VBP causes the displacement of NLRP1 from DPP9 resulting in C-terminal release. Three additional mechanisms have been described in human only. (C) The LRR domain of hNLRP1 senses dsRNA inducing ATP hydrolysis by the NACHT domain, conformational change and inflammasome activation. (D) KSHV ORF45 binds to the disordered linker region of hNLRP1 which displaces the N-terminal fragment from the C-terminal fragment. (E) Ultraviolet B radiation triggers ribotoxic stress response leading to ZAK α -dependent hyperphosphorylation of hNLRP1 disordered linker region and further inflammasome activation. Figure created with BioRender.

1.3.2.3 AIM2 inflammasome

The AIM2 protein is composed of two domains, a PYD domain and a hematopoietic interferon-inducible nuclear (HIN) domain [Figure 1.6]. Crystal structures of AIM2 reveal that the HIN domain binds to non-specific DNA of at least 80 base pairs [157]. The PYD domain promotes this interaction, leading to the oligomerisation of AIM2 along the length of DNA and the formation of filamentous structures [158]. The binding is optimal in the presence of long dsDNA, of around 300 base pairs. Additionally, at high concentrations,

AIM2 can oligomerise and initiate the formation of filaments without DNA interaction [158]. Thereby, Morrone and colleagues suggested that dsDNA acts as a one-dimensional ruler to increase the local concentration of AIM2 [158]. Then AIM2 interacts with the PYD domain of ASC and forms an inflammasome complex that in turn activates caspase-1 and leads to the secretion of IL-1 β [159]. The AIM2 inflammasome is activated after recognition of DNA viruses, cytosolic bacteria or self-DNA [159, 160]. Interestingly RNA virus infection can also trigger this inflammasome. For instance, IAV promotes release of oxidised mtDNA into the cytoplasm of infected macrophages which is then recognised by AIM2 receptor triggering AIM2 inflammasome and IL-1 β secretion [161]. However AIM2 inflammasome activation during IAV infection seems to be cell specific as IL-1 β release is impaired in the absence of AIM2 in primary human alveolar macrophages but not in primary human alveolar type II epithelial cells [162]. The outcome of the AIM2 inflammasome activation for an RNA virus infection is still under debate. One report showed that IAV-infected mice lacking AIM2 had an increased survival rate and showed less inflammation and lung injury [162] whereas another study showed that *Aim2*^{-/-} mice infected with IAV had an enhanced mortality rate and inflammatory responses [163]. However these variations may be due to the differences in IAV stock production and in the dose used as well as the microbiome of the animal [162–164]. Importantly, both studies show that the absence of AIM2 expression doesn't affect viral replication [162, 163].

1.3.2.4 RIG-I inflammasome

During viral infection, RIG-I detects and binds to viral RNA via its C-terminal domain and DExD/H-Box RNA helicase domain, and interacts with MAVS via their CARD domains [**Figure 1.6**]. This induces antiviral responses by activating NF- κ B and IRF3 transcription factors. However two studies have shown that RIG-I can also act as an inflammasome sensor. In mouse bone marrow-derived dendritic cells (BMDCs) infected with vesicular stomatitis virus or stimulated with 5'-triphosphate RNA agonist, RIG-I induces the synthesis of pro-IL-1 β in a MAVS, CARD9, Bcl10 and NF- κ B-dependent manner [50]. In parallel, RIG-I interacts with ASC to activate caspase-1 which then processes pro-IL-1 β into its mature form [50]. This step is independent of MAVS, type I IFN and NLRP3, but seems to require potassium efflux [50]. Interaction between RIG-I and ASC has also been detected in primary human bronchial epithelial cells infected with IAV [165].

1.3.2.5 MxA inflammasome

Mx genes, which are found in almost all vertebrates, are upregulated after binding of IFN to IFNAR receptors and have antiviral properties especially against RNA viruses. Two *Mx* proteins are found in humans and have different functions. While MxA localises in the cytoplasm and is involved in antiviral responses, MxB resides in the nucleus and is implicated in cellular processes. MxA targets different steps of the viral life cycle of different families of viruses, namely negative and positive ssRNA viruses, dsRNA viruses but also dsDNA viruses, and protects the cell from viruses that replicate both in the nucleus and the cytoplasm [166].

A recent IFN-dependent inflammasome involving MxA has been described during IAV infection in respiratory epithelial cells. During viral infection, targeted cells trigger an IFN response to induce an antiviral state within the infected cell and in neighbouring non-infected cells. Once secreted, IFN binds to its receptor and induces the transcription of antiviral ISGs including MxA which, after translation, recognised IAV nucleoprotein in infected cells. MxA was then shown to interact with ASC to form an inflammasome complex which leads to caspase-1 activation and IL-1 β secretion. This MxA-dependent IL-1 β secretion is not found in myeloid cells [147].

1.3.3 Inflammasome assembly

The inflammasome is composed of a sensor, the adaptor protein ASC and the effector pro-caspase-1 [Figure 1.9]. ASC has two domains, namely a N-terminal PYD domain and a C-terminal CARD domain [Figure 1.6]. Pro-caspase-1 is found under an inactive state within the cells and contains the following domains: an amino-terminal CARD domain, a central large catalytic domain (p20) and a carboxy-terminal small catalytic subunit domain (p10) [116] [Figure 1.6].

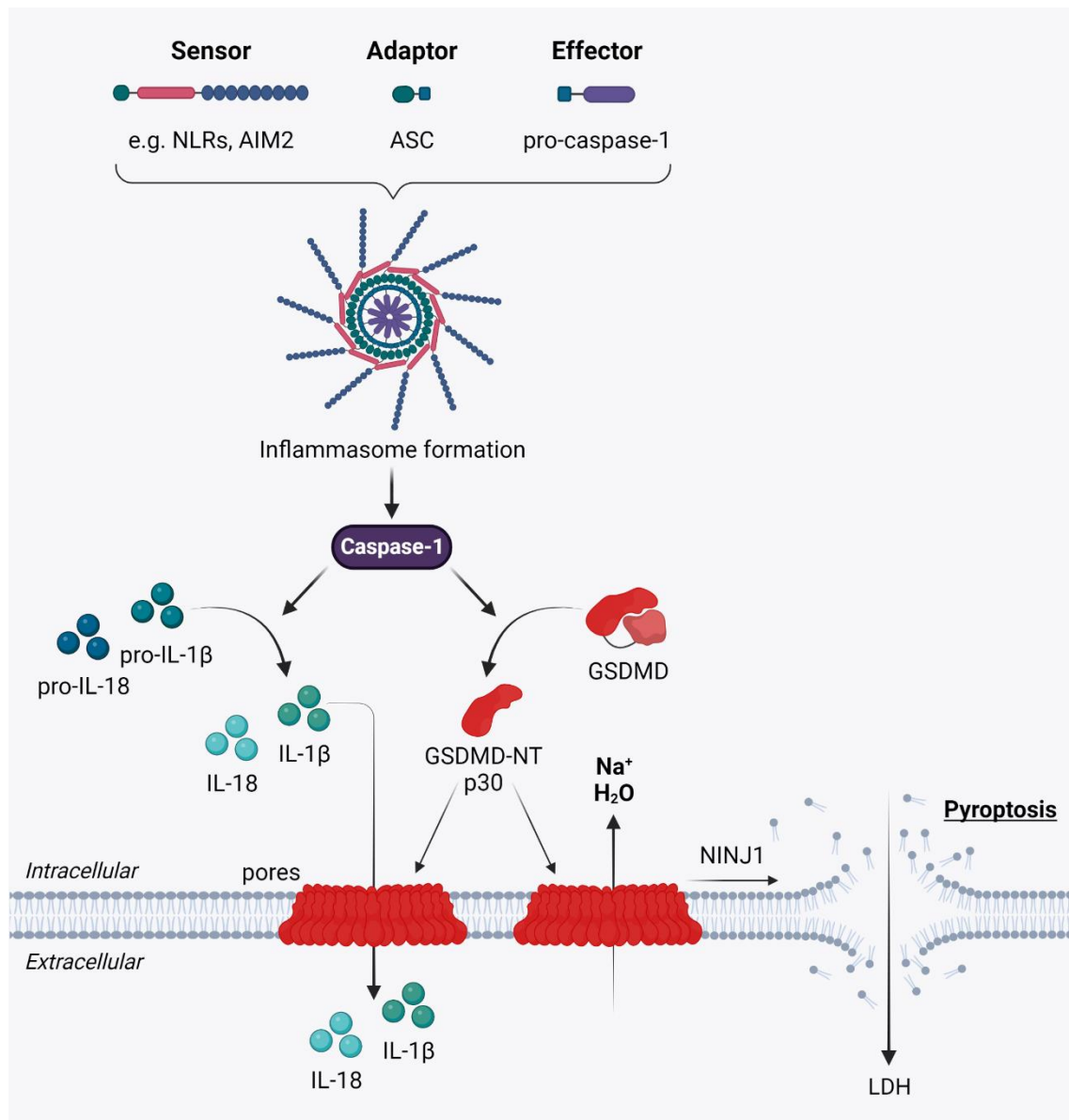


Figure 1.9: Inflammasome formation leads to GSDMD pore formation, cytokine secretion and pyroptotic cell death.

The inflammasome is a multiprotein complex that contains a sensor, the adaptor protein ASC and the effector pro-caspase-1. Autoprocessing of pro-caspase-1 leads to active caspase-1 that cleaves pro-IL-1 β and pro-IL-18 into their mature forms IL-1 β and IL-18. Caspase-1 also processes GSDMD in its linker region to release the N-terminal domain from the repressive C-terminal domain. The N-terminal fragment then inserts to the plasma membrane and oligomerises to form pores that allow the release of mature IL-1 β and IL-18. The entry of sodium and water into the cell induces cell swelling and membrane rupture mediated by NINJ1 leading to pyroptotic cell death. Larger cytosolic contents such as LDH can then be released. Figure created with BioRender.

The first step of the inflammasome formation following DAMPs or PAMPs recognition is the receptor oligomerisation. Nevertheless this step is specific for the sensor. For instance, upon stimulation or infection, NLRP3 NACHT domains interact together which induce receptor oligomerisation [116]. On the contrary, AIM2 protein doesn't contain an oligomerisation domain. It has been shown that DNA bound to AIM2 serves as an oligomerisation platform allowing the formation of AIM2 inflammasome [157]. Next, sensor oligomers interact with ASC *via* their respective PYD domains which results in the polymerisation of ASC into long fragments, known as ASC specks [168]. Since MxA protein doesn't have any PYD domain, the interaction between the oligomerised sensor and the adaptor protein is mediated by the GTPase domain of MxA [167]. In addition hNLRP1 and mNLRP1B bound to ASC through their C-terminal CARD domain instead of their PYD domain [169] which shows a key difference between NLRP1 inflammasome and other NLRP inflammasomes. Finally ASC recruits pro-caspase-1 through CARD-CARD interactions leading to proximity-induced caspase-1 autocleavage and activation [168]. Indeed this proximity increases the local concentration of caspase-1 and facilitates its dimerisation [170]. Nevertheless, while hNLRP1 requires ASC to form an inflammasome, mNLRP1B can either interact with ASC or directly associate with caspase-1 to induce further signaling [171]. Full-length caspase-1 (p46) can cleave selected substrates such as GSDMD but requires a self-cleavage event that forms a p33/p10 complex to be fully active [172]. This complex can then cleave many substrates such as GSDMD, pro-IL-1 β and pro-IL-18. Then a second self-cleavage removes the CARD domain which forms p20/p10 tetramer. The instability of this tetramer abrogates protease activity. The rapidity of caspase-1 deactivation is different according to the cell types and inflammasome complex size [172].

1.3.4 Effector mechanisms of the inflammasome

1.3.4.1 GSDMD pore formation

Inflammasome activation leads to pro-inflammatory cytokine release and/or pyroptosis [**Figure 1.9**]. In 2015 GSDMD protein was identified as the executioner of this programmed lytic cell death [173–175]. GSDMD is composed of a pore-forming N-terminal fragment and a repressive C-terminal fragment bound together by an interdomain linker [**Figure 1.9**]. GSDMD has been identified as a substrate of the inflammatory caspases, namely human caspase-1, -4, -5 or mouse caspase-11 [173, 174]. Thus following the inflammasome assembly, the inflammatory caspases cleave GSDMD within the interdomain

linker at position Asp275 releasing the N-terminal domain from the autoinhibitory C-terminal domain. The N-terminal domain then binds to phosphatidylinositol phosphates and phosphatidylserine present in the inner leaflet of the cell membrane, and cardiolipin in mitochondria [176] leading to oligomerisation of the N-terminal fragment. The resulting pre-pore configuration inserts into the lipid bilayer to form a pore of 10 to 20 nm [177, 178] causing cytosolic contents release including mature IL-1 β and IL-18. Simultaneously, extracellular sodium gets into the cell through GSDMD pores leading to water influx, which results in cell swelling and plasma membrane rupture [174] **[Figure 1.9]**. Pyroptosis allows the release of larger cytosolic content such as lactate dehydrogenase (LDH) [179] or ASC specks which increase maturation of IL-1 β [180]. The release of the GSDMD N-terminal domain during pyroptosis doesn't induce cell death in the bystander cells given that this fragment binds to the inner leaflet of the cell membrane [176]. Until recently, this lytic cell death was thought to be a passive event but the discovery of ninjurin-1 (NINJ1) protein that acts as a mediator of the plasma membrane rupture showed that pyroptosis is actively regulated [181] **[Figure 1.9]**. GSDM-mediated pyroptosis could either be protective or detrimental for the host (further described in **section 5.1**). Nevertheless the formation of GSDMD pores doesn't necessarily leads to cell membrane rupture and thus allows cytokine secretion from living cells [182]. In addition, GSDMD pores induce calcium influx that recruits the endosomal sorting complexes required for transport-III (ESCRT-III) repair machinery at the plasma membrane which prevents pyroptotic cell death by removing GSDMD pores *via* exocytosis [183].

1.3.4.2 Mechanisms of GSDMD-dependent cytokine release

Inflammasome-dependent caspase-1 activation leads to the maturation of the pro-inflammatory cytokines IL-1 β and IL-18 **[Figure 1.9]**. Many cytokines are released through conventional secretory pathways; however, the IL-1 and IL-18 cytokines lack a signal sequence meaning that they are secreted *via* an unconventional protein secretion pathway. Many hypothesis have been proposed to explain the secretion of these cytokines [184]. The discovery of the GSDMD pores formation in 2015 brought major change in the field implicating as it did a role for these pores in IL-1 β and IL-18 cytokine secretion [174, 175]. This secretion was first thought to be dependent on GSDMD-mediated pyroptosis [174], however IL-1 β secretion can be uncoupled from cell death and be secreted from living cells during the sublytic phase in a GSDMD-dependent manner [182]. Thus the current understanding of IL-1 secretion is that GSDMD pores, and not necessarily pyroptotic cell

death, is required [Figure 1.9]. IL-1 β maturation triggers its relocalisation at the plasma membrane in the proximity of GSDMD pores [185]. Indeed positively charged mature IL-1 β associates with negatively charged phosphatidylinositol 4,5-bisphosphate containing plasma membrane where the cytokine can be released through negatively charged GSDMD pores [185, 186]. The presence of acidic residues in the pro domain deters the release of pro-IL-1 β through GSDMD despite its small size [185, 186]. Similar mechanism is involved in IL-18 secretion [186]. However the presence of acidic residues in both pro-IL-1 α and IL-1 α suggests that this cytokine isn't released through GSDMD pores [186]. Nevertheless these pores are important for IL-1 α maturation since calcium influx through GSDMD pores activates calpain which then cleaves pro-IL-1 α into its mature form [187].

1.4 Aims

Inflammasomes are multiprotein complexes activated after infection or cellular stress that induce the secretion of IL-1 β and/or pyroptotic cell death. To date it is assumed that it is mainly the NLRP3 inflammasome and GSDMD pores that are formed during RNA virus infection. However most of the studies have been done in macrophage mouse cells and to a lesser extent in human myeloid cells, even though airway epithelial cells are the first cells that sense the presence of respiratory viruses. In addition the function of GSDM in innate immune responses to respiratory viral infection in barrier epithelial cells is still unclear. Thus it is crucial to investigate the mechanism of inflammasome activation and GSDM pore formation in response to viruses and viral PAMPs in respiratory epithelial cells.

Thus the aims of this project are:

1. Define the best cellular model to study inflammasome activation in human undifferentiated respiratory epithelial cells;
2. Determine which inflammasome and GSDM are activated during viral dsRNA delivery and respiratory virus infection in basal respiratory epithelial cells;
3. Dissect molecular mechanisms of GSDM activation in basal respiratory epithelial cells;
4. Assess the contribution of inflammasomes and GSDM pores in IL-1 β and IL-1 α secretion in basal respiratory epithelial cells.

Chapter 2. Materials and methods

2.1 Materials

2.1.1 Buffers and solutions

Buffer	Composition
10X phosphate buffered saline (PBS)	1.45 M NaCl
	39 mM NaH ₂ PO ₄
	227 mM Na ₂ HPO ₄
10X tris-buffered saline (TBS)	1.37M NaCl
	250 mM Tris
	27 mM KCl pH 7.5
10X running buffer - Western blot	250 mM Tris
	1.9 M Glycine
	35 mM SDS
10X transfer buffer - Western blot	250 mM Tris
	1.9 M Glycine
1X transfer buffer - Western blot	10 % (v/v) 10X transfer buffer
	20 % (v/v) Methanol
Washing buffer - Western blot	1X PBS or TBS/0.1 % (v/v) Tween
Washing buffer - ELISA	1X PBS/0.05 % (v/v) Tween
3X sample buffer - Western blot	30 % (v/v) Glycerol
	6 % (w/v) SDS
	0.3 % (w/v) Bromophenol blue
	187.5 mM Tris pH 6.8
Crystal violet	9.5 % Crystal violet (1 % solution in water)
	19 % EtOH
Lysis oligomerisation buffer	50 mM HEPES
	100 mM NaCl
	1 mM EDTA
	10 % Glycerol
	1% NP40
	1% Aprotinin
	1 mM Sodium orthovanadate
	1 mM PMSF – <i>Add just prior to use</i>
Flu Overlay	0.7X MEM
	0.3 % BSA V (7.5 % solution)
	2.8 mM L-Glutamine
	0.2 % Sodium bicarbonate (7.5 % solution)
	14 mM 1 M HEPES
	0.007 % DEAE dextran
	142 U/ml penicillin
	142 µg/ml streptomycin

Table 2.1: Buffers and solutions.

2.1.2 Cells and cell culture

2.1.2.1 Cells

THP-1 and A549 cells were purchased from the European Collection of Cell Cultures. BEAS-2B cells were a kind gift from Dr Sinead Miggin (National University of Ireland Maynooth, Ireland). NuLi-1 cells were kindly provided by Prof Jose Bengoechea (Queen's University Belfast, Northern Ireland). Vero-118 cells were a kind gift from Dr Bernadette Van den Hoogen (Erasmus MC, The Netherlands). Madin-Darby canine kidney (MDCK) cells were kindly provided by Dr Kim Roberts (Trinity College Dublin, Ireland). HEK-Blue IFN- α/β cells were kindly provided by Prof Luke O'Neill (Trinity College Dublin, Ireland). Primary normal human bronchial epithelial (NHBE) cells with retinoic acid, were purchased from Lonza. Two different NHBE cells from healthy donors were used: a 66-year-old Hispanic female and a 56-year-old Caucasian male. In this project, experiments were performed in undifferentiated NHBE cells and not in differentiated NHBE cells at air liquid interface. No significant differences in cellular responses to stimulants and infections were observed between the two donors.

2.1.2.2 Cell culture reagents

Dulbecco's Modified Eagle's Medium (DMEM) with GlutaMAX, Roswell Park Memorial Institute (RPMI) 1640 with GlutaMAX, Iscove's Modified Dulbecco's Medium (IMDM), 10X Minimum Essential Medium (MEM), Opti-MEM, bovine albumin fraction (BSA) V (7.5 % solution), sodium bicarbonate (7.5 % solution), fetal calf serum (FCS) and L-glutamine were purchased from Gibco. Bronchial epithelial cell growth basal medium (BEBM) complete with BEGM SingleQuots kit and subculture reagents for NHBE cells, namely HEPES, trypsin-EDTA and trypsin neutralizing solution, were purchased from Lonza. Trypsin inhibitor, for NuLi-1 cell subculture, was purchased from Roche. PBS, HEPES, dimethyl sulfoxide (DMSO), trypsin-EDTA, penicillin-streptomycin, human placental collagen type IV and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich. Normocin, blasticidin, zeocin, lipofectamine 2000 and QUANTI-Blue were purchased from Invivogen. CytoTox 96 non-radioactive cytotoxicity assay was purchased from Promega. Cell culture plastics were purchased from Corning Life Sciences and Greiner Bio-One.

2.1.2.3 Cell culture ligands

Ligand	Receptor	Concentration	Company
SA-LTA	TLR2	2.5 µg/ml	Invivogen
High molecular weight poly(I:C)	Naked: TLR3; Transfected: cytosolic receptors	2.5 µg/ml or 10 µg/ml (detailed in figure legends)	Invivogen
Low molecular weight poly(I:C)	Naked: TLR3; Transfected: cytosolic receptors	2.5 µg/ml or 10 µg/ml (detailed in figure legends)	Invivogen
LPS from <i>E.coli</i> , serotype EH100	TLR4	200 ng/ml	Enzo Life Sciences
3p-hpRNA	RIG-I	100 ng/ml	Invivogen

Table 2.2: List of ligands and their receptors.

2.1.2.4 Cell culture inhibitors

Inhibitor	Target	Concentration	Company
Ac-YVAD-cmk	Caspase-1	25 µM	Sigma-Aldrich
VX-765	Caspase-1	20 µM	Selleckchem
Z-DEVD-FMK	Caspase-3	20 µM	MBL
Z-IETD-FMK	Caspase-8	20 µM	MBL
Z-LEHD-FMK	Caspase-9	20 µM	MBL
Z-VAD-FMK	Pan-caspase	25 µM	Sigma-Aldrich
GSK'872	RIPK3	3 µM	Merck
MCC950	NLRP3	25 µM	Prof Luke O'Neill laboratory
C16	PKR	2 µM	Sigma-Aldrich
Cycloheximide	Translation	50 µg/ml	Fisher Scientific

Table 2.3: List of inhibitors and their target.

2.1.2.5 Inflammasome inducers

Inducer	Inflammasome	Concentration	Company
Nigericin	NLRP3	20 µM	Invivogen
poly(dA:dT)	AIM2	2.5 µg/ml	Sigma-Aldrich
Val-boroPro	NLRP1 and CARD8	3 µM	Tocris

Table 2.4: List of inflammasome inducers and the inflammasome activated.

2.1.3 Virus strains

RSV A2 strain was a kind gift from Dr Bernadette Van den Hoogen (Erasmus MC, The Netherlands). Influenza A/WSN/33 (H1N1) was a kind gift from Prof Ron Fouchier (Erasmus MC, The Netherlands).

2.1.4 Virus propagation and titration

Sucrose was purchased from Sigma-Aldrich. 35 ml centrifuge tubes for viral purification were purchased from Beckman. For immunofluorescence titration, primary antibody RSV 2F7 was purchased from Santa Cruz (sc-101362) and FITC-labelled secondary antibody was purchased from Dako (F0313).

2.1.5 Small interfering RNA (siRNA) target sequence

Gene	Sequence	Concentration	Company
ASC	CGGGAAGGTCCTGACGGATGA	1 nM	Qiagen
MAVS	T ^T AAAGGAGT ^T TATCGATGTA	5 nM	Qiagen
TRIF	CAGGACGCCATAGACCACTCA	3 nM	Qiagen
NLRP1	CAGGGTGGAGCTGCATCACAT	3 nM	Qiagen
GSDMA	CCGGTACGTCCGCACCGACTA	3 nM	Qiagen
GSDMB	CACCAGTGGGACCTCT ^T TAAA	3 nM	Qiagen
GSDMC	ATGGGTAGAGACT ^T TAGGAAA	3 nM	Qiagen
GSDMD	CAGGAGCT ^T TCCACT ^T TCTACGA	3 nM	Qiagen
GSDME	GCGGTCCTATT ^T TGATGATGAA	3 nM	Qiagen

Table 2.5: List of siRNA target sequences.

2.1.6 Enzyme-linked immunosorbent assay (ELISA)

Human IL-1 β (IL-1F2), IL-1 α (IL-1F1), IL-6 and IP-10 (CXCL10) DuoSet ELISA kits were purchased from R&D Systems Europe Ltd. BD OptEIA™ TMB substrate reagents set was purchased from BD Biosciences. BSA was purchased from Sigma-Aldrich. High binding 96-well plates were purchased from SPL Life Sciences Ltd.

2.1.7 Quantitative real-time polymerase chain reaction (qRT-PCR)

High Pure RNA Isolation kits were obtained from Roche. dNTPs were purchased from New England Biolabs. M-MLV reverse transcriptase and 5x M-MLV buffer were purchased from Promega. RNaseOUT was purchased from Invitrogen. 8-strip PCR tubes and PCR tubes caps were purchased from Fisher Scientific. PowerUp SYBR green master mix, qPCR plate seals and MicroAmp fast optical 96-well qPCR plates were purchased from Applied Biosystems. Random hexamers and primers were synthesised by IDT. The primer sequences used for qRT-PCR are listed in **Table 2.6**.

Gene	Sequence 5'-3'
<i>gapdh</i>	F: TCTTTTGGCTCGCCAGCCGAG R: ACCAGGCGCCCAATACGACCA
<i>il1β</i>	F: GGAGCAACAAGTGGTGTCTCTCC R: TGGGATCTACACTCTCCAGCTGTA
<i>il1a</i>	F: GGATGAAGCAGTGAAATTTGACATGGG R: CAGGCATCTCCTTCAGCAGCA
<i>ticam1</i> (TRIF)	F: GGATCCCTGATCTGCTTGGGC R: TGGAGGTGGTGAAGGCATGTTC
<i>mavs</i>	F: GTGCCTACTAGCATGGTGCTC R: GACCCAAGGCCCTATCT
<i>pypard</i> (ASC)	F: ATCCAGGCCCTCCTCAGT R: CGTTTGTGACCCTCGCGATAAGC
<i>nlrp1</i>	F: CTTTCAGCAGACGGAAACCAAGTGT R: CCGCCCTCTCTGATCCGA
<i>gsdma</i>	F: TTGAGCTCGCACTTGAAGGGG R: GGCTTCACCTAGCTCTGTTAGGGC
<i>gsdmb</i>	F: GCCCTGCTAGAGCTGTCTG R: GACAGATTTCACCTGGTCCTTCAAC
<i>gsdmc</i>	F: AGCCATAATGGTGCTGAGTGACTT R: GATGCTCCTTACCAGCTCCTGTTG
<i>gsdmd</i>	F: CAGCACCTCGCATTCGG R: AGAGAAGGACGTCCAAGTCAGAGTC
<i>gsdme</i>	F: CCAAGACGGTGCAGGTGTCAG R: AGAACTCGAACTGGCCGTCCAG

Table 2.6: List of primer sequences used for qRT-PCR.

2.1.8 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

HEPES, glycerol, NP-40, aprotinin, sodium orthovanadate, phenylmethanesulfonyl fluoride (PMSF), glycine, tris, sodium dodecyl sulphate (SDS), ammonium persulfate (APS), N,N,N',N'-Tetramethylethylenediamine (TEMED), bromophenol blue, tween 20 and skimmed milk were purchased from Sigma-Aldrich. Protogel acrylamide was purchased from National Diagnostics. Disuccinimidyl suberate (DSS), dithiothreitol (DTT), pageRuler plus prestained protein ladder, 3MM Whatmann filter papers and nitrocellulose membrane were purchased from Fisher Scientific. StrataClean resin was purchased from Agilent Technologies. A list of primary antibodies used for immunoblotting is given in **Table 2.7**. IRDye 680 RD and 800 CW secondary antibodies were purchased from LI-COR.

Antibody, anti-	Specie	Dilution	Company	Catalogue number
β -actin	Mouse	1 in 5000	Sigma-Aldrich	A5316
Histone H3	Mouse	1 in 1000	Upstate technologies	05-499
IL-1 β	Goat	1 in 1000	R&D systems	AF-201-NA
IL-1 α	Rabbit	1 in 1000	PeptoTech	500-P21A
GSDMD	Rabbit	1 in 1000	Sigma-Aldrich	HPA044487
GSDME	Rabbit	1 in 1000	Abcam	ab215191
Caspase-1	Mouse	1 in 1000	Adipogen	AG-20B-0048
Cleaved caspase-3	Rabbit	1 in 1000	Cell Signaling	9661
ASC	Mouse	1 in 500	Santa Cruz	sc-514414
MAVS	Mouse	1 in 500	Santa Cruz	sc-166583
TRIF	Rabbit	1 in 500	Cell Signaling	4596
AIM2	Mouse	1 in 1000	Adipogen	AG-20B-0040
NLRP3	Mouse	1 in 1000	AdipoGen	AG-20B-0014
NLRP1	Mouse	1 in 200	BioLegend	679802
Total PKR	Rabbit	1 in 100	Santa Cruz	sc-708
Phospho-PKR	Rabbit	1 in 1000	Abcam	ab32036
RIG-I	Rabbit	1 in 1000	Cell Signaling	3743
MDA-5	Rabbit	1 in 1000	Cell Signaling	5321
H1N1 IAV nucleocapsid	Rabbit	1 in 1000	Abcam	ab104870

Table 2.7: List of antibodies used in immunoblotting.

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Maintaining and differentiating THP-1 cells

The non-adherent human monocytic THP-1 cell line was grown in RPMI 1640 with GlutaMAX medium containing 10% (v/v) FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml normocin and cells were kept at 37°C with 5 % CO₂. Cells were split or plated out by centrifugation of the cell suspension at 220 g for 5 min. The supernatant was discarded, and cells were resuspended in fresh pre-warmed complete RPMI with GlutaMAX medium. Cells were either split into 75 cm² flasks or counted using an automated cell counter (Biorad, TC20). For experiments, THP-1 cells were seeded at a density of 5x10⁵ cells/ml in culture plates and were differentiated for 48 h into a macrophage-like phenotype by adding 100 nM PMA to the cell suspension.

2.2.1.2 Maintaining and seeding Vero118 cells

Adherent monkey Vero118 cells were grown in IMDM medium supplemented with 10 % (v/v) FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Cells were kept at 37°C with 5 % CO₂ and were split or plated out upon reaching 80-90 % confluency. In order to do so, the medium was removed and cells were washed once with 1X PBS. Then trypsin-EDTA was added and the flask was incubated at 37°C for 5 min. Then pre-warmed complete IMDM medium was added and cells were centrifugated at 220 g for 5 min. The supernatant was discarded, and cells were resuspended in fresh pre-warmed complete IMDM medium. Cells were either split into 75 cm² flasks or counted using an automated cell counter (Biorad, TC20). Vero118 cells were seeded at a density of 1x10⁵ cells/ml in 96-well plates and were incubated overnight at 37°C with 5 % CO₂.

2.2.1.3 Maintaining and seeding HEK-Blue IFN α / β cells

Adherent HEK-Blue IFN α / β cells were grown in DMEM with GlutaMAX medium supplemented with 10 % (v/v) FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml normocin, 30 µg/ml blasticin, and 100 µg/ml Zeocin (growth medium). Cells were kept at 37°C with 5 % CO₂ and were split or plated out upon reaching 70-80 % confluency. In order to do so, the medium was removed and cells were washed two times with pre-warmed 1X PBS. Then cells were detached in the presence of PBS by tapping the

flask and the cell suspension was centrifugated at 220 g for 5 min. The supernatant was discarded and cells were resuspended either in fresh pre-warmed growth medium for further passages or in fresh pre-warmed test medium (DMEM with GlutaMAX medium supplemented with 10 % (v/v) FCS, 100 U/ml penicillin and 100 µg/ml streptomycin) for experiments. For the HEK-Blue IFN α /β bioassay, HEK-Blue IFN α /β cells were counted using an automated cell counter (Biorad, TC20) and were seeded at a density of 2.8×10^5 cells per well in 96-well plates and were incubated overnight at 37°C with 5 % CO₂.

2.1.1.4 Maintaining and seeding A549, BEAS-2B and MDCK cells

Adherent human epithelial cell lines A549 and BEAS-2B cells, and adherent MDCK cells were grown in DMEM with GlutaMAX medium supplemented with 10 % (v/v) FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml normocin. Cells were kept at 37°C with 5 % CO₂ and were split or plated out upon reaching 80-90 % confluency. In order to do so, the medium was removed and cells were washed one time with 1X PBS. Then trypsin-EDTA was added and the flask was incubated at 37°C for several minutes. Once cells were detached, pre-warmed complete DMEM with GlutaMAX medium was added and cells were centrifugated at 220 g for 5 min. The supernatant was discarded and cells were resuspended in fresh pre-warmed complete DMEM with GlutaMAX medium. Cells were either split into 75 cm² flasks or counted using an automated cell counter (Biorad, TC20). A549 cells were seeded at a density of 5×10^4 cells/ml in 24-well plates. BEAS-2B cells were seeded at a density of 8×10^4 cells/ml in 24-well plates. MDCK cells were seeded at a density of 2×10^5 cells/ml in 12-well plates for IAV titration. Cells were incubated overnight at 37°C with 5 % CO₂.

2.2.1.5 Maintaining and seeding NuLi-1 cells

All the flasks, plates and petri dishes were pre-coated with 60 µg/mL sterile solution of human placental collagen type IV for 24 h, then washed three times with 1X PBS and air-dried and stored at 4°C. Adherent NuLi-1 cell line was grown in BEBM medium completed with supplements and growth factors (Lonza). Cells were kept at 37°C with 5 % CO₂ and were split or plated out upon reaching 80 % confluency. In order to do so, the medium was removed, cells were washed one time with 1X PBS and trypsin-EDTA was added. Cells were incubated at 37°C for 5 min. Then trypsin inhibitor was added and cell suspension was centrifuged at 220 g for 5 min. The supernatant was discarded and cells were resuspended in

fresh pre-warmed complete BEBM medium. Cells were either split into 75 cm² flasks or counted using an automated cell counter (Biorad, TC20). NuLi-1 cells were seeded at a density of 1.5×10^5 cells/ml in 24-well plates and were incubated overnight at 37°C with 5 % CO₂.

2.2.1.6 Maintaining and seeding primary NHBE cells

Adherent undifferentiated primary NHBE cells were grown in BEBM completed with supplements and growth factors (Lonza) until passage 6 and cultured following the manufacturer's instructions. Cells were kept at 37°C with 5 % CO₂ and were split or plated out upon reaching 80 % confluency, namely every four days. In order to do so, the medium was removed, cells were washed one time with HEPES and trypsin-EDTA solution was added. Cells were incubated at 37°C for 5 min maximum. Then trypsin neutralizing solution were added and cell suspension was centrifuged at 220 g for 5 min. The supernatant was discarded and cells were resuspended in fresh pre-warmed complete BEBM medium. Cells were either split at a density of 3×10^5 cells per 75 cm² flasks or counted using an automated cell counter (Biorad, TC20). NHBE cells were seeded at a density of 3.5×10^4 cells per well in 24-well plates or at a density of 1.5×10^5 cells per well in 6-well plates and were incubated for three days at 37°C with 5 % CO₂. Medium was refreshed the day after seeding.

2.2.2 Stimulation and inoculation of cells

2.2.2.1 Stimulation, transfection and inhibition of cells

Before cell stimulation, the medium was removed and appropriate serum free medium (SFM) was added for all cell types except NHBE or NuLi-1 when fresh BEGM was added. All the TLR ligands (LTA, poly(I:C) and LPS) and inflammasome inducers (nigericin, VBP and poly(deoxyadenylic-deoxythymidylic acid sodium salt (poly(dA:dT))) were diluted in SFM or Opti-MEM as required and were added directly onto the cells at appropriate concentrations. Poly(I:C), 3p-hpRNA and poly(dA:dT) were transfected using lipofectamine 2000. For transfection, for every ml of cells, a mix containing 50 µl SFM and 1 µl of lipofectamine was made in a 1.5 ml microcentrifuge tube and was allowed to stand for 5 min at room temperature. In another 1.5 ml microcentrifuge tube, the appropriate amount of RNA or DNA ligand was added into 50 µl of SFM. After 5 min, the two tubes were mixed and the resulting mix was incubated for a further 20 min at room temperature before addition

to the cells. All inhibitors were diluted in SFM if required and were added directly onto the cells at appropriate concentrations 1 h prior to stimulation or infection. Inhibitors were not washed off cells during the entire experiment.

2.2.2.2 RSV A2 and IAV WSN inoculation

NHBE cells were seeded in a 24-well plate or a 6-well plate. Since NHBE cells were allowed to grow for three days before experiments, an extra well was seeded to count the cells before inoculation. The volume of virus needed for an experiment was calculated as follows:

$$\text{volume of virus (ml)} = \frac{\text{number of cells per well} * \text{MOI}}{\text{viral titre}}$$

The multiplicity of infection (MOI) used is indicated in the figure legends. The inoculum consisted of a mix containing the required amount of virus and BEGM media. The medium was removed, cells were gently washed with 1X PBS and the inoculum was added to the cells. Cells were incubated at 37°C with 5 % CO₂ for 1 h for IAV WSN and for 2 h for RSV A2. The inoculum was then removed, cells were gently washed with 1X PBS and BEGM was added. Cells were then incubated at 37°C with 5 % CO₂ for the time indicated in the figure legends.

2.2.2.3 RSV A2 replication kinetics

NHBE cells were seeded in 25 cm² flasks. The medium was removed and cells were gently washed with 1X PBS. The inoculum consisted of a mix containing RSV A2 at MOI 1 and BEGM media. Cells were incubated for 2 h at 37°C with 5 % CO₂. Then the inoculum was removed, cells were gently washed with 1X PBS three times and BEGM was added. 100 µl of cell supernatants were harvested and mixed with 100 µl 50 % (w/v) sucrose at 2 h, 12 h, 24 h, 48 h and 72 h post inoculation and stored at -80°C until further use. Viral titre was determined using 50 % tissue culture infective dose (TCID₅₀) method (detailed in **section 2.2.3.3**).

2.2.2.4 IAV WSN replication kinetics

NHBE cells were seeded in 6-well plates. The medium was removed and cells were gently washed with 1X PBS. The inoculum consisted of a mix containing IAV WSN at MOI 0.1 and BEGM media. Cells were incubated for 1 h at 37°C with 5 % CO₂. Then the inoculum was removed, cells were gently washed three times with 1X PBS and BEGM was added. 100 µl supernatants were harvested at 2 h, 8 h, 24 h and 48 h post inoculation and stored at -80°C until further use. Viral titre was determined using the plaque assay method (detailed in **section 2.2.3.5**).

2.2.3 Propagation and titration of RSV A2 and IAV WSN stocks

2.2.3.1 Propagation of RSV A2 stock

The day before inoculation, ten 175 cm² flasks of Vero118 cells were seeded from four 175 cm² flasks in IMDM medium supplemented with 10 % (v/v) FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Cells were incubated overnight at 37°C with 5 % CO₂. The next day, cells were gently washed with 1X PBS and inoculated with RSV A2 passage 1 stock at MOI 0.01 in 25 ml of IMDM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Cells were returned to the incubator and medium was refreshed every other day. Cells were harvested when 80 % cytopathic effect was reached. To avoid the formation of defective interfering particles, the medium was refreshed the night before harvesting. The following day the cells were scraped, and the medium containing virus particles and cells was transferred to 50 ml tubes and frozen at -80°C until purification. RSV passage 1 stock was produced by reverse genetics in the laboratory of Dr Bernadette Van den Hoogen (Erasmus MC, The Netherlands).

2.2.3.2 Purification of RSV A2 stock

In the week of harvesting, a sucrose gradient purification of RSV A2 stock was performed. After being thawed, 50 ml tubes were centrifugated at 800 g for 10 min at 4°C. Supernatants were added to 35 ml centrifuge tubes containing 0.5 ml of 60 % (w/v) sucrose on the bottom. The tubes were then placed into the ultracentrifuge buckets, placed on a scale and topped up with basal medium or 1X PBS to have the exact same weight. Tubes were centrifugated at 27,000 rpm for 2 h at 4°C with brakes at the maximum. Then 30 ml of supernatant was removed and the pellet was resuspended in remaining medium. In new

35 ml centrifuge tubes, 10 ml of 30 % (w/v) sucrose was added on top of a 0.5 ml of 60 % (w/v) sucrose layer and the virus suspension was added to the gradient. Tubes were then placed into the ultracentrifuge buckets, placed on a scale and topped off with basal medium or 1X PBS to have the exact same weight. Tubes were centrifugated at 27,000 rpm for 2 h at 4°C with brakes at the maximum (scale 9). Then 30 ml of supernatant were removed and the pellet was resuspended in IMDM supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin and 2 mM glutamine at ratio 1:1. The virus suspension was transferred to a 50 ml tube, sonicated three times for 10 seconds in ice cold water, aliquoted in cryovials and kept at -80°C.

2.2.3.3 Titration of RSV A2 stock using TCID₅₀ method

Vero118 cells were seeded in 96-well plates at concentration 1×10^5 cells/ml and incubated overnight at 37°C with 5 % CO₂ to form a confluent monolayer. The next day, 10-fold serial dilutions of virus stock were prepared in IMDM containing 2 % (v/v) FCS and each dilution was done in triplicate. Cells were gently washed with 1X PBS and 100 µl of the virus dilution was added to the cells. Cells were incubated for three days at 37°C with 5 % CO₂. Then cell supernatant was removed, cells were gently washed with 1X PBS and 50 µl of 80 % (v/v) acetone was added. Cells were incubated at least 15 min at -20°C. After this step, all the procedure was continued outside of a BSL2 cabinet. After 15 min, acetone was removed in a fume hood and cells were washed once with 1X PBS. Primary antibody (RSV 2F7, sc-101362) diluted in PBS was added for 45 min to 1 h and cells were incubated at 37°C with 5 % CO₂. Then cells were washed once with 1X PBS. Secondary antibody (anti-mouse FITC, Dako F0313) diluted in PBS was added for 45 min to 1 h and cells were incubated at 37°C with 5 % CO₂. Then cells were washed once with 1X PBS. Finally endpoint dilution was determined using a fluorescence microscope and Reed-Muench method was used to calculate viral titre.

2.2.3.4 Propagation and titration of IAV WSN stock

PhD student Pau Ribó-Molina from the team of Prof Ron Fouchier (Erasmus MC, The Netherlands) propagated IAV WSN in MDCK cells and determined viral titre by hemagglutination assay as previously described [188, 189].

2.2.3.5 Measurement of IAV WSN viral titre using the plaque assay method

MDCK cells were seeded in 12-well plates at a concentration 2×10^5 cells/ml and incubated overnight at 37°C with 5 % CO₂ to form a confluent monolayer. The next day, 10-fold serial dilutions of the virus stock were prepared in serum free DMEM with GlutaMAX medium. Cells were gently washed with 1X PBS and 100 µl of the virus dilution was added to the well. Each dilution was used in duplicate to inoculate cells. Cells were incubated for 1 h at 37°C with 5 % CO₂. Every 15 min, the plate was tapped to spread the virus evenly over the cells and to prevent the cells from drying out. After 1 h incubation, 1X PBS was added to the cells. Then 2 % (w/v) agarose (Oxoid) was melted, and inoculum in PBS was removed. For every 12-well plate, 7.5 ml of melted agarose was added to 17.5 ml of warm Flu Overlay and 1 ml overlay was added per well. Agarose was allowed to set, and plates were placed upside down at 37°C with 5 % CO₂ for three days to enable plaques to form. After three days, plaques were counted before crystal violet staining. Then agarose was removed, and cells were stained with crystal violet for 20 min. Crystal violet was discarded, cells were washed with water to remove remaining crystal violet, and plaques were recounted. Viral titre was calculated using the following equation:

$$\text{Viral titre (PFU/ml)} = \frac{\text{mean of number of plaques} \times \text{dilution}}{0.1}$$

Where mean of number of plaques is the mean of the plaques count of the duplicated wells; dilution is the serial dilution used to count the plaques; 0.1 is the volume in ml of virus dilution used to inoculate the cells.

2.2.4 siRNA silencing

siRNA silencing was performed following a “two hit protocol”: siRNA were transfected using lipofectamine 2000 at both 24 h and 48 h after NHBE cells seeding. The day after seeding, the medium was removed and fresh BEGM medium was added. A transfection mix consisting of lipofectamine 2000, the required concentration of siRNA (given in **Table 2.5**) and Opti-MEM was prepared as described in **section 2.2.2.1**. The mix was then added to the wells and the plate was incubated at 37°C for further 24 h. The following day, the siRNA transfection was repeated, without changing the medium, and the plate was incubated at 37°C for further 24 h. The following day, the plate was used for experiments.

2.2.5 LDH assay

LDH assay was performed using CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's protocol. Briefly, lysis buffer (100 μ l per 1 ml of cell supernatant) was added to untreated cells to elicit maximum LDH release (100 % positive control) 30 min prior to harvesting. At the end of the experiment, cell supernatants were transferred into a new 96-well plate and centrifugated at 400 g for 5 min. 40 μ l of cell supernatant and positive control were added into a new 96-well plate and 40 μ l of CytoTox 96 reagent was added. The reaction plate was kept in the dark and was incubated until the positive control turned red. The reaction was stopped by addition of 40 μ l of Stop solution. NB: for infected cells, all the steps were performed in a class II biological safety cabinet in a CL2 lab. The absorbance was measured at 490 nm using a VersaMax microplate reader (Molecular Devices) and analysed with SoftMax Pro v7.0.3 software. The data were normalised to untreated lysed cells which were set at 100%. All experiments were performed in triplicate wells for each condition.

2.2.6 PI uptake

To monitor membrane permeability as a surrogate marker for GSDM pore formation after poly(I:C) transfection, NHBE cells were seeded in a 24-well plate. 1 μ l of propidium iodide (PI, Invitrogen, 1 μ g/ml) was added to the cells directly after transfecting poly(I:C). The plate was then placed in an incubator containing IncuCyte S3 live-cell analysis system (Sartorius) that allows automated fluorescence imaging. The number of red cells, which are PI positive cells, was acquired over a 20-hour period and analysed with IncuCyte S3 v2019B software.

2.2.7 ELISA

Human IL-1 β /IL-1F2, IL-1 α /IL-1F1, IL-6 and IP-10 (CXCL10) protein concentrations in cell supernatants were detected using the appropriate DuoSet R&D ELISA kit following the manufacturer's instructions and corresponding lot-specific Certificate of Analysis. ELISA was performed in a high binding 96-well plate (Greiner Bio-One). The plate was coated with 50 μ l of capture antibody diluted in 1X PBS in each well and incubated overnight at 4°C. The plate was then washed three times with ELISA wash buffer (1X PBS/0.05 % (v/v) Tween) and tapped dry. Non-specific protein binding was blocked with 200 μ l of reagent diluent (1X PBS/1% (w/v) BSA) per well for 1 h at room temperature.

Blocking solution was then removed and the plate was tapped dry. Standards were prepared at the appropriate concentration in triplicates and were serially diluted 1 in 2 to generate a standard curve. Blanks containing reagent diluent alone were included. 50 µl of cell supernatants were added to the plate at the appropriate dilution in triplicate. The plate was incubated for 2 h at room temperature and then washed three times and tapped dry. Detection antibodies were diluted in reagent diluent and 50 µl were added per well. The plate was incubated for 2 h at room temperature followed by washing and drying. Next 50 µl of streptavidin-HRP diluted in reagent diluent was added to each well and the plate was incubated in the dark for 20 min at room temperature followed by washing and drying. Finally, 50 µl of 1:1 mixture of TMB substrate solution (BD biosciences) was added to each well until the standards were visible and the reaction was stopped by the addition of 25 µl of 0.16 M sulphuric acid (H₂SO₄). NB: for virus containing samples, all the steps from the addition of standards and samples to the revelation were performed in a class II biological safety cabinet in a CL2 lab. The plate was read using a VersaMax microplate reader (Molecular Devices) at absorbance 450 nm and 540 nm. The data were analysed using SoftMax Pro v7.0.3 software.

2.2.8 HEK-Blue IFN α / β bioassay

IFN α / β concentration was quantified using HEK-Blue IFN α / β cells (Invivogen) according to the manufacturer's protocol. Human IFN β was used as standard and was serially diluted 1 in 2 in test medium (DMEM with GlutaMAX medium supplemented with 10 % (v/v) FCS, 100 U/ml penicillin and 100 µg/ml streptomycin). 20 µl of standards and cell supernatants from previous experiments were added to a 96-well plate. HEK-Blue IFN α / β cells were harvested and counted using an automated cell counter (Biorad, TC20) and a cell suspension of 2.8×10^5 cells/ml was prepared. 180 µl of cell suspension was added to wells containing standards and supernatants. Cells were incubated at 37°C with 5 % CO₂ overnight. The following day, 20 µl of HEK-Blue IFN α / β supernatants and 180 µl of QUANTI-Blue solution were added in a new 96-well plate. Plate was incubated at 37°C for 5 to 30 min until the standard were visible. All experiments were performed in triplicate. The absorbance was measured at 620 nm using VersaMax microplate reader (Molecular Devices) and analysed with SoftMax Pro v7.0.3 software.

2.2.9 RT-qPCR

2.2.9.1 RNA isolation

Total RNA was isolated using a High Pure RNA isolation kit (Roche) according to the manufacturer's protocol. Experiments were performed in 24-well plate and cells were harvested by adding a mix of 100 µl of 1X PBS and 200 µl of Lysis/Binding buffer. The lysates were either directly processed for RNA isolation or stored at -80°C until further use. Isolated RNA was stored at -80°C until reverse transcription was carried out.

2.2.9.2 Reverse transcription

Reverse transcription was performed to synthesise cDNA from isolated RNA. Isolated RNA was quantified using NanoDrop 2000 spectrophotometer and the lower concentration was used to normalise all RNA concentrations. For one reaction, a master mix was prepared consisting of 2 µl 5X M-MLV Buffer, 2 µl dNTPs (2.5 mM each), 0.5 µl Random hexamer primer (1 µg/ml), 0.25 µl RNaseOUT and 0.25 µl M-MLV reverse transcriptase enzyme. Master mix was gently mixed and 5 µl of master mix was dispensed into 8-strip PCR tubes. 5 µl of RNA was added to each PCR strip tube. The tubes were briefly centrifuged and the reverse transcription reaction was performed in a mastercycler nexus gradient thermal cycler (Eppendorf) using the following program: 25°C for 10 min, 42°C for 50 min, and 95°C for 3 min, then hold at 4°C. The cDNA samples were then diluted 1 in 2 by adding 10 µl of ultrapure water and stored at -20°C until real-time qPCR analysis.

2.2.9.3 Real-time quantitative PCR (qPCR)

Real-time qPCR was performed from cDNA samples. For one reaction, a master mix was prepared consisting of 5 µl Syber Green, 2 µl ultrapure water, 0.5 µl forward primer (5 µM) and 0.5 µl reverse primer (5 µM). The master mix was gently mixed and 8 µl of master mix was dispensed per well into a 96-well qPCR plate. Then 2 µl of cDNA was added to each well. All experiments were performed in duplicate. The plate was sealed with an adhesive slip and was briefly centrifuged. The plate was run in a QuantStudio3 (Applied Biosystems) with the fast program consisting of the following steps: 95°C for 2 min, and then 40 cycles of 95°C for 10 s and 60°C for 30 s. Results were analysed using comparative C_T method and *gapdh* was used as the housekeeping gene.

2.2.10 Western blot

2.2.10.1 Cell lysate preparation

After appropriate cell stimulation the cell culture plate was placed on ice. Cell supernatants were removed and either discarded or transferred into 1.5 ml microcentrifuge tube in order to be concentrated (detailed in **section 2.2.10.2**). Cells were washed in ice-cold 1X PBS and detached by scrapping. Suspension cells were transferred into 1.5 ml microcentrifuge tube and centrifuged at 2,400 g for 5 min at 4°C. Then PBS was removed, lysis buffer consisting of 1X sample buffer and DTT 1M (150 µl/ml) was added, and lysates were boiled for 5 min at 95°C. The lysate samples were stored at -20°C until further use. NB: infected cells were processed in a class II biological safety cabinet in a CL2 lab until the samples were boiled.

2.2.10.2 Supernatant concentration

Proteins in the cell supernatants were concentrated by addition of 10 µl of StrataClean resin (Agilent) per ml of supernatant. Supernatants were then vortexed and rotated overnight at 4°C. The next day, supernatants were centrifuged at 2,400 g for 5 min at 4°C. Supernatant were removed and discarded, and the resin was resuspended in lysis buffer consisting of 1X sample buffer and DTT 1M (150 µl/ml). Samples were then boiled for 5 min at 95°C and were stored at -20°C until further use. NB: infected supernatants were processed in a class II biological safety cabinet in a CL2 lab until the samples were boiled.

2.2.10.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were made using Bio-Rad apparatus and consisted of a stacking gel cast over a resolving gel. The percentage of the resolving gel was dependent on the molecular weight of the protein of interest. The composition of the 1.5 mm resolving and stacking gels are given in **Table 2.8**. Once poured, the top of the resolving gel was covered with ultrapure water and allowed to solidify. Then ultrapure water was removed and stacking gel was poured on top of the resolving gel. The comb was inserted, and the gel was left to solidify. Samples were boiled for 5 min at 95°C prior to loading followed by brief centrifugation. A pre-stained molecular weight marker was loaded in one well and 15-20 µl of samples were loaded in the remaining wells. The gel ran in 1X running buffer at 80 Volts, until the proteins and

molecular weight marker passed through the stacking gel, and then at 120 Volts using a Mini-Protean Tetra cell system (Bio-Rad) until proteins and MWMs were suitably resolved.

Reagents for two gels	8 % resolving gel	12 % resolving gel	15 % resolving gel	Stacking gel
ultrapure water	9.3 ml	6.6 ml	4.6 ml	5.5 ml
30 % acrylamide	5.3 ml	8 ml	10 ml	1.3 ml
1.5 M Tris pH 8.8	5 ml	5 ml	5 ml	-
1 M Tris pH 6.8	-	-	-	1 ml
10 % SDS	200 μ l	200 μ l	200 μ l	80 μ l
10 % APS	200 μ l	200 μ l	200 μ l	80 μ l
TEMED	12 μ l	8 μ l	8 μ l	8 μ l

Table 2.8: Resolving and stacking gel compositions.

2.2.10.4 Semi-dry transfer

The resolved proteins were transferred to 0.45 μ m nitrocellulose membrane using Biometra semi-dry transfer system. A transfer sandwich was made by laying three sheets of Whatman filter papers soaked in cold 1X transfer buffer on the surface of semi-dry machine followed by nitrocellulose membrane soaked in cold transfer buffer 1X, SDS-PAGE gel and three additional filter papers soaked in cold transfer buffer 1X. Air bubbles were removed by rolling a cylinder over the transfer sandwich. The semi-dry transfer ran at 75 mA per gel for 90 min.

2.2.10.5 Immunoblotting

The nitrocellulose membrane was incubated in 5 % (w/v) skimmed milk powder in 1X PBS/0.1 % (v/v) Tween for 1 h to avoid non-specific antibody binding. Then, the membrane was incubated rolling overnight at 4°C in primary antibody diluted in 5 % (w/v) skimmed milk powder in 1X PBS/0.1 % (v/v) Tween. The membrane was then washed three times in 1X PBS/0.1 % (v/v) Tween for 5 to 10 min. The appropriate fluorescent-labelled secondary antibody was added to 5 % (w/v) skimmed milk powder in 1X PBS/0.1% (v/v) Tween and the membrane was incubated for 1 h at room temperature. The membrane was then washed three times in 1X PBS/0.1 % (v/v) Tween for 5 to 10 min. To detect phosphorylated protein, 5 % (w/v) skimmed milk powder in 1X PBS/0.1 % (v/v) Tween

was replaced with 3 % (w/v) BSA in 1X TBS/0.1 % (v/v) Tween, and the membrane was washed in 1X TBS/0.1 % (v/v) Tween. The membrane was developed using the Odyssey Infrared Imaging System (Li-COR) and analysed with Image studio lite version 5.2 software.

2.2.11 ASC oligomerisation assay

The plate was placed on ice except where specified. Cell supernatants were removed and discarded, and cells were washed in ice-cold 1X PBS and detached by scrapping. Suspension cells were transferred into 1.5 ml microcentrifuge tubes and centrifuged at 2,400 g for 5 min at 4°C. Then PBS was removed and 50 µl ice-cold lysis oligomerisation buffer was added for 30 min. After incubation, the lysates were centrifuged at 14,000 g for 5 min at 4°C and the supernatants containing the soluble fraction were transferred to a fresh 1.5 ml microcentrifuge tube. Mix of soluble fraction, 3X sample buffer and DTT 1M (150 µl/ml) was performed. Soluble fraction samples were then boiled for 5 min at 95°C and stored at -20°C until further use. Then the insoluble pellets were crosslinked in 100 µl of a 2 mM solution of DSS in PBS and were incubated at room temperature for 45 min on a rotator. DSS stock (50 mM) was freshly prepared each time. The samples were then centrifuged at 2,400 g for 5 min at 4°C. The supernatant was removed and 1X sample buffer and DTT 1M (150 µl/ml) was added to the pellet. Insoluble fraction samples were boiled for 5 min at 95°C and stored at -20°C until further use. Soluble and insoluble fraction samples were subjected to SDS-PAGE and immunoblotting.

2.2.12 GSDME oligomerisation assay

To detect the interaction between GSDME monomers, non-reducing and denaturing conditions were used. Cell lysates and supernatants were prepared as described in **sections 2.2.10.1 and 2.2.10.2** except that no DTT was added to the sample buffer to conserve protein interactions. Samples were then subjected to SDS-PAGE and immunoblotting as previously described.

2.2.13 Statistical analysis

Statistical analyses were performed using unpaired Student's t test when comparing two groups and one-way ANOVA when comparing more than two groups. To determine the effect of different stimulations and of time on pro-IL-1β mRNA upregulation; and the

impact of GSDM siRNA and of time on viral replication, two-way ANOVA was used. Data are presented as mean $\pm$ standard error of the mean (SEM) of three independent experiments, except for cytokine and LDH release time course where data are presented as mean $\pm$ standard deviation (SD) of a representative experiment of three independent experiments. All the details are indicated in the figure legends. Regarding experiments with NHBE cells, two donors were used during this project that gave similar cellular responses to stimulants and infections. Thereby each dot represents an independent experiment performed with one of these donors. GraphPad Prism 9 was used to calculate p values and significance was defined as follows: ns: not significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Chapter 3. Results I - Determination of the best cellular model to study the inflammasome activation in respiratory epithelial cells

3.1 Introduction

The innate immune response is essential to mount an antiviral response to fight viral infection. Inflammasomes are multiprotein complexes that are formed following detection of PAMPs or DAMPs. Inflammasomes include members of the NLR and ALR protein families, interact with the adaptor protein ASC which recruits pro-caspase-1 leading to caspase-1 autoprocessing and activation. Caspase-1 can then cleave pro-IL-1 β into mature IL-1 β and GSDMD into a pore forming fragment to enable IL-1 β secretion and pyroptotic cell death [174, 175]. Thereby, inflammasome activation leads to a pro-inflammatory environment that participates in the recruitment of immune cells to the site of infection. For instance, rhinovirus infection triggers IL-1 secretion from human epithelial cells, which allows the recruitment of immune cells to the lung [112]. Nevertheless, dysregulation in inflammasome formation can trigger constitutive activation leading to a basal pro-inflammatory state which contributes to autoimmune diseases.

Mouse macrophages have been extensively used to uncover the mechanism of inflammasome activation, cytokine secretion and pyroptotic cell death [174, 175, 182]. To assess these findings in human myeloid cells, the acute monocytic leukemia THP-1 cell line is often used [147, 190, 191]. THP-1 cells are usually differentiated into a macrophage-like phenotype using phorbol 12-myristate 13-acetate (PMA), to model primary human macrophages [192]. Macrophages and monocytes are essential components of the innate immune response against pathogens. Indeed, in addition to inflammasome activation, their PRRs recognise PAMPs and DAMPs which promote the secretion of pro-inflammatory chemokines and cytokines leading to the recruitment of effector cells to the site of infection. In addition, macrophages phagocytose particles to eliminate pathogens.

For a long time, respiratory epithelial cells have been described as a passive physical barrier in the defence against infections. Nevertheless, it is now clear that epithelial cells have an active role in initiating innate immune responses. Given that respiratory epithelial cells are the first site of infection of respiratory viruses such as IAV and RSV, it is essential to understand how epithelial cells respond to virus infection. Most of our knowledge on inflammasome activation come from studies performed in murine myeloid cells, yet mechanisms described in mouse cells cannot always be translated to human cells. For instance, ultraviolet irradiation results in inflammasome activation in human keratinocytes but not in mouse keratinocytes [193]. Moreover NEK7 is not required for NLRP3 inflammasome activation in human monocytes, unlike murine myeloid cells [120]. This

emphasises the need to study human barrier epithelial cells, especially respiratory epithelial cells.

Besides inflammasome-dependent IL-1 β secretion, barrier epithelial cells also secrete IL-1 α during viral infection [194]. Interestingly, inflammasome formation and IL-1 β are important to trigger the release of mature IL-1 α from myeloid cells [195] but during IAV infection, the secretion of IL-1 α is also needed for optimal IL-1 β release [114]. This shows a synergy between IL-1 β and IL-1 α cytokines. Given that IL-1 β and IL-1 α share the same receptor, it is of interest to determine whether respiratory epithelial cells secrete both IL-1 β and IL-1 α upon viral stimulation or viral infection.

Thus, the aim of this chapter is to determine which respiratory epithelial cells can be used as a cellular model for studies into virus-induced inflammasome activation in human epithelial cells.

3.2 Results

3.2.1 Validation of the tools to characterise inflammasome activation in differentiated THP-1 cells

I initially investigated inflammasome activation in differentiated THP-1 (dTHP-1) cells, with a view to assessing potential inflammasome activators in these well characterised cells, prior to studies in epithelial cells. Priming is an important step to upregulate the transcription of the inflammasome components and of pro-IL-1 β mRNA. Thereby, to determine which stimuli can prime dTHP-1 cells, I measured the expression of pro-IL-1 β mRNA following addition of PAMPs. Both lipoteichoic acid (LTA), a TLR2 agonist, and 5'-triphosphate hairpin RNA (hpRNA), a RIG-I ligand, significantly upregulated pro-IL-1 β mRNA expression at 16 h [Figure 3.1A, B]. TLR4 stimulation with lipopolysaccharide (LPS) increased pro-IL-1 β mRNA expression at 16 h but didn't reach significance. Neither the delivery of naked high molecular weight (HMW) or low molecular weight (LMW) poly(I:C), both of which are TLR3 agonists, or transfected HMW or LMW poly(I:C), which stimulate cytosolic RNA sensors, induced pro-IL-1 β mRNA expression [Figure 3.1A, B]. Thus, TLR2, RIG-I and potentially TLR4 stimulation, but not TLR3 or other cytosolic RNA receptors, lead to pro-IL-1 β mRNA induction in dTHP-1 cells.

Inflammasome activation results in the recruitment and autoprocessing of pro-caspase-1 into its fully active p33/p10 complex and subsequently p20/p10 tetramers [172]. NLRP3 inflammasome activation requires a priming step to induce pro-IL-1 β and NLRP3 gene expression and an activation step to trigger the inflammasome assembly [116]. Thereby I pretreated dTHP-1 cells with LPS before adding nigericin, the NLRP3 activator, and confirmed that caspase-1 was activated as shown by the detection of p33 and p20 [Figure 3.2, lane 4]. Interestingly, nigericin alone caused caspase-1 maturation similarly to LPS and nigericin treatment [Figure 3.2, lane 3 compared to lane 4], suggesting that in PMA-treated cells, nigericin alone is sufficient to trigger caspase-1 activation. Then I validated that the pharmaceutical inhibitors YVAD, the caspase-1 inhibitor, and ZVAD, the pan-caspase inhibitor, diminished caspase-1 activation [Figure 3.2, lanes 6, 7]. Finally, treatment with MCC950, the potent and specific NLRP3 inhibitor [196], prevented the activation of caspase-1 [Figure 3.2, lane 8], confirming that NLRP3 inflammasome can be activated in dTHP-1 cells.

Once processed, caspase-1 cleaves pro-IL-1 β into a minor 26-kDa fragment and into its biologically active 17-kDa (p17) form [197] which is an important biological consequence

of inflammasome activation. Both nigericin alone and LPS and nigericin led to the secretion of IL-1 β maturation [Figure 3.3A, lanes 3, 4]. This process was inhibited in the presence of YVAD, ZVAD or MCC950 [Figure 3.3A, lanes 6-8]. Using ELISA as another readout of cytokine release, I confirmed that the secretion of IL-1 β was significantly diminished in the presence of YVAD or MCC950 [Figure 3.3B], demonstrating that LPS and nigericin lead to NLRP3 inflammasome-dependent IL-1 β secretion. The level of TNF- α secretion, an inflammasome-independent cytokine, was not impacted by the addition of the inhibitors showing their specificity and the absence of toxicity [Figure 3.3C].

Besides pro-IL-1 β , caspase-1 also cleaves the executioner protein of pyroptotic cell death GSDMD [174, 175]. Stimulation with LPS and nigericin, but also nigericin alone, caused GSDMD cleavage into its pore-forming fragment (p30) [Figure 3.4A, lanes 3, 4]. This cleavage was inhibited by addition of YVAD, ZVAD or MCC950 [Figure 3.4A, lanes 6-8]. Interestingly, addition of MCC950 led to GSDMD cleavage into a p43 fragment which is a marker of GSDMD inactivation [Figure 3.4A, lane 8]. Inflammasome activation mediates pyroptotic cell death which releases large cytosolic components into the extracellular space [182]. Thereby measurement of LDH release in the cell supernatant is a marker of plasma membrane damage, and hence lytic cell death. LPS and nigericin stimulation released LDH in the supernatant [Figure 3.4B], which was significantly decreased by addition of YVAD or MCC950 [Figure 3.4B]. Together these results confirm that NLRP3 inflammasome activation mediates GSDMD cleavage and lytic cell death in human myeloid cells.

Thus, several readouts can be used to assess inflammasome activation, namely quantification of IL-1 β and LDH release into the supernatant by ELISA and LDH assay respectively, and visualisation of GSDMD cleavage into pore-forming fragments by immunoblot.

3.2.2 Measurement of inflammasome activation in A549 cells

A549 is a cancerous alveolar epithelial cell line that is often used to study the IFN response to respiratory viral infection. To determine whether A549 cells are a good model to study inflammasome activation, I first measured pro-IL-1 β mRNA expression following TLR agonist and cytosolic RNA delivery. Among TLRs ligands (LPS, LTA and dsRNA), only LTA significantly increased pro-IL-1 β mRNA at 16 h [Figure 3.5A]. Transfection of HMW poly(I:C), LMW poly(I:C) and hpRNA induced significant pro-IL-1 β mRNA

upregulation at 16 h [**Figure 3.5B**]. Overall, TLR2 and cytosolic RNA sensor stimulation can prime A549 cells, but not TLR3 nor TLR4 activation.

Next, inflammasome activation was monitored by measuring IL-1 β cytokine secretion and LDH release. Since LPS and nigericin treatment is a potent NLRP3 inflammasome activator, I used these to investigate whether NLRP3 could also be triggered in alveolar epithelial cells. LPS and nigericin didn't induce IL-1 β secretion [**Figure 3.6A**] nor LDH release [**Figure 3.6C**] in A549 cells. Priming the cells with naked HMW or LMW poly(I:C), instead of LPS, before nigericin treatment didn't enhance these responses [**Figure 3.6A, C**]. I next stimulated the cells with LTA and naked HMW or LMW poly(I:C) which also failed to elicit a cytokine or lytic cell death response [**Figure 3.6A, C**]. To determine whether A549 cells were responsive to TLRs ligands, I measured the secretion of IL-6, whose expression is enhanced following stimulation and infection [97]. None of the TLRs stimulation triggered IL-6 release [**Figure 3.6B**] suggesting that A549 cells might not be responsive to TLR2, TLR4 or TLR3 stimulation. Next, I investigated whether cytosolic RNA delivery could activate inflammasomes in A549 cells. I stimulated the cells with transfected HMW poly(I:C), LMW poly(I:C) and hpRNA. None of these conditions resulted in IL-1 β secretion [**Figure 3.7A**] nor LDH release [**Figure 3.7C**] but all of these stimulations triggered IL-6 secretion [**Figure 3.7B**], showing that A549 cells are responsive to cytosolic RNA. **Figure 3.5B** showed that cytosolic RNA delivery induced high levels of pro-IL-1 β mRNA, thereby I primed A549 cells with these RNA before nigericin stimulation. Nevertheless, none of the conditions enhanced IL-1 β or IL-6 secretion nor lytic cell death [**Figure 3.7A-C**]. Thus, these results demonstrate that A549 cells are not a suitable model to study inflammasome activation.

3.2.3 Measurement of inflammasome activation in BEAS-2B cells

Another cell line used to study respiratory viral infection is the transformed bronchial epithelial cell line BEAS-2B. Similar to A549 cells, I first measured pro-IL-1 β mRNA expression following TLR or cytosolic RNA stimulation. Among TLR stimulants, only LTA induced pro-IL-1 β mRNA upregulation, at 4 h and 16 h [**Figure 3.8A**], whereas cytosolic delivery of all RNAs tested significantly enhanced pro-IL-1 β mRNA expression at 16 h [**Figure 3.8B**]. Thereby, BEAS-2B cells can be primed with TLR2 and cytosolic RNA sensor agonists.

Next, I measured IL-1 β secretion and LDH release after TLR stimulation and cytosolic RNA delivery and found that none of the conditions triggered IL-1 β secretion nor lytic cell death [Figures 3.9A, C; Figure 3.10A, C]. However, LTA, naked HMW poly(I:C), transfected poly(I:C) and hpRNA led to significant IL-6 secretion [Figures 3.9B; Figure 3.10B] showing that BEAS-2B cells are responsive to TLR2, TLR3, and cytosolic RNA sensor stimulation. Priming BEAS-2B with transfected RNA before nigericin treatment didn't trigger inflammasome activation [Figures 3.9A, C; Figure 3.10A, C]. Thus BEAS-2B cells, similar to A549 cells, are not a good model to study inflammasome activation.

3.2.4 Measurement of inflammasome activation in NuLi-1 cells

Lee and colleagues showed that IAV induces inflammasome activation in pre-cancerous cell lines but not in cancerous cell lines such as A549 cells [198]. For this reason, I performed similar experiments in the non-cancerous immortalised bronchial epithelial cell line, NuLi-1. I first determined which stimulation could act as the priming step. LTA, naked HMW poly(I:C) and transfected HMW poly(I:C) significantly induced pro-IL-1 β mRNA upregulation at both 4 h and 16 h [Figure 3.11]. LPS and hpRNA treatment displayed decreased pro-IL-1 β mRNA expression at 4 h compared to Lipofectamine alone [Figure 3.11]. Thus, TLR2, TLR3 and cytosolic RNA sensor stimulation can prime NuLi-1 cells.

I next measured cytokine secretion and lytic cell death in response to TLR stimulation. Neither LTA, LPS nor naked poly(I:C) triggered IL-1 β secretion [Figure 3.12A]. In addition, priming with LPS or non-transfected poly(I:C) before nigericin treatment didn't induce IL-1 β release [Figure 3.12A]. Nevertheless, detection of IL-6 in the supernatant following naked HMW poly(I:C) showed that NuLi-1 cells responded to TLR3 stimulation [Figure 3.12B]. Thereby, TLR stimulation doesn't prime or activate inflammasomes in NuLi-1 cells. Then, I examined whether cytosolic RNA delivery could trigger cytokine secretion and lytic cell death. Even though transfected HMW poly(I:C) and high concentration of transfected LMW poly(I:C) enhanced IL-1 β secretion, it didn't reach significance [Figure 3.13A]. Similarly, IL-6 release was increased, but not significantly, following delivery of all RNAs tested [Figure 3.13B]. Priming NuLi-1 cells with cytosolic RNA didn't increase cytokine secretion [Figure 3.13A, B]. Nevertheless, transfected poly(I:C), but not hpRNA, triggered significant LDH release [Figure 3.13C]. Based on the IL-1 β and LDH results, these data suggest that transfected HMW poly(I:C) and high concentration of transfected LMW poly(I:C) may activate the inflammasome in NuLi-1 cells.

Given that **Figures 3.12 and 3.13** showed that the NLRP3 inflammasome activator nigericin had no effect on cells, and that transfected poly(I:C) might activate an inflammasome, I next investigated whether other inflammasomes could be triggered in NuLi-1 cells. Poly(dA:dT) is the main activator of the AIM2 inflammasome [159] and Val-boroPro (VBP) causes NLRP1 and CARD8 inflammasomes formation [147, 148, 199]. Here, poly(dA:dT), but not VBP, led to IL-1 β and IL-6 secretion and LDH release [**Figure 3.14A, C, D**]. Since inflammasome and IL-1 β are needed for mature IL-1 α secretion in mouse myeloid cells [195] and that IL-1 α released is important for optimal IL-1 β secretion during IAV infection [114], I measured IL-1 α release following stimulation by ELISA. I found that poly(dA:dT) but not VBP triggered IL-1 α secretion [**Figure 3.14B**], showing that there is a correlation between IL-1 β and LDH release, and IL-1 α secretion. Thus, AIM2 inflammasome but not NLRP1 nor CARD8 is likely present and active in NuLi-1 cells.

3.2.5 Measurement of inflammasome activation in primary bronchial epithelial cells

I sought to compare the results from NuLi-1 cells to a more physiologically relevant cell type using undifferentiated primary normal human bronchial epithelial cells (NHBE). NHBE cells, isolated from healthy donors, were purchased from Lonza and were cultured until passage 6. To determine whether stimulation would enhance pro-IL-1 β transcription, I stimulated NHBE cells with the ligands previously used. Naked and transfected HMW poly(I:C) induced pro-IL-1 β mRNA upregulation whereas LTA, LPS and hpRNA didn't have any significant effect [**Figure 3.15A, B**]. Then I examined if pro-IL-1 α mRNA expression could also be enhanced following stimulation. Similarly to pro-IL-1 β mRNA, only naked and transfected HMW poly(I:C) induced pro-IL-1 α mRNA expression at both 4 h and 16 h [**Figure 3.15C, D**]. Thus, TLR3 and cytosolic RNA receptors stimulation can prime NHBE cells.

Next, I measured cytokine secretion and LDH release in response to TLR stimulation in NHBE cells. LTA and LPS didn't induce IL-1 β , IL-1 α or IL-6 secretion nor lytic cell death [**Figure 3.16A-D**]. Naked HMW poly(I:C) triggered IL-6 secretion but not IL-1 β or IL-1 α secretion nor cell death [**Figure 3.16A-D**], showing that NHBE cells are responsive to TLR3 stimulation. Then I stimulated NHBE cells with cytosolic RNA delivery. Transfected HMW poly(I:C) and hpRNA, led to IL-1 β , IL-1 α and IL-6 secretion and LDH release but IL-1 β secretion only reach significance for HMW poly(I:C) transfection [**Figure 3.17A-D**]. Interestingly the secretion of IL-1 β and IL-1 α was similar following poly(I:C) transfection

[**Figure 3.17A, B**]. IL-1 β release elicited by cytosolic RNA delivery was almost ten times higher than in NuLi-1 cells [**Figure 3.17A compared to Figure 3.13A**]. Together, these results show that cytosolic poly(I:C) activates inflammasome in NHBE cells.

Next, I examined which inflammasomes are expressed and activated in undifferentiated NHBE cells. Firstly, I looked at the NLRP3 inflammasome. Immunoblot analysis showed that NLRP3 was neither basally nor inducibly detectable in NHBE cells [**Figure 3.18A**]. Nevertheless NLRP3 was detectable in dTHP-1 cells confirming that the antibody was working [**Figure 3.18A**]. Stimulation of NHBE cells with LPS and nigericin failed to trigger cytokine secretion or lytic cell death [**Figure 3.18B-E**]. **Figure 3.15** showed that TLR3 and cytosolic dsRNA delivery were both good inducers of pro-IL-1 β and pro-IL-1 α mRNA, therefore I primed NHBE cells with naked HMW poly(I:C) or transfected HMW poly(I:C) or hpRNA before nigericin treatment. However, none of these conditions increased cytokine secretion nor lytic cell death [**Figures 3.16A-D, 3.17A-D**]. Thus, these results show that NLRP3 inflammasome is not activated in NHBE cells. Other inflammasome receptors, such as NLRP1 and AIM2, are expressed in epithelial cells [200]. I next looked at NLRP1 expression by immunoblot which showed that NLRP1 was constitutively expressed in NHBE cells [**Figure 3.19A**]. Unlike NuLi-1 cells, VBP treatment resulted in IL-1 β and IL-1 α secretion and LDH release but didn't trigger IL-6 secretion [**Figure 3.19B-E**], suggesting that NLRP1 inflammasome is active in NHBE cells. Finally, I looked at the AIM2 inflammasome. Immunoblot analysis showed that AIM2 was not basally expressed in NHBE cells but was induced by poly(dA:dT) stimulation [**Figure 3.20A**]. Poly(dA:dT) triggered IL-1 β , IL-1 α and IL-6 release and lytic cell death [**Figure 3.20B-E**], showing that AIM2 inflammasome can be activated in NHBE cells. Overall, these results demonstrate that cytosolic dsRNA triggers inflammasome activation and that several inflammasomes are present in undifferentiated NHBE cells which demonstrates that NHBE cells are a good model to study the inflammasome activation in respiratory epithelial cells.

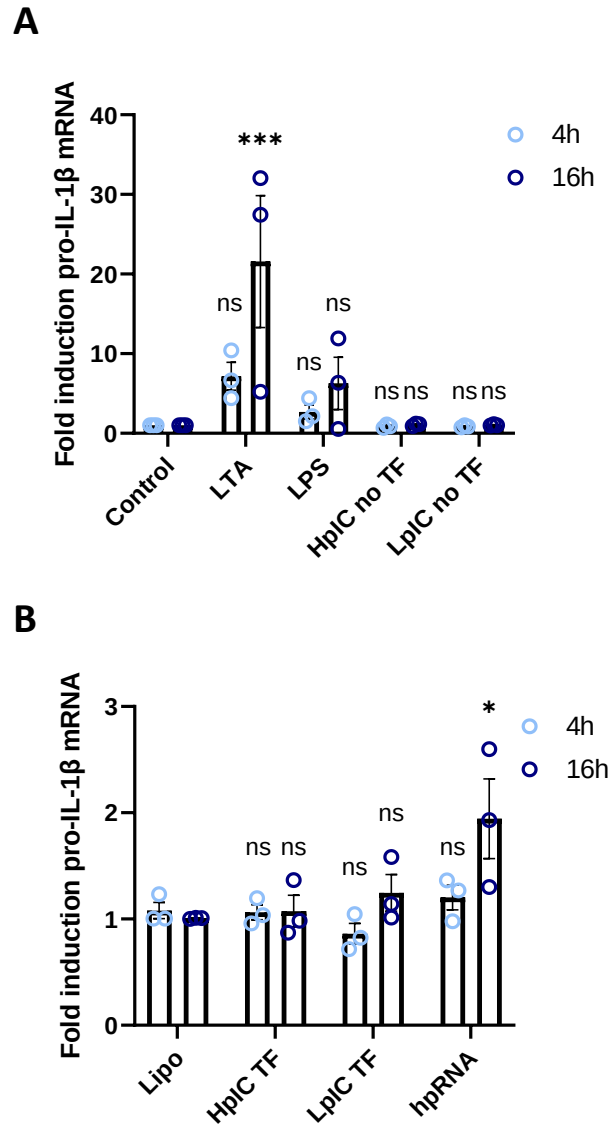


Figure 3.1: TLR and RIG-I stimulation induces pro-IL-1 β mRNA expression in dTHP-1 cells.

THP-1 cells (5×10^5 cells/ml) were differentiated with PMA (100 nM) for 48 h prior to stimulation (dTHP-1 cells). Cells were treated with LTA (2.5 μ g/ml), LPS (200ng/ml), high molecular weight poly(I:C) (HpIC, 10 μ g/ml), low molecular weight poly(I:C) (LpIC, 10 μ g/ml) (A) or transfected (TF) with HpIC (10 μ g/ml), LpIC (10 μ g/ml), hpRNA (100 ng/ml) (B) for 4 h and 16 h. Cell lysates were harvested and pro-IL-1 β mRNA expression was analysed by RT-qPCR. Data are mean $\pm$ SEM of three independent experiments. ns: not significant, * $p < 0.05$ and *** $p < 0.001$ by two-way ANOVA, compared to control (A) or lipo (B).

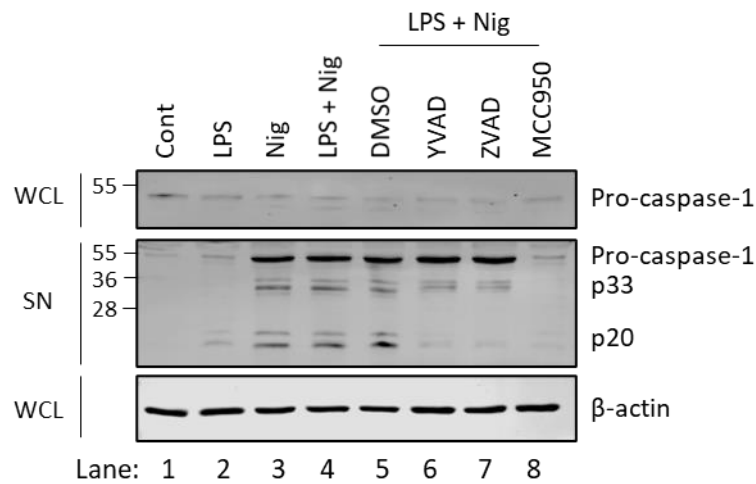


Figure 3.2: Caspase and NLRP3 inhibitors decrease caspase-1 activation in nigericin treated LPS-primed dTHP-1 cells.

dTHP-1 cells (5×10^5 cells/ml) were treated with LPS (200 ng/ml) for 4 h. After 3 h, DMSO, YVAD or MCC950 (both 25 μ M) was added for 1 h. Nigericin (Nig, 10 μ M) was subsequently added for 2 h. Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of pro-caspase-1, active caspase-1 (p33, p20) and β -actin. Immunoblots are representative of three independent experiments.

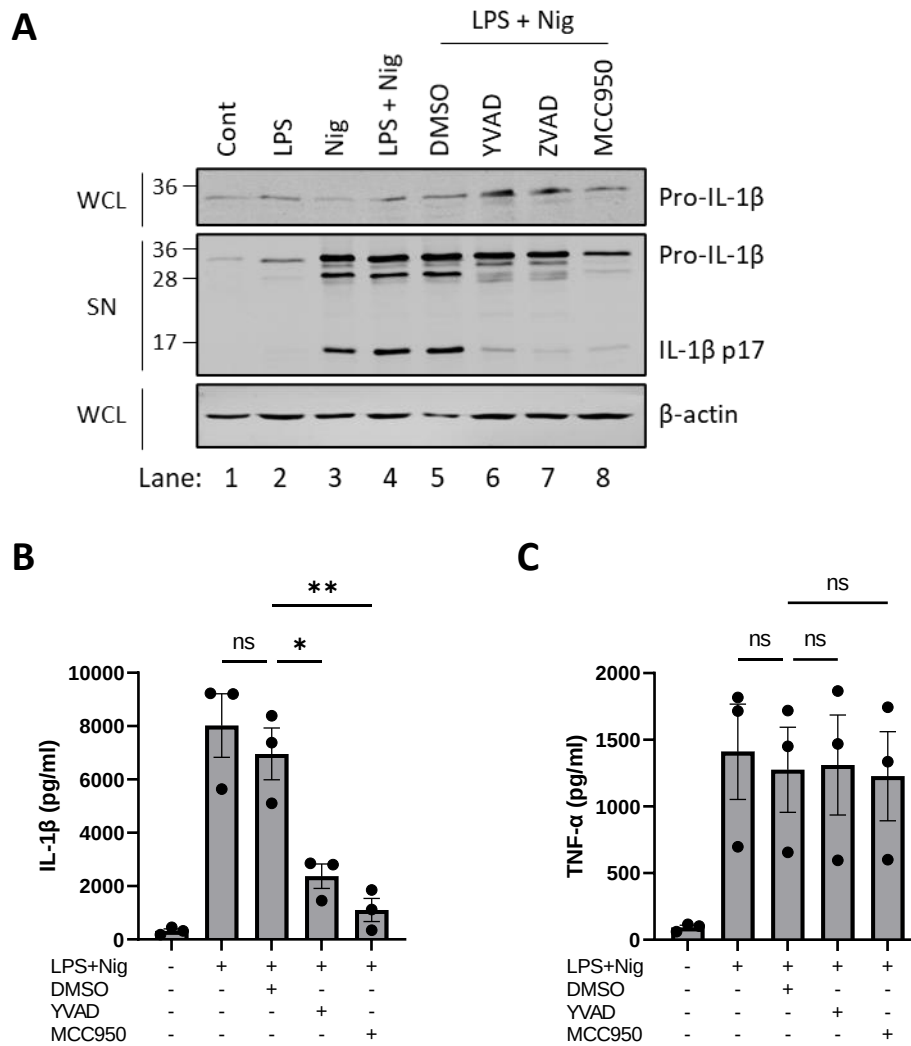


Figure 3.3: Caspase and NLRP3 inhibitors decrease IL-1 β maturation and secretion in nigericin treated LPS-primed dTHP-1 cells.

dTHP-1 cells (5×10^5 cells/ml) were treated with LPS (200 ng/ml) for 4 h. After 3 h, DMSO, YVAD or MCC950 (both 25 μ M) was added for 1 h. Nigericin (Nig, 10 μ M) was subsequently added for 2 h.

(A) Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of pro-IL-1 β , mature IL-1 β (p17) and β -actin. Immunoblots are representative of three independent experiments.

(B, C) IL-1 β (B) and TNF- α (C) secretion was measured by ELISA. Data are mean $\pm$ SEM of three independent experiments. ns: not significant, * $p < 0.05$ and ** $p < 0.01$ by one-way ANOVA.

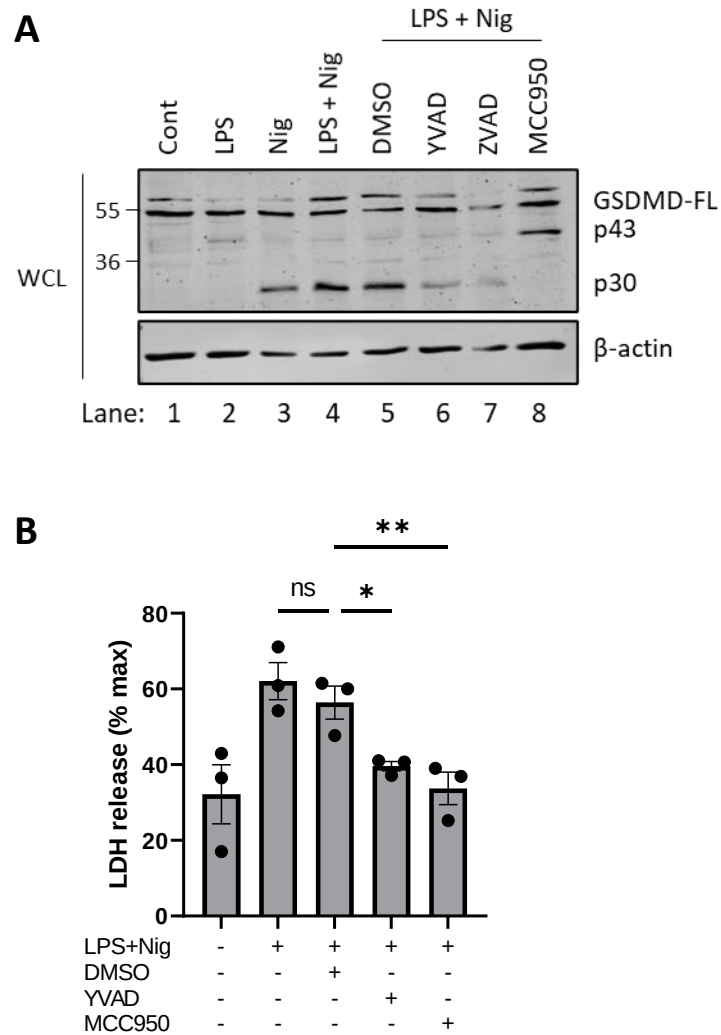


Figure 3.4: Caspase and NLRP3 inhibitors decrease GSDMD cleavage and lytic cell death in nigericin treated LPS-primed dTHP-1 cells.

dTHP-1 cells (5×10^5 cells/ml) were treated with LPS (200 ng/ml) for 4 h. After 3 h, DMSO, YVAD or MCC950 (both 25 μ M) was added for 1 h. Nigericin (Nig, 10 μ M) was subsequently added for 2 h.

(A) Cell lysates (WCL) were immunoblotted for the expression of full length (FL), inactivated (p43) and activated (p30) GSDMD and β -actin. Immunoblots are representative of three independent experiments.

(B) LDH present in the supernatant was quantified by LDH assay. Data are mean $\pm$ SEM of three independent experiments. ns: not significant, * $p < 0.05$ and ** $p < 0.01$ by one-way ANOVA.

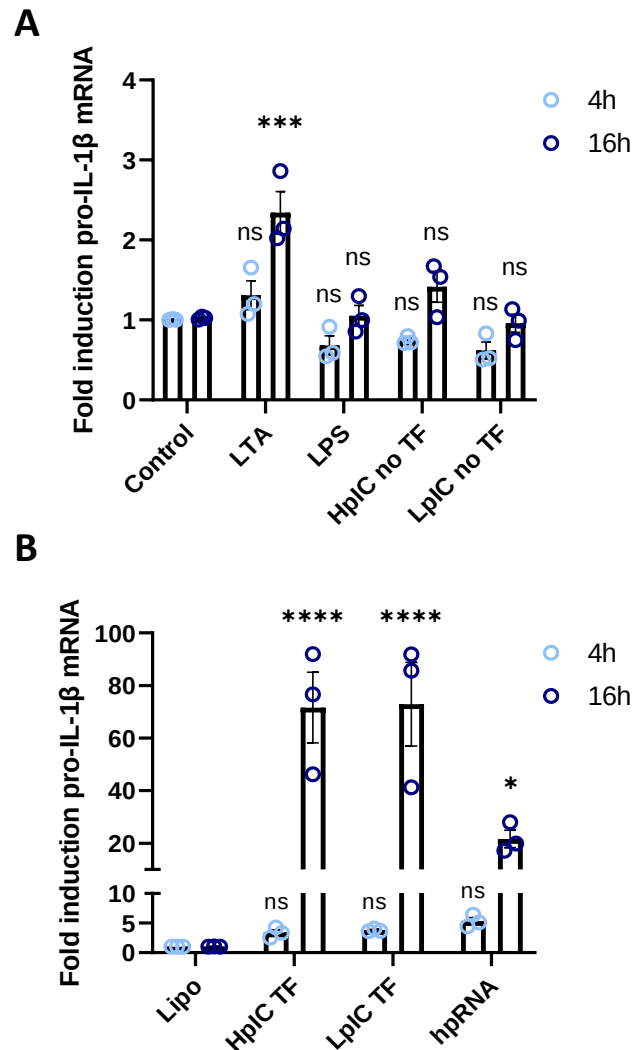


Figure 3.5: TLR2 and cytosolic RNA delivery induces pro-IL-1 β mRNA expression in A549 cells.

A549 cells (5×10^4 cells/ml) were treated with LTA (2.5 μ g/ml), LPS (200 ng/ml), high molecular weight poly(I:C) (HpIC, 2.5 μ g/ml), low molecular weight poly(I:C) (LpIC, 2.5 μ g/ml) (A) or transfected (TF) with HpIC (2.5 μ g/ml), LpIC (2.5 μ g/ml), hpRNA (100 ng/ml) (B) for 4 h and 16 h. Cell lysates were harvested and pro-IL-1 β mRNA expression was analysed by RT-qPCR. Data are mean $\pm$ SEM of three independent experiments. ns: not significant, * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$ by two-way ANOVA, compared to control (A) or lipo (B).

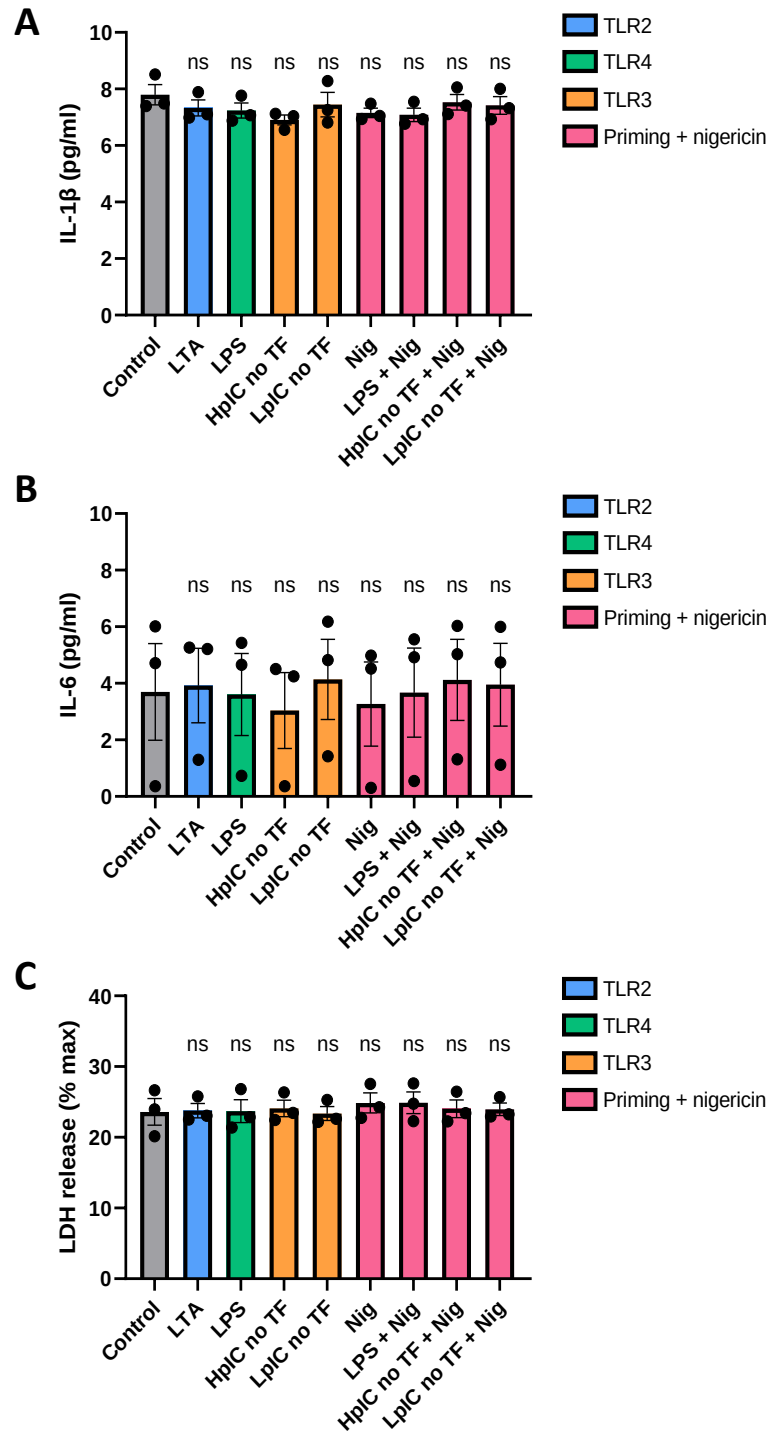


Figure 3.6: TLR ligands fail to cause IL-1 β secretion or trigger lytic cell death in A549 cells.

A549 cells (5×10^4 cells/ml) were primed with LPS (200 ng/ml) for 4 h followed by stimulation with LTA (2.5 μ g/ml), high molecular weight poly(I:C) (HpIC, 2.5 μ g/ml) or low molecular weight poly(I:C) (LpIC, 2.5 μ g/ml) for 16 h. Nigericin (Nig, 10 μ M) was subsequently added for 2 h in the indicated conditions. IL-1 β (A) and IL-6 (B) secretion was measured by ELISA. LDH present in the supernatant was quantified by LDH assay (C). Data are mean $\pm$ SEM of three independent experiments. ns: not significant by one-way ANOVA, TLR2, TLR4, TLR3 and nigericin compared to control, and “priming + nigericin” compared to respective TLRs.

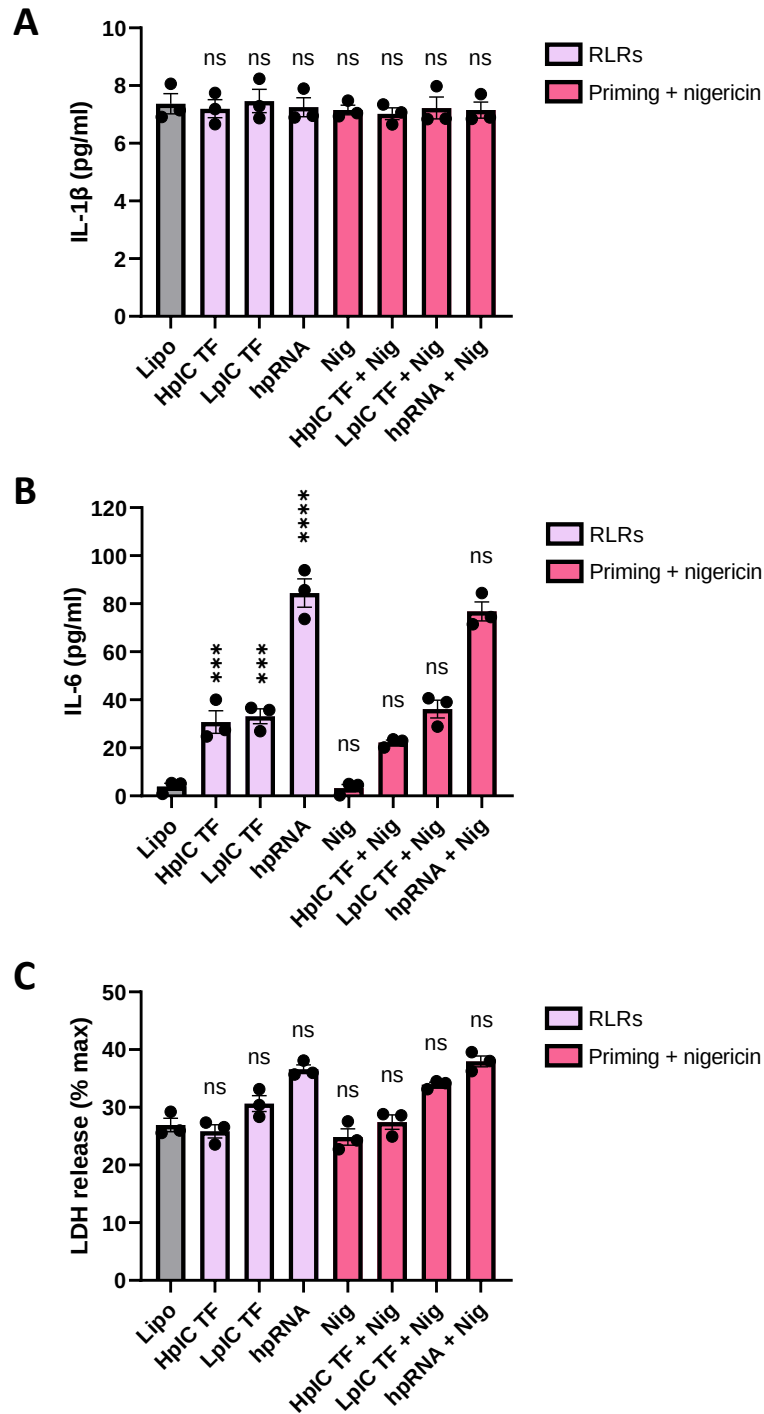


Figure 3.7: Cytosolic RNA delivery triggers lytic cell death but not IL-1 β secretion in A549 cells.

A549 cells (5×10^4 cells/ml) were transfected (TF) with high molecular weight poly(I:C) (HpIC, 2.5 μ g/ml), low molecular weight poly(I:C) (LpIC, 2.5 μ g/ml) or hpRNA (100 ng/ml) for 16 h. Nigericin (Nig, 10 μ M) was subsequently added for 2 h in the indicated conditions. IL-1 β (A) and IL-6 (B) secretion was measured by ELISA. LDH present in the supernatant was quantified by LDH assay (C). Data are mean $\pm$ SEM of three independent experiments. ns: not significant, *** $p < 0.001$ and **** $p < 0.0001$ by one-way ANOVA, RLRs and nigericin compared to lipo, and “priming + nigericin” compared to respective RLRs.

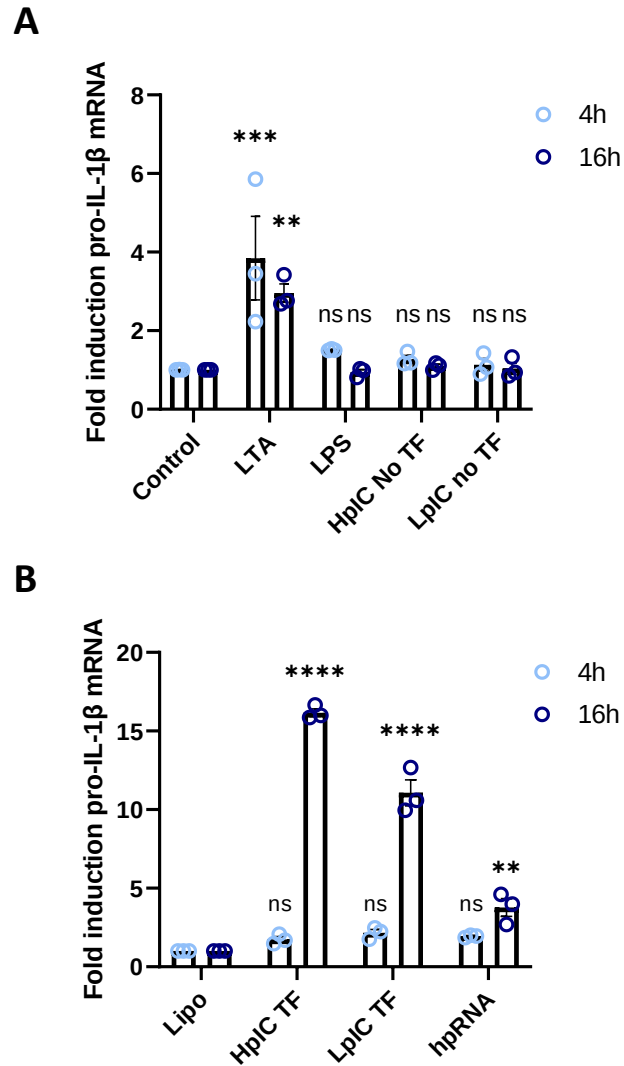


Figure 3.8: TLR2 and cytosolic RNA delivery induces pro-IL-1 β mRNA expression in BEAS-2B cells.

BEAS-2B cells (8×10^4 cells/ml) were stimulated with LTA (2.5 μ g/ml), LPS (200 ng/ml), high molecular weight poly(I:C) (HpIC, 2.5 μ g/ml), low molecular weight poly(I:C) (LpIC, 2.5 μ g/ml) (A) or transfected (TF) with HpIC (2.5 μ g/ml), LpIC (2.5 μ g/ml), hpRNA (100 ng/ml) (B) for 4 h and 16 h. Cell lysates were harvested and pro-IL-1 β mRNA expression was analysed by RT-qPCR. Data are mean $\pm$ SEM of three independent experiments. ns: not significant, **p < 0.01, ***p < 0.001 and ****p < 0.0001 by two-way ANOVA, compared to control (A) or lipo (B).

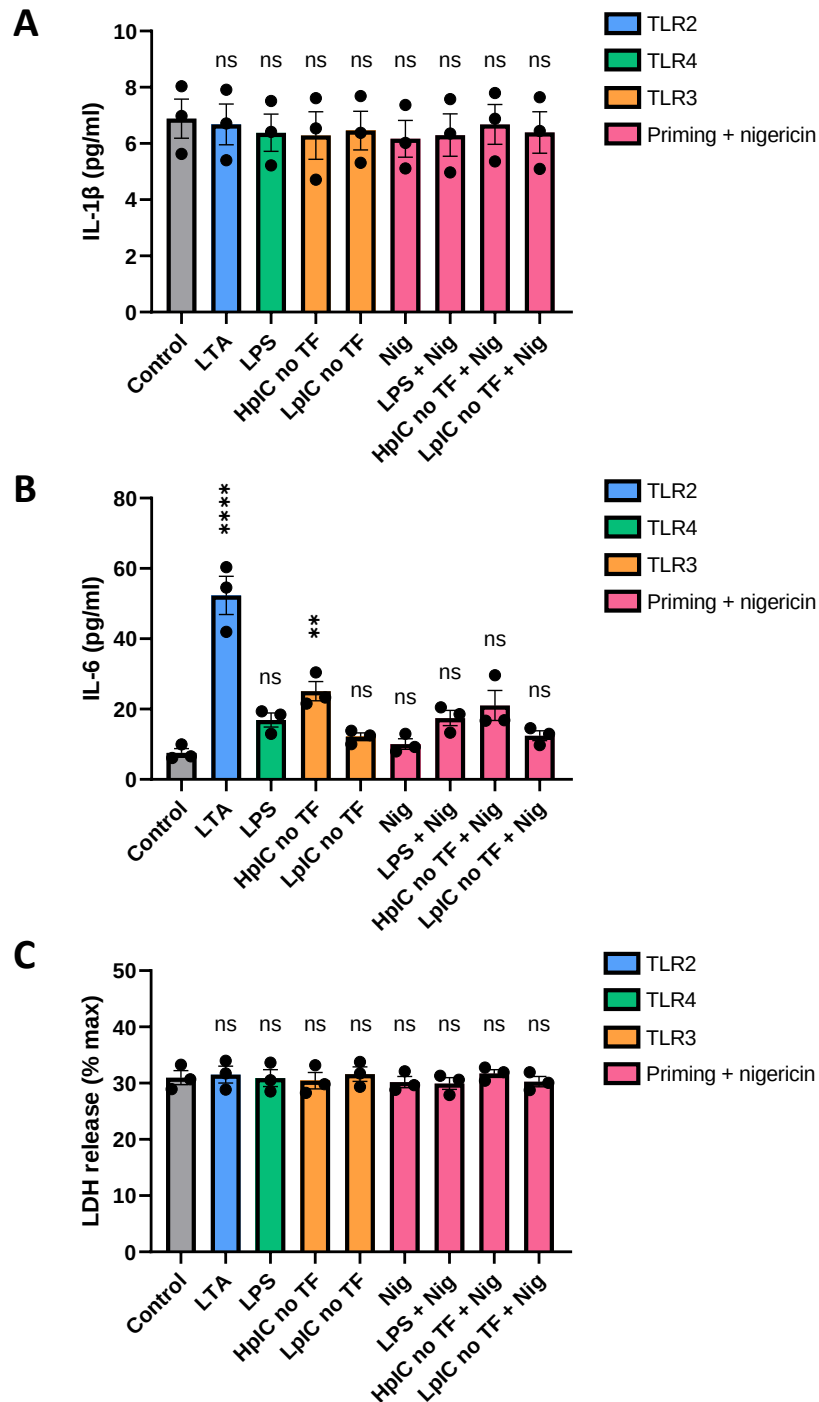


Figure 3.9: TLR ligands fail to cause IL-1 β secretion or trigger lytic cell death in BEAS-2B cells.

BEAS-2B cells (8×10^4 cells/ml) were primed with LPS (200 ng/ml) for 4 h followed by stimulation with LTA (2.5 μ g/ml), high molecular weight poly(I:C) (HpIC, 2.5 μ g/ml) or low molecular weight poly(I:C) (LpIC, 2.5 μ g/ml) for 16 h. Nigericin (Nig, 10 μ M) was subsequently added for 2 h in the indicated conditions. IL-1 β (A) and IL-6 (B) secretion was measured by ELISA. LDH present in the supernatant was quantified by LDH assay (C). Data are mean $\pm$ SEM of three independent experiments. ns: not significant, ** $p < 0.01$ and **** $p < 0.0001$ by one-way ANOVA, TLR2, TLR4, TLR3 and nigericin compared to control, and “priming + nigericin” compared to respective TLRs.

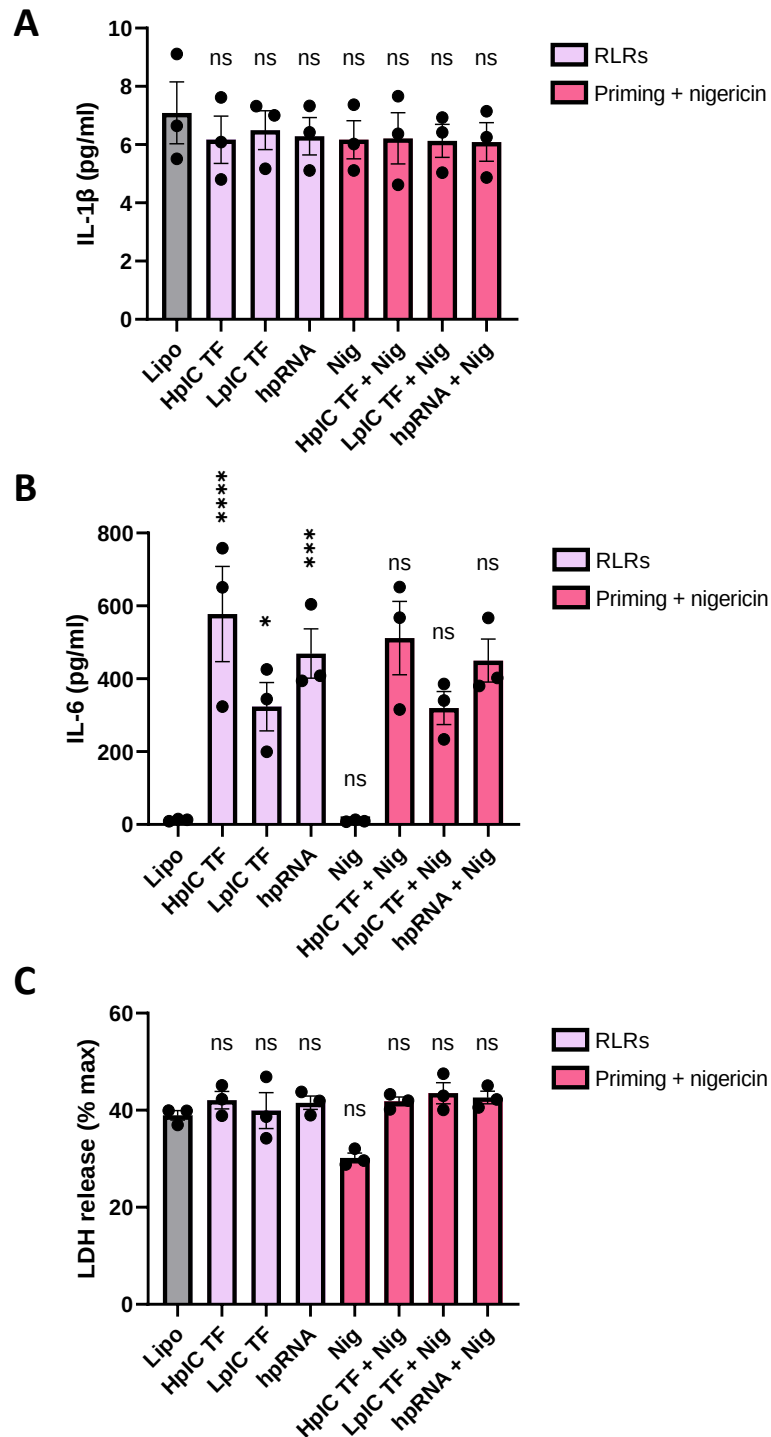


Figure 3.10: Cytosolic RNA delivery fails to cause IL-1 β secretion or trigger lytic cell death in BEAS-2B cells.

BEAS-2B cells (8×10^4 cells/ml) were transfected (TF) with high molecular weight poly(I:C) (HpIC, 2.5 μ g/ml), low molecular weight poly(I:C) (LpIC, 2.5 μ g/ml) or hpRNA (100 ng/ml) for 16 h. Nigericin (Nig, 10 μ M) was subsequently added for 2 h in the indicated conditions. IL-1 β (A) and IL-6 (B) secretion was measured by ELISA. LDH present in the supernatant was quantified by LDH assay (C). Data are mean $\pm$ SEM of three independent experiments. ns: not significant, * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$ by one-way ANOVA, RLRs and nigericin compared to lipo, and “priming + nigericin” compared to respective RLRs.

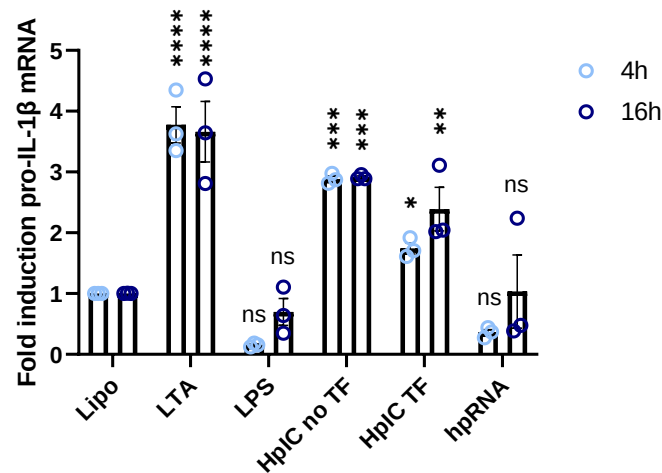


Figure 3.11: TLR ligands and cytosolic RNA delivery induce pro-IL-1 β mRNA expression in NuLi-1 cells.

NuLi-1 cells (1.5×10^5 cells/ml) were treated with LTA (2.5 μ g/ml), LPS (200 ng/ml), high molecular weight poly(I:C) (HpIC, 2.5 μ g/ml) or transfected (TF) with HpIC (2.5 μ g/ml), hpRNA (100 ng/ml) for 4 h and 16 h. Cell lysates were harvested and pro-IL-1 β mRNA expression was analysed by RT-qPCR. Data are mean $\pm$ SEM of three independent experiments. ns: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ by two-way ANOVA, compared to lipo.

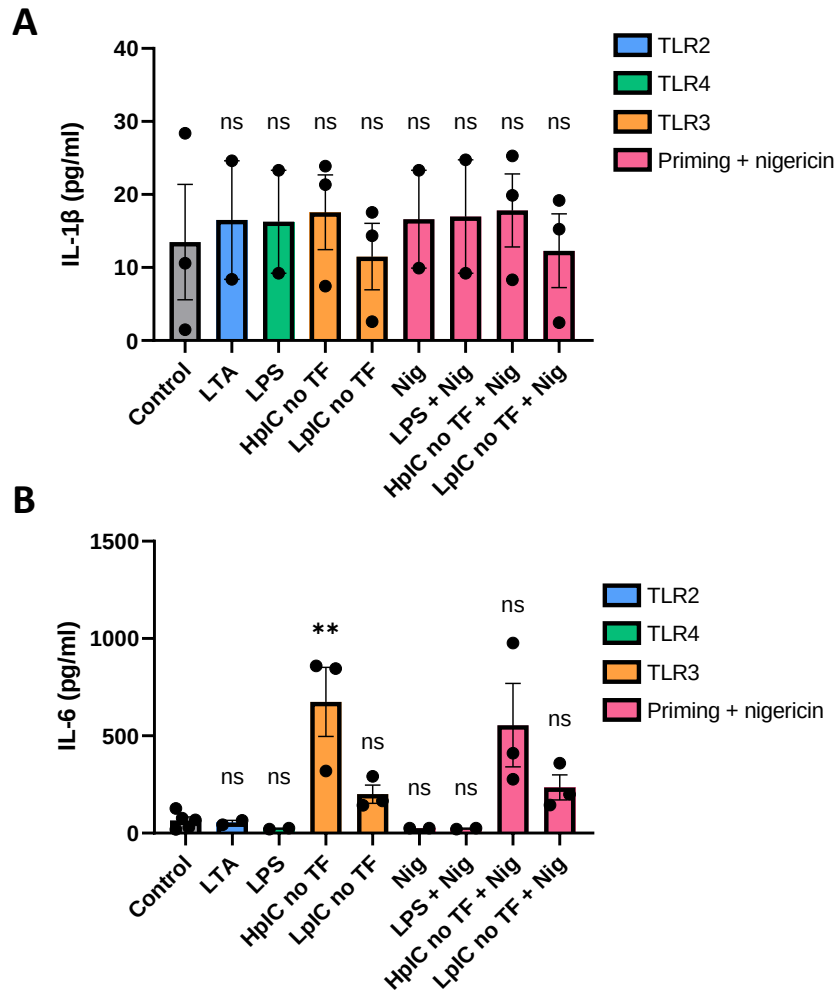


Figure 3.12: TLR ligands fail to cause IL-1 β secretion in NuLi-1 cells.

NuLi-1 cells (1.5×10^5 cells/ml) were primed with LPS (200 ng/ml) for 4 h followed by stimulation with LTA (2.5 μ g/ml), high molecular weight poly(I:C) (HpIC, 2.5 or 10 μ g/ml) or low molecular weight poly(I:C) (LpIC, 2.5 or 10 μ g/ml) for 16 h. Nigericin (Nig, 10 μ M) was subsequently added for 2 h in the indicated conditions. IL-1 β (A) and IL-6 (B) secretion was measured by ELISA. Data are mean $\pm$ SEM of three independent experiments. ns: not significant and ** $p < 0.01$ by one-way ANOVA, TLR2, TLR4, TLR3 and nigericin compared to control, and “priming + nigericin” compared to respective TLRs.

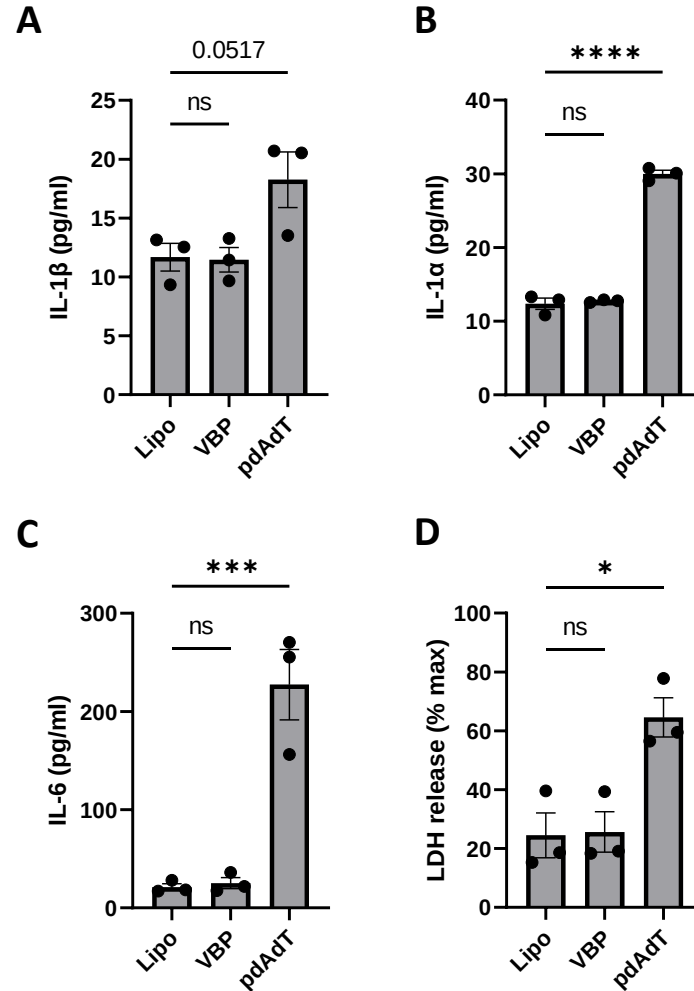


Figure 3.14: Poly(dA:dT), but not VBP, triggers cytokine secretion and lytic cell death in NuLi-1 cells.

NuLi-1 cells (1.5×10^5 cells/ml) were treated with Val-boro-Pro (VBP, 3 μ M) or transfected with poly(dA:dT) (pdAdT, 2.5 μ g/ml) for 16 h. IL-1 β (A), IL-1 α (B) and IL-6 (C) secretion was measured by ELISA. LDH present in the supernatant was quantified by LDH assay (D). Data are mean $\pm$ SEM of three independent experiments. ns: not significant, * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$ by one-way ANOVA.

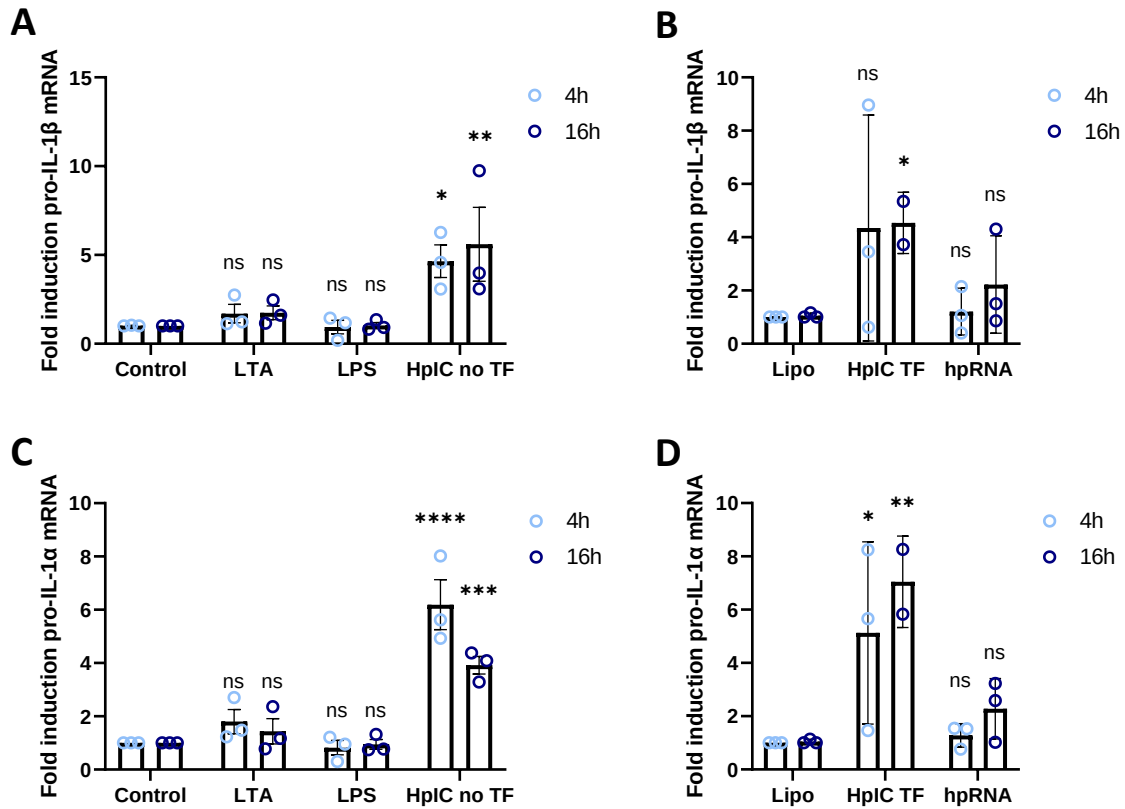


Figure 3.15: Transfected and naked dsRNA induces pro-IL-1 β and pro-IL-1 α mRNA expression in normal human bronchial epithelial (NHBE) cells.

NHBE cells were treated LTA (2.5 μ g/ml), LPS (200 ng/ml), high molecular weight poly(I:C) (HpIC, 2.5 μ g/ml) or transfected (TF) with HpIC (2.5 μ g/ml), hpRNA (100 ng/ml) for 4 h and 16 h. Cell lysates were harvested and pro-IL-1 β (A, B) and pro-IL-1 α (C, D) mRNA expression was analysed by RT-qPCR. Data are mean $\pm$ SEM of three independent experiments. ns: not significant, * p < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001 by two-way ANOVA, compared to control (A, C) or lipo (B, D).

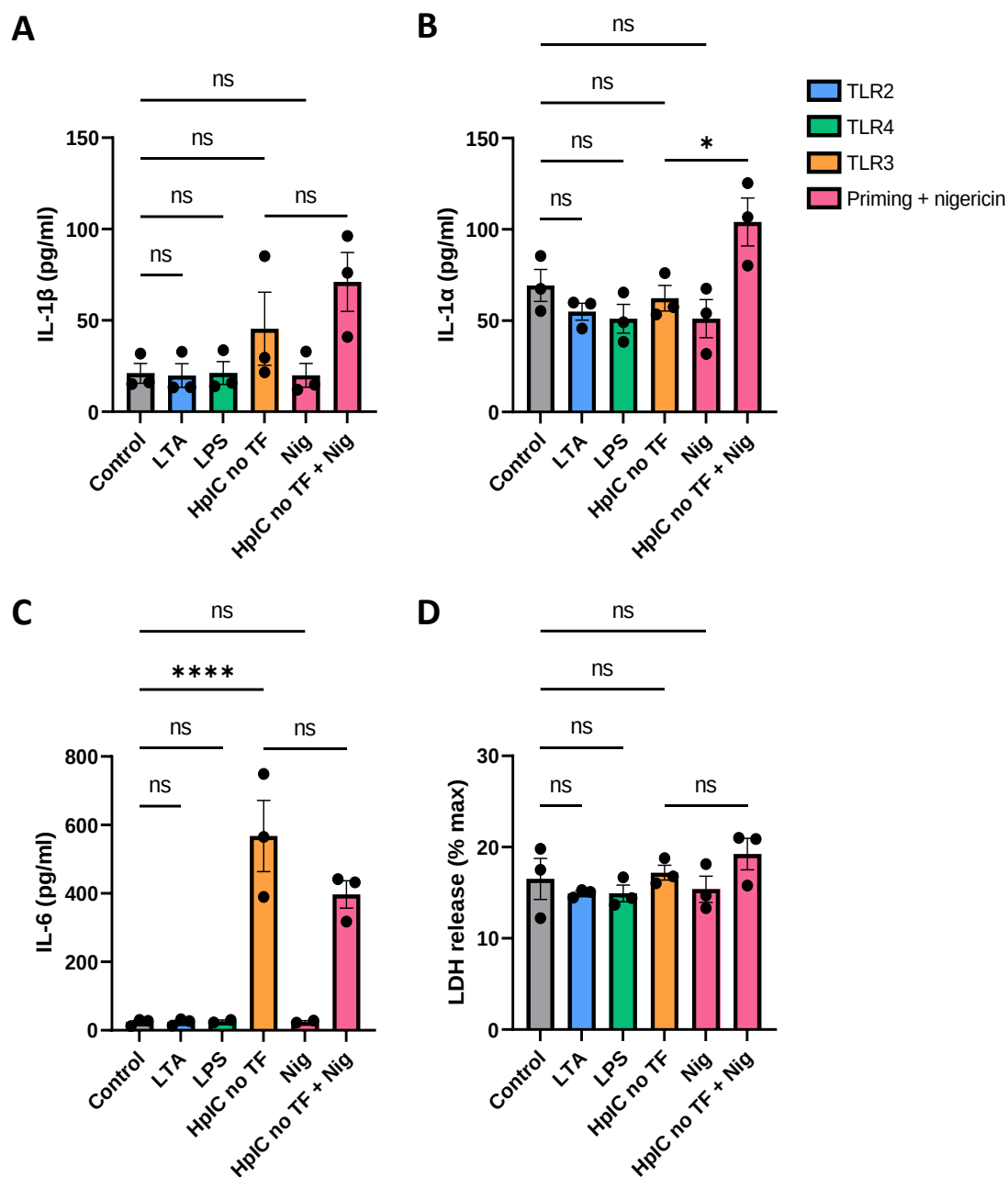


Figure 3.16: TLR stimulations fail to secrete IL-1 β and trigger lytic cell death in NHBE cells.

NHBE cells were treated with LTA (2.5 μ g/ml), LPS (200 ng/ml) or high molecular weight poly(I:C) (HpIC, 2.5 μ g/ml) for 16 h. Nigericin (Nig, 10 μ M) was subsequently added for 2 h in the indicated conditions. IL-1 β (A), IL-1 α (B) and IL-6 (C) secretion was measured by ELISA. LDH present in the supernatant was quantified by LDH assay (D). Data are mean $\pm$ SEM of two or three independent experiments. ns: not significant, * p < 0.05 and **** p < 0.0001 by one-way ANOVA.

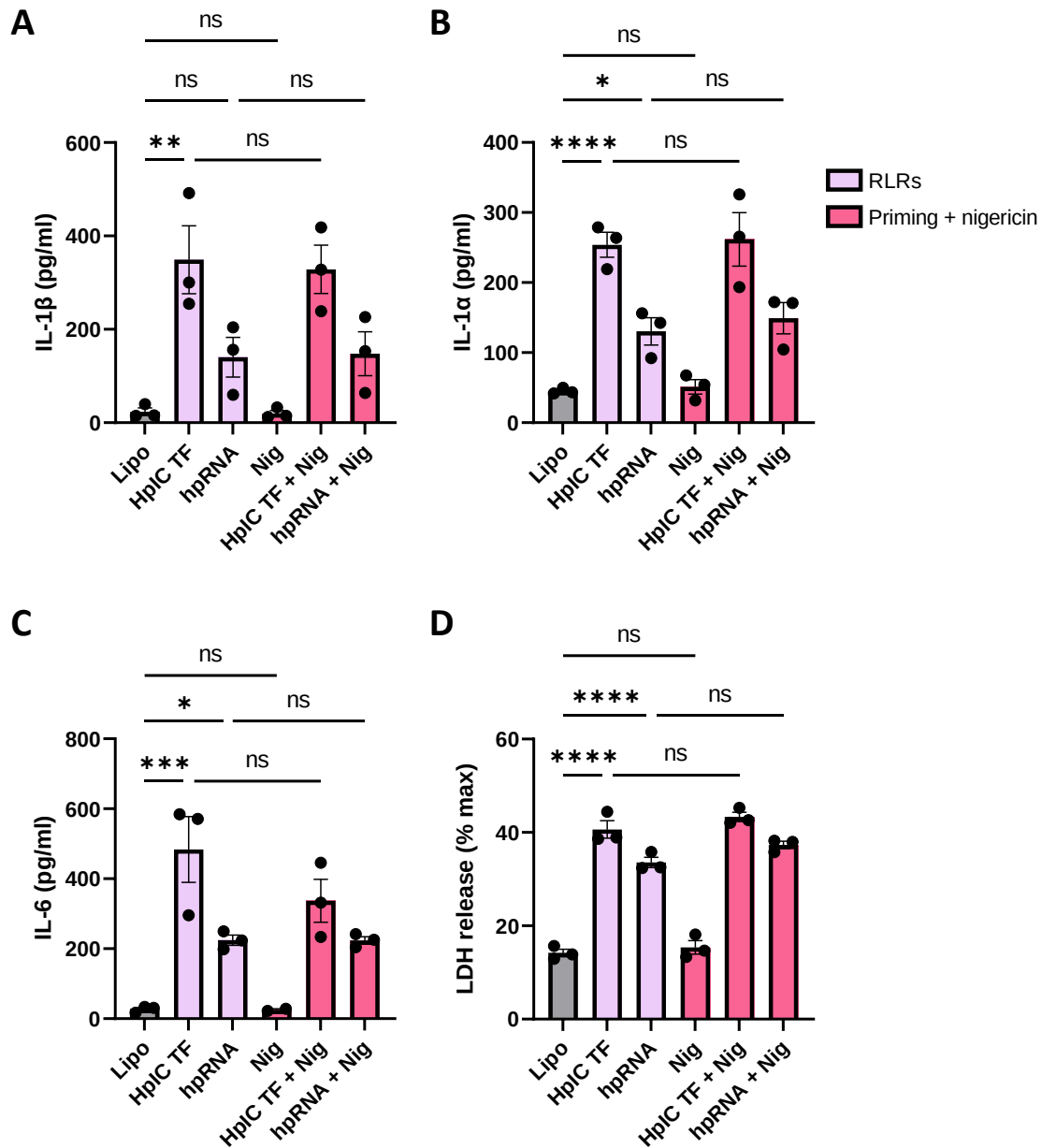


Figure 3.17: Cytosolic dsRNA delivery leads to IL-1 secretion and lytic cell death in NHBE cells.

NHBE cells were transfected (TF) with high molecular weight poly(I:C) (HpIC, 2.5 μ g/ml) or hpRNA (100 ng/ml) for 16 h. Nigericin (Nig, 10 μ M) was subsequently added for 2 h in the indicated conditions. IL-1 β (A), IL-1 α (B) and IL-6 (C) secretion was measured by ELISA. LDH present in the supernatant was quantified by LDH assay (D). Data are mean $\pm$ SEM of three independent experiments. ns: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ by one-way ANOVA.

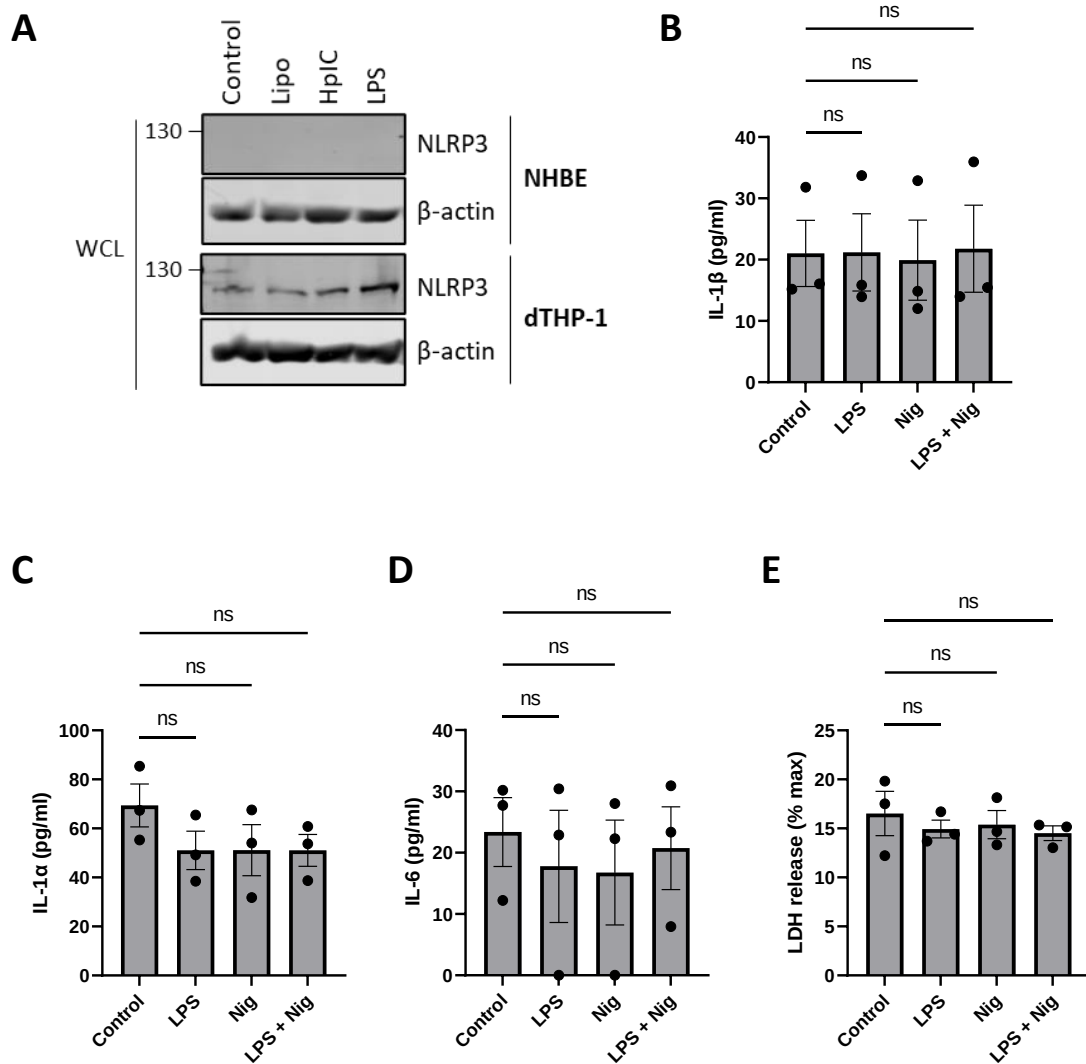


Figure 3.18: NLRP3 inflammasome is not activated in NHBE cells.

(A) NHBE cells were transfected with high molecular weight poly(I:C) (HpIC, 2.5 µg/ml) or treated with LPS (200 ng/ml) for 16 h. dTHP-1 cells (5×10^5 cells/ml) cells were transfected with high molecular weight poly(I:C) (HpIC, 10 µg/ml) or treated with LPS (200 ng/ml) for 16 h. Cell lysates (WCL) were immunoblotted for the expression of NLRP3 and β-actin. Immunoblots are representative of three independent experiments.

(B-E) NHBE cells were treated with LPS (200 ng/ml) for 4 h. Nigericin (Nig, 10 µM) was subsequently added for 2 h. IL-1β (B), IL-1α (C) and IL-6 (D) secretion was measured by ELISA. LDH present in the supernatant was quantified by LDH assay (E). Data are mean ± SEM of three independent experiments. ns: not significant by one-way ANOVA.

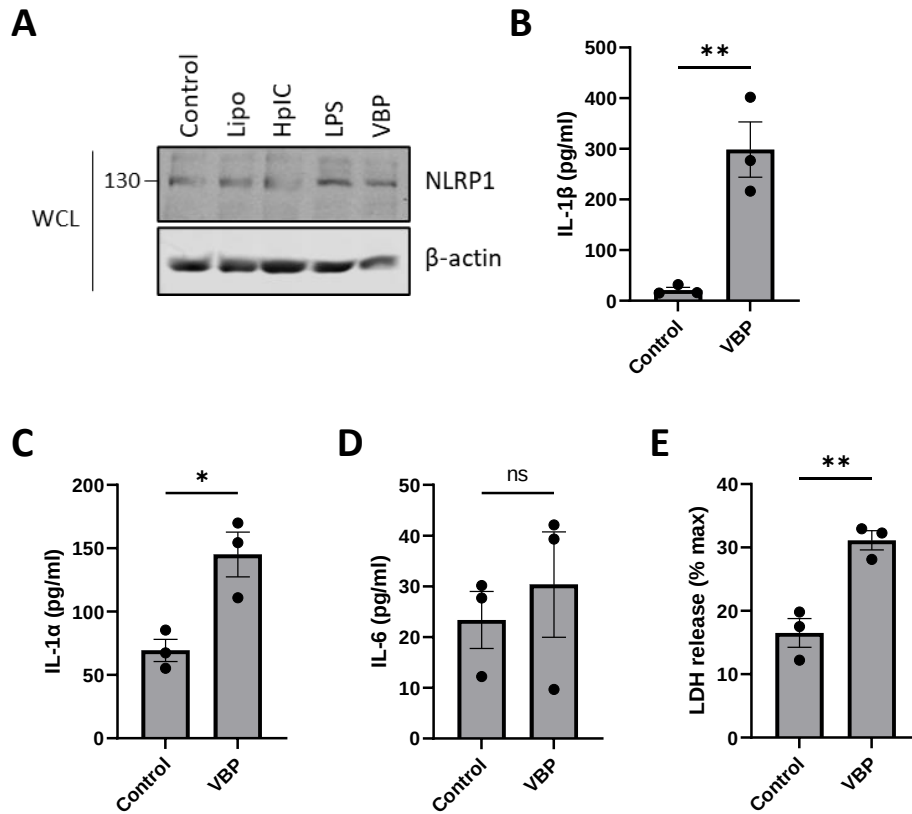


Figure 3.19: VBP triggers IL-1 secretion and lytic cell death in NHBE cells.

(A) NHBE cells were transfected with high molecular weight poly(I:C) (HpIC, 2.5 µg/ml) or treated with LPS (200 ng/ml), Val-boro-Pro (VBP, 3 µM) for 16 h. Cell lysates (WCL) were immunoblotted for the expression of NLRP1 and β-actin. Immunoblots are representative of three independent experiments.

(B-E) NHBE cells were treated with Val-boro-Pro (VBP, 3 µM) for 16 h. IL-1β (B), IL-1α (C) and IL-6 (D) secretion was measured by ELISA. LDH present in the supernatant was quantified by LDH assay (E). Data are mean ± SEM of three independent experiments. ns: not significant, *p < 0.05 and **p < 0.01 by unpaired Student's t test.

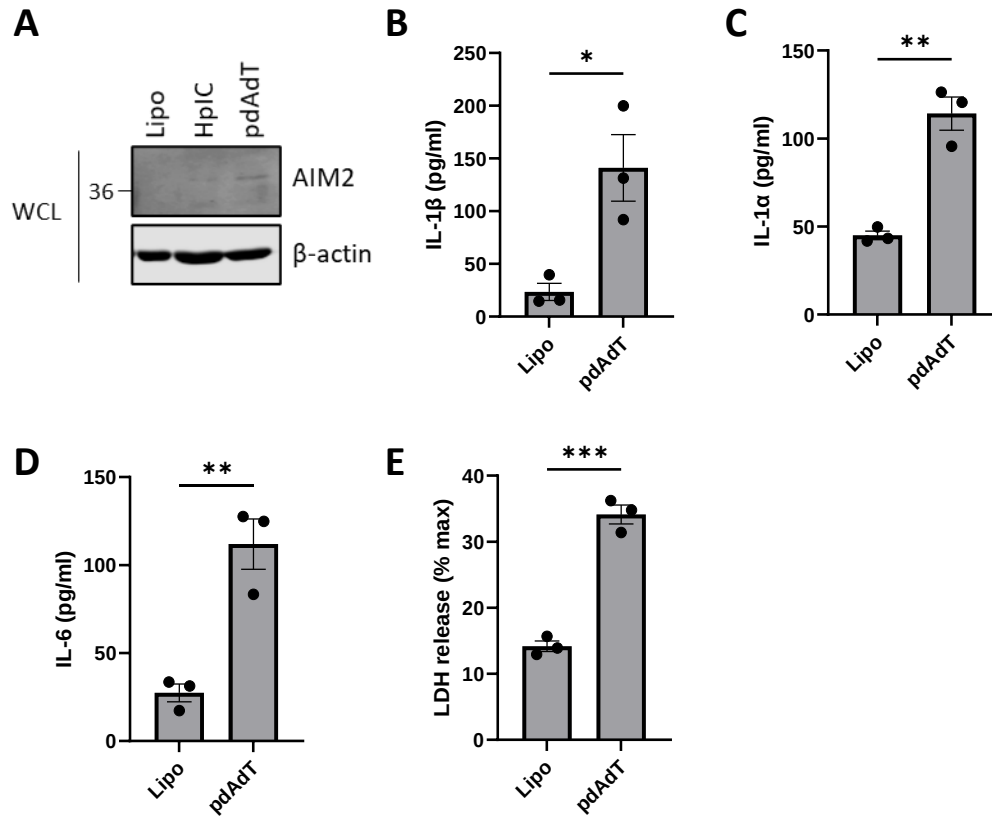


Figure 3.20: Poly(dA:dT) triggers IL-1 secretion and lytic cell death in NHBE cells.

(A) NHBE cells were transfected with high molecular weight poly(I:C) (HpIC, 2.5 µg/ml) or poly(dA:dT) (pdAdT, 2.5 µg/ml) for 16 h. Cell lysates (WCL) were immunoblotted for the expression of AIM2 and β-actin. Immunoblots are representative of three independent experiments.

(B-E) NHBE cells were transfected with poly(dA:dT) (pdAdT, 2.5 µg/ml) for 16 h. IL-1β (B), IL-1α (C) and IL-6 (D) secretion was measured by ELISA. LDH present in the supernatant was quantified by LDH assay (E). Data are mean ± SEM of three independent experiments. ns: not significant, *p < 0.05, **p < 0.01 and ***p < 0.001 by unpaired Student's t test.

3.3 Discussion

3.3.1 dTHP-1 cells are a good model to study NLRP3 inflammasome

Our results demonstrated that LPS and nigericin treatment activated NLRP3 inflammasome which led to GSDMD cleavage, IL-1 β secretion and LDH release in dTHP-1 cells. This is consistent with previous studies in mouse myeloid cells [174] and dTHP-1 cells [201]. Furthermore, the use of MCC950, the NLRP3 inhibitor, decreased the secretion of pro-caspase-1 in the supernatant and increased its expression in the WCL compared to YVAD and ZVAD, the caspase-1 inhibitor and pan-caspase inhibitor respectively, which further showed the involvement of NLRP3 inflammasome in the pyroptotic cell death.

Priming is an important step that upregulates pro-IL-1 β and NLRP3 expression [116]. Unlike LTA and hpRNA, LPS didn't significantly enhance pro-IL-1 β mRNA expression. This result was surprising since LPS is expected to upregulate pro-IL-1 β transcription in differentiated THP-1 cells [202]. It is possible that there was a problem with the stock of LPS used to perform these experiments. However immunoblots showed that both pro-IL-1 β and NLRP3 proteins were constitutively expressed in dTHP-1 cells. Interestingly, nigericin treatment alone triggered caspase-1 activation, GSDMD cleavage into a pore-forming fragment, pro-IL-1 β maturation and IL-1 β secretion in dTHP-1 cells. These data show that priming is not a necessary step to trigger NLRP3 inflammasome assembly in dTHP-1 cells, which is in line with a previous report showing that priming was not necessary in human monocyte cells [201]. It is worth noting that NLRP3 expression was increased in LPS-treated dTHP-1 cells suggesting that priming could still be important to enhance NLRP3-dependent response in dTHP-1 cells.

Our results also showed that in nigericin treated LPS-primed dTHP-1 cells, YVAD and ZVAD diminished caspase-1 p20, and to a lesser extent p33 appearance, but that MCC950 completely abolished pro-caspase-1 autoprocessing into mature caspase-1. Similarly, while YVAD and ZVAD decreased pro-IL-1 β maturation and secretion, this was abolished in the presence of MCC950. One explanation is that in the presence of YVAD and ZVAD, NLRP3 inflammasome can still be formed which results in caspase-1 activation that is not completely inhibited by YVAD and ZVAD, whereas in the presence of the potent MCC950, NLRP3 inflammasome is not formed and therefore pro-caspase-1 is not autoprocessed preventing pro-IL-1 β maturation. Moreover, our results demonstrated in the presence of MCC950, GSDMD p30 cleavage was inhibited, as expected, but that GSDMD

was cleaved into a p43 fragment instead. This cleavage is a marker of GSDMD inactivation and is mediated by caspase-3 [203]. Interestingly a study reported that in the absence of caspase-1, stimulation of the NLRP3 inflammasome triggered activation of caspase-3 in mouse and human macrophages [204]. Given that MCC950 prevented caspase-1 activation and that dTHP-1 cells were stimulated with LPS and nigericin, it is possible that these conditions induce caspase-3-mediated GSDMD inactivation.

3.3.2 A549 and BEAS-2B cells are not a suitable model to study inflammasome in human epithelial cells

Cancerous alveolar A549 cells and transformed bronchial BEAS-2B cells failed to elicit lytic cell death or IL-1 β secretion following stimulation, revealing that they are not a good model to study inflammasome activation. Since all TLR stimulations also failed to induce the secretion of IL-6 in A549 cells, another inflammasome-independent cytokine, such as IL-8, could have been measured to control the efficacy of the stimulations. Nevertheless, transfected poly(I:C) and hpRNA did induce IL-6 secretion in both cell types, but not IL-1 β , showing that the cells can respond to cytosolic RNA delivery. Moreover transfection of poly(I:C) induced IL-1 β secretion and LDH release in NHBE cells, strengthening the conclusion that A549 and BEAS-2B cells are not a suitable model for inflammasome study. Consistent with these results, a report showed that precancerous and primary bronchial epithelial cells secreted IL-1 β following IAV infection, unlike A549 cells [198]. The authors suggested that to avoid being removed by immune cells, tumor cells such as A549 cells had downregulated pathways that could lead to inflammatory cell death such as pyroptosis [198]. A recent paper confirmed that A549 cells indeed didn't express NLRP1 receptor unlike NHBE cells [145].

3.3.3 NHBE cells are a better model than NuLi-1 to study inflammasome activation

None of the TLR agonists triggered inflammasome activation in the precancerous NuLi-1 cells nor in the undifferentiated primary NHBE cells. Stimulation of RIG-I with hpRNA ligand caused lytic cell death in both NuLi-1 and NHBE cells but only induced IL-1 β secretion in NHBE cells. In addition, cytosolic poly(I:C) delivery triggered significant IL-1 β secretion and lytic cell death in NuLi-1 and NHBE cells. Despite being low, the levels of IL-1 β release in NuLi-1 cells are consistent with a previous report for a human precancerous respiratory epithelial cell line called PL16T [167]. Nevertheless, the release of

IL-1 β was stronger and reached significance in NHBE cells. Indeed IL-1 β secretion was 10-times higher in NHBE compared to NuLi-1 cells following dsRNA sensing. Since NHBE and NuLi-1 are both bronchial epithelial cells, one possible explanation for the difference in IL-1 β secretion is that the immortalisation process could have downregulated the basal expression of pro-IL-1 β and/or the inflammasome component proteins. Indeed, VBP didn't trigger IL-1 β release nor lytic cell death in NuLi-1 cells, unlike NHBE cells, suggesting that NLRP1 and/or CARD8 inflammasome can't be activated in NuLi-1. In addition, cytosolic RNA ligands primed NuLi-1 cells and NHBE cells in a similar way, suggesting that gene transcription isn't impacted. Thereby, determining the expression of pro-IL-1 β and inflammasome component proteins by immunoblot in NuLi-1 cells could help to understand this difference.

Interestingly, IL-1 β and IL-1 α secretion followed a similar pattern in NuLi-1 and NHBE cells. For instance, while VBP treatment neither released IL-1 β nor IL-1 α , poly(dA:dT) triggered the release of both IL-1 β and IL-1 α at similar levels in NuLi-1 cells. Similarly in NHBE cells, when a ligand induced IL-1 β secretion, such as cytosolic dsRNA, VBP and poly(dA:dT), such ligand also released a similar quantity of IL-1 α . Inflammasome and IL-1 β enhance mature IL-1 α secretion in mouse myeloid cells [195], since VBP and poly(dA:dT) are inflammasome activators, it is possible that a similar mechanism exists in respiratory epithelial cells. Another explanation is that IL-1 α is passively released from cells during lytic cell death. For instance, IL-1 α is basally expressed in epithelial cells [96], thereby plasma membrane rupture triggered by inflammasome activation could release pro-IL-1 α and mature IL-1 α in the extracellular space. In addition, IL-6 secretion was usually enhanced when IL-1 β and IL-1 α were released. This is in accordance with a study showing that once secreted from RSV-infected pulmonary epithelial cells, IL-1 β can act in an autocrine manner to trigger the secretion of IL-6 [205]. In addition a report demonstrated that IL-1 α can elicit IL-6 release from epithelial cells [206]. Thereby measuring IL-6 secretion in NuLi-1 and NHBE cells could be an indirect marker of IL-1 release, and subsequently inflammasome activation.

Overall, I provided evidence that NHBE cells are a suitable model to characterise inflammasome activation in basal respiratory epithelial cells.

3.3.4 Several inflammasome receptors are expressed in NHBE cells

I failed to detect NLRP3 protein expression in NHBE cells and LPS and nigericin treatment didn't lead to IL-1 β secretion nor lytic cell death, showing a key difference between myeloid and respiratory epithelial cells. Only a few papers have implicated NLRP3 inflammasome as being active in epithelial cells. For instance, siRNA against NLRP3 in IAV-infected NHBE cells showed that IL-1 β release was NLRP3 inflammasome [165]. However, in that study the protein expression of NLRP3 at the basal level or after siRNA treatment wasn't assessed. Thus, our results are consistent with the majority of the papers showing that NLRP3-independent inflammasomes are activated in epithelial cells [143, 153, 167].

VBP and poly(dA:dT) stimulation triggered the release of IL-1 β and LDH in NHBE cells. While VBP-mediated pyroptosis is NLRP1 inflammasome-dependent in human barrier epithelial cells [148, 153], VBP can also activate CARD8 inflammasome in human myeloid cells and T cells [147, 199]. In addition, VBP triggers both NLRP1 and CARD8 inflammasome in endothelial cells [207]. Since immunoblot analysis showed that NLRP1 was expressed in NHBE cells, NLRP1 inflammasome most likely triggers VBP-mediated cytokine release and pyroptotic cell death. Yet a role for CARD8 inflammasome cannot be excluded. In addition, poly(dA:dT) is recognised by the cytosolic sensor AIM2 which forms the AIM2 inflammasome [159]. However, RNA polymerase III can transcribe poly(dA:dT) into 5'-triphosphate dsRNA which triggers RIG-I receptors [208]. Since our results showed that hpRNA, the RIG-I agonist, caused IL-1 β secretion and lytic cell death in NHBE cells, it's possible that part of poly(dA:dT) response is RIG-I-dependent. RIG-I can indeed form an inflammasome in response to vesicular stomatitis virus infection or 5'-triphosphate RNA stimulation in mouse BMDCs [50]. Thereby it will be interesting to downregulate RIG-I expression using siRNA to evaluate the contribution of RIG-I inflammasome in poly(dA:dT) and hpRNA-mediated responses.

In NuLi-1 cells, I showed that HMW poly(I:C) and high concentration of LMW poly(I:C) led to similar IL-1 β and LDH release. Given that RIG-I or MDA5 senses short and long cytosolic poly(I:C) respectively [41] and that MDA5 has not been directly involved in inflammasome activation, it's likely that neither RIG-I or MDA5 forms an inflammasome following cytosolic dsRNA delivery. Nevertheless, transfected poly(I:C) directly triggered IL-1 β secretion, suggesting that dsRNA can act both as a priming step and activator of the inflammasome in basal respiratory epithelial cells. Thereby, RLRs receptors could sense poly(I:C) and induce the priming step. Moreover, since NLRP1 is expressed and activated by VBP stimulation in NHBE cells, it is of interest to further investigate the contribution of

NLRP1 *versus* RIG-I/MDA5 in cytosolic dsRNA-mediated cytokine secretion and lytic cell death. Indeed, increasing the concentration of HMW poly(I:C) didn't impact IL-1 β secretion in NuLi-1 cells, whereas low concentration of LMW poly(I:C) induced less IL-1 β secretion than higher concentration. NLRP1 receptor binds to dsRNA of minimum 500 bp length such as HMW poly(I:C) [153]. The size of LMW poly(I:C) varies between 200 bp and 1 kb. Thereby, higher concentrations of LMW poly(I:C) could increase the availability of longer dsRNA that can then be recognised by NLRP1. However even though AIM2 expression is difficult to detect by immunoblot, its role in inflammasome activation shouldn't be ruled out.

Since we are particularly interested in the downstream consequences of virus-induced inflammasome activation in epithelial cells, and since the data here showed that transfected dsRNA, which is a well-known mimic of viral infection, activated the inflammasome, in the next chapter I focused on characterising in detail inflammasome-dependent dsRNA responses in undifferentiated NHBE cells, especially GSDM cleavage.

**Chapter 4. Results II - Mechanisms of gasdermin
cleavage in response to dsRNA sensing in
basal respiratory epithelial cells**

4.1 Introduction

In the last chapter I showed that poly(I:C) treatment of NHBE cells led to IL-1 β secretion and LDH release. This allowed me to use these cells to examine in more detail in epithelial cells the consequences of activation of inflammasome pathways. The formation of GSDMD pores after inflammasome activation is critical for release of IL-1 and as a step towards cell death. Therefore in this chapter I focused on investigating the mechanism of GSDM cleavage in response to cytosolic dsRNA sensing in basal respiratory epithelial cells.

The GSDM family is composed of six proteins members, namely GSDMA, GSDMB, GSDMC, GSDMD, GSDME and Pejvakin (PJVK). These proteins have a N-terminal pore-forming domain, except PJVK, and a C-terminal autoinhibitory domain that are connected through a linker region [209, 210]. The C-terminal domain interacts with the N-terminal domain to avoid constitutive activation of GSDM proteins that would trigger excessive cell death [209]. Most of our current knowledge on the GSDM family has come from the study of GSDMD protein. Downstream of the inflammasome, autoprocessed caspase-1 cleaves GSDMD within the linker region at residue Asp275 to release the pyroptotic N-terminal domain from the repressor C-terminal domain [174, 175] **[Figure 4.1]**. The N-terminal domain can then bind to the plasma membrane, oligomerise and form pores from 10 to 20 nm [177, 178] that allows the release of pro-inflammatory IL-1 β and IL-18 cytokines [174, 175], but also IL-1 α [187]. Even though the ESCRT machinery can repair the plasma membrane by removing the pores during the sublytic phase [183], the presence of pores can lead to plasma membrane rupture resulting in pyroptosis [174, 175]. In addition to caspase-1, human caspase-4 and human caspase-5, mouse caspase-11 can also cleave GSDMD into a pore-forming fragment [173, 174, 211] **[Figure 4.1]**.

Although the role of inflammatory caspases in pyroptosis has been well established, the apoptotic caspase-8 can also cleave GSDMD at position Asp275, the same site used by caspase-1, to generate a pore-forming fragment that allows IL-1 β secretion and pyroptotic cell death in murine macrophages [212–215] **[Figure 4.1]**. Additionally, it has recently been reported that apoptotic cells can progress to pyroptosis in the presence of caspase-3 [216, 217]. The authors demonstrated that caspase-3 cleaves GSDME after Asp270 into a N-terminal and a C-terminal fragment **[Figure 4.1]**. The GSDME p30 N-terminal fragment then forms pores either by inserting into the plasma membrane leading to pyroptotic cell death [216, 217], or into the mitochondrial membrane leading to cytochrome c release that further activates caspase-3 through the apoptosome [218]. However, besides promoting

GSDME processing, caspase-3 can also cleave the full length and the pore-forming fragment (p30) of GSDMD into a p43 and p20 fragment respectively resulting in GSDMD inactivation [203, 214, 215] [Figure 4.1]. GSDME processing into active pores illustrates a dual role for caspase-8 and caspase-3 in apoptotic and pyroptotic cell death. The involvement of non-inflammatory caspases in induction of pyroptotic cell death and the presence of pore-forming activity in GSDM family proteins encouraged the Nomenclature Committee on Cell Death to propose a new definition of pyroptosis: “a type of regulated cell death that critically depends on the formation of plasma membrane pores by members of the gasdermin protein family, often but not always as a consequence of inflammatory caspase activation” [219].



Figure 4.1: Summary of GSDMD and GSDME cleavage sites.

Cleavage at Asp275 by caspase-1, caspase-4, caspase-5 and to a lesser extent caspase-8 leads to GSDMD activation. Cleavage at Asp87 by caspase-3 results in GSDMD inactivation. Cleavage at Asp270 by caspase-3 generates GSDME pore-forming fragment.

GSDME, also called deafness autosomal dominant 5 (DFNA5) was first described as a gene causing autosomal dominant non-syndromic hearing loss [220]. Thereafter, identification of structural and sequence similarities between DFNA5 and GSDM proteins classified DFNA5 into the GSDM family upon which it was renamed GSDME [220]. GSDME is thought to have a tumour suppressive activity by causing pyroptosis in tumour cells [217, 221]. Indeed chemotherapy drugs usually induce apoptosis, but high levels of GSDME in cancer cells switches apoptosis to pyroptosis [217]. It has also recently been highlighted that GSDME leads to pyroptosis during viral infection [145, 194, 222]. This will be further described in **section 5.1**. In addition, GSDME pores allow the secretion of IL-1 β independently of pyroptosis during the sublytic phase in human GSDMD-deficient macrophages treated with nigericin, the activator of the NLRP3 inflammasome [223]. This

shows that GSDME, like GSDMD, can induce cytokine secretion from living cells. In that study, the authors also showed that higher expression of GSDME triggered GSDME-dependent lytic cell death, which further enhanced cytokine release [223]. In addition, in Pam3CSK4-primed mouse and human macrophages treated with nigericin, in the absence of caspase-1, ASC promotes caspase-8 activation which mediates caspase-3-dependent GSDME pore formation allowing IL-1 α secretion [204]. Since GSDME appears to have similar acidic residues as GSDMD [186], IL-1 α is likely released by GSDME-mediated lytic cell death rather than secreted through GSDME pores. However the contribution of GSDME pores in cytokine secretion in respiratory epithelial cells remains unclear.

In the previous chapter, I mimicked viral infection of epithelial cells by transfection of poly(I:C), which led to lytic cell death and IL-1 secretion in primary bronchial epithelial cells. Therefore in this chapter, I investigated the potential contribution of GSDM cleavage to these responses following dsRNA sensing in basal respiratory epithelial cells.

4.2 Results

4.2.1 Cytosolic dsRNA sensing triggers GSDM cleavage in NHBE cells

In the previous chapter I showed that transfected HMW poly(I:C) led to IL-1 secretion and lytic cell death, therefore here I examined GSDMD, another marker of inflammasome activation and potential explanation for how IL-1 would get released from cells. Hereafter all experiments were performed with HMW poly(I:C) unless otherwise stated. NHBE cells were transfected with poly(I:C) for 16 h and whole cell lysates and cell supernatants were subsequently immunoblotted to visualise GSDMD cleavage. Analysis showed that cytosolic poly(I:C) generated a pyroptotic p30 GSDMD fragment but also a p43 fragment that is a marker of GSDMD inactivation [**Figure 4.2**]. A recent report showed that GSDME can also be cleaved in barrier epithelial cells [194]. Thereby, I next investigated whether GSDME was also activated in basal respiratory epithelial cells in response to dsRNA. Immunoblot analysis indicated that GSDME was cleaved into a pyroptotic p30 fragment following transfection of poly(I:C) [**Figure 4.2**]. Importantly, naked poly(I:C) stimulation barely triggered GSDME pore formation and didn't induce GSDMD cleavage [**Figure 4.2**] indicating that GSDM processing is specific to detection of cytosolic dsRNA. In addition, GSDM fragments were detected in both the whole cell lysates and cell supernatants, showing that poly(I:C) transfection is a major inducer of lytic cell death. Therefore, I decided to carry on with analysing both the lysate and cell supernatant to monitor the effect of GSDM on cell lysis. To understand the kinetics of GSDM processing directly following cytosolic dsRNA, I performed a poly(I:C) transfection time course, looking at time points prior to 16 h. GSDMD cleavage into a p30 pore-forming fragment became prominently visible at 6 h post-transfection [**Figure 4.3**]. The inactivated p43 fragment of GSDMD was also faintly visible at 6 h post-transfection, but was more prominent by 10 h [**Figure 4.3**]. This shows that GSDMD is first activated and then inactivated. GSDME active pore-forming fragment appeared at 6 h and increased by 10 h [**Figure 4.3**]. To confirm the ability of GSDME to self-assemble into larger molecular complexes, as a surrogate marker for GSDME pore formation, I used non-reducing SDS-PAGE as previously described [9]. **Figure 4.4** showed that cytosolic poly(I:C) led to the dimerisation of GSDME, confirming that the p30 GSDME fragment generated is a pore-forming fragment [**Figure 4.4**]. Thus these results demonstrate that cytosolic dsRNA sensing triggers both GSDMD and GSDME processing into a pore-forming fragment in basal respiratory epithelial cells but that GSDMD is then mostly inactivated over time.

4.2.2 GSDM pores elicit cytokine secretion and lytic cell death following cytosolic dsRNA sensing in NHBE cells

Next I investigated the contribution of GSDM in dsRNA-stimulated membrane permeabilisation, by measuring PI uptake. Since I was working with primary cells, I took advantage of siRNA gene silencing to downregulate GSDM expression. qPCR and immunoblot analysis showed that both mRNA and protein levels of GSDMD and GSDME were effectively diminished in the presence of their respective siRNA in NHBE cells [Figure 4.5]. Poly(I:C) transfection engendered PI uptake over time that plateaued from 12 h [Figure 4.6]. PI uptake was decreased in the presence of GSDMD and GSDME siRNA up to 8 h compared to the presence of control siRNA, whereas from 12 h, PI uptake was mostly diminished upon GSDME downregulation but not GSDMD downregulation [Figure 4.6]. Importantly, treatment of cells with lipofectamine and control siRNA failed to induce PI positive cells over time, confirming that the results are specific to dsRNA sensing and are not due to the detection of siRNA [Figure 4.6]. Thereby cytosolic dsRNA sensing led to both GSDMD and GSDME pore formation at early times but mostly triggered GSDME pore formation at later times, which is in accordance with previous results showing that GSDMD is inactivated over time.

Given that poly(I:C) sensing generated GSDM pores and that GSDM pores trigger pyroptosis [174, 216], I next quantified the release of LDH, a marker of lytic cell death, in the cell supernatant over time. LDH release increased over time following poly(I:C) transfection and diminished at 8 h post-transfection in the presence of GSDMD and GSDME siRNA, however this decrease only reached significance for siRNA against GSDME [Figure 4.7A, B]. At 24 h, only GSDME knockdown decreased LDH secretion [Figure 4.7B]. These results show that GSDME is required for lytic cell death in dsRNA-stimulated NHBE cells.

In addition to lytic cell death, GSDMD pores enable IL-1 β secretion [174, 175]. It has recently been revealed that GSDME pores also release IL-1 β in macrophages [223]. Furthermore, IL-1 α secretion is mediated by GSDMD and GSDME pores in mouse and human macrophages [187, 204]. Thus I next measured IL-1 β and IL-1 α secretion over time by ELISA to determine the contribution of GSDMD and GSDME pores to cytokine release. Analysis showed that IL-1 β secretion was diminished in the presence of both GSDMD and GSDME siRNA at 8 h but that only GSDME was required for IL-1 β secretion at 24 h [Figure 4.8A, B]. However silencing of GSDMD and GSDME decreased the release of IL-1 α at both 8 h and 24 h [Figure 4.8C, D]. Next, I measured the secretion of IL-6 whose

release is enhanced by IL-1 [205]. While there was less IL-6 secretion in the presence of GSDMD and GSDME siRNA at both 8 h and 24 h, it reached significance only for GSDME knockdown [**Figure 4.8E, F**].

Overall, these data indicate that GSDME is required for both cytokine secretion and lytic cell death, and that GSDMD contributes to cytokine release, especially at early times in dsRNA-stimulated NHBE cells.

4.2.3 Both caspase-1 and caspase-3 are required for GSDM cleavage and cytokine secretion following cytosolic dsRNA sensing in NHBE cells

Inflammasome formation leads to caspase-1 activation that further mediates GSDMD pore formation [174, 175]. Nevertheless instead of being cleaved in its linker region by caspase-1, GSDMD can also be processed in its N-terminal domain by apoptotic caspase-3 into an inactivated p43 fragment [203]. Moreover, activated caspase-3 cleaves GSDME into a pyroptotic fragment [216]. Consequently, I next examined the activation of caspase-1 and caspase-3 in response to dsRNA sensing by detecting their activated forms, namely p33 and p20 for caspase-1, and p17 and p12 for caspase-3, in the cell supernatant. Immunoblot analysis indicated that caspase-1 was activated from 6 h post-transfection and that caspase-3 was visible at 6 h post-transfection but was more predominant by 8 h [**Figure 4.9**] which correlates with GSDMD and GSDME cleavage over time [**Figure 4.3**]. Thereby I next treated NHBE cells with VX765, a caspase-1 inhibitor, and DEVD, a caspase-3 inhibitor, before poly(I:C) transfection, to determine the involvement of these caspases in GSDM cleavage. I first confirmed that VX765 and DEVD inhibitors decreased the activation of caspase-1 and caspase-3 respectively at 6 h post-transfection [**Figure 4.10**]. It is worth nothing that the decrease of caspase-1 active form in the presence of DEVD was likely a consequence of DEVD limiting caspase-3-dependent secretion of cellular proteins, rather than a direct role of caspase-3 in caspase-1 maturation [**Figure 4.10; Figure 4.11**]. Then, immunoblot analysis showed that GSDMD and GSDME pore-forming fragments were diminished in the presence of VX765 and DEVD respectively [**Figure 4.10**] and that DEVD abolished the appearance of GSDMD inactivated p43 fragment [**Figure 4.10**]. Thereby these results show that caspase-1 mediates GSDMD cleavage into a pore-forming fragment and that caspase-3 leads to GSDMD inactivation and GSDME activation. I next investigated the contribution of caspase-1 and caspase-3 in lytic cell death and cytokine secretion. The caspase-3 inhibitor DEVD diminished dsRNA-stimulated LDH release,

unlike the caspase-1 inhibitor VX765 [Figure 4.11], showing that lytic cell death is mediated by caspase-3 but not by caspase-1. However ELISA and immunoblot analysis showed both VX765 and DEVD decreased the secretion of IL-1 β and IL-1 α [Figure 4.12A-D] demonstrating that both caspase-1 and caspase-3 are required for optimal IL-1 release. Importantly the addition of VX765 and DEVD did not affect IL-1 β and IL-1 α pro-forms expression [Figure 4.12B, D]. Measurement of IL-6 in the cell supernatant showed that caspase-1 was not involved in IL-6 release and that caspase-3 slightly increased its secretion [Figure 4.12E]. Overall these results indicate that caspase-1 is important for GSDMD pore formation and the secretion of IL-1 cytokine but not for lytic cell death, and that caspase-3 mediates GSDMD inactivation, GSDME activation, lytic cell death and IL-1 secretion.

4.2.4 ASC is involved in GSDMD cleavage, lytic cell death, IL-1 β and early IL-1 α secretion following cytosolic dsRNA sensing in NHBE cells

Given that caspase-1 is activated following inflammasome formation, I next investigated potential upstream mechanisms of caspase activation by first looking at inflammasome activation. Most inflammasomes contain the adaptor protein ASC that polymerises into long fragments within the multiprotein complex [168] and these ASC oligomers can be crosslinked and visualised by immunoblot. Here, ASC monomers were detectable in the soluble cell lysate, and poly(I:C) delivery enhanced ASC dimerisation and oligomerisation in the insoluble pellet fraction [Figure 4.13]. This indicates that dsRNA sensing enables the formation of inflammasomes containing ASC in NHBE cells. To determine the contribution of the inflammasome in GSDM cleavage, I used siRNA to downregulate ASC mRNA and protein expression [Figure 4.14]. The presence of ASC siRNA decreased the release of caspase-1 p33 and p20 forms in the cell supernatant and prevented the cleavage of GSDMD into its pyroptotic p30 fragment at both 6 h and 16 h [Figure 4.15A, B]. However, ASC downregulation didn't affect caspase-3 activation, GSDME pore formation nor GSDMD inactivation [Figure 4.15A, B]. Then I assessed the involvement of ASC in lytic cell death and cytokine secretion. LDH release was diminished at both 6 h and to a lesser extent at 16 h when ASC expression was reduced by siRNA [Figure 4.16A, B], showing that ASC mediates lytic cell death. ASC siRNA also decreased IL-1 β secretion at both 6 h and 16 h but didn't affect the expression of pro-IL-1 β [Figure 4.17A-C]. In addition, ASC downregulation decreased IL-1 α release at 6 h but not at 16 h [Figure 4.17D] and ASC siRNA didn't impact IL-6 secretion [Figure 4.17E]. Overall these results show that ASC is required for caspase-1 activation, GSDMD pore formation, lytic

cell death, IL-1 β and early IL-1 α secretion. This suggests that sensing of cytosolic dsRNA activates an inflammasome in NHBE cells.

4.2.5 MAVS is not required for GSDM cleavage, lytic cell death nor cytokine secretion following cytosolic dsRNA sensing in NHBE cells

Given that cytosolic dsRNA can be recognised by RIG-I and MDA5 [41] leading to MAVS-dependent signaling, and that MAVS plays a role in inflammasome formation in response to cytosolic dsRNA [224], this prompted us to evaluate the contribution of MAVS in GSDM cleavage. I first confirmed that MAVS siRNA did decrease MAVS mRNA and protein levels [Figure 4.18]. Then immunoblot analysis showed that MAVS expression downregulation didn't prevent GSDM cleavage nor caspases activation [Figure 4.19A, B]. In addition, the presence of MAVS siRNA didn't affect LDH release [Figure 4.20A, B], nor IL-1 or IL-6 secretion [Figure 4.21A-E]. These results show that GSDM cleavage, lytic cell death and cytokine secretion are not mediated by a MAVS-dependent response. Nevertheless, even though MAVS expression was decreased by gene silencing [Figure 4.18], the remaining protein could still be sufficient to trigger MAVS-mediated signaling response. Thereby, I transfected hpRNA, the potent RIG-I ligand, in the presence of control siRNA or MAVS siRNA and measured IFN α/β release in cell supernatant. IFN α/β secretion was almost abolished in the presence of MAVS siRNA compared to control siRNA [Figure 4.22], indicating that MAVS siRNA efficiently blocks signaling pathway downstream of MAVS, which therefore confirms that MAVS is not involved in the above measured dsRNA-mediated responses.

4.2.6 TRIF is involved in early GSDME cleavage, lytic cell death and cytokine secretion following cytosolic dsRNA sensing in NHBE cells

TRIF is the key adaptor for TLR3 which senses dsRNA in endosomes and TRIF has also been described to be involved in the NLRP3 inflammasome activation by LPS in human monocytes [225], but whether TRIF is required for GSDM cleavage is still unclear. Thereby, I next examined whether TRIF-dependent signaling pathway was involved in GSDM cleavage. Measurement of TRIF mRNA levels after treatment with TRIF siRNA confirmed that TRIF expression was downregulated in NHBE cells [Figure 4.23A]. However, TRIF protein expression was not detectable by immunoblot at endogenous level [Figure 4.23B]. Nonetheless, immunoblot analysis showed that GSDME pore formation in response to

transfected dsRNA was diminished when TRIF mRNA expression was suppressed, especially at 6 h and that GSDMD inactivated p43 fragment was slightly decreased at 6 h post-transfection [Figure 4.24A, B]. However TRIF siRNA didn't affect GSDMD pore-forming fragment processing [Figure 4.24A, B]. In addition, TRIF knockdown diminished the detection of caspase-1 and caspase-3 active forms at 6 h but not at 16 h [Figure 4.24A, B]. Then I further characterised the contribution of TRIF in lytic cell death and cytokine secretion. LDH release in cell supernatants was decreased at both 6 h and 16 h in the presence of TRIF siRNA [Figure 4.25A, B] indicating that TRIF is involved in lytic cell death. In addition, TRIF knockdown diminished the release of IL-1 β , IL-1 α and IL-6 at both 6 h and 16 h [Figure 4.26A-E]. Immunoblot analysis showed that expression of pro-IL-1 β wasn't affected by treatment with TRIF siRNA [Figure 4.26B, C], confirming that TRIF is required for IL-1 β secretion. Together these data demonstrate that TRIF is involved in early caspase-1 and caspase-3 activation, early GSDME pore formation, early GSDMD inactivation, lytic cell death and cytokine secretion in dsRNA-stimulated NHBE cells.

4.2.7 NLRP1 is required for GSDMD cleavage, early lytic cell death and cytokine secretion following cytosolic dsRNA sensing in NHBE cells

The data thus far show that ASC and TRIF, but not MAVS are involved in dsRNA-mediated GSDM responses. I next focused on revealing which inflammasome is activated upstream of ASC following cytosolic dsRNA sensing. I first considered the NLRP3 inflammasome. Even though this inflammasome is mostly detected in myeloid cells, some reports have revealed a role for NLRP3 during viral infection in respiratory epithelial cells [69]. In addition, cytosolic dsRNA delivery triggers NLRP3 inflammasome activation in macrophages [224]. Thereby although I couldn't detect NLRP3 protein expression in NHBE cells [Figure 3.18A] and that LPS and nigericin treatment failed to induce IL-1 β and LDH release [Figure 3.18B, E], I next used MCC950, the potent NLRP3 inhibitor [196], to clarify the involvement of NLRP3 inflammasome in dsRNA-stimulated NHBE cells. However MCC950 didn't affect LDH nor IL-1 β release following poly(I:C) transfection [Figure 4.27A, B], demonstrating that NLRP3 inflammasome is not involved in the dsRNA-mediated response.

Recently, NLRP1 has been shown to bind to dsRNA leading to inflammasome formation in barrier epithelial cells [153] and also to sense SARS-CoV-2 infection which mediates GSDME pore formation in respiratory epithelial cells [145]. Therefore, I next

explored the contribution of NLRP1 inflammasome in GSDM cleavage. I first validated that NLRP1 siRNA decreased both NLRP1 mRNA and protein expression [Figure 4.28]. Then, immunoblot analysis showed that caspase-1 p33 and p20 forms were diminished at both 6 h and 16 h dsRNA treatment in the presence of NLRP1 siRNA [Figure 4.29 A, B]. This indicates that NLRP1 is required for caspase-1 activation and thus that NLRP1 forms an inflammasome in response to cytosolic dsRNA. In addition, GSDMD p30 fragment was diminished upon NLRP1 knockdown [Figure 4.29 A, B] which is in accordance with a decrease in caspase-1 activation. However NLRP1 siRNA didn't affect dsRNA-stimulated GSDME cleavage, GSDMD p43 fragment appearance, nor caspase-3 activation [Figure 4.29 A, B]. Next I investigated whether NLRP1 was involved in lytic cell death and cytokine secretion over time. LDH release in cell supernatants was decreased in the presence of NLRP1 siRNA at 8 h but not at 24 h [Figure 4.30A, B], indicating that NLRP1 inflammasome leads to early lytic cell death during dsRNA sensing. Further, downregulation of NLRP1 expression almost completely impaired the secretion of IL-1 β , IL-1 α and IL-6 at both 8 h and 24 h [Figure 4.31A, B; 4.32A-D], showing an important contribution of NLRP1 inflammasome in dsRNA-stimulated cytokine secretion. It is worth nothing that dsRNA-stimulated pro-IL-1 β expression, was also inhibited in the presence of NLRP1 siRNA [Figure 4.31C, D], suggesting that NLRP1 is required for pro-IL-1 β expression, as well as pro-IL-1 β processing by caspase-1. Overall these results indicate that an NLRP1-ASC-caspase-1 inflammasome mediates GSDMD pore formation, early lytic cell death and cytokine secretion. Surprisingly, the data also show that NLRP1 is required for dsRNA-stimulated pro-IL-1 β expression too.

4.2.8 Involvement of signaling pathways in type I IFN secretion following cytosolic dsRNA sensing in NHBE cells

Detection of cytosolic dsRNA by RLRs triggers type I IFN secretion that is essential to restrict viral infection [226], however here I found that similar to the lack of a role for MAVS in dsRNA-stimulated GSDMD responses, MAVS siRNA also did not affect dsRNA-stimulated type I IFN expression [Figure 4.33A]. Nevertheless NLRs can also contribute to or regulate this antiviral response [227]. Therefore, since NLRP1 seemed to have a role in dsRNA-stimulated pro-IL-1 β expression I tested whether this was also the case for type I IFN expression. Indeed, downregulation of NLRP1 expression did diminish type I IFN secretion, however not significantly [Figure 4.33B]. Any effect that NLRP1 did have on type I IFN expression was not inflammasome-mediated since ASC siRNA showed no inhibition

[**Figure 4.33C**]. Surprisingly then, type I IFN release was decreased in the presence of either GSDMD or GSDME siRNA [**Figure 4.33D**]. This demonstrates that GSDMD and GSDME pores enhance type I IFN release following cytosolic dsRNA delivery, although ASC is not involved.

4.2.9 PKR is involved in GSDME cleavage, GSDMD inactivation, lytic cell death, IL-1 β and late IL-1 α secretion following cytosolic dsRNA sensing in NHBE cells

Our data established that cytosolic dsRNA sensing activated the NLRP1 inflammasome leading to GSDMD pore formation, and that TRIF was involved in early GSDME pore formation and was partially required for GSDMD inactivation. Given that MAVS-dependent signaling is not necessary for processing of GSDM, the next step was to investigate whether another signaling pathway was implicated in GSDM cleavage, especially at later times during intracellular dsRNA sensing. I hypothesised that GSDME processing was mediated by PKR for three reasons. Firstly, a recent study demonstrated that translation inhibition mediated caspase-3-dependent GSDME processing in keratinocytes [194]. Secondly, activation of PKR mostly triggers translation shut down, and finally PKR is activated by binding cytosolic dsRNA [59]. To test this hypothesis, I first determined whether poly(I:C) transfection triggered PKR activation in NHBE cells. Immunoblot analysis showed that poly(I:C) transfection did indeed cause phosphorylation of PKR, and that this was suppressed in the presence of the PKR inhibitor C16 [228], in a dose-dependent manner [**Figure 4.34**]. Next I examined the contribution of PKR in GSDM cleavage using C16. **Figure 4.35** shows that C16 diminished dsRNA-stimulated GSDME pore formation and GSDMD p43 fragment generation, and also decreased the appearance of active caspase-3 p17 and p12 forms at both 6 h and 16 h [**Figure 4.35A, B**]. However, the PKR inhibitor did not affect the appearance of the GSDMD active p30 fragment, nor caspase-1 activation [**Figure 4.35A, B**]. It is worth noting that C16 slightly decreased pro-caspase-1 expression at both 6 h and 16 h [**Figure 4.35A, B**]. This demonstrates that cytosolic dsRNA sensing triggers PKR activation that subsequently leads to caspase-3-dependent GSDME activation and GSDMD inactivation. I thus investigated if PKR was required for dsRNA-stimulated lytic cell death and cytokine secretion. C16 inhibitor decreased LDH release at both 8 h and 24 h [**Figure 4.36A, B**] showing that PKR contributes to lytic cell death. In addition, C16 treatment diminished IL-1 β secretion at both 8 h and 24 h [**Figure 4.37A, B**]. Interestingly pro-IL-1 β expression, but not β -actin, was decreased in the presence of C16 at both 6 h and 16 h [**Figure 4.37C, D**]. Measurement of IL-1 α and IL-6 secretion showed that C16

significantly diminished the release of both cytokines at 24 h but not at 8 h [Figure 4.38A-D]. These data show that PKR mediates the release of IL-1 β and contributes to IL-1 α and IL-6 secretion at later times. Finally, quantification of type I IFN secretion indicated that the decrease of IFN- α/β release in the presence of C16 inhibitor didn't reach significance [Figure 4.38E] showing that PKR is not predominantly required for dsRNA-mediated IFN secretion. Overall these results show that PKR is required for GSDME pore formation, GSDMD inactivation, lytic cell death, IL-1 β and late IL-1 α and IL-6 secretion in dsRNA-stimulated NHBE cells.

4.2.10 Mechanisms of caspase-3 activation following cytosolic dsRNA sensing in NHBE cells

The data thus far show that dsRNA stimulates PKR-mediated caspase-3 activation to generate GSDME pores, but how exactly caspase-3 is activated downstream of PKR in NHBE cells is unclear. Caspase-3 can be activated by caspase-8 and caspase-9 through the extrinsic and intrinsic apoptotic pathways respectively [229]. To gain more insights into how caspase-3 is activated following dsRNA sensing, I next examined GSDM cleavage in the presence of IETD, a caspase-8 inhibitor, and LEHD, a caspase-9 inhibitor. Immunoblot analysis confirmed that the appearance of caspase-3 active forms stimulated by dsRNA were suppressed in the presence of the caspase 3 inhibitor DEVD, but also in the presence of IETD and LEHD [Figure 4.39]. As shown previously, the caspase-1 inhibitor VX765 had no effect on dsRNA-stimulated caspase-3 activation [Figure 4.39]. Consistent with their effects on caspase-3, DEVD, IETD and LEHD, but not VX765, decreased the appearance of GSDME pore-forming fragment and GSDMD inactive p43 fragment [Figure 4.39]. Nevertheless, the active GSDMD p30 fragment wasn't affected by the presence of the inhibitors [Figure 4.39]. Caspase-1 p33 form was decreased in the presence of VX765 whereas all inhibitors diminished caspase-1 p20 fragment secretion [Figure 4.39]. The fact that caspase-1 inhibitor didn't decrease GSDMD cleavage into a p30 fragment could be explain by the timing of the experiment which was performed at 16 h post-transfection. Indeed, Figure 4.11 showed that caspase-1 wasn't involved at later times in lytic cell death, unlike caspase-3. I next determined the involvement of caspase-8 and caspase-9 in lytic cell death and cytokine secretion. Treatment with IETD and LEHD diminished LDH release after 16 h dsRNA stimulation [Figure 4.40A]. Moreover, secretion of IL-1 β was significantly diminished in the presence of both caspase-8 and caspase-9 inhibitors [Figure 4.40B, C], and this decrease was not due to an impaired expression of pro-IL-1 β [Figure 4.40C]. IETD

and LEHD decreased the secretion of IL-1 β at the same extent as caspase-3 inhibitor, DEVD [Figure 4.40C]. IL-1 α secretion was diminished in the presence of both inhibitors but didn't reach significance [Figure 4.40D]. Finally IL-6 secretion was not impacted by the presence of the inhibitors [Figure 4.40E]. Overall these data show that caspase-8 and caspase-9 mediate dsRNA stimulated caspase-3 activation that can further cleaves GSDM and trigger lytic cell death and IL-1 β secretion.

Further, a recent study demonstrated that translation inhibition mediated by viral infection or protein synthesis inhibitors triggered caspase-3-dependent GSDME cleavage in keratinocytes [194], which would provide a rationale as to how PKR activates caspase-3 for GSDME cleavage. Thus to investigate whether translation inhibition could directly lead to caspase-3 and GSDME activation in basal respiratory epithelial cells, I stimulated NHBE cells with cycloheximide and puromycin to inhibit translation. Immunoblot analysis showed that both treatments triggered GSDME cleavage into a p30 fragment and the appearance of active caspase-3 p17 form [Figure 4.41]. Similarly to the previous report [194], puromycin was a better inducer of GSDME activation than cycloheximide [Figure 4.41]. This result shows that translation inhibition can directly cause GSDME pore formation in bronchial epithelial cells.

4.2.11 Cytosolic dsRNA sensing does not trigger necroptosis but leads to GSDMA pore formation in NHBE cells

Figure 4.7 showed that downregulation of GSDME expression using siRNA didn't completely diminish LDH release at 8 h and that most of lytic cell death is independent of GSDME at 24 h. Giving that necroptosis also leads to plasma membrane rupture resulting in LDH release [230] and that inflammasome-dependent GSDMD pores can trigger necroptosis through the release of mitochondrial ROS [231], I next determined whether necroptosis contributed to cytosolic dsRNA-triggered cell lysis, by using GSK'872, the RIPK3 kinase and necroptosis inhibitor. However, GSK'872 had no effect on LDH nor IL-1 β release at 16 h, whereas ZVAD, the pan caspase inhibitor, decreased both LDH and IL-1 β secretion as expected [Figure 4.42A, B], suggesting that necroptosis is not involved in lytic cell death nor IL-1 β release in response to cytosolic dsRNA sensing.

Next, I investigated whether other GSDM family members could trigger pore formation following dsRNA delivery. I found that, as well as GSDMD and GSDME, I could also detect expression of GSDMA, GSDMB and GSDMC in NHBE cells by qPCR [Figure

4.43]. After validating that siRNA against GSDMA, GSDMB and GSDMC decreased their respective mRNA expression [**Figure 4.43**], I measured dsRNA-stimulated PI uptake over time, in the presence of each siRNA. Only suppression of GSDMA expression significantly decreased the number of PI positive cells from 7 h [**Figure 4.44**], suggesting that dsRNA delivery not only triggers GSDMD and GSDME cleavage but also leads to GSDMA pore formation from 7 h.

4.2.12 Both NLRP1 and PKR enhance RLRs expression following cytosolic dsRNA sensing in NHBE cells

Even though our data showed that MAVS-dependent signaling is not involved in dsRNA-mediated GSDM cleavage nor type I IFN secretion in NHBE cells, it is likely that RLRs will have some function in dsRNA responses in these cells, perhaps at later time points than were assessed here. Further, transfection of cells with dsRNA led to a robust increase in expression of both RIG-I and MDA5 [**Figure 4.45A, B**]. Therefore I examined whether NLRP1 or PKR, which I showed both sense dsRNA, had any impact on RLR expression. Interestingly, RIG-I and MDA5 expression was diminished when NLRP1 expression was suppressed, or when PKR was inhibited [**Figure 4.45C**], whereas TRIF siRNA had no effect on dsRNA-stimulated RLR expression [**Figure 4.45D**]. Together, these results show that dsRNA-stimulated RLR expression is dependent on NLRP1 and PKR.

Overall these data indicate that both NLRP1 and PKR have critical roles in sensing cytosolic dsRNA in NHBE cells, not only for caspase and GSDM activation, but also for protein expression (pro-IL-1 β , type I IFN, RLRs).

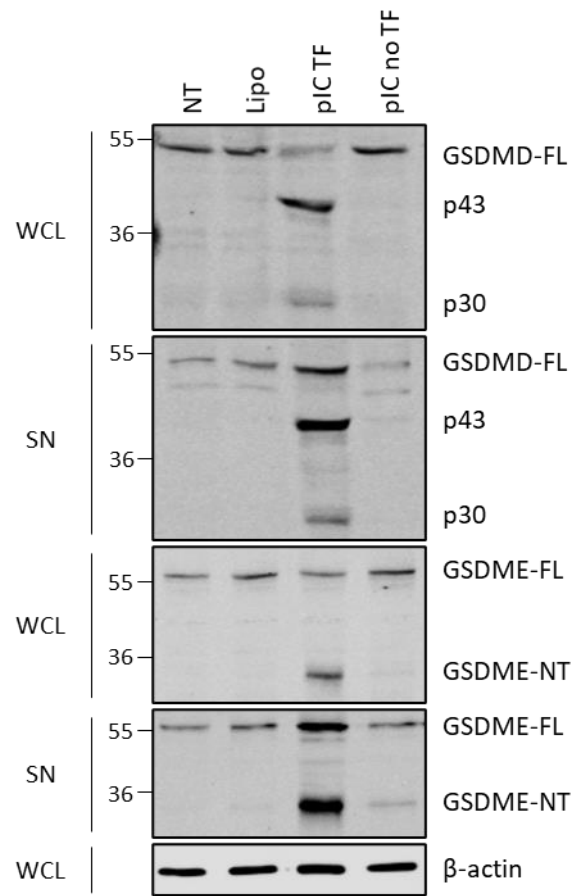


Figure 4.2: dsRNA transfection leads to GSDMD inactivation and GSDME activation in NHBE cells.

NHBE cells were treated or transfected with poly(I:C) (pIC, 2.5 µg/ml) for 16 h. Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of full length (FL), inactivated (p43) and activated (p30) GSDMD; full length (FL) and activated (p30) GSDME and β-actin. Immunoblots are representative of three independent experiments.

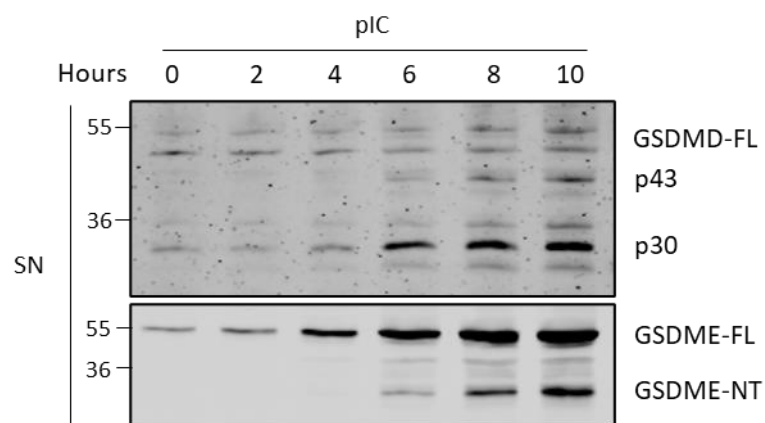


Figure 4.3: Time course of dsRNA-stimulated GSDMD and GSDME cleavage in NHBE cells.

NHBE cells were transfected with poly(I:C) (pIC, 2.5 µg/ml). Supernatants (SN) were harvested at indicated time points and immunoblotted for the expression of full length (FL), inactivated (p43) and activated (p30) GSDMD; and full length (FL) and activated (p30) GSDME. Immunoblots are representative of three independent experiments.

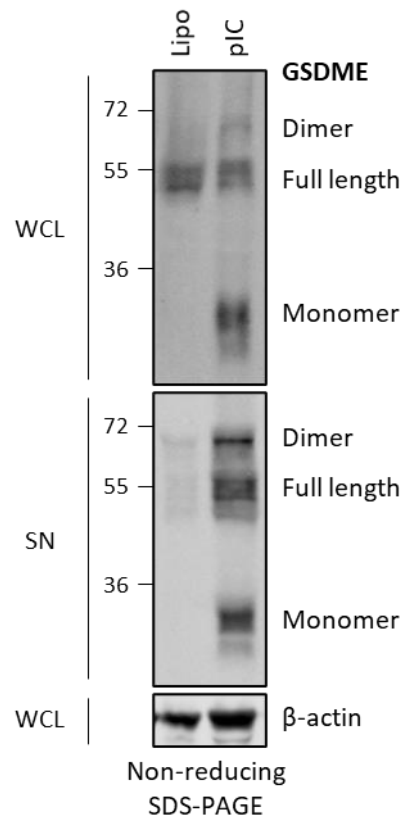


Figure 4.4: dsRNA transfection leads to GSDME oligomerisation in NHBE cells.

NHBE cells were transfected with poly(I:C) (pIC, 2.5 µg/ml) for 16 h. Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of dimer, full length and monomer GSDME and β-actin. Non-reducing SDS-PAGE was used to preserve self-protein interactions. Immunoblots are representative of three independent experiments.

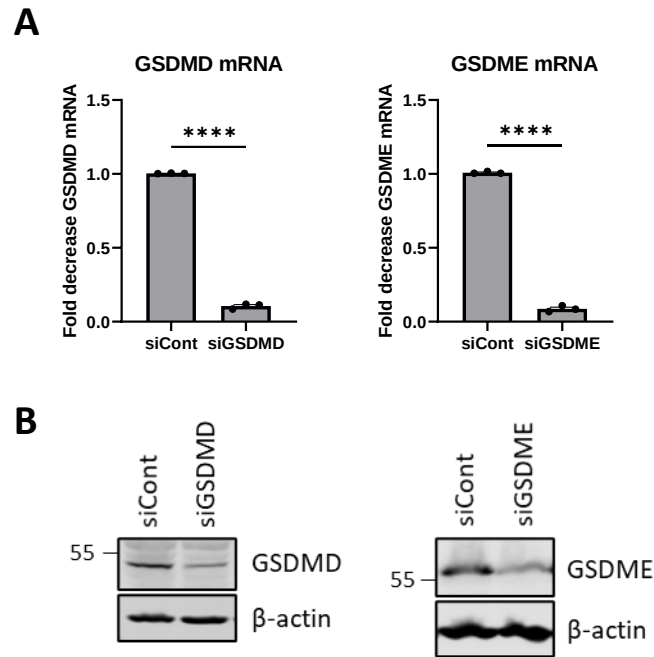


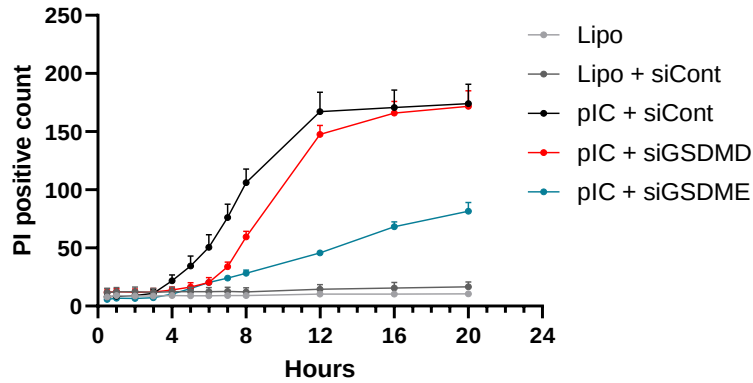
Figure 4.5: Validation of GSDMD and GSDME gene silencing by siRNA in NHBE cells.

NHBE cells were transfected with siRNA targeting GSDMD (siGSDMD) or GSDME (siGSDME), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells.

(A) GSDMD and GSDME mRNA expression was quantified 48 h after seeding by qPCR to confirm the knockdown. Data are mean $\pm$ SEM of three independent experiments.

**** $p < 0.0001$ by unpaired Student's *t* test.

(B) Cell lysates were immunoblotted for the expression of GSDMD, GSDME and β -actin. Immunoblots are representative of three independent experiments.



Statistics	<5h	6h	7h	8h	12h	16h	20h
pIC + siGSDMD	ns	*	*	**	ns	ns	ns
pIC + siGSDME	ns	*	**	***	***	***	**

Figure 4.6: Cytosolic dsRNA sensing leads to GSDMD and GSDME pore formation in NHBE cells.

NHBE cells were transfected with siRNA targeting GSDMD (siGSDMD) or GSDME (siGSDME), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with poly(I:C) (pIC, 2.5 µg/ml). Pore formation at the plasma membrane was assessed by PI uptake. The number of PI positive cells was analysed by time-lapse microscopy over a 20-hour period. Data are mean ± SEM of three independent experiments. ns: not significant, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by one-way ANOVA, compared to pIC + siCont. For clarity purposes, statistics are indicated in a table below the graph.

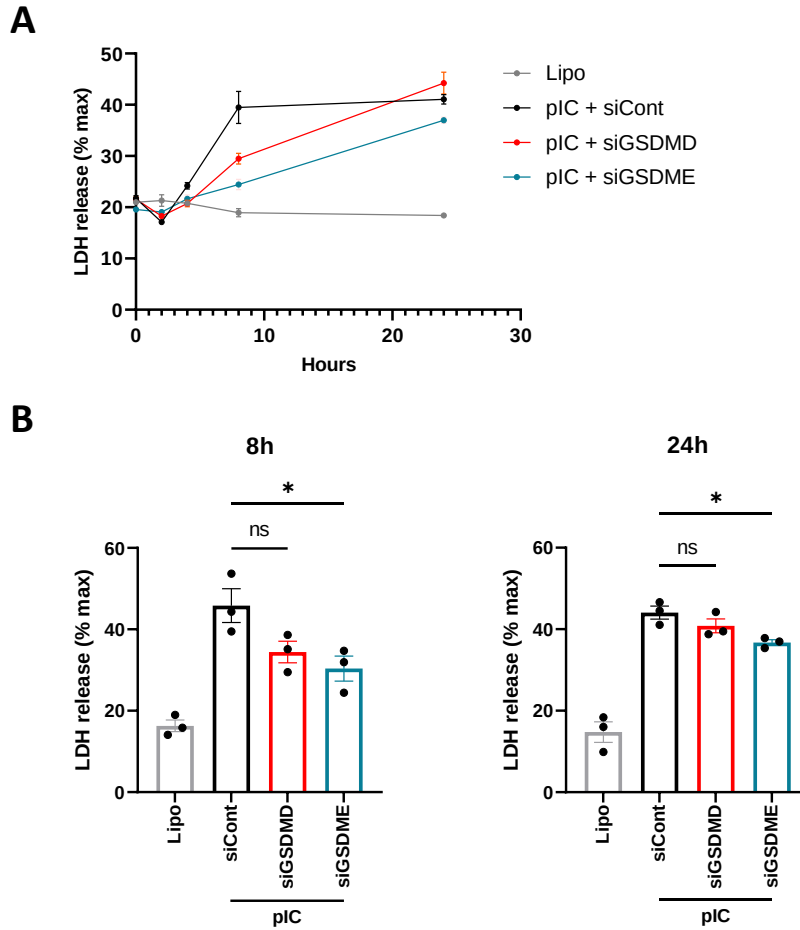


Figure 4.7: GSDME pores elicit lytic cell death in dsRNA-stimulated NHBE cells.

NHBE cells were transfected with siRNA targeting GSDMD (siGSDMD) or GSDME (siGSDME), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with poly(I:C) (pIC, 2.5 μ g/ml).

(A) Representative time course of LDH release into the supernatant.

(B) LDH present in the supernatant was quantified by LDH assay at 8 h and 24 h.

Data are mean $\pm$ SD (A) of a representative experiment of three independent experiments or mean $\pm$ SEM (B) of three independent experiments. ns: not significant and * $p < 0.05$ by one-way ANOVA.

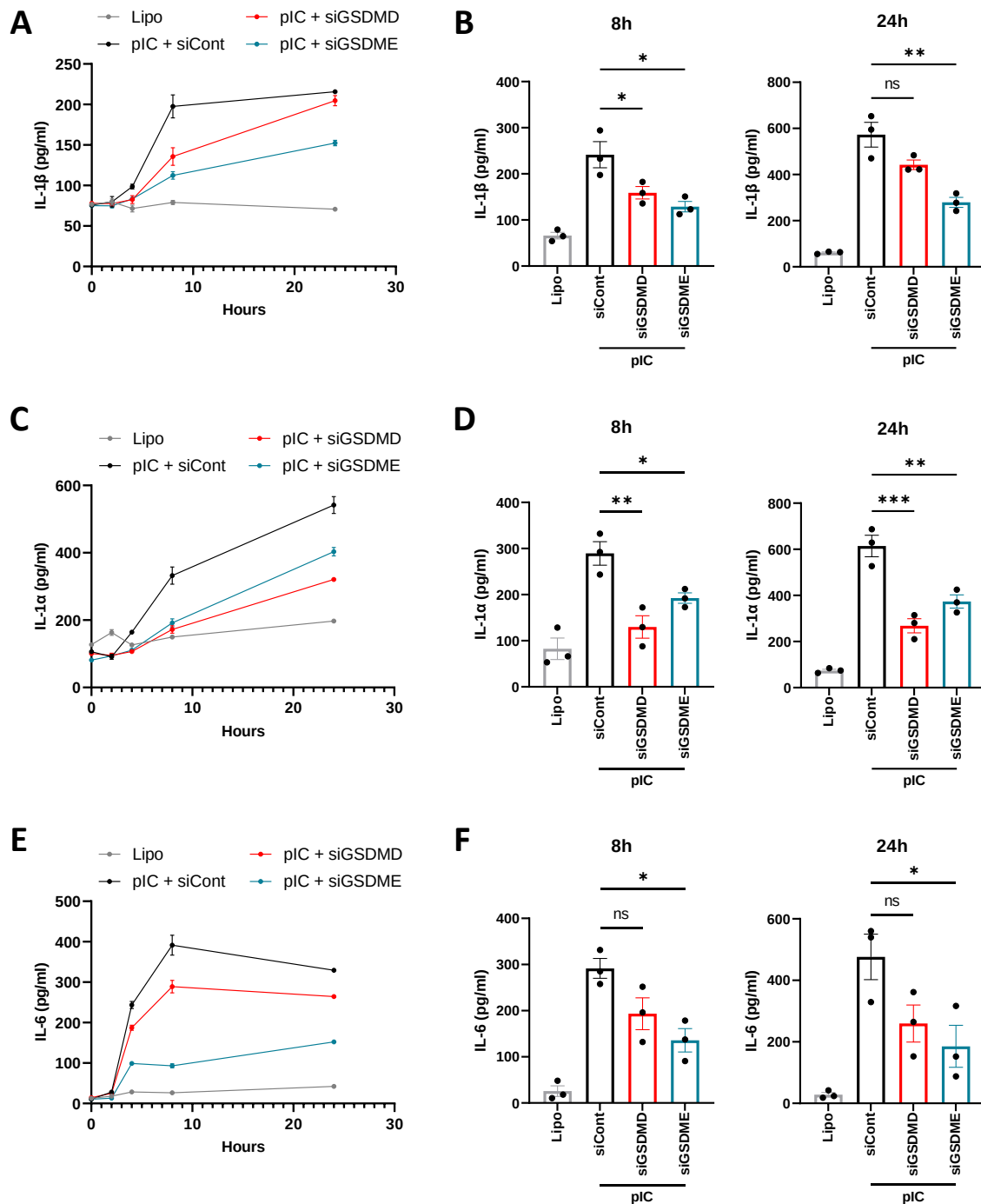


Figure 4.8: GSDM pores are required for cytokine secretion in response to dsRNA sensing in NHBE cells.

NHBE cells were transfected with siRNA targeting GSDMD (siGSDMD) or GSDME (siGSDME), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with poly(I:C) (pIC, 2.5 μ g/ml).

(A, C, E) Representative time course of IL-1 β (A), IL-1 α (C) and IL-6 (E) secretion.

(B, D, F) IL-1 β (B), IL-1 α (D) and IL-6 (F) secretion was measured by ELISA at 8 h and 24 h.

Data are mean $\pm$ SD (A, C, E) of a representative experiment of three independent experiments or mean $\pm$ SEM (B, D, F) of three independent experiments. ns: not significant, * p < 0.05, ** p < 0.01 and *** p < 0.001 by one-way ANOVA.

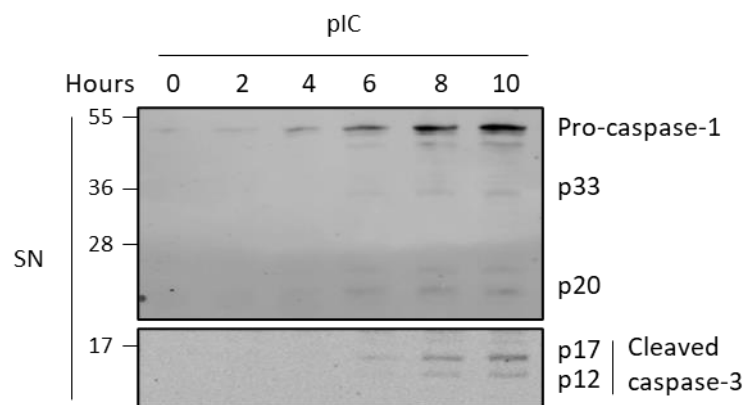


Figure 4.9: Time-dependent activation of caspase-1 and caspase-3 by cytosolic dsRNA in NHBE cells.

NHBE cells were transfected with poly(I:C) (pIC, 2.5 $\mu\text{g}/\text{ml}$). Supernatants (SN) were harvested at indicated time points and immunoblotted for the expression of pro-caspase-1, active caspase-1 (p33 and p20) and cleaved caspase-3 (p17 and p12). Immunoblots are representative of three independent experiments.

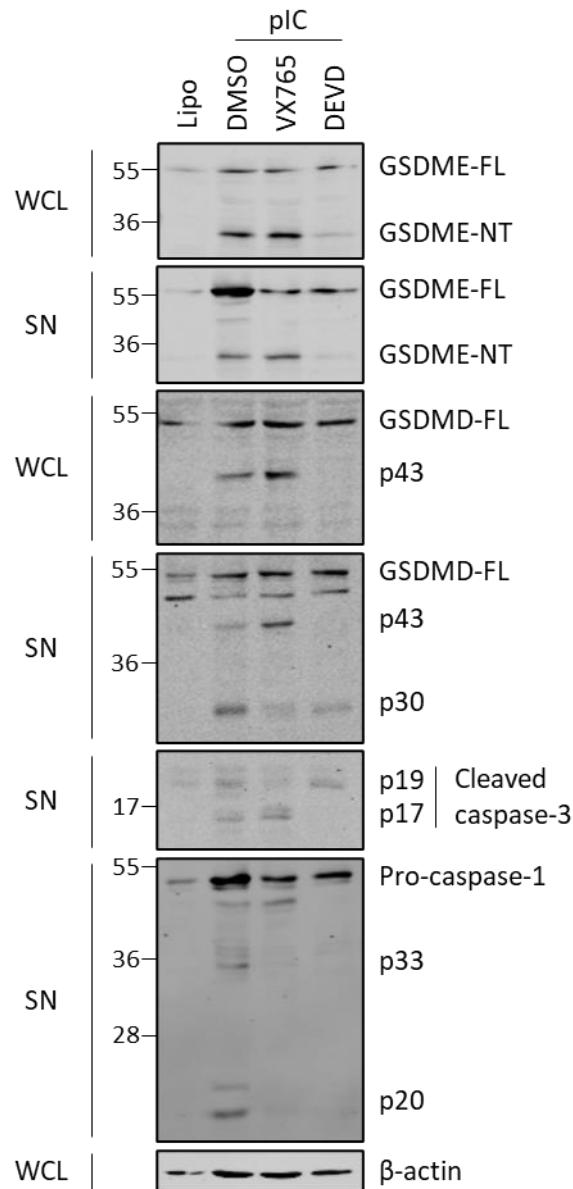


Figure 4.10: Caspase-1 and caspase-3 are required for GSDMD and GSDME cleavage in dsRNA-stimulated NHBE cells.

NHBE cells were treated with DMSO, caspase-1 inhibitor (VX765) or caspase-3 inhibitor (DEVD, both 20 μ M) for 1 h before transfection of poly(I:C) (pIC, 2.5 μ g/ml) for 6 h. Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of full length (FL), inactivated (p43) and activated (p30) GSDMD; full length (FL) and activated (p30) GSDME; pro-caspase-1, active caspase-1 (p33 and p20); cleaved caspase-3 (p19 and p17) and β -actin. Immunoblots are representative of three independent experiments.

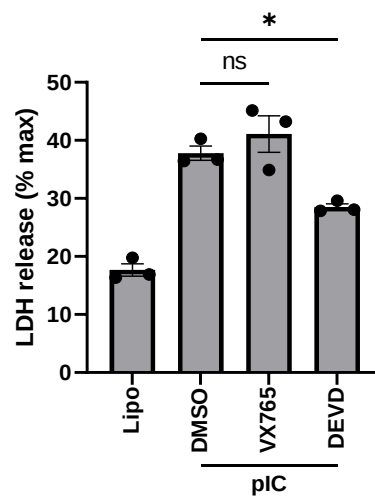


Figure 4.11: Caspase-3 is involved in lytic cell death following dsRNA sensing in NHBE cells.

NHBE cells were treated with DMSO, caspase-1 inhibitor (VX765) or caspase-3 inhibitor (DEVD, both 20 μ M) for 1 h before transfection of poly(I:C) (pIC, 2.5 μ g/ml) for 16 h. LDH present in the supernatant was quantified by LDH assay. Data are mean $\pm$ SEM of three independent experiments. ns: not significant and *p < 0.05 by one-way ANOVA.

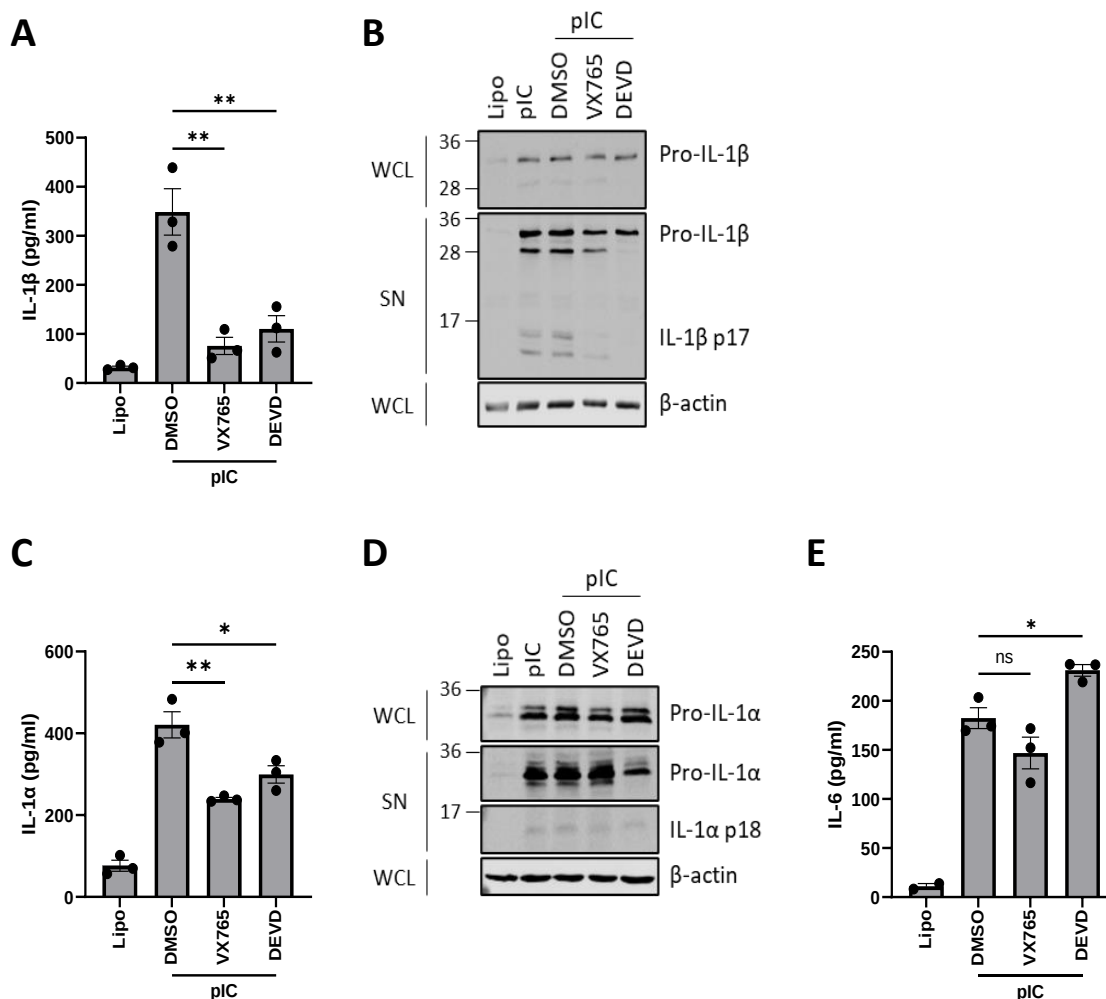


Figure 4.12: Both caspase-1 and caspase-3 are required for IL-1 secretion in dsRNA-stimulated NHBE cells.

NHBE cells were treated with DMSO, caspase-1 inhibitor (VX765) or caspase-3 inhibitor (DEVD, both 20 μ M) for 1 h before transfection of poly(I:C) (pIC, 2.5 μ g/ml) for 16 h. (A, C, E) IL-1 β (A), IL-1 α (C) and IL-6 (E) secretion was measured by ELISA. Data are mean $\pm$ SEM of three independent experiments. ns: not significant, * $p < 0.05$ and ** $p < 0.01$ by one-way ANOVA.

(B, D) Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of pro-IL-1 β , mature IL-1 β (p17) (B); pro-IL-1 α , mature IL-1 α (p18) (D) and β -actin. Immunoblots are representative of three independent experiments.

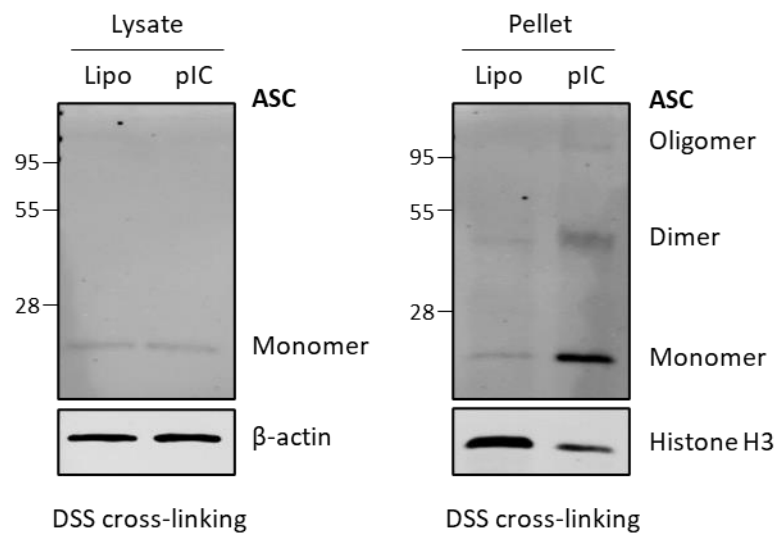


Figure 4.13: dsRNA stimulation leads to ASC oligomerisation in NHBE cells.

NHBE cells were transfected with poly(I:C) (pIC, 2.5 µg/ml) for 16 h. Cells were lysed and centrifugated to separate the soluble cell lysates from the cell pellets. Cross-linking reagent DSS was added for 45 min to the cell pellet. Cell lysate and pellet were immunoblotted for the expression of ASC, β -actin and histone H3, respectively. Immunoblots are representative of three independent experiments.

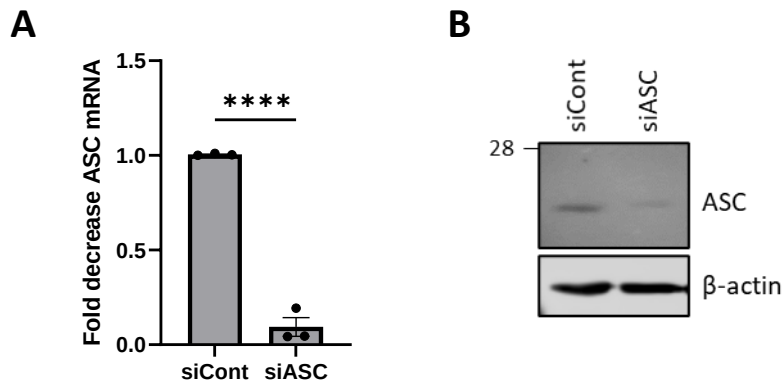


Figure 4.14: Validation of ASC gene silencing by siRNA in NHBE cells.

NHBE cells were transfected with siRNA targeting ASC (siASC), or control siRNA (siCont) (1 nM) at 24 h and 48 h post-seeding of cells.

(A) ASC mRNA expression was quantified 48 h after seeding by qPCR to confirm the knockdown. Data are mean $\pm$ SEM of three independent experiments. **** $p < 0.0001$ by unpaired Student's *t* test.

(B) Cell lysates were immunoblotted for the expression of ASC and β -actin. Immunoblots are representative of three independent experiments.

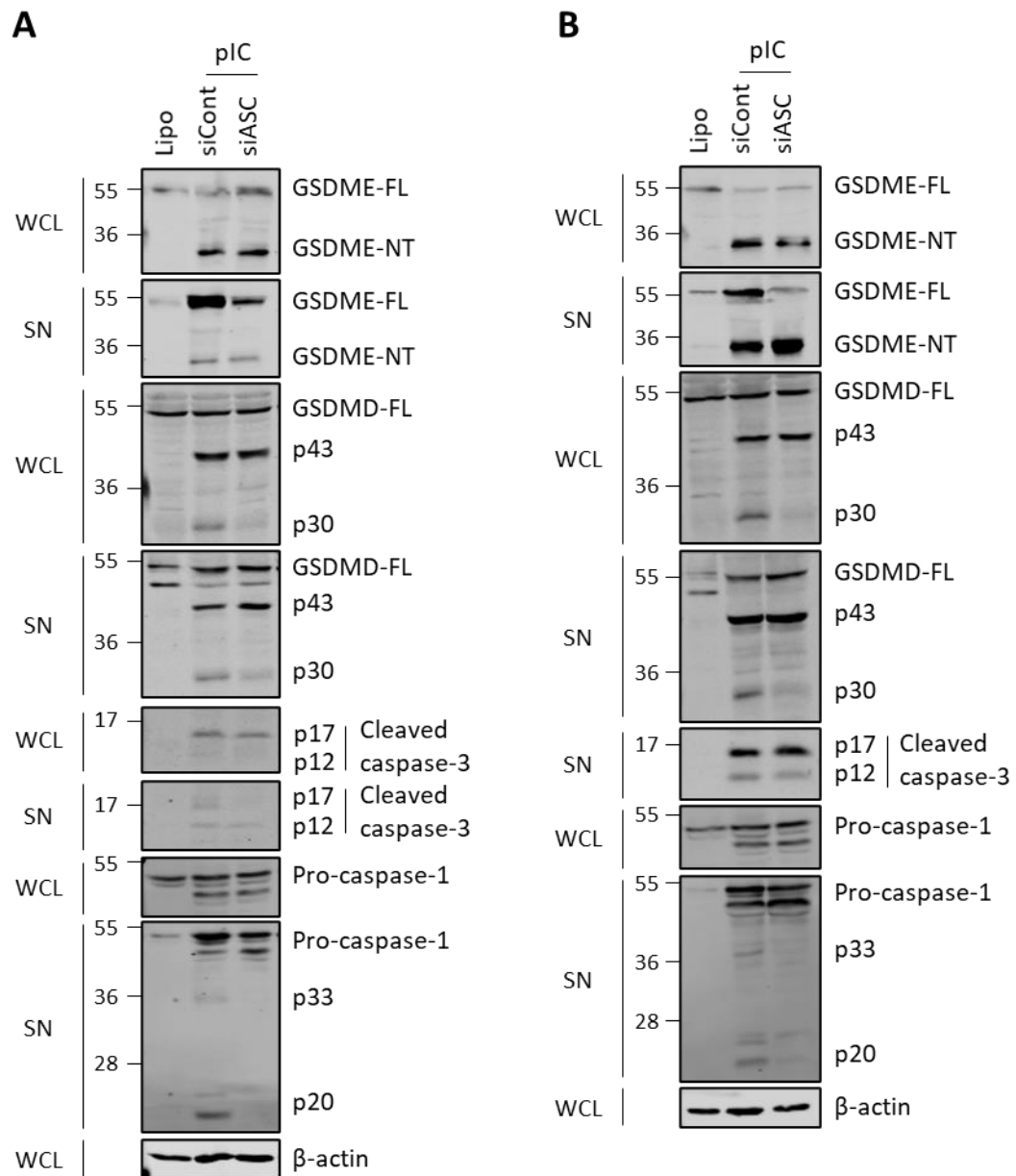


Figure 4.15: GSDMD pore formation and caspase-1 activation are inflammasome-dependent in dsRNA-stimulated NHBE cells.

NHBE cells were transfected with siRNA targeting ASC (siASC), or control siRNA (siCont) (1 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with poly(I:C) (pIC, 2.5 µg/ml) for 6 h (A) or 16 h (B). Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of full length (FL), inactivated (p43) and activated (p30) GSDMD; full length (FL) and activated (p30) GSDME; pro-caspase-1, active caspase-1 (p33 and p20); cleaved caspase-3 (p17 and p12) and β-actin. Immunoblots are representative of three independent experiments.

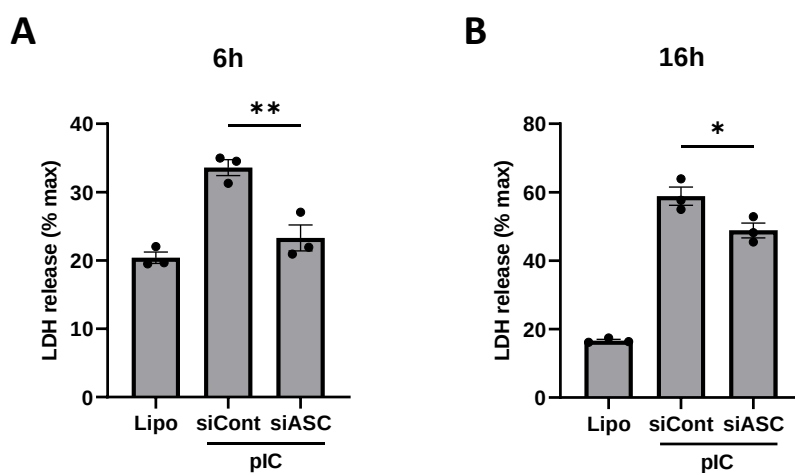


Figure 4.16: ASC is required for lytic cell death in dsRNA-stimulated NHBE cells.

NHBE cells were transfected with siRNA targeting ASC (siASC), or control siRNA (siCont) (1 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with poly(I:C) (pIC, 2.5 μ g/ml) for 6 h (A) or 16 h (B). LDH present in the supernatant was quantified by LDH assay. Data are mean $\pm$ SEM of three independent experiments. * $p < 0.05$ and ** $p < 0.01$ by unpaired Student's t test.

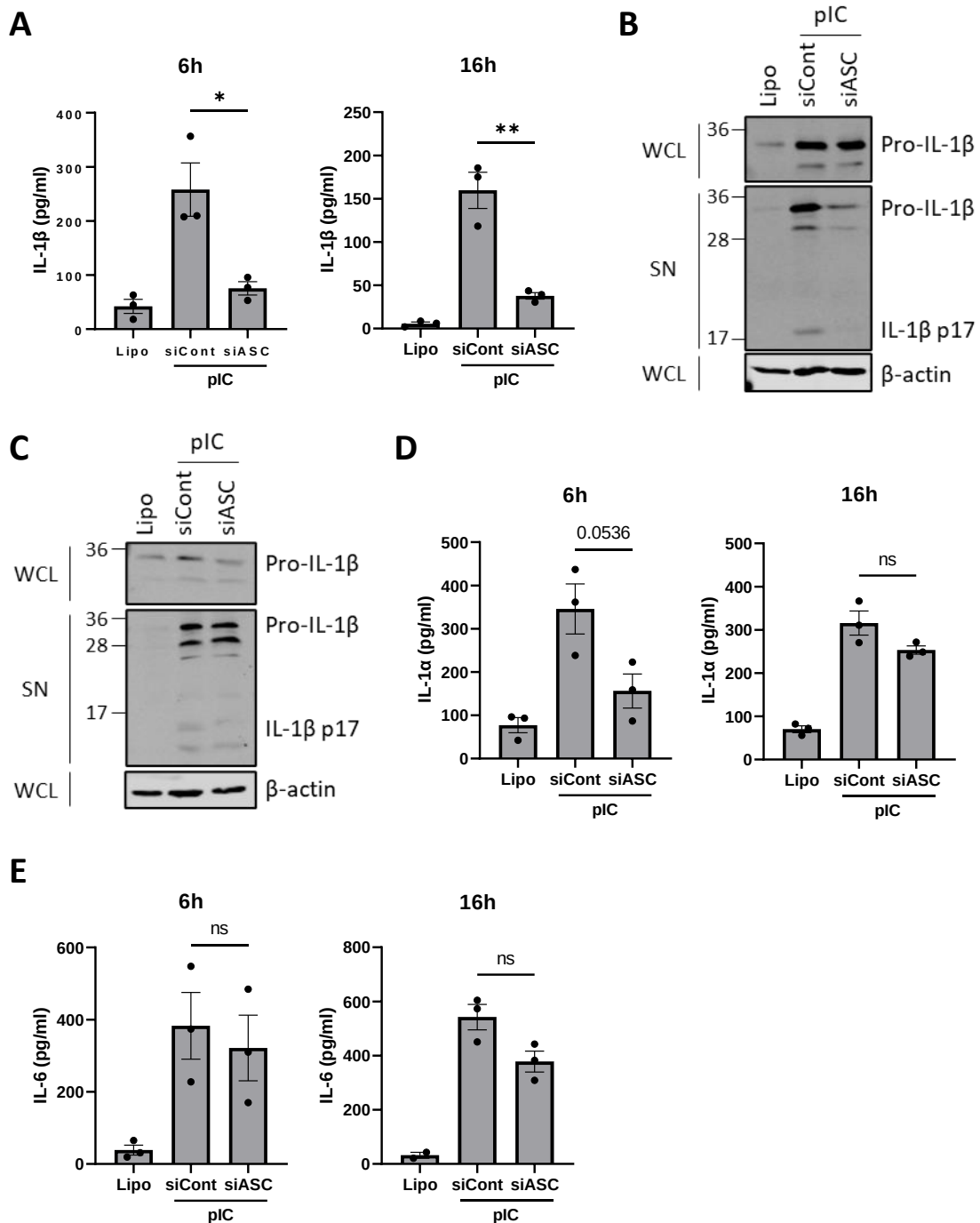


Figure 4.17: IL-1 β secretion is ASC-dependent in dsRNA-stimulated NHBE cells.

NHBE cells were transfected with siRNA targeting ASC (siASC), or control siRNA (siCont) (1 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with poly(I:C) (pIC, 2.5 μ g/ml) for 6 h or 16 h.

(A, D, E) IL-1 β (A), IL-1 α (D) and IL-6 (E) secretion was measured by ELISA.

Data are mean $\pm$ SEM of three independent experiments. ns: not significant, * $p < 0.05$ and ** $p < 0.01$ by unpaired Student's *t* test.

(B, C) Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of pro-IL-1 β , mature IL-1 β (p17) and β -actin at 6 h (B) and 16 h (C). Immunoblots are representative of three independent experiments.

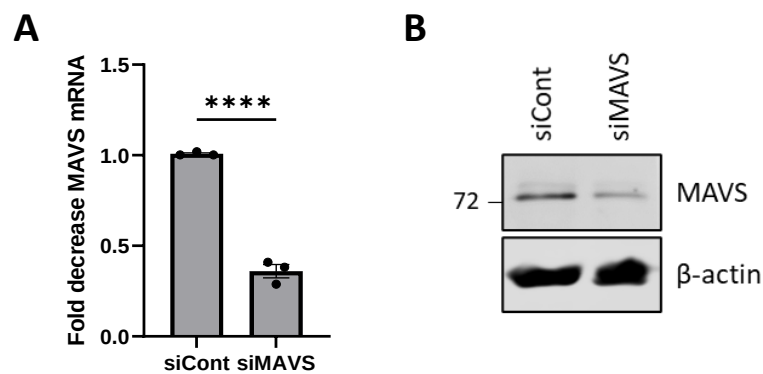


Figure 4.18: Validation of MAVS gene silencing by siRNA in NHBE cells.

NHBE cells were transfected with siRNA targeting MAVS (siMAVS), or control siRNA (siCont) (5 nM) at 24 h and 48 h post-seeding of cells.

(A) MAVS mRNA expression was quantified 48 h after seeding by qPCR to confirm the knockdown. Data are mean $\pm$ SEM of three independent experiments. **** $p < 0.0001$ by unpaired Student's *t* test.

(B) Cell lysates were immunoblotted for the expression of MAVS and β -actin. Immunoblots are representative of three independent experiments.

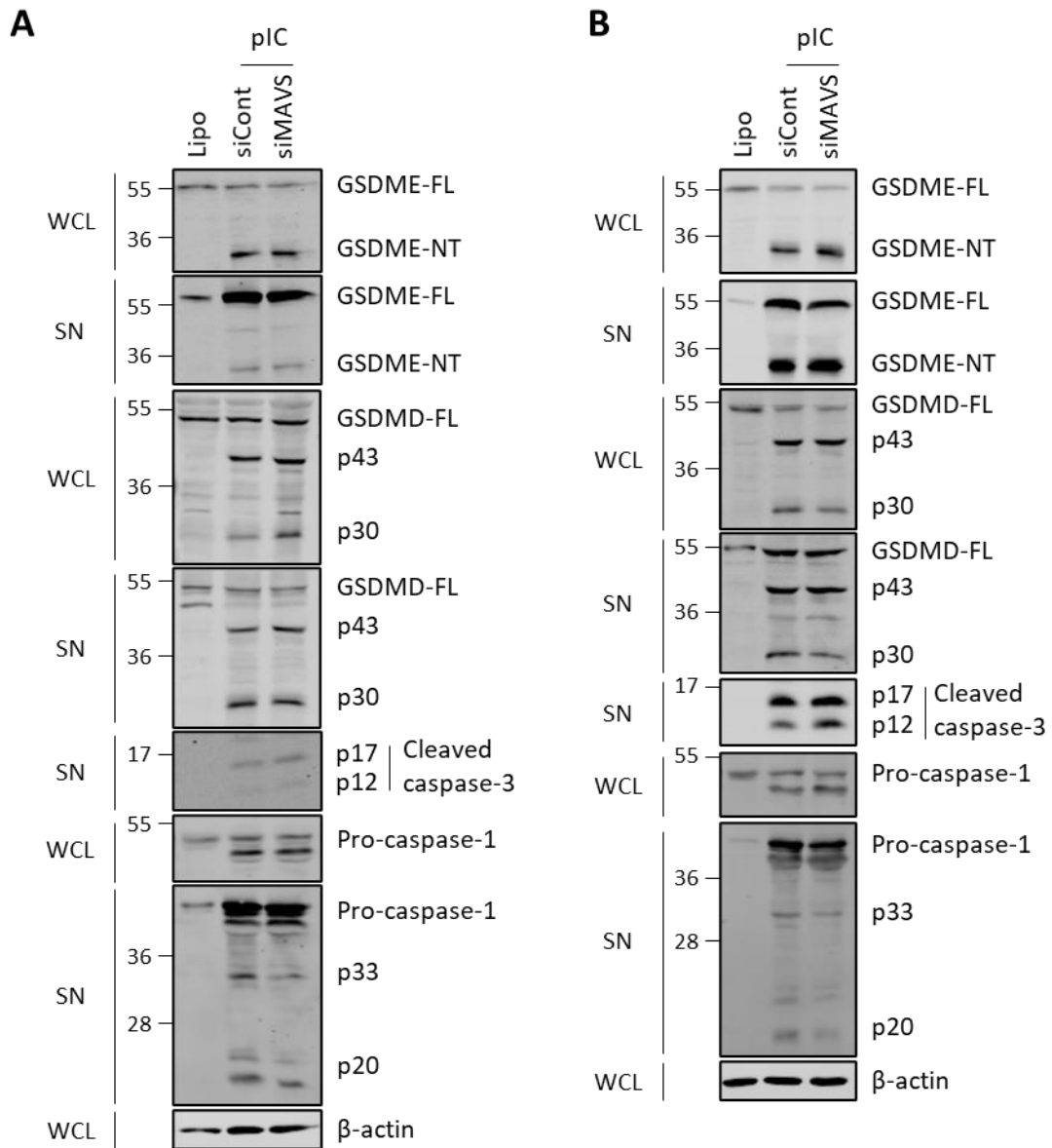


Figure 4.19: MAVS is not involved in GSDM processing nor caspase activation in dsRNA-stimulated NHBE cells.

NHBE cells were transfected with siRNA targeting MAVS (siMAVS), or control siRNA (siCont) (5 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with poly(I:C) (pIC, 2.5 µg/ml) for 6 h (A) or 16 h (B). Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of full length (FL), inactivated (p43) and activated (p30) GSDMD; full length (FL) and activated (p30) GSDME; pro-caspase-1, active caspase-1 (p33 and p20); cleaved caspase-3 (p17 and p12) and β-actin. Immunoblots are representative of three independent experiments.

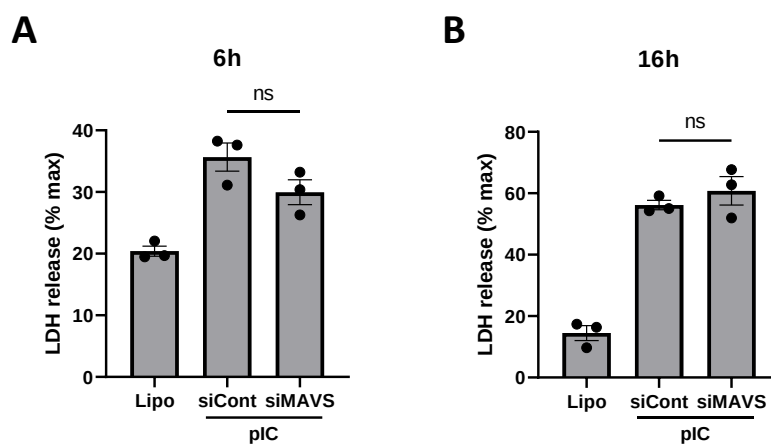


Figure 4.20: Lytic cell death is MAVS-independent in dsRNA-stimulated NHBE cells.

NHBE cells were transfected with siRNA targeting MAVS (siMAVS), or control siRNA (siCont) (5 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with poly(I:C) (pIC, 2.5 μ g/ml) for 6 h (A) or 16 h (B). LDH present in the supernatant was quantified by LDH assay. Data are mean $\pm$ SEM of three independent experiments. ns: not significant by unpaired Student's t test.

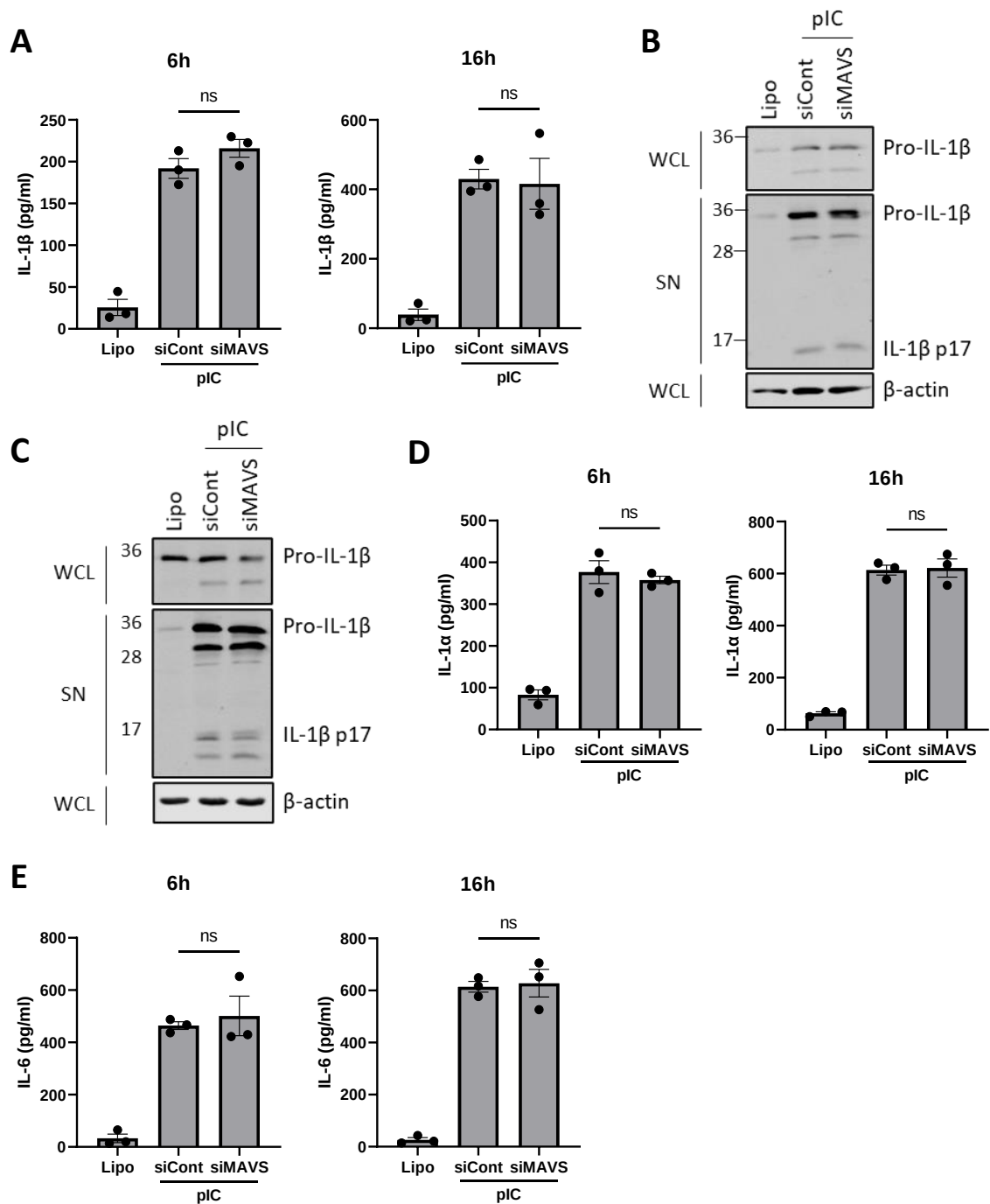


Figure 4.21: Cytokine secretion is MAVS-independent in dsRNA-stimulated NHBE cells.

NHBE cells were transfected with siRNA targeting MAVS (siMAVS), or control siRNA (siCont) (5 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with poly(I:C) (pIC, 2.5 μ g/ml) for 6 h or 16 h.

(A, D, E) IL-1 β (A), IL-1 α (D) and IL-6 (E) secretion was measured by ELISA.

Data are mean $\pm$ SEM of three independent experiments. ns: not significant by unpaired Student's t test.

(B, C) Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of pro-IL-1 β , mature IL-1 β (p17) and β -actin at 6 h (B) and 16 h (C). Immunoblots are representative of three independent experiments.

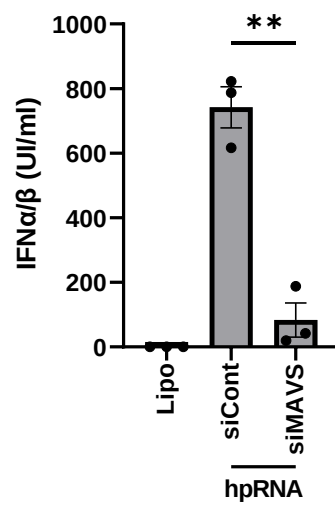


Figure 4.22: MAVS is required for IFN- α/β secretion in hpRNA-stimulated NHBE cells.

NHBE cells were transfected with siRNA targeting MAVS (siMAVS), or control siRNA (siCont) (5 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with hpRNA (100 ng/ml) for 6 h. IFN- α/β secretion was measured by HEK Blue IFN- α/β bioassay. Data are mean $\pm$ SEM of three independent experiments. **p < 0.01 by unpaired Student's t test.

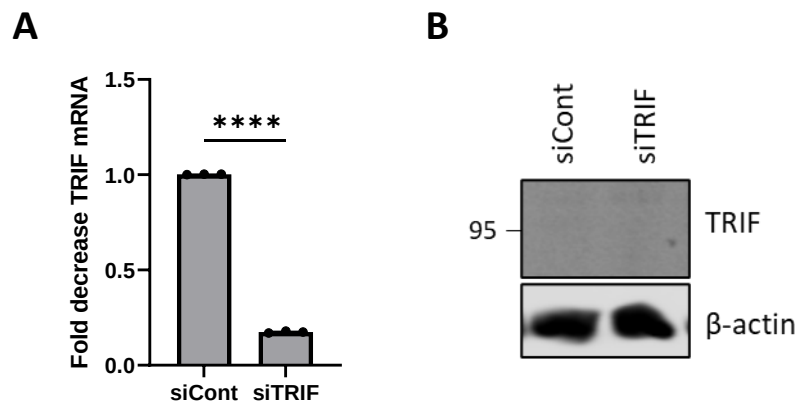


Figure 4.23: Validation of TRIF gene silencing by siRNA in NHBE cells.

NHBE cells were transfected with siRNA targeting TRIF (siTRIF), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells.

(A) TRIF mRNA expression was quantified 48 h after seeding by qPCR to confirm the knockdown. Data are mean $\pm$ SEM of three independent experiments. **** $p < 0.0001$ by unpaired Student's *t* test.

(B) Cell lysates were immunoblotted for the expression of TRIF and β -actin. Immunoblots are representative of three independent experiments.

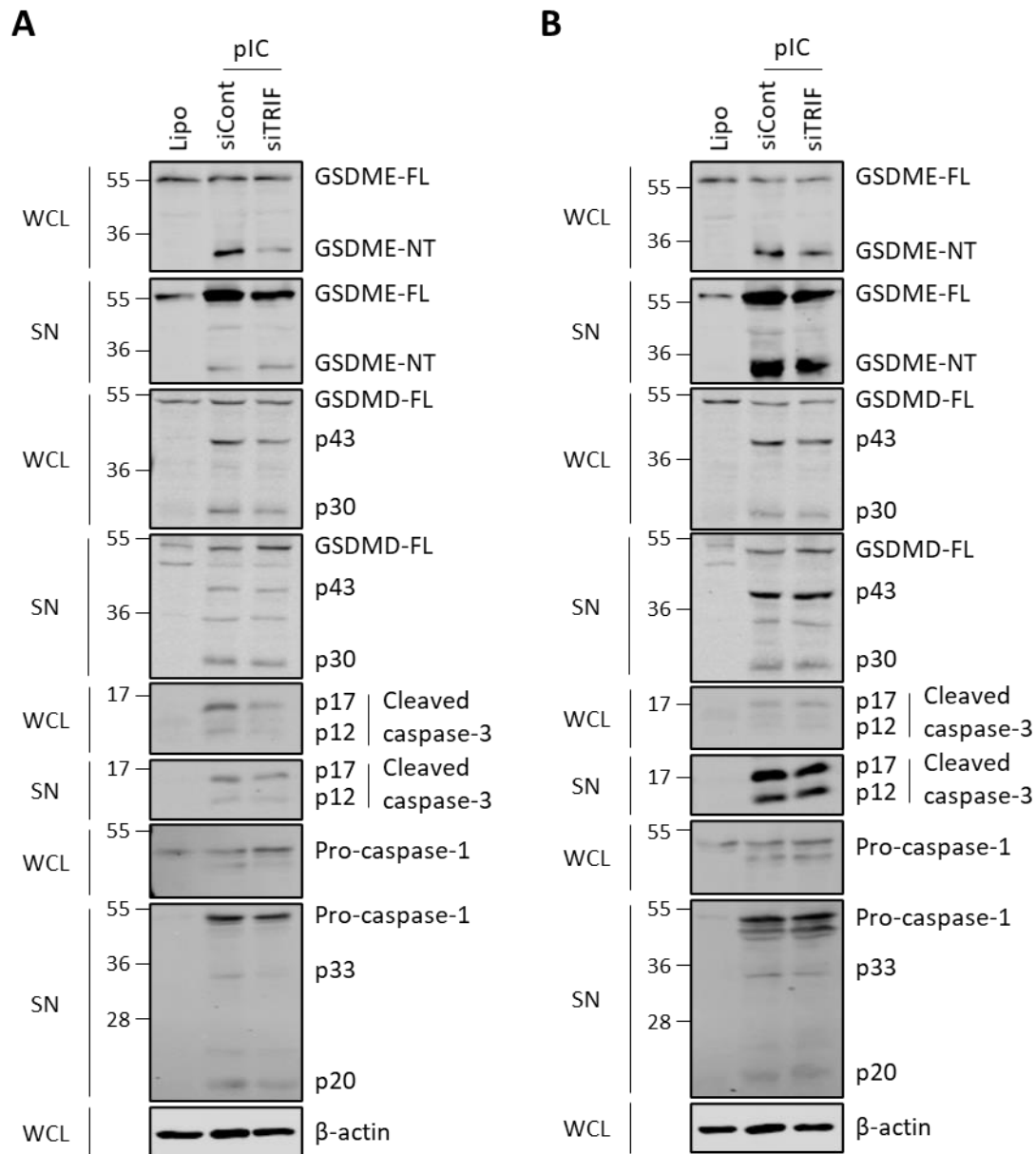


Figure 4.24: TRIF is involved in early GSDME pore formation and caspase-3 activation in dsRNA-stimulated NHBE cells.

NHBE cells were transfected with siRNA targeting TRIF (siTRIF), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with poly(I:C) (pIC, 2.5 µg/ml) for 6 h (A) or 16 h (B). Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of full length (FL), inactivated (p43) and activated (p30) GSDMD; full length (FL) and activated (p30) GSDME; pro-caspase-1, active caspase-1 (p33 and p20); cleaved caspase-3 (p17 and p12) and β-actin. Immunoblots are representative of three independent experiments.

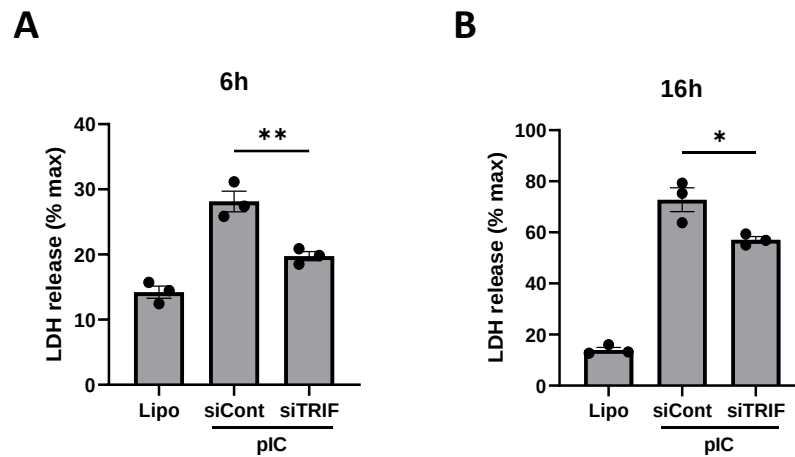


Figure 4.25: TRIF is involved in lytic cell death in dsRNA-stimulated NHBE cells. NHBE cells were transfected with siRNA targeting TRIF (siTRIF), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with poly(I:C) (pIC, 2.5 μ g/ml) for 6 h (A) or 16 h (B). LDH present in the supernatant was quantified by LDH assay. Data are mean $\pm$ SEM of three independent experiments. * $p < 0.05$ and ** $p < 0.01$ by unpaired Student's t test.

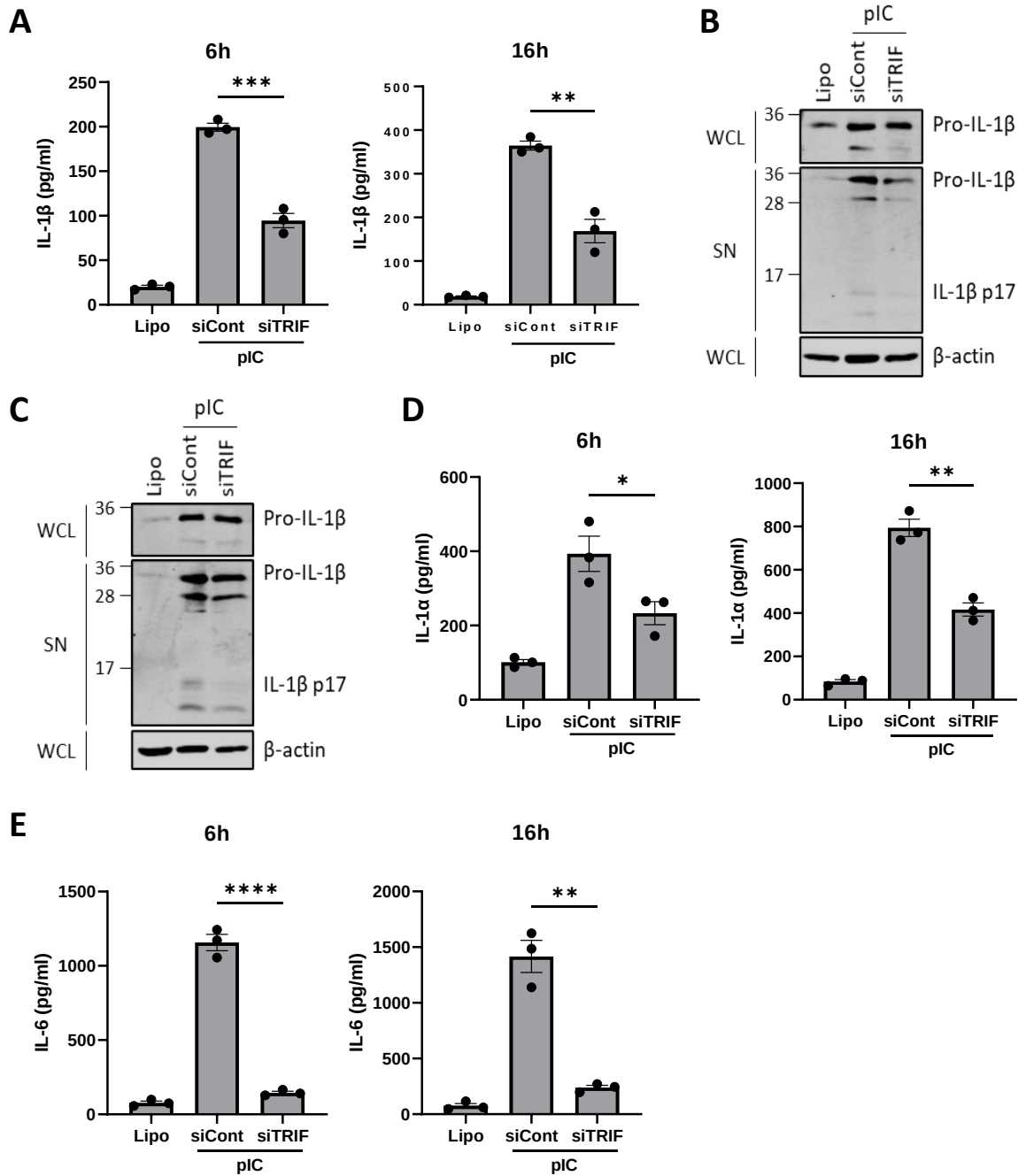


Figure 4.26: TRIF is required for cytokine secretion in dsRNA-stimulated NHBE cells.

NHBE cells were transfected with siRNA targeting TRIF (siTRIF), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with poly(I:C) (pIC, 2.5 μ g/ml) for 6 h or 16 h.

(A, D, E) IL-1 β (A), IL-1 α (D) and IL-6 (E) secretion was measured by ELISA.

Data are mean $\pm$ SEM of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001 by unpaired Student's t test.

(B, C) Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of pro-IL-1 β , mature IL-1 β (p17) and β -actin at 6 h (B) and 16 h (C). Immunoblots are representative of three independent experiments.

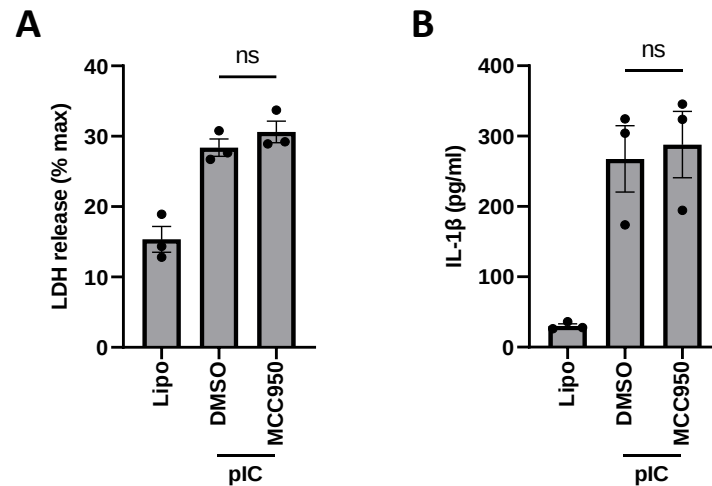


Figure 4.27: NLRP3 inflammasome does not elicit lytic cell death nor IL-1 β secretion following cytosolic dsRNA sensing in NHBE cells.

NHBE cells were treated with DMSO or MCC950 (25 μ M) for 1 h before transfection of poly(I:C) (pIC, 2.5 μ g/ml) for 6 h.

(A) LDH present in the supernatant was quantified by LDH assay.

(B) IL-1 β secretion was measured by ELISA.

Data are mean $\pm$ SEM of three independent experiments. ns: not significant by unpaired Student's t test.

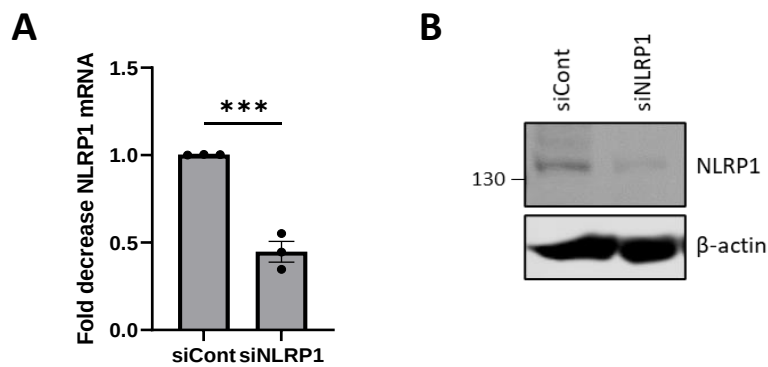


Figure 4.28: Validation of NLRP1 gene silencing by siRNA in NHBE cells.

NHBE cells were transfected with siRNA targeting NLRP1 (siNLRP1), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells.

(A) NLRP1 mRNA expression was quantified 48 h after seeding by qPCR to confirm the knockdown. Data are mean $\pm$ SEM of three independent experiments. *** $p < 0.001$ by unpaired Student's *t* test.

(B) Cell lysates were immunoblotted for the expression of NLRP1 and β -actin. Immunoblots are representative of three independent experiments.

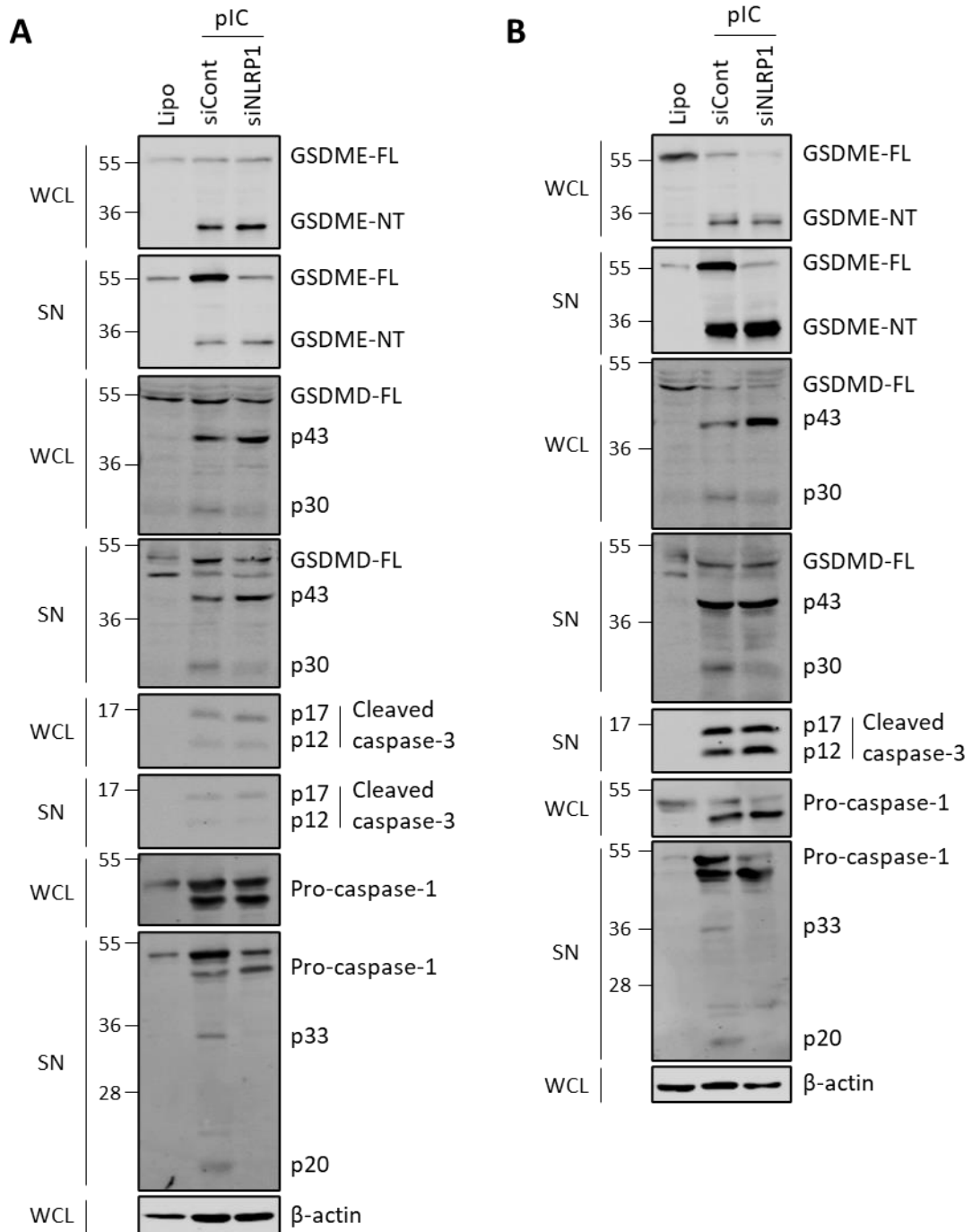


Figure 4.29: NLRP1 inflammasome leads to GSDMD pore formation and caspase-1 activation in dsRNA-stimulated NHBE cells.

NHBE cells were transfected with siRNA targeting NLRP1 (siNLRP1), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with poly(I:C) (pIC, 2.5 µg/ml) for 6 h (A) or 16 h (B). Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of full length (FL), inactivated (p43) and activated (p30) GSDMD; full length (FL) and activated (p30) GSDME; pro-caspase-1, active caspase-1 (p33 and p20); cleaved caspase-3 (p17 and p12) and β-actin. Immunoblots are representative of three independent experiments.

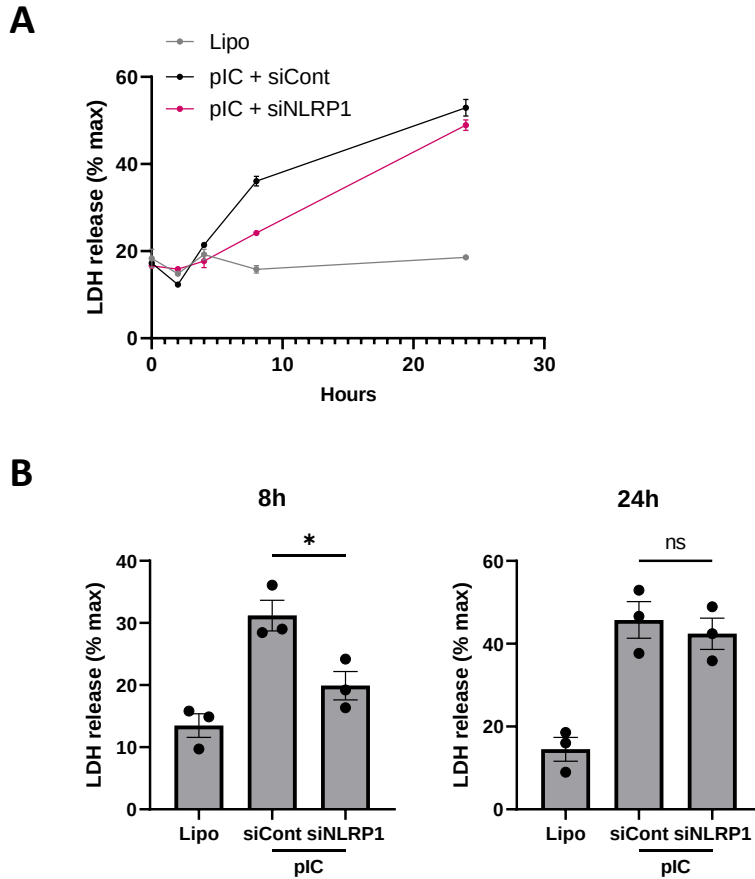


Figure 4.30: NLRP1 inflammasome elicits early lytic cell death in dsRNA-stimulated NHBE cells.

NHBE cells were transfected with siRNA targeting NLRP1 (siNLRP1), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with poly(I:C) (pIC, 2.5 μ g/ml).

(A) Representative time course of LDH release in the supernatant.

(B) LDH present in the supernatant was quantified by LDH assay at 8 h and 24 h.

Data are mean $\pm$ SD (A) of a representative experiment of three independent experiments or mean $\pm$ SEM (B) of three independent experiments. ns: not significant and * $p < 0.05$ by unpaired Student's *t* test.

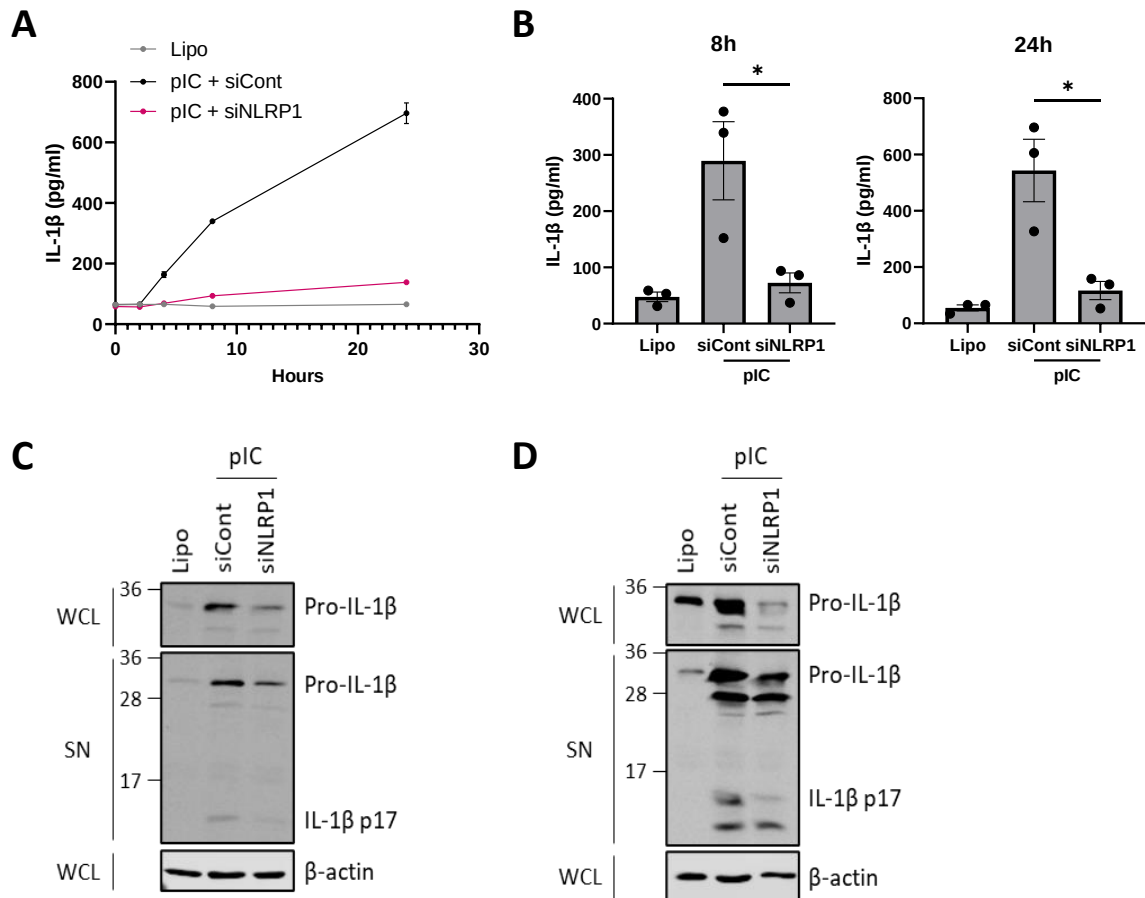


Figure 4.31: IL-1 β secretion is NLRP1-dependent in dsRNA-stimulated NHBE cells.

NHBE cells were transfected with siRNA targeting NLRP1 (siNLRP1), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with poly(I:C) (pIC, 2.5 μ g/ml).

Representative time course of IL-1 β secretion.

(B) IL-1 β secretion was measured by ELISA at 8 h and 24 h.

Data are mean $\pm$ SD (A) of a representative experiment of three independent experiments or mean $\pm$ SEM (B) of three independent experiments. *p < 0.05 by unpaired Student's t test.

(C, D) Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of pro-IL-1 β , mature IL-1 β (p17) and β -actin at 6 h (C) and 16 h (D). Immunoblots are representative of three independent experiments.

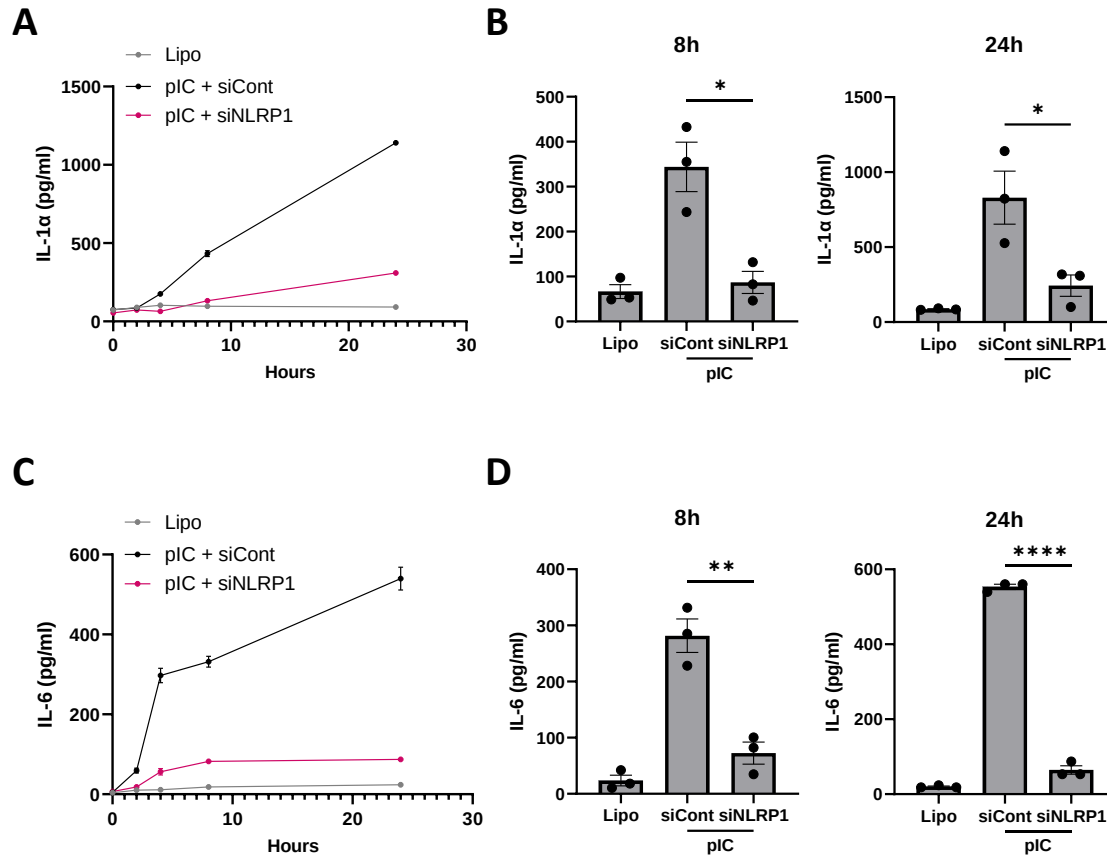


Figure 4.32: IL-1 α and IL-6 secretion is NLRP1-dependent in dsRNA-stimulated NHBE cells.

NHBE cells were transfected with siRNA targeting NLRP1 (siNLRP1), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with poly(I:C) (pIC, 2.5 μ g/ml).

(A, C) Representative time course of IL-1 α (A) and IL-6 (C) secretion.

(B, D) IL-1 α (B) and IL-6 (D) secretion was measured by ELISA at 8 h and 24 h.

Data are mean $\pm$ SD (A, C) of a representative experiment of three independent experiments or mean $\pm$ SEM (B, D) of three independent experiments. * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$ by unpaired Student's t test.

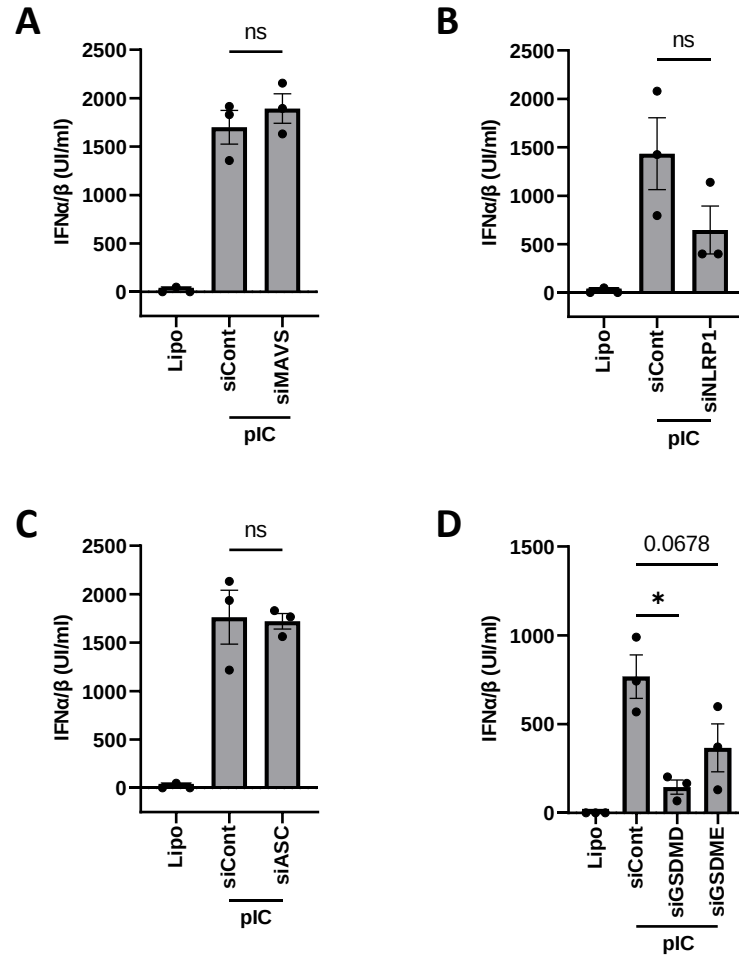


Figure 4.33: GSDM pores contribute to IFN- α/β secretion in dsRNA-stimulated NHBE cells.

NHBE cells were transfected with siRNA targeting ASC (siASC, 1 nM), MAVS (siMAVS, 5 nM), NLRP1 (siNLRP1, 3 nM), GSDMD (siGSDMD, 3 nM), GSDME (siGSDME, 3 nM) or control siRNA (siCont) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with poly(I:C) (pIC, 2.5 μ g/ml) for 8 h. IFN- α/β secretion was measured by HEK Blue IFN- α/β bioassay. Data are mean $\pm$ SEM of three independent experiments. ns: not significant and * $p < 0.05$ by unpaired Student's t test (A-C) or one-way ANOVA (D).

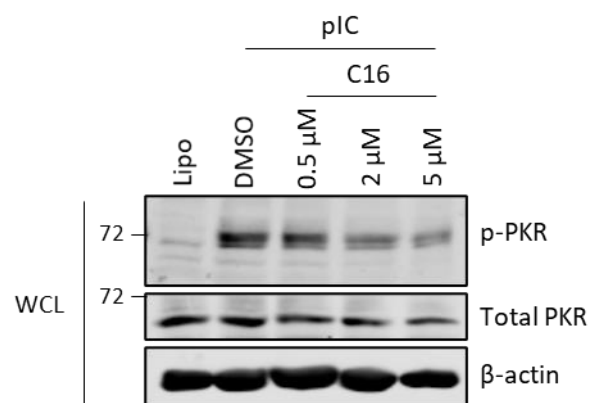


Figure 4.34: C16 inhibits dsRNA-stimulated phosphorylation of PKR in NHBE cells.

NHBE cells were treated with DMSO or PKR inhibitor (C16, 0.5, 2 or 5 μ M) for 1 h before transfection of poly(I:C) (pIC, 2.5 μ g/ml) for 6 h. Cell lysates (WCL) were immunoblotted for the expression of phospho-PKR (p-PKR), total PKR and β -actin. Immunoblots are representative of three independent experiments.

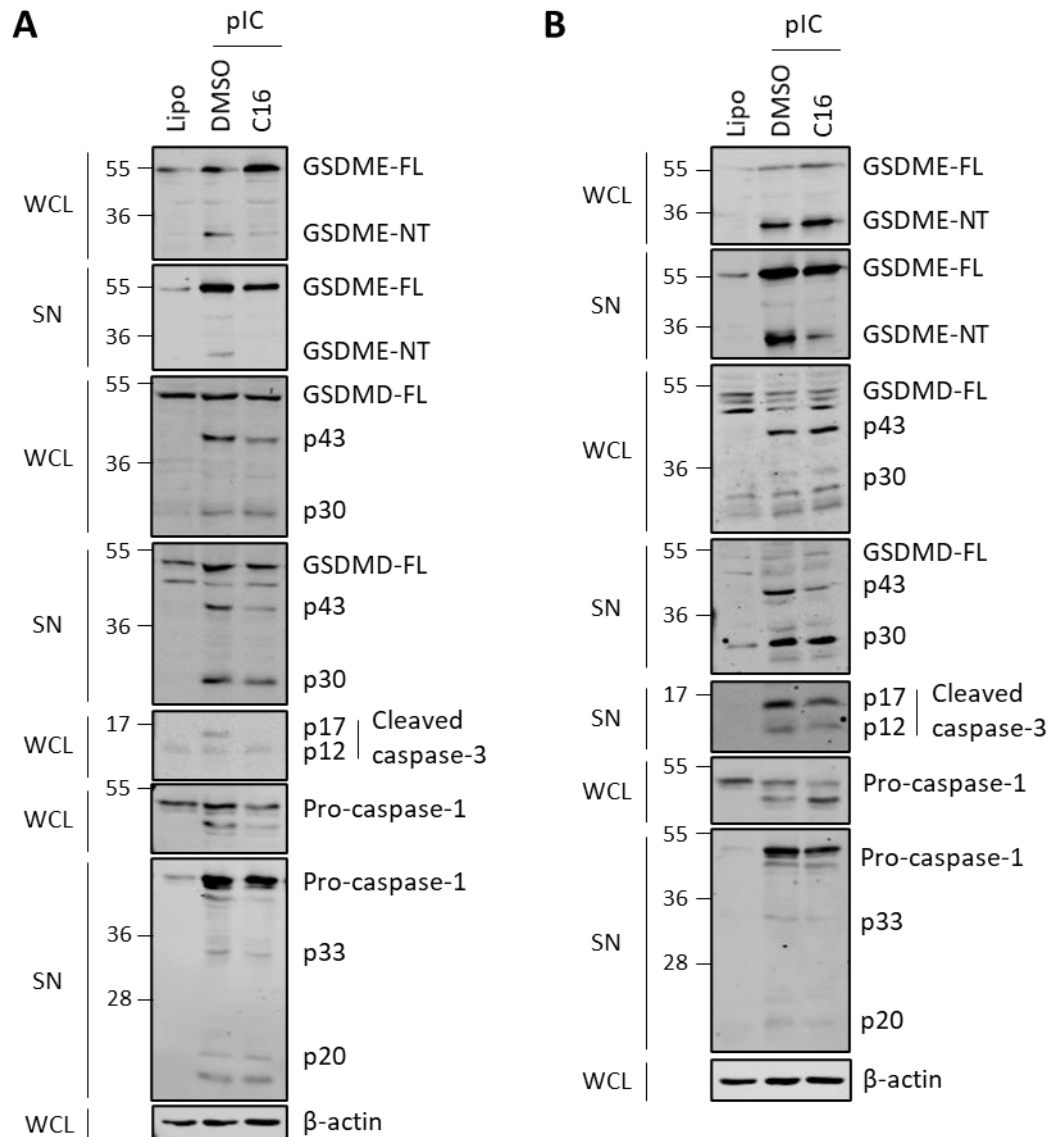


Figure 4.35: PKR is required for GSDME pore formation and caspase-3 activation in dsRNA-stimulated NHBE cells.

NHBE cells were treated with DMSO or PKR inhibitor (C16, 2 μ M) for 1 h before transfection of poly(I:C) (pIC, 2.5 μ g/ml) for 6 h (A) or 16 h (B). Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of full length (FL), inactivated (p43) and activated (p30) GSDMD; full length (FL) and activated (p30) GSDME; pro-caspase-1, active caspase-1 (p33 and p20); cleaved caspase-3 (p17 and p12) and β -actin. Immunoblots are representative of three independent experiments.

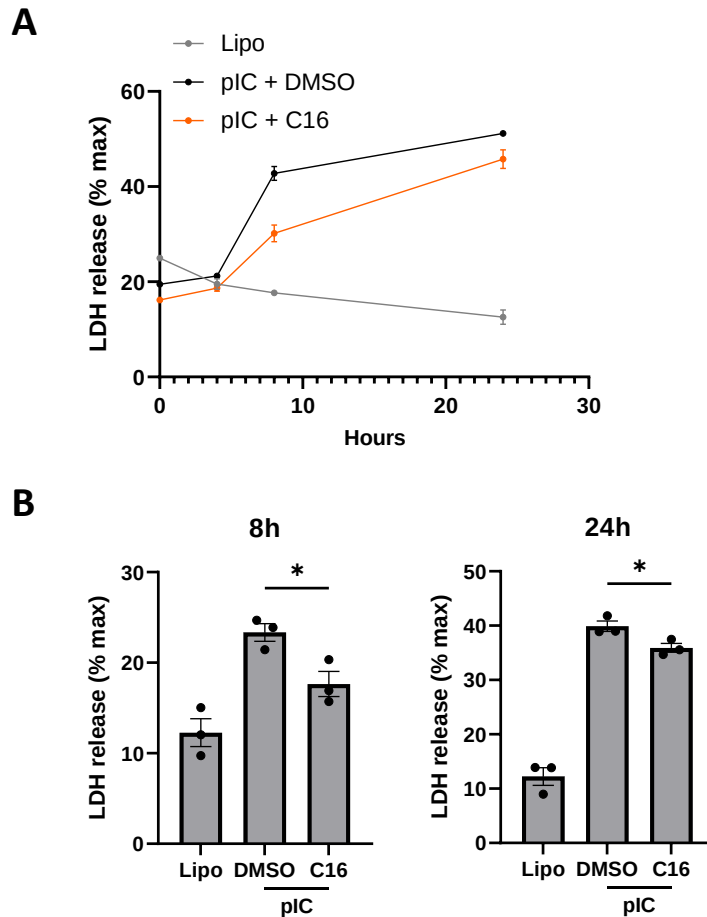


Figure 4.36: PKR is involved in lytic cell death in dsRNA-stimulated NHBE cells.

NHBE cells were treated with DMSO or PKR inhibitor (C16, 2 μ M) for 1 h before transfection of poly(I:C) (pIC, 2.5 μ g/ml).

(A) Representative time course of LDH release in the supernatant.

(B) LDH present in the supernatant was quantified by LDH assay at 8 h and 24 h. Data are mean $\pm$ SD (A) of a representative experiment of three independent experiments or mean $\pm$ SEM (B) of three independent experiments. * p < 0.05 by unpaired Student's t test.

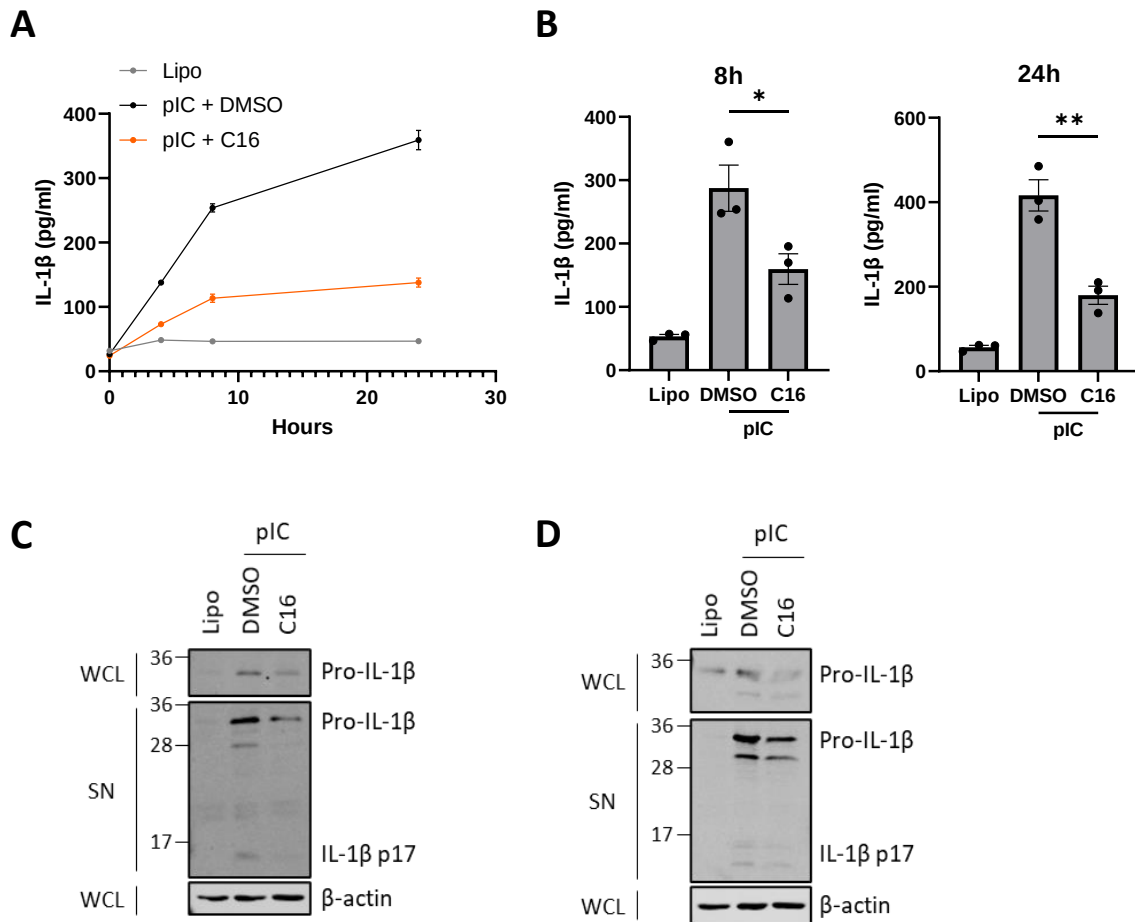


Figure 4.37: PKR is required for IL-1 β secretion in dsRNA-stimulated NHBE cells.

NHBE cells were treated with DMSO or PKR inhibitor (C16, 2 μ M) for 1 h before transfection of poly(I:C) (pIC, 2.5 μ g/ml).

(A) Representative time course of IL-1 β secretion.

(B) IL-1 β secretion was measured by ELISA at 8 h and 24 h.

Data are mean $\pm$ SD (A) of a representative experiment of three independent experiments or mean $\pm$ SEM (B) of three independent experiments. * p < 0.05 and ** p < 0.01 by unpaired Student's t test.

(C, D) Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of pro-IL-1 β , mature IL-1 β (p17) and β -actin at 6 h (B) and 16 h (C). Immunoblots are representative of three independent experiments.

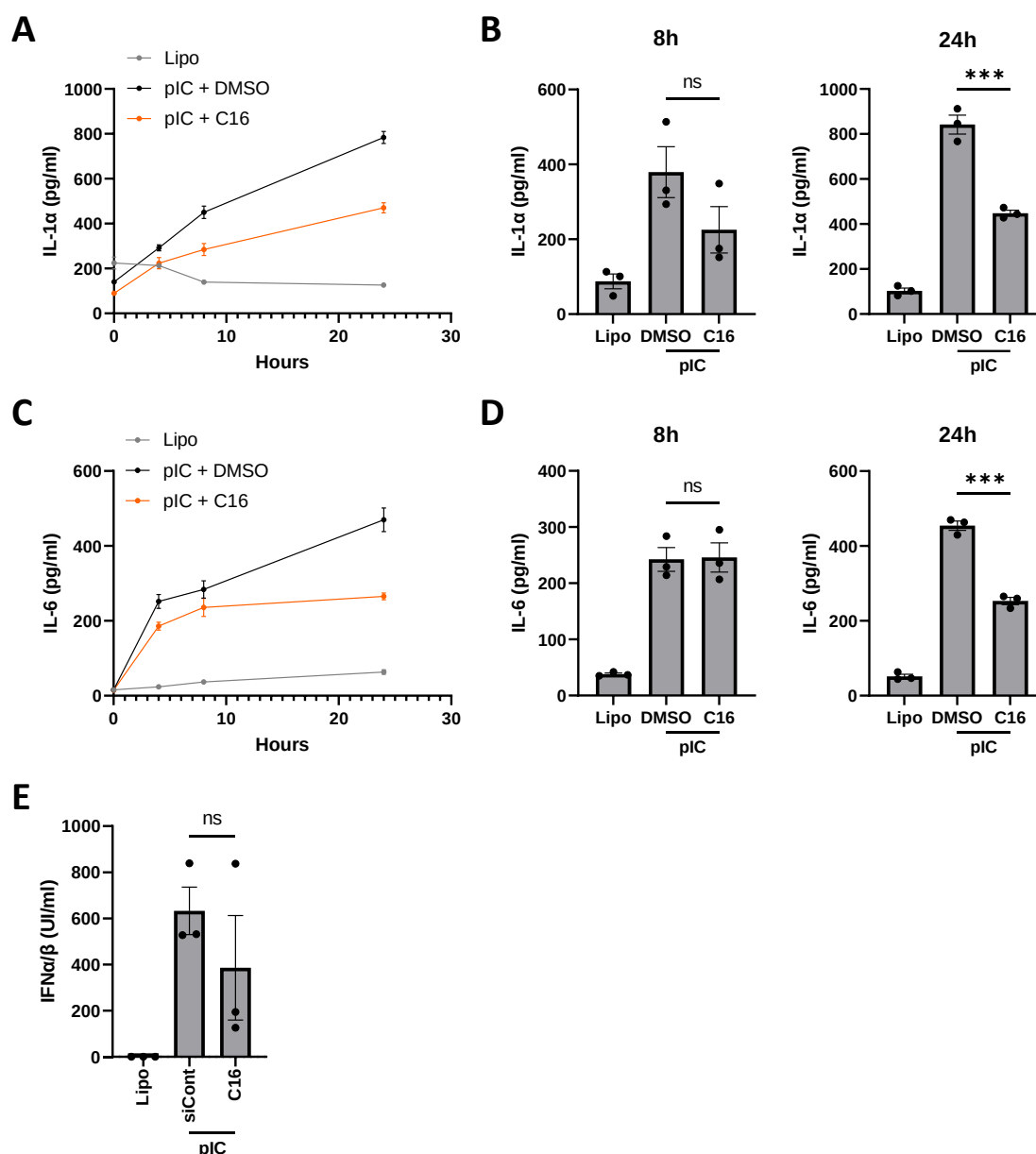


Figure 4.38: PKR contributes to late IL-1 α and IL-6 secretion in dsRNA-stimulated NHBE cells.

NHBE cells were treated with DMSO or PKR inhibitor (C16, 2 μ M) for 1 h before transfection of poly(I:C) (pIC, 2.5 μ g/ml).

(A, C) Representative time course of IL-1 α (A) and IL-6 (C) secretion.

(B, D) IL-1 α (B) and IL-6 (D) secretion was measured by ELISA at 8 h and 24 h.

(E) IFN- α/β secretion was measured by HEK Blue IFN- α/β bioassay.

Data are mean $\pm$ SD (A, C) of a representative experiment of three independent experiments or mean $\pm$ SEM (B, D, E) of three independent experiments. ns: not significant and *** p < 0.001 by unpaired Student's t test.

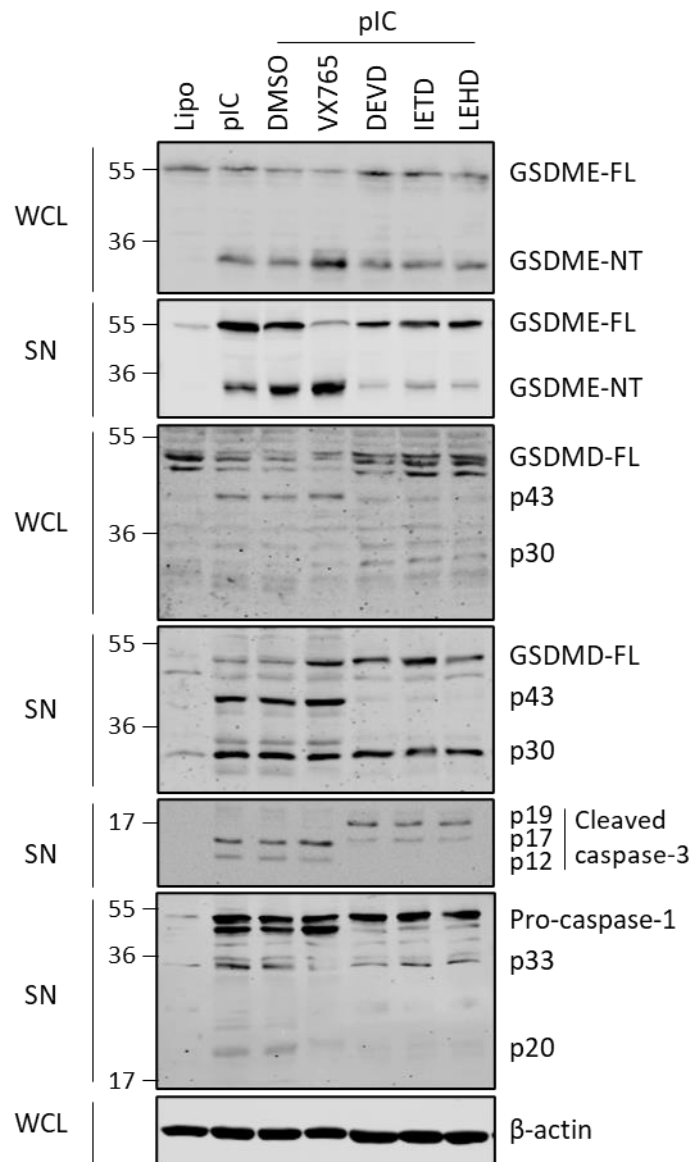


Figure 4.39: Caspase-8 and caspase-9 are required for GSDMD and GSDME cleavage in dsRNA-stimulated NHBE cells.

NHBE cells were treated with DMSO, caspase-1 inhibitor (VX765), caspase-3 inhibitor (DEVD), caspase-8 inhibitor (IETD) or caspase-9 inhibitor (LEHD, all 20 μ M) for 1 h before transfection of poly(I:C) (pIC, 2.5 μ g/ml) for 16 h. Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of full length (FL), inactivated (p43) and activated (p30) GSDMD; full length (FL) and activated (p30) GSDME; pro-caspase-1, active caspase-1 (p33 and p20); cleaved caspase-3 (p19, p17 and p12) and β -actin. Immunoblots are representative of three independent experiments.

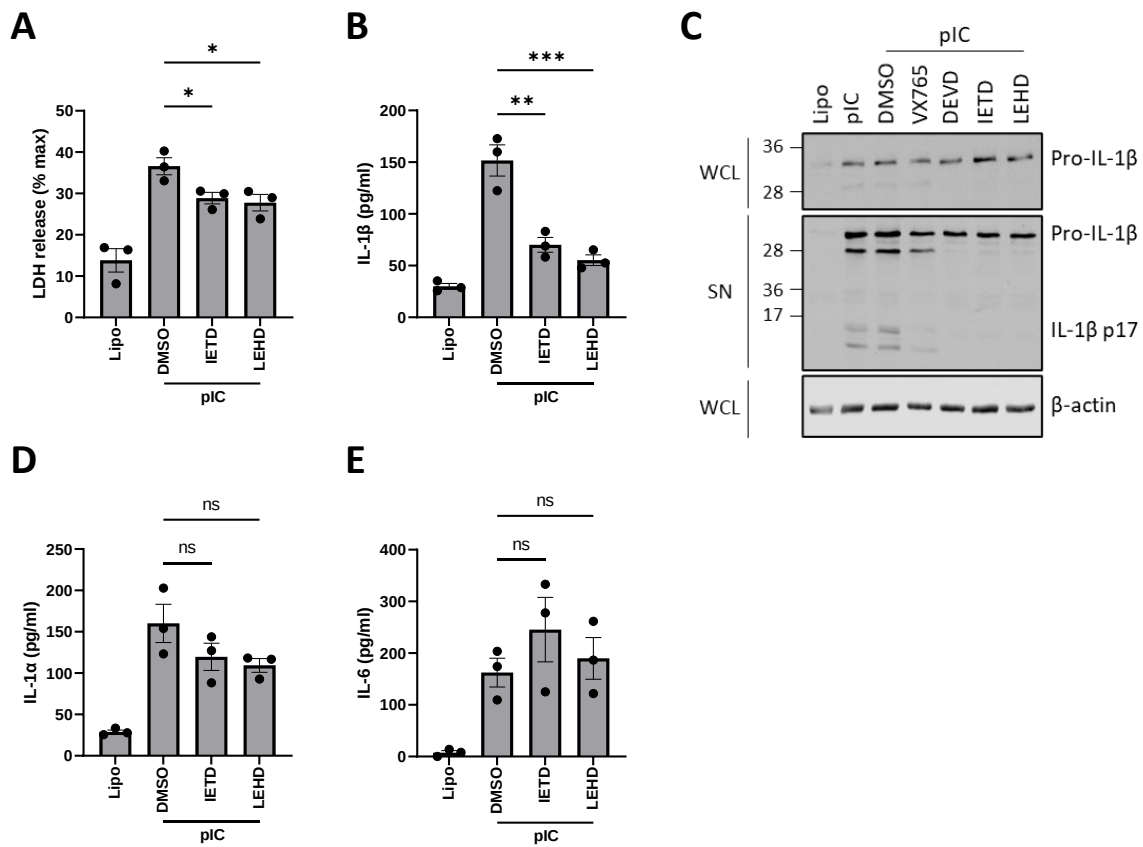


Figure 4.40: Both caspase-8 and caspase-9 are involved in lytic cell death and IL-1 β secretion in dsRNA-stimulated NHBE cells.

NHBE cells were treated with DMSO, caspase-1 inhibitor (VX765), caspase-3 inhibitor (DEVD), caspase-8 inhibitor (IETD) or caspase-9 inhibitor (LEHD, all 20 μ M) for 1 h before transfection of poly(I:C) (pIC, 2.5 μ g/ml) for 16 h.

(A) LDH present in the supernatant was quantified by LDH assay.

(B, D, E) IL-1 β (B), IL-1 α (D) and IL-6 (E) secretion was measured by ELISA.

Data are mean $\pm$ SEM of three independent experiments. ns: not significant, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by one-way ANOVA.

(C) Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of pro-IL-1 β , mature IL-1 β (p17) and β -actin. Immunoblots are representative of three independent experiments.

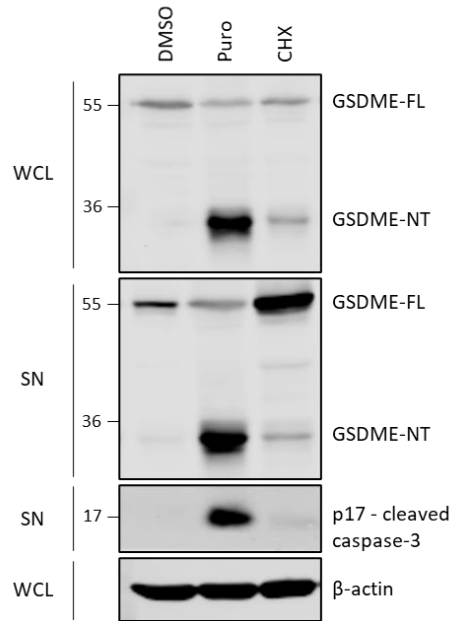


Figure 4.41: Translation inhibition leads to GSDME cleavage in NHBE cells.

NHBE cells were treated with DMSO, puromycin (Puro, 2.5 $\mu\text{g}/\text{ml}$) or cycloheximide (CHX, 50 $\mu\text{g}/\text{ml}$) for 16 h. Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of full length (FL) and activated (p30) GSDME; cleaved caspase-3 (p17) and β -actin. Immunoblots are representative of two independent experiments.

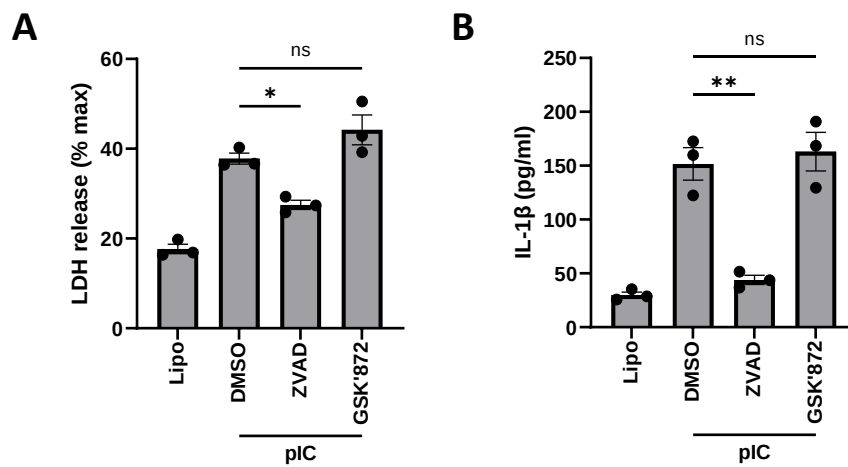


Figure 4.42: Necroptosis is not involved in lytic cell death nor IL-1 β secretion in dsRNA-stimulated NHBE cells.

NHBE cells were treated with DMSO, pan-caspase inhibitor (ZVAD, 25 μ M) or RIPK3 inhibitor (GSK'872, 3 μ M) for 1 h before transfection of poly(I:C) (pIC, 2.5 μ g/ml) for 16 h. (A) LDH present in the supernatant was quantified by LDH assay.

(B) IL-1 β secretion was measured by ELISA.

Data are mean $\pm$ SEM of three independent experiments. ns: not significant, *p < 0.05 and **p < 0.01 by one-way ANOVA.

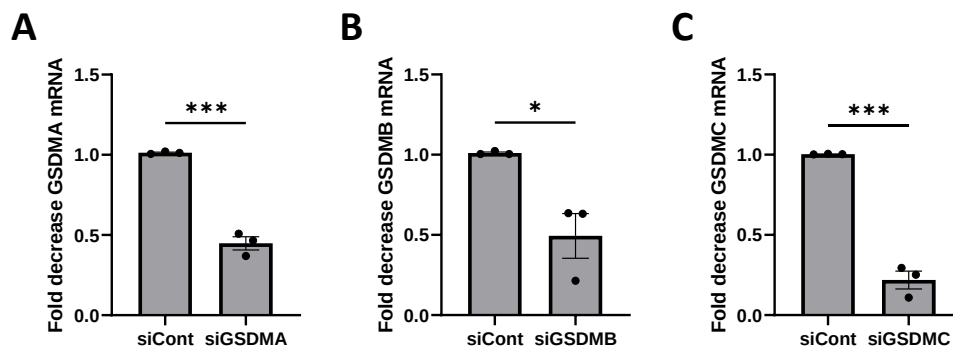
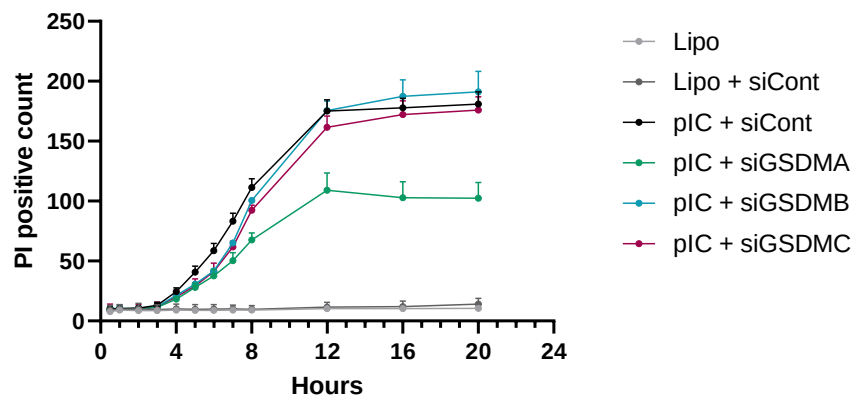


Figure 4.43: Validation of GSDMA, GSDMB and GSDMC gene silencing by siRNA in NHBE cells.

NHBE cells were transfected with siRNA targeting GSDMA (siGSDMA) or GSDMB (siGSDMB) or GSDMC (siGSDMC), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells. GSDMA (A), GSDMB (B) and GSDMC (C) mRNA expression was quantified 48 h after seeding by qPCR to confirm the knockdown. Data are mean $\pm$ SEM of three independent experiments. * $p < 0.05$ and *** $p < 0.001$ by unpaired Student's t test.



Statistics	<6h	7h	8h	12h	16h	20h
pIC + siGSDMA	ns	*	***	**	**	**
pIC + siGSDMB	ns	ns	ns	ns	ns	ns
pIC + siGSDMC	ns	ns	ns	ns	ns	ns

Figure 4.44: dsRNA leads to GSDMA pore formation at later times in NHBE cells.

NHBE cells were transfected with siRNA targeting GSDMA (siGSDMA) or GSDMB (siGSDMB) or GSDMC (siGSDMC), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were transfected with poly(I:C) (pIC, 2.5 µg/ml). Pore formation at the plasma membrane was assessed by PI uptake. The number of PI positive cells was analysed by time-lapse microscopy over a 20-hour period. Data are mean $\pm$ SEM of three independent experiments. ns: not significant, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by one-way ANOVA, compared to pIC + siCont. For clarity purposes, statistics are indicated in a table below the graph.

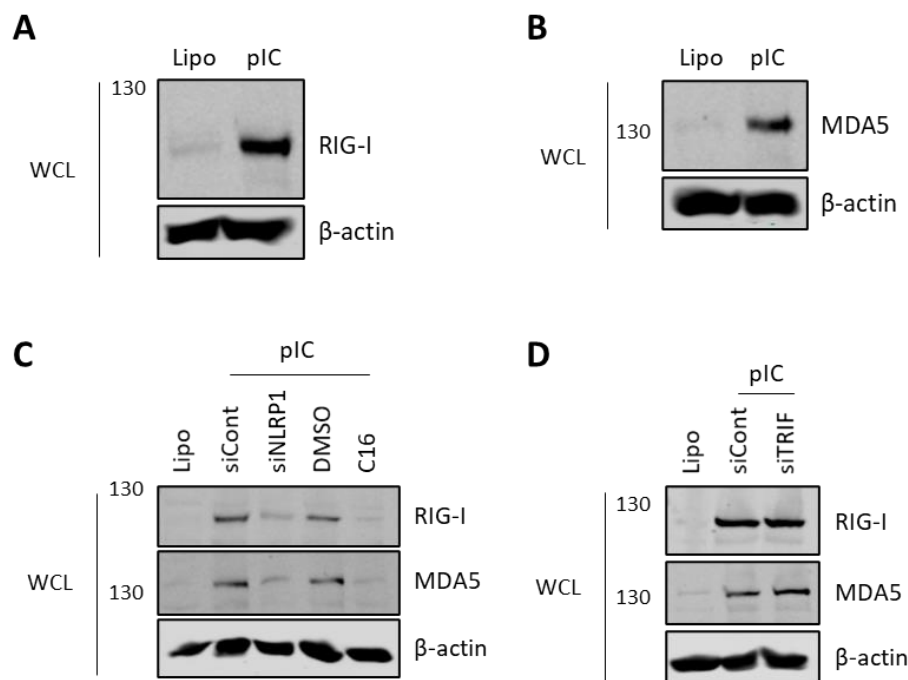


Figure 4.45: Regulation of RLR expression by other dsRNA responsive pathways.

(A, B) NHBE cells were transfected with poly(I:C) (pIC, 2.5 µg/ml) for 16 h. Cell lysates (WCL) were immunoblotted for the expression of RIG-I (A), MDA5 (B) and β-actin. Immunoblots are representative of two independent experiments.

(C, D) NHBE cells were transfected with siRNA targeting TRIF (siTRIF), NLRP1 (siNLRP1), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were treated with DMSO or PKR inhibitor (C16, 2 µM) for 1 h before transfection of poly(I:C) (pIC, 2.5 µg/ml) for 24 h. Cell lysates (WCL) were immunoblotted for the expression of RIG-I (A), MDA5 (B) and β-actin. Immunoblots are representative of three independent experiments.

4.3 Discussion

In this chapter our results showed that dsRNA delivery triggered NLRP1 inflammasome formation resulting in caspase-1-mediated GSDMD cleavage into a pore-forming fragment. In parallel, dsRNA also activated PKR leading to caspase-3-dependent GSDME pore formation and GSDMD inactivation. In addition, our results showed that TRIF, caspase-8 and caspase-9 were involved in GSDME cleavage, and that dsRNA sensing by NLRP1 and PKR also contributed to upregulation of expression of innate proteins such as pro-IL-1 β and RLRs. The main findings are summarised in **Figure 4.46**.

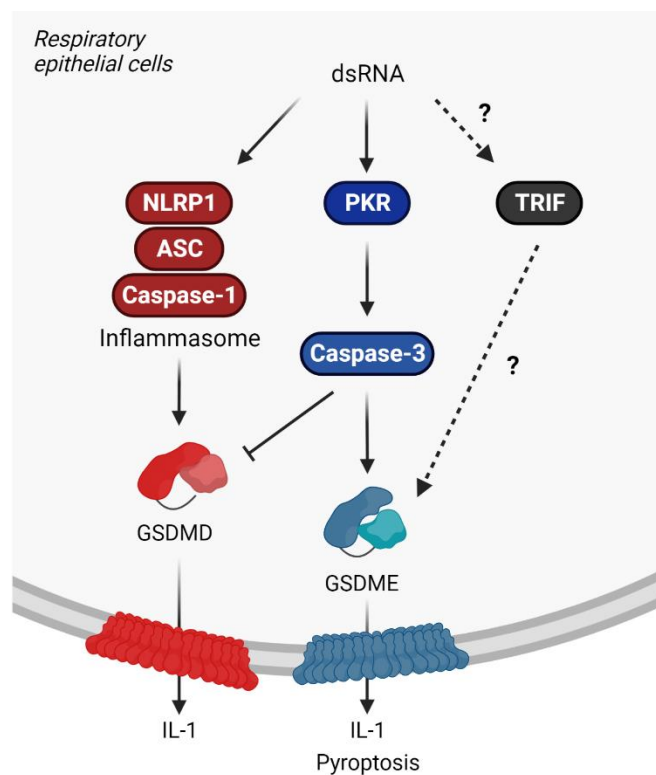


Figure 4.46: Cytosolic dsRNA leads to GSDM pore formation via different pathways in NHBE cells.

Cytosolic dsRNA triggers NLRP1 inflammasome formation and caspase-1 activation which mediates GSDMD pore formation. In parallel dsRNA is also recognised by PKR resulting in caspase-3-dependent GSDME cleavage into a pore-forming fragment and GSDMD inactivation. TRIF is also involved in GSDME pore formation following dsRNA delivery. GSDME pores is involved in lytic cell death whereas both GSDME and GSDME pores are required for cytokine secretion.

4.3.1 dsRNA sensing triggers caspase-mediated GSDM cleavage in NHBE cells

Our study showed that dsRNA sensing triggered caspase-1-dependent GSDMD activation and caspase-3-mediated GSDME pore formation and GSDMD inactivation in basal respiratory epithelial cells. This is in accordance with the literature showing that caspase-1 and caspase-3 cleave GSDMD and GSDME respectively within their linker regions to release the p30 fragment from the repressor domain [174, 175, 216]. In addition the apoptotic caspase-3 has been reported to cleave GSDMD in its N-terminal domain leading to an inactive p43 fragment [203].

Interestingly, caspase-3 can be activated by caspase-1 leading to GSDMD inactivation and apoptosis [203]. However our data showed that the presence of caspase-1 inhibitor didn't impact the generation of active caspase-3 forms. Our data also showed that caspase-3 inhibitor decreased the appearance of caspase-1 p33 and p20 forms in the supernatant. Given that GSDMD was activated before the appearance of the active form of caspase-3, and that caspase-3 mediated lytic cell death which allows the release of larger molecules [179], it is more likely that active caspase-1 appearance in supernatants was decreased due to the inhibition of caspase-3-dependent membrane rupture rather than a direct role of caspase-3 in caspase-1 processing. This indicates that caspase-1 and caspase-3 are probably activated in parallel rather than one after another.

Although caspase-8 can cleave GSDMD into a pore-forming fragment [212, 213], we showed that caspase-8 was not involved in GSDMD activation in dsRNA-stimulated NHBE cells. However our results indicated that caspase-8 and caspase-9 were involved in the generation of GSDME active p30 fragment and GSDMD inactive p43 fragment. Caspase-3 can be activated by both caspase-8 and caspase-9 through the extrinsic and intrinsic mitochondrial pathways respectively [229]. Since PKR-mediated translation arrest could trigger apoptosis [59], caspase-8 and caspase-9 could then activate caspase-3 following dsRNA sensing and thereby be indirectly involved in GSDM cleavage.

4.3.2 GSDM pores are required for lytic cell death and cytokine secretion in response to dsRNA sensing in NHBE cells

After being processed, GSDMD and GSDME can mediate pyroptotic cell death [174, 175, 216]. Our results demonstrated that GSDME is required for in lytic cell death but not GSDMD, which is in accordance with our data showing that caspase-3/8/9 were involved in LDH release unlike caspase-1. Additionally, GSDMD and GSDME pore

formation enables IL-1 β and IL-1 α secretion in myeloid cells [174, 175, 187, 204, 223]. Our data showed that GSDME was required for IL-1 secretion whereas GSDMD pores were involved in IL-1 α and early IL-1 β release in basal respiratory epithelial cells. This is in line with studies showing that GSDMD pores allow secretion of IL-1 β from living macrophages cells without or prior to pyroptotic cell death [182].

In this chapter our results further demonstrated that secretion of IL-1 β was caspase-1, -3, -8, -9-dependent following dsRNA sensing in basal respiratory epithelial cells. Since caspase-1 is required for pro-IL-1 β processing and secretion [197], the involvement of this inflammatory caspase is not surprising. In addition, since caspase-3 induces GSDME pore forming fragment, the presence of caspase-3 inhibitor would diminish the quantity of pores in the plasma membrane and thus GSDME-mediated cytokine release. Also, since caspase-8 and caspase-9 can activate caspase-3 [229], their inactivation would prevent caspase-3-dependent GSDME pore formation and thereby cytokine secretion. Thus these three caspases are probably indirectly involved in cytokine secretion. Nonetheless a direct role of caspase-8 in cytokine secretion shouldn't be completely ruled out. Indeed, caspase-8 can also cleave pro-IL-1 β into its mature form [232] but there is no evidence that caspase-9 nor caspase-3 can do that. Another report showed that caspase-3 triggered caspase-8 activation leading to caspase-8-dependent IL-1 β cleavage [233]. Our results showed that inhibition of caspase-3, -8, -9, all prevented caspase-3 activation, but it will be interesting to examine whether caspase-3 inhibitor would also affect caspase-8 activation.

IL-1 α is usually described as an alarmin released during passive cell lysis [94]. However our results showed that IL-1 α secretion is partially dependent on caspase-1 and caspase-3 in epithelial cells. Since caspase-1 doesn't cleave pro-IL-1 α [234], the requirement for caspase-1 is most likely indirect. Caspase-1 is indeed required for GSDMD pore formation that allows calcium influx leading to the activation of calpain which then cleaves pro-IL-1 α into its mature form [187]. Since our data showed that caspase-1-mediated GSDMD pores didn't significantly affect lytic cell death, caspase-1 could be involved in IL-1 α secretion through calpain activation in respiratory epithelial cells. Regarding the involvement of apoptotic caspases, caspases-mediated GSDME pores could also allow calpain activation through calcium influx or IL-1 α could be released during caspases-mediated GSDME-dependent lytic cell death.

Interestingly our data showed that IL-1 α and IL-1 β had similar secretion kinetics following dsRNA sensing. This co-secretion has also been reported in another study, and

was caspase-1-dependent following stimulation of different inflammasome such as NLRP3, NLRP1 and AIM2 in BMDCs [235]. Moreover, IL-6 secretion was usually similar to the secretion of IL-1 in NHBE cells. Given that IL-6 release is enhanced by IL-1 β secretion [205] and that IL-1 β requires caspase-1 for its maturation [197], IL-1-dependent IL-6 secretion was expected to be caspase-1-dependent. However here caspase-1 was not involved in IL-6 secretion. In addition, since the secretion of IL-6 was decreased in the presence of GSDME siRNA, addition of caspase-3 inhibitor was expected to decrease IL-6 secretion, but this was not the case here. Thereby further investigation is needed to understand how IL-6 is released from dsRNA-stimulated NHBE cells.

The role of GSDM pores in type I IFN secretion is still debated. One paper showed that GSDMD pores prevented cGAS-mediated type I IFN through K⁺ efflux, for DNA sensing [236], whereas another report indicated that GSDMD enabled the secretion of IFN- β that further limited viral replication in swine testis cells [237]. Our study showed that both GSDMD and GSDME were involved in type I IFN secretion in dsRNA-stimulated epithelial cells.

4.3.3 dsRNA sensing mediates GSDMA pore formation but not necroptosis in NHBE cells

Lytic cell death wasn't fully GSDME-dependent as the LDH readout only showed partial restoration of plasma membrane integrity at 24 h in the presence of GSDME siRNA. This led us to investigate whether necroptosis or other GSDM protein could be involved in late lytic cell death. Necroptosis and pyroptosis can both release a large amount of cytoplasmic content such as LDH during plasma membrane rupture [182]. Also although IL-1 β secretion is an hallmark of GSDM pore formation, necroptosis can also lead to GSDMD-independent IL-1 β cytokine secretion [238]. However using a pharmaceutical inhibitor, I ruled out that necroptosis is involved in late lytic cell death and IL-1 β secretion in basal respiratory epithelial cells. In addition our data showed that caspase-8 was involved in GSDM cleavage, cell death and IL-1 β release whereas necroptosis is activated in the absence of caspase-8, which strengthen the conclusion that dsRNA sensing doesn't induce necroptosis in basal respiratory epithelial cells [239]. Interestingly, our results indicated that GSDMA formed pore at the plasma membrane from 12 h. Since GSDMA pore formation led to pyroptosis in keratinocytes following bacterial infection [240], this suggests that GSDMA might be involved in lytic cell death following dsRNA sensing in basal respiratory

epithelial cells. However PI uptake data suggested that membrane permeabilisation was not fully dependent on GSDMA and GSDME pores at 20 h. Thereby it will be interesting to assess the contribution of GSDMA pores in lytic cell death, especially at 24 h, to determine if another pathway is involved in plasma membrane damages such as necrosis. Our data also showed that neither GSDMB nor GSDMC were involved in dsRNA-induced pore formation. In line with this, GSDMB mRNA is highly expressed in differentiated NHBE cells compared to undifferentiated NHBE cells [241] and our results were generated in undifferentiated NHBE cells. However to completely rule out the involvement of GSDMB and GSDMC in pore formation in dsRNA-stimulated NHBE cells, their protein levels should be assessed to confirm that the knockdown was effective.

4.3.4 NLRP1 inflammasome leads to GSDMD pore formation in response to dsRNA sensing in NHBE cells

The recognition of infection or cellular damage by inflammasome complexes induces oligomerisation of the sensor protein which allows them to interact with the adaptor protein ASC. Once polymerised, ASC recruits pro-caspase-1 leading to its autocleavage and activation [168]. Our results showed that dsRNA sensing induced ASC oligomerisation and pro-caspase-1 maturation, thereby inflammasome formation in NHBE cells. One study showed that the NLRP3 inflammasome was activated following cytosolic dsRNA delivery in macrophages [224] but our data demonstrated that NLRP3 inflammasome inhibition didn't impact lytic cell death nor IL-1 β release in basal respiratory epithelial cells. This is in accordance with results from Chapter 3 showing that NLRP3 was not activated in NHBE cells by LPS and nigericin treatment. Further investigation using siRNA demonstrated that dsRNA activated an NLRP1 inflammasome, that led to GSDMD activation, early lytic cell death and cytokine secretion. This is consistent with the recent discovery that NLRP1 is dsRNA-sensing inflammasome in keratinocytes [153] and the emerging idea that NLRP1 is the predominant inflammasome in barrier epithelial cells such as keratinocyte and lung epithelial cells [143, 145, 153].

4.3.5 PKR is required for GSDME pore formation and GSDMD inactivation in response to dsRNA sensing in NHBE cells

Our study showed for the first time that PKR triggered caspase-3-dependent GSDME cleavage into a pore-forming fragment following dsRNA delivery. This is

consistent with PKR being a primary sensor of cytosolic dsRNA in NHBE cells, even though to date there has been more focus on PKR being an IFN-inducible dsRNA-responsive effector protein after initial sensing of dsRNA by other PRRs [59]. Previous study demonstrated that translation inhibition led to caspase-3-mediated GSDME cleavage during viral infection [194]. Given that PKR activation suppresses protein synthesis [59] and that cycloheximide and puromycin treatment triggered caspase-3 activation and GSDME cleavage in NHBE cells, it is likely that PKR activation leads to translation shut down that further mediates caspase-3-dependent GSDME cleavage into a pore-forming fragment during dsRNA sensing in basal respiratory epithelial cells. However in this study, translation inhibition mediated caspase-3 activation independently of caspase-8 [194] whereas our results here showed that caspase-8 was involved in caspase-3 activation and GSDM cleavage. Interestingly, PKR can activate both caspase-8 and caspase-9 following cytosolic poly(I:C) delivery [242] which could explain how caspase-3 is activated downstream of PKR activation. Nevertheless it is possible that both pathways act in concert to activate caspase-3. Thereby further investigation is needed to uncover the mechanism of caspase-3 activation downstream of PKR.

Several studies have reported that PKR is involved in type I IFN [243, 244] but our data showed that inhibition of PKR activation didn't significantly affect IFN release in dsRNA-stimulated NHBE cells indicating that PKR doesn't have a major contribution in type I IFN response.

4.3.6 TRIF is involved in early GSDME pore formation in response to dsRNA sensing in NHBE cells

Our results demonstrated that TRIF was required for lytic cell death, cytokine secretion and for early caspase-1 and caspase-3 activation, early GSDME pore formation and modest GSDMD inactivation. I also showed that untransfected dsRNA caused some GSDME cleavage. TRIF is the adaptor protein that is recruited by TLR3 to mediate downstream signaling response [38]. It is possible that not all dsRNAs were internalised into liposomes during preparation which would result in small fraction of extracellular dsRNA that can then be endocytosed and recognised by TLR3. Nevertheless most of the dsRNA should be transfected into NHBE cells, and given that our results demonstrated an important contribution of TRIF in dsRNA-induced responses, the potential recognition of naked dsRNA doesn't explain all the phenotype. Interestingly, TLR3 can also recognise cytosolic

long dsRNA and trigger apoptosis [242, 245]. For instance, transfection of poly(I:C) induces apoptosis in a RLRs and MAVS-independent manner but TLR3-dependent manner [245]. In addition poly(I:C) delivery can be recognised by PKR but also by TLR3 which both activate caspase-8 that further promotes caspase-9 activation and apoptotic cell death [242]. Thereby, TLR3 could be partially involved in cytosolic dsRNA sensing and contribute to caspase-8 activation upstream of caspase-3-dependent GSDME cleavage through the adaptor TRIF in respiratory epithelial cells. Interestingly DExD/H-box helicases can sense dsRNA and trigger dsRNA-stimulated responses through the adaptor TRIF. For instance DDX1, DDX21, and DHX36 helicases sense intracellular poly(I:C) in the cytosol leading to NF- κ B and IRF3 activation through the adaptor TRIF in dendritic cells [246]. Since NF- κ B upregulates the transcription of pro-IL-1 β and pro-caspase-1 [247, 248], this would explain how TRIF contributes to pro-inflammatory cytokine secretion and caspase-1 activation in dsRNA-stimulated respiratory epithelial cells. The authors also demonstrated that poly(I:C) can bind to PKR as well as the RNA helicases complex [246], indicating that this TRIF-dependent pathway can coexist with PKR-dependent responses.

4.3.7 MAVS is not involved in GSDM cleavage in response to dsRNA sensing in NHBE cells

Our study revealed that the mechanism of GSDM cleavage, lytic cell death and cytokine secretion was independent of the adaptor MAVS. Interestingly dsRNA-induced IFN secretion was also MAVS-independent whereas hpRNA, the RIG-I ligand, triggered a potent MAVS-dependent IFN release. This result confirmed that MAVS siRNA was efficient in decreasing MAVS-dependent signaling and that MAVS was indeed not required for dsRNA-induced responses.

RLRs could be involved in dsRNA-mediated response independently of the adaptor MAVS. RLRs can indeed promote interaction between dsRNA and PKR by removing viral proteins bound to dsRNA which leads to translation inhibition [249]. However our results showed that both RIG-I and MDA5 were barely expressed in NHBE cells at steady state and that their expression was enhanced only after 16 h of poly(I:C) treatment, in a NLRP1 and PKR-dependent manner. Yet GSDM cleavage, lytic cell death and cytokine secretion were detectable from 6 h. This suggests that RLRs are not involved as primary sensors in dsRNA-stimulated responses in respiratory epithelial cells.

4.3.8 NLRP1 and PKR, but not RLRs, are primary sensors of cytosolic dsRNA in NHBE cells

Our data indicated that NLRP1 and PKR activation are required to upregulate RIG-I and MDA5 expression upon dsRNA sensing in NHBE cells. Even though NLRP1 and PKR didn't significantly impact type I IFN release, treatment with NLRP1 siRNA and C16 modestly decreased IFN secretion. Since RIG-I and MDA5 are ISGs whose expression is upregulated by IFN [105] and since both GSDMD and GSDME were required for IFN secretion, NLRP1 and PKR could trigger type I IFN release in small amount through GSDM pores which then enhances RLR expression. However it is still an open question as to how NHBE cells elicit type I IFN following dsRNA sensing. Given that DDXs and TRIF can form a complex in the cytosol in the presence of intracellular dsRNA and promote IRF3-dependent response [246], such a complex might be required for type I IFN release in respiratory epithelial cells.

Our data also demonstrated that IL-1 β and early IL-1 α secretion were ASC-dependent, whereas IL-1 β , IL-1 α and IL-6 release were all NLRP1-dependent, suggesting that NLRP1 could have an ASC-independent role in epithelial cells. Moreover pro-IL-1 β but not pro-caspase-1 expression was decreased in the presence of NLRP1 siRNA, showing that NLRP1 specifically contributes to pro-IL-1 β expression. Even though a role for NLRP1 in transcription or translation has not been unveiled yet, NLRP1 could enhance the transcription of select genes besides forming an inflammasome. Indeed, other NLR proteins have been shown to have transcriptional activity [66]. For instance, CIITA that belongs to the NLRA family is a transactivator that regulates the expression of major histocompatibility complex class II [66]. Thereby NLRP1 might increase the transcription through direct or indirect recruitment to the promoters of genes. Interestingly, PKR inhibitor decreased the expression of pro-IL-1 β and pro-caspase-1 at 6 h and 16 h but not the expression of full length GSDM, indicating that PKR specifically contributes to pro-IL-1 β and pro-caspase-1 expression as well as RLR expression. It has been reported that the transcription factor NF- κ B is needed to enhance pro-IL-1 β and pro-caspase-1 transcription and expression [247, 248] and that PKR can promote NF- κ B activation [250, 251]. Thereby PKR might also be involved in transcription following dsRNA sensing in respiratory epithelial cells. This further highlights a previously underappreciated role of PKR and NLRP1 as primary innate immune sensors of cytosolic dsRNA in NHBE cells.

Overall in this chapter I demonstrated that dsRNA sensing triggered NLRP1 inflammasome-dependent GSDMD pore formation whereas PKR activation led to

caspase-3-mediated GSDME activation and GSDMD inactivation. In addition, TRIF, caspase-8 and caspase-9 were required for GSDME pore formation. While both GSDMD and GSDME pores were necessary for cytokine secretion, only GSDME was required for lytic cell death. In the next chapter I will examine how relevant these pathways are for live viruses in undifferentiated NHBE cells.

**Chapter 5. Results III – Mechanisms of gasdermin
cleavage in response to respiratory virus infection in
basal respiratory epithelial cells**

5.1 Introduction

Respiratory virus infections cause epidemics each year resulting in severe respiratory tract diseases in young, elderly and immunocompromised population and potentially death. Besides replicating in humans, some respiratory viruses such as IAV and coronaviruses have animal reservoirs which increase the risk of an animal virus appearing in the human population. This could then create a pandemic as recently illustrated with the COVID-19 pandemic. In this chapter I am interested in IAV and RSV since their infections are responsible for the majority of hospitalisations and deaths during annual epidemics [1]. Both IAV and RSV are enveloped viruses containing negative-sense single-stranded RNAs, but while RSV has a linear genome, the IAV genome is segmented into eight RNAs. Additionally, IAV replicates in the nucleus whereas RSV replicates in the cytoplasm of infected airway epithelial cells from the upper respiratory tract. Nevertheless these viruses can also target epithelial cells from the lower respiratory tract which then trigger a robust pro-inflammatory response resulting in severe diseases such as pneumonia and bronchiolitis [3, 8]. Viral replication in lower epithelial cells can also promote bacterial coinfections which enhance the severity of the infection [3, 8].

Respiratory epithelial cells express many PRRs, including inflammasome receptors, that are essential to mount an innate immune response against IAV and RSV viruses. Nevertheless, inflammasome activation can also contribute to exacerbate harmful inflammation at later stages of the infection [128]. IAV infection mostly triggers the NLRP3 inflammasome in myeloid cells [124, 125] but can also activate an IFN-dependent inflammasome, called the MxA inflammasome, that recognises IAV nucleoprotein in infected bronchial epithelial cells [167]. IAV infection also mediates GSDMD and GSDME pore formation in mouse myeloid cells and lung epithelial cells respectively, that leads to pyroptosis and cytokine secretion [252, 253]. A study reported that IAV led to apoptosis at early stage of infection in human bronchial epithelial cells but then secretion of IFN suppresses apoptosis and caused pyroptosis that released pro-inflammatory cytokines [198]. GSDM-dependent lytic cell death triggered by RNA virus infection can favour the host. For instance, rotavirus replication is enhanced in GSDMD-deficient mice compared to wild-type mice [254] demonstrating that for some viruses at least GSDMD pores can limit viral replication. Furthermore, several studies have highlighted viral evasion mechanisms that target GSDMD to prevent pore formation and pyroptotic cell death, which emphasises a role for GSDMD pores in the antiviral responses. 3C protease of enterovirus 71 and SARS-CoV-2 cleaves GSDMD at position 193 leading to its inactivation [145, 255]. Also

SARS-CoV-2 nucleoprotein blocks caspase-1-mediated GSDMD activation by binding to the GSDMD linker region [256]. This interaction thereby prevents caspase-1 cleavage which decreases IL-1 β release and pyroptosis [256]. In contrast, Zika virus NS2B3 protease cleaves GSDMD at position 249 into an active N-terminal fragment and promotes pyroptosis [257] which suggests that pyroptosis could favour Zika virus infection. Importantly, lytic cell death can also be detrimental to the host as demonstrated by the positive correlation between LDH detection in the serum of patients and the severity of the disease during the pandemic 2009 H1N1 influenza virus [258]. The correlation between extracellular LDH concentration and disease severity has also been established for COVID-19 [259].

Although lung epithelial cells are the first line of defence against respiratory RNA viruses, little is known about how lytic cell death is caused in these cells and how it impacts viral replication. Thereby understanding the mechanism of GSDM pore formation is essential to understand how lytic cell death and cytokine secretion are mediated in respiratory epithelial cells following viral infection. In the previous chapter I demonstrated that NLRP1 inflammasome led to GSDMD activation and that PKR triggered GSDME pore formation during dsRNA sensing. Thus in this chapter I investigated whether these pathways also contributed to GSDM cleavage during IAV and RSV infection in basal respiratory epithelial cells.

5.2 Results

5.2.1 IAV infection triggers GSDME pore formation and mainly GSDMD inactivation in NHBE cells

Before investigating whether IAV leads to GSDM cleavage, I first determined if NHBE cells were permissive to infection with the WSN strain of IAV. Initially cells were infected at an MOI of 0.1, and replication measured by plaque assay at different times post-infection. This showed that the viral titre increased over time, plateauing at 24 h [Figure 5.1], demonstrating that NHBE cells are permissive for productive IAV infection. Consistent with productive replication, infection of NHBE cells at MOI 0.1 and MOI 1 showed expression of IAV nucleoprotein at 24 h post infection (hpi) [Figure 5.2]. Interestingly, immunoblot analysis then showed that IAV infection at MOI 0.1 mainly triggered GSDME cleavage into a pore-forming fragment, whereas IAV infection at MOI 1 caused strong cleavage of GSDME, appearance of the GSDMD p43 fragment and faint generation of the GSDMD p30 fragment [Figure 5.2]. This indicates that IAV infection mostly leads to GSDME pore formation and GSDMD inactivation in NHBE cells.

5.2.2 Caspase-3 mediates GSDME activation and GSDMD inactivation following IAV infection in NHBE cells

Next I examined whether IAV infection mediated caspase-1 and caspase-3 activation. Immunoblot analysis showed potent caspase-3 activation whereas active caspase-1 p33 and p20 forms were barely detectable [Figure 5.3]. I further characterised the requirement of caspase-3 and caspase-1 in GSDM processing using their respective inhibitors VX765 and DEVD. The decrease in appearance of caspase-3 active forms in the presence of DEVD [Figure 5.3] demonstrated that the caspase-3 inhibitor was still effective after viral infection. Then, immunoblot analysis revealed that DEVD diminished both GSDME cleavage and GSDMD p43 appearance [Figure 5.3]. DEVD also diminished the detection of GSDMD pore-forming fragment in cell supernatant [Figure 5.3], however this is likely due to the decrease of caspase-3-dependent cell death in the presence of the inhibitor, rather than a direct role of caspase-3 in GSDMD processing. Interestingly, VX765 didn't affect GSDM processing, particularly GSDMD p30 generation [Figure 5.3]. These results suggest that IAV infection triggers caspase-3-dependent GSDME activation and GSDMD inactivation but also moderates caspase-1-independent GSDMD pore formation.

5.2.3 Caspase-3 and caspase-8, but not caspase-1, trigger lytic cell death and IL-1 β secretion following IAV infection in NHBE cells

Then I assessed whether caspase-1 and caspase-3 were involved in lytic cell death following IAV infection. Measurement of LDH release in cell supernatant showed that caspase-3 but not caspase-1 mediated virus-stimulated lytic cell death [Figure 5.4]. Given that VSV infection triggered IL-1 α secretion in keratinocytes [194] and that I demonstrated that NHBE cells can release IL-1 and IL-6 cytokine upon cytosolic dsRNA sensing, I next examined whether IAV infection caused cytokine secretion. Figure 5.5 showed that NHBE cells secreted modest amounts of IL-1 β [Figure 5.5A] but didn't release IL-1 α nor IL-6 [Figure 5.5B, C]. Since respiratory epithelial cells also secrete IP-10 during IAV infection [15], I next measured the release of this cytokine. Figure 5.5 showed that NHBE cells released IP-10 in response to IAV infection [Figure 5.5D]. Then I examined the involvement of caspase-1 and caspase-3 in cytokine release. Interestingly, IL-1 β release was decreased in the presence of DEVD but not VX765 [Figure 5.5A], however IP-10 secretion was not decreased by DEVD nor VX765 [Figure 5.5D], indicating that caspase-3, but not caspase-1, is required for the secretion of IL-1 β during IAV infection.

It has been established that IL-1 β is secreted from cells following caspase-1-dependent maturation of pro-IL-1 β [197]. However caspase-8 can also contribute to IL-1 β release by processing pro-IL-1 β into IL-1 β at the same site as caspase-1 [260]. In addition, similar to caspase-1, caspase-8 can cleave GSDMD into a pore-forming fragment [212, 213]. Thereby I examined if caspase-8 contributed to lytic cell death and cytokine secretion using the caspase-8 inhibitor IETD. Importantly, IETD diminished the release of LDH and IL-1 β , but not IP-10, in the cell supernatants [Figure 5.4; Figure 5.5A, D]. Thus, caspase-8 rather than caspase-1 is required for lytic cell death and IL-1 β secretion during IAV infection in NHBE cells.

5.2.4 GSDM cleavage is independent of ASC, NLRP1 and MAVS following IAV infection in NHBE cells

Since RIG-I is known to respond to IAV RNA in some cells, I next determined if MAVS was involved in IAV-mediated GSDM processing. In addition, although I demonstrated that caspase-1 was not involved in GSDM cleavage, lytic cell death nor IL-1 β secretion in IAV-infected NHBE cells, I assessed whether NLRP1 or ASC were involved in IAV-mediated GSDM cleavage in epithelial cells. Thereby I downregulated MAVS, ASC and

NLRP1 expression using their respective siRNAs. However none of these siRNAs affected IAV-stimulated GSDM nor caspase-3 cleavage [Figure 5.6] suggesting that GSDM processing is independent of MAVS, ASC and NLRP1 in basal respiratory epithelial cells during IAV infection.

5.2.5 Lytic cell death and IL-1 β secretion are independent of ASC, NLRP1, MAVS and TRIF following IAV infection in NHBE cells

Then I further characterised the involvement of MAVS, ASC and NLRP1 in lytic cell death and cytokine secretion. Since I demonstrated that TRIF was involved in the dsRNA-stimulated response in epithelial cells, I also looked at the contribution of TRIF in IAV-mediated responses. Neither MAVS, ASC, NLRP1 nor TRIF siRNA impacted LDH release nor IL-1 β secretion [Figure 5.7A, B]. These results show that IAV infection triggers inflammasome-, TRIF- and MAVS-independent cell lysis and IL-1 β release in NHBE cells. Nevertheless I then measured the release of IP-10 in the presence of these siRNA since our data showed that NLRP1 can have an ASC-independent role in protein expression and secretion during dsRNA sensing. Also MAVS signaling is important for IP-10 secretion during viral infection [261]. Figure 5.7C showed that ASC siRNA didn't affect IP-10 release whereas NLRP1 siRNA diminished this secretion in a non-significant manner [Figure 5.7C], suggesting that NLRP1 might be involved in this process. In addition IP-10 release was enhanced in the presence of TRIF siRNA but was almost significantly decreased when MAVS expression was suppressed [Figure 5.7C], indicating that TRIF inhibits IAV-mediated IP-10 release whereas MAVS is likely required for this secretion.

5.2.6 PKR is required for GSDME activation, GSDMD inactivation, lytic cell death and cytokine secretion following IAV infection in NHBE cells

Given that VSV-mediated translation inhibition leads to caspase-3-mediated GSDME cleavage in keratinocytes [194] and that I demonstrated in the previous chapter that PKR triggered caspase-3-dependent GSDME pore formation following dsRNA sensing in NHBE cells, I examined whether PKR was required for IAV-mediated GSDM cleavage. Interestingly, addition of C16, the PKR inhibitor, prevented the generation of the GSDME pore-forming fragment, the GSDMD p43 fragment and the active p17 and p12 forms of caspase-3 [Figure 5.8], indicating that PKR leads to caspase-3-mediated GSDME activation and GSDMD inactivation during IAV infection. Furthermore, C16 abrogated the release of

LDH, IL-1 β and IP-10 in IAV-infected NHBE cells [Figure 5.9; Figure 5.10A, B], demonstrating PKR triggers lytic cell death and cytokine secretion following IAV infection. Overall these results illustrate that PKR is required for GSDME activation and GSDMD inactivation in IAV-infected NHBE cells.

5.2.7 GSDM pores contribute to lytic cell death, IL-1 β secretion and constrain viral replication following IAV infection in NHBE cells

Our results showed that IAV induced robust generation of the GSDME pore-forming fragment and modest GSDMD activation [Figure 5.2]. To distinguish the contribution of GSDME pores *versus* GSDMD pores in lytic cell death and cytokine secretion, I downregulated their expression using siRNA. While LDH release was only decreased upon GSDME downregulation [Figure 5.11], silencing of either GSDME or GSDMD diminished the secretion of IL-1 β [Figure 5.12A]. However IP-10 release wasn't significantly impacted by either siRNA treatment [Figure 5.12B]. Together these results demonstrate that even though only GSDME is required for virus-mediated lytic cell death, both GSDME and GSDMD pores are important for IL-1 β secretion in IAV-infected NHBE cells.

Lastly, to assess whether GSDM pores had an intrinsic antiviral function in respiratory epithelial cells, I infected NHBE cells at a low MOI and assessed the replication kinetics in the presence of control or GSDM siRNA. While viral titres were not impacted by GSDM downregulation at 24 hpi, viral replication was significantly lower at 48 hpi in NHBE cells treated with control siRNA compared to cells silenced for GSDMD or GSDME [Figure 5.13]. Thereby, GSDM pores contribute to constrain IAV replication in basal respiratory epithelial cells.

5.2.8 Potential contribution of PKR in GSDME cleavage following RSV infection in NHBE cells

So far our data showed that PKR is required for caspase-3-mediated GSDME activation and GSDMD inactivation following IAV infection. To determine whether other respiratory viruses could activate this PKR-caspase-3-GSDME axis, I infected NHBE cells with RSV. Replication kinetics showed an increase of the viral titre up to 24 hpi followed by a decrease over time [Figure 5.14]. This indicates that NHBE cells are permissive to

productive RSV infection. The decrease in viral titre after 24 h was probably due to cell death induced by viral infection which limited the number of cells available for the virus to replicate. Immunoblot analysis showed that RSV infection resulted in the appearance of GSDME pore-forming fragment, GSDMD inactivated p43 fragment and caspase-3 active forms at 30 hpi [**Figure 5.15**]. Treatment with PKR inhibitor mostly decreased GSDME cleavage in the cell supernatant and caspase-3 activation [**Figure 5.15**]. Even though C16 diminished GSDMD cleavage into a p43 fragment in the cell supernatant, this fragment was more detectable in the cell lysate compared to DMSO [**Figure 5.15**] which makes it difficult to interpret if PKR is involved in GSDMD inactivation. However this suggests that C16 protects cells from virus-induced lysis. Together the results suggest that PKR could be required for GSDME and caspase-3 processing or for GSDM-independent lytic cell death.

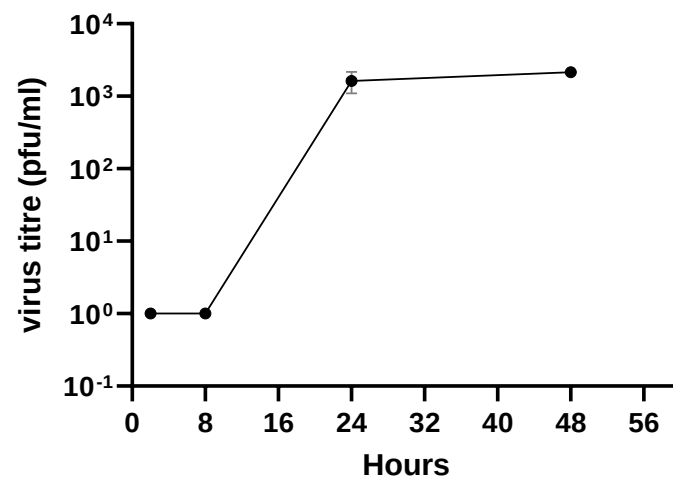


Figure 5.1: IAV replication kinetic in NHBE cells.

NHBE cells were infected with IAV at MOI 0.1 and supernatant was collected at 2 h, 8 h, 24 h and 48 h post infection (hpi). Infectious viral particles production was measured by plaque assay method. Data are mean $\pm$ SEM of two independent experiments.

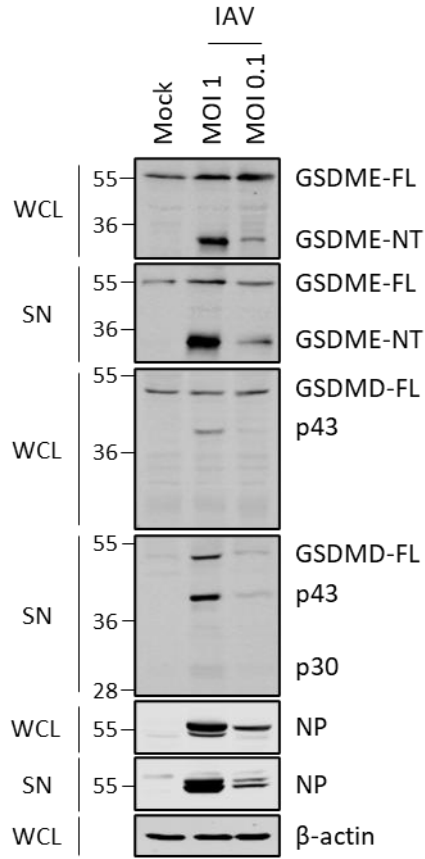


Figure 5.2: IAV infection leads to GSDME activation and GSDMD inactivation in NHBE cells.

NHBE cells were infected with IAV at MOI 1 for 24 h. Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of full length (FL), inactivated (p43) and activated (p30) GSDMD; full length (FL) and activated (p30) GSDME; viral nucleoprotein (NP) and β -actin. Immunoblots are representative of three independent experiments.

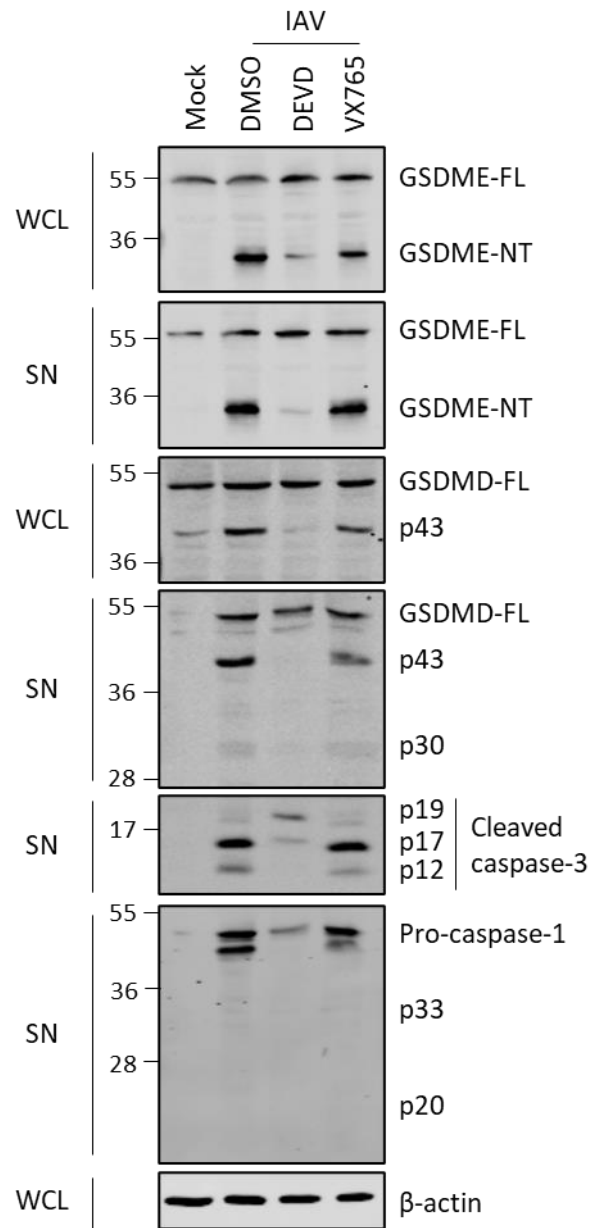


Figure 5.3: Caspase-3 mediates GSDME activation and GSDMD inactivation in response to IAV infection in NHBE cells.

NHBE cells were treated with DMSO, caspase-1 inhibitor (VX765) or caspase-3 inhibitor (DEVD, both 20 μ M) for one hour before IAV infection at MOI 1 for 24 h. Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of full length (FL), inactivated (p43) and activated (p30) GSDMD; full length (FL) and activated (p30) GSDME; pro-caspase-1, active caspase-1 (p33 and p20); cleaved caspase-3 (p19, p17 and p12) and β -actin. Immunoblots are representative of three independent experiments.

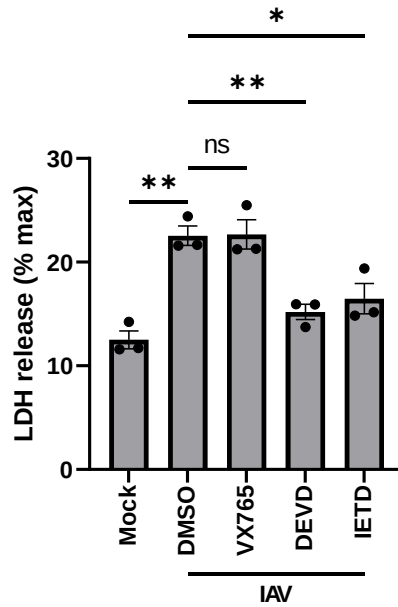


Figure 5.4: Caspase-3 and caspase-8, but not caspase-1, trigger lytic cell death in response to IAV infection in NHBE cells.

NHBE cells were treated with DMSO, caspase-1 inhibitor (VX765), caspase-3 inhibitor (DEVD) or caspase-8 inhibitor (IETD, all 20 μ M) for one hour before IAV infection at MOI 1 for 24 h. LDH present in the supernatant was quantified by LDH assay. Data are mean $\pm$ SEM of three independent experiments. ns: not significant, * $p < 0.05$ and ** $p < 0.01$ by one-way ANOVA.

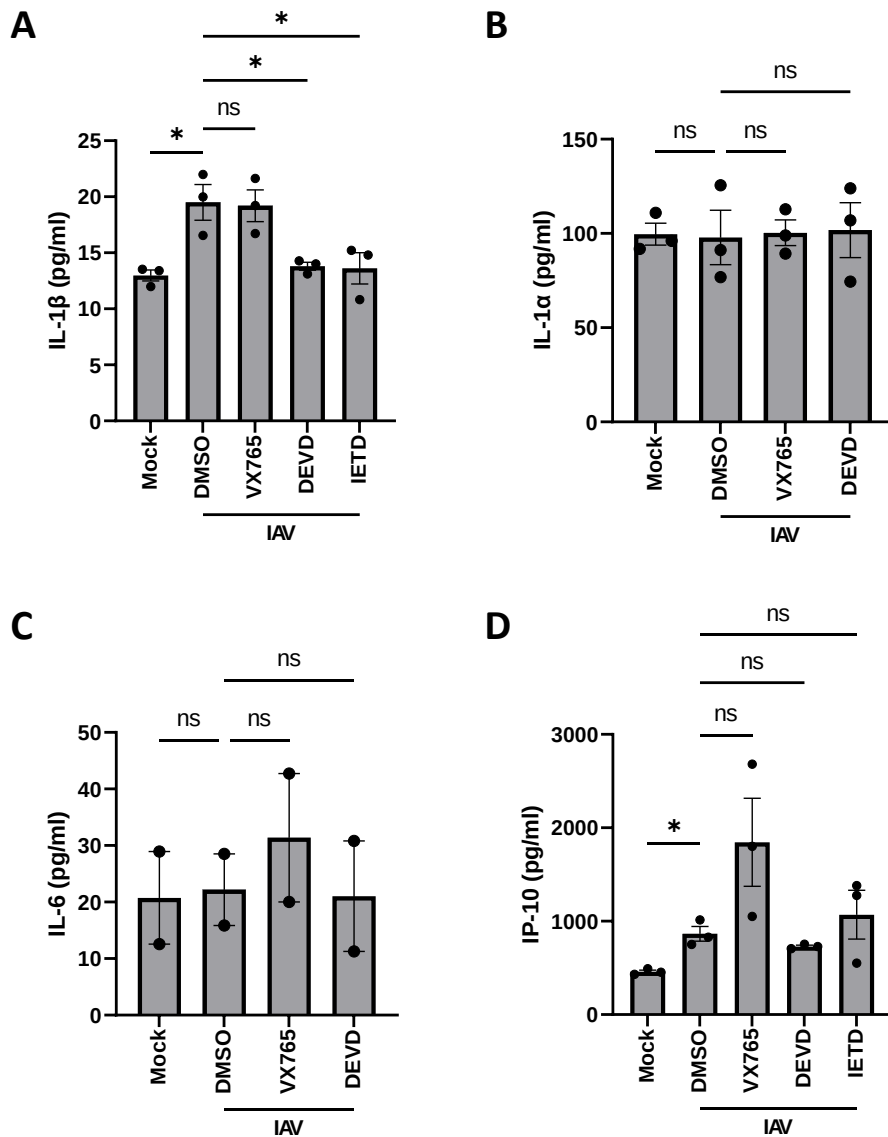


Figure 5.5: Caspase-3 and caspase-8, but not caspase-1, trigger IL-1 β secretion in response to IAV infection in NHBE cells.

NHBE cells were treated with DMSO, caspase-1 inhibitor (VX765), caspase-3 inhibitor (DEVD) or caspase-8 inhibitor (IETD, all 20 μ M) for one hour before IAV infection at MOI 1 for 24 h. IL-1 β (A), IL-1 α (B), IL-6 (C) and IP-10 (D) secretion was measured by ELISA. Data are mean $\pm$ SEM of three independent experiments. ns: not significant and *p < 0.05 by one-way ANOVA.

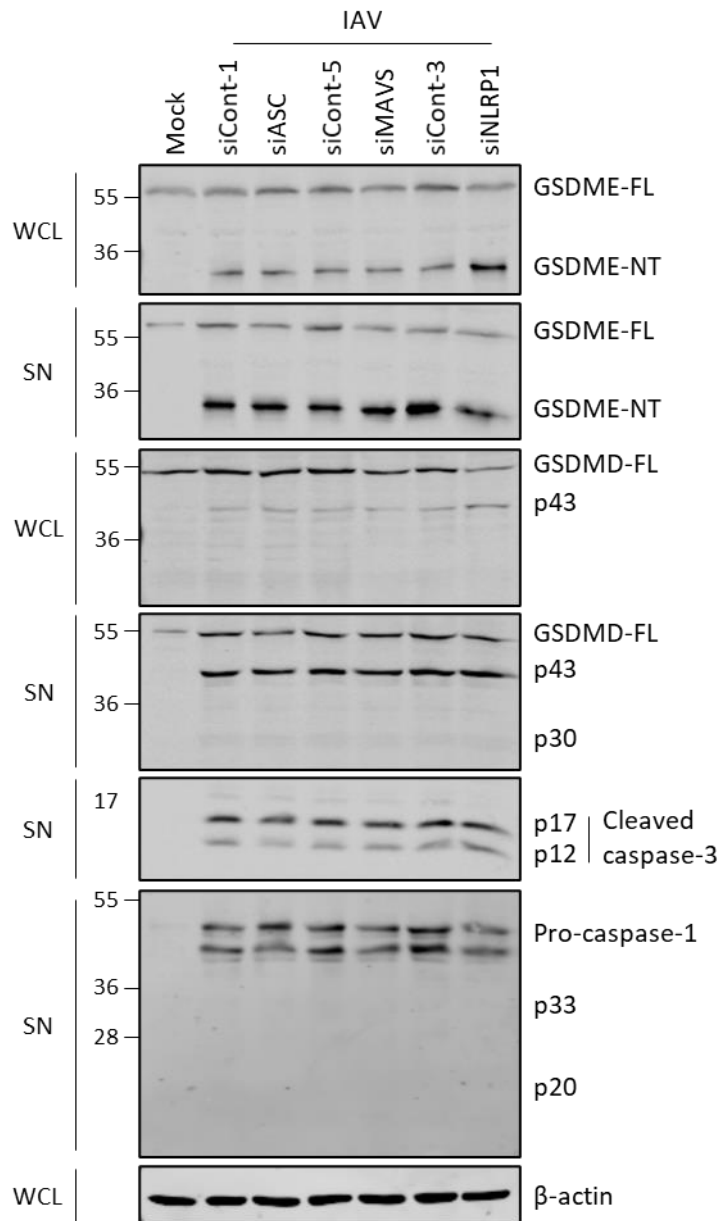


Figure 5.6: GSDM cleavage is independent of ASC, NLRP1 and MAVS in response to IAV infection in NHBE cells.

NHBE cells were transfected with siRNA targeting ASC (siASC, 1 nM), MAVS (siMAVS, 5 nM), NLRP1 (siNLRP1, 3 nM) or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were infected with IAV at MOI 1 for 24 h. Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of full length (FL), inactivated (p43) and activated (p30) GSDMD; full length (FL) and activated (p30) GSDME; pro-caspase-1, active caspase-1 (p33 and p20); cleaved caspase-3 (p17 and p12) and β -actin. Immunoblots are representative of three independent experiments.

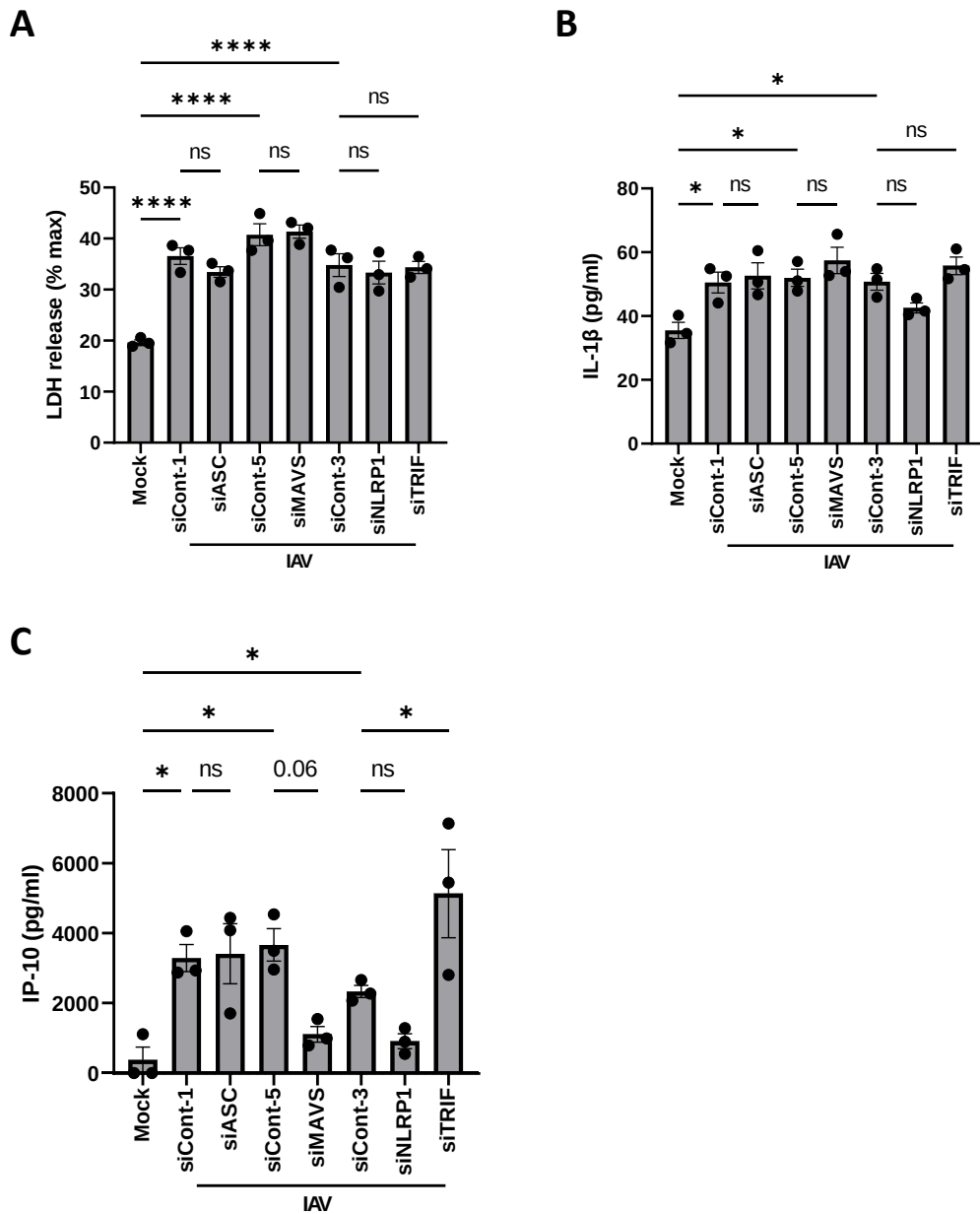


Figure 5.7: Lytic cell death and IL-1 β secretion are independent of ASC, NLRP1, MAVS and TRIF in response to IAV infection in NHBE cells.

NHBE cells were transfected with siRNA targeting ASC (siASC, 1 nM), MAVS (siMAVS, 5 nM), NLRP1 (siNLRP1, 3 nM) or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were infected with IAV at MOI 1 for 24 h. (A) LDH present in the supernatant was quantified by LDH assay.

(B, C) IL-1 β (B) and IP-10 (C) secretion was measured by ELISA.

Data are mean $\pm$ SEM of three independent experiments. ns: not significant, * $p < 0.05$ and **** $p < 0.0001$ by one-way ANOVA.

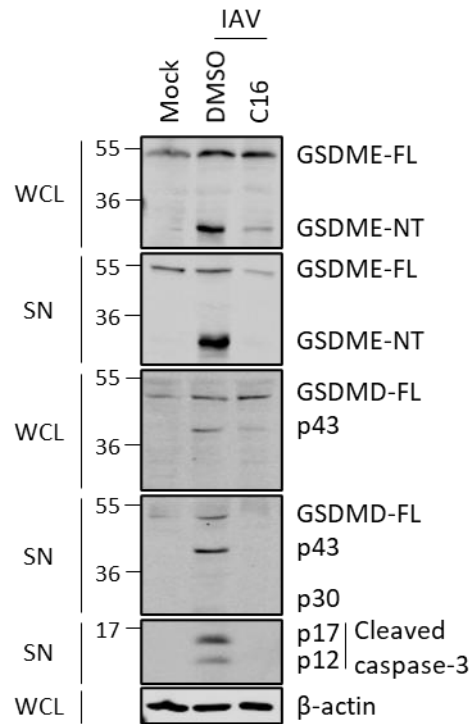


Figure 5.8: PKR is required for GSDME pore formation, GSDMD inactivation and caspase-3 activation in response to IAV infection in NHBE cells.

NHBE cells were treated with DMSO or PKR inhibitor (C16, 2 μ M) for one hour before IAV infection at MOI 1 for 24 h. Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of full length (FL), inactivated (p43) and activated (p30) GSDMD; full length (FL) and activated (p30) GSDME; cleaved caspase-3 (p17 and p12) and β -actin. Immunoblots are representative of three independent experiments.

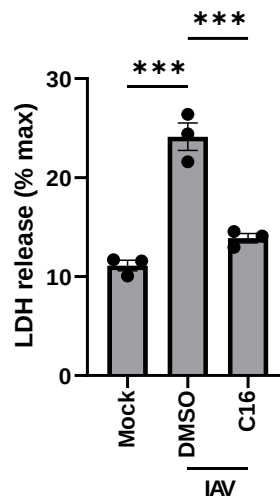


Figure 5.9: PKR is required for lytic cell death in response to IAV infection in NHBE cells.

NHBE cells were treated with DMSO or PKR inhibitor (C16, 2 μ M) for one hour before IAV infection at MOI 1 for 24 h. LDH present in the supernatant was quantified by LDH assay. Data are mean $\pm$ SEM of three independent experiments. *** $p < 0.001$ by one-way ANOVA.

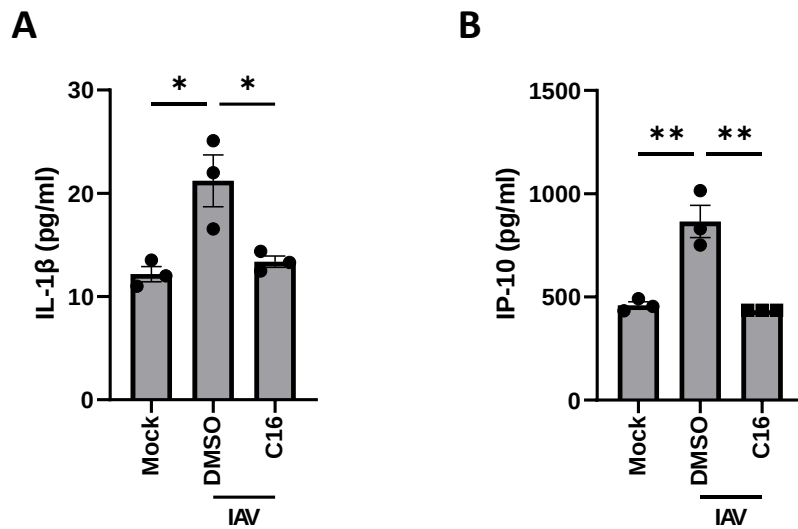


Figure 5.10: PKR is required for cytokine secretion in response to IAV infection in NHBE cells.

NHBE cells were treated with DMSO or PKR inhibitor (C16, 2 μ M) for one hour before IAV infection at MOI 1 for 24 h. IL-1 β (A) and IP-10 (B) secretion was measured by ELISA. Data are mean $\pm$ SEM of three independent experiments. *p < 0.05 and **p < 0.01 by one-way ANOVA.

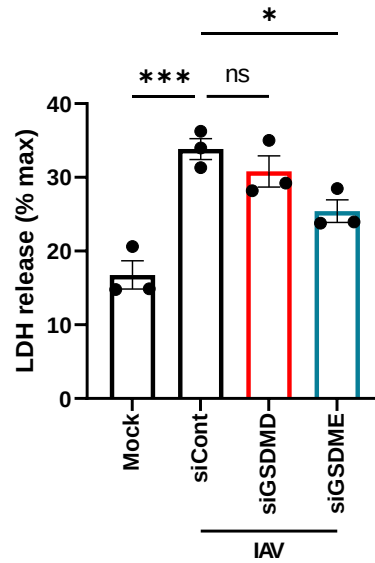


Figure 5.11: GSDME pores contribute to lytic cell death in response to IAV infection in NHBE cells.

NHBE cells were transfected with siRNA targeting GSDMD (siGSDMD) or GSDME (siGSDME), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were infected with IAV at MOI 1 for 24 h. LDH present in the supernatant was quantified by LDH assay. Data are mean $\pm$ SEM of three independent experiments. ns: not significant, * $p < 0.05$ and *** $p < 0.001$ by one-way ANOVA.

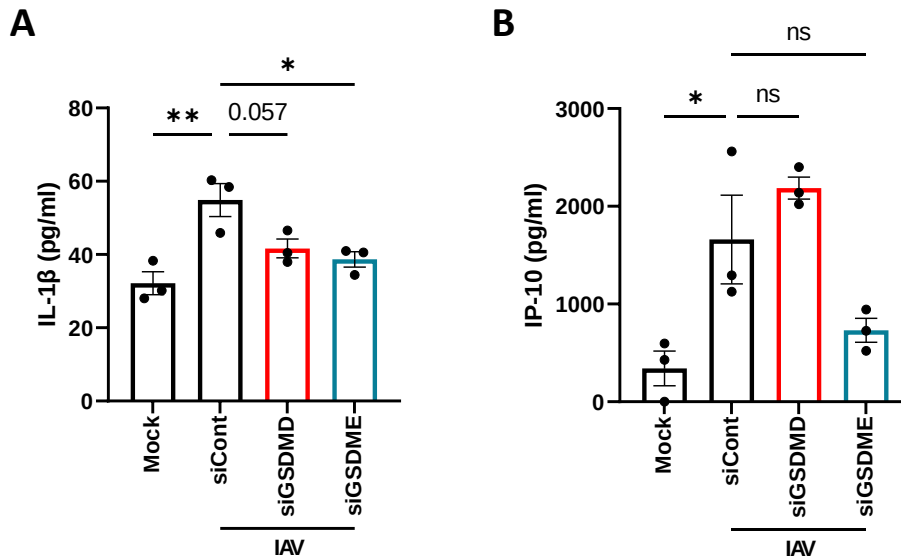


Figure 5.12: GSDM pores contribute to IL-1 β secretion in response to IAV infection in NHBE cells.

NHBE cells were transfected with siRNA targeting GSDMD (siGSDMD) or GSDME (siGSDME), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were infected with IAV at MOI 1 for 24 h. IL-1 β (A) and IP-10 (B) secretion was measured by ELISA. Data are mean $\pm$ SEM of three independent experiments. ns: not significant, * p < 0.05 and ** p < 0.01 by one-way ANOVA.

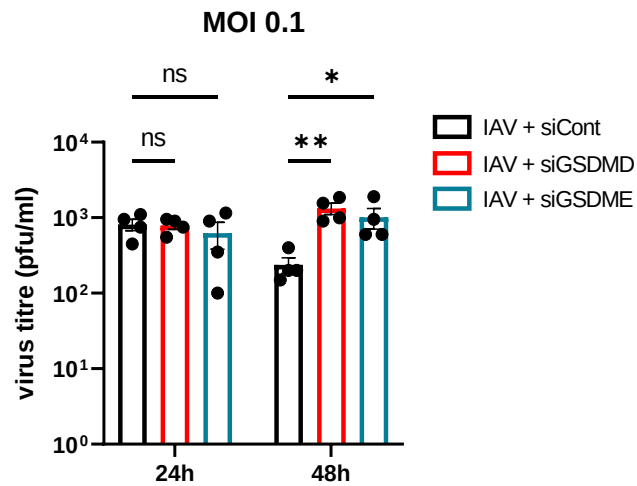


Figure 5.13: GSDM pores constrain IAV replication in NHBE cells.

NHBE cells were transfected with siRNA targeting GSDMD (siGSDMD) or GSDME (siGSDME), or control siRNA (siCont) (3 nM) at 24 h and 48 h post-seeding of cells. The following day, NHBE cells were infected with IAV at MOI 0.1 and supernatant was collected at 24 and 48 hpi. Infectious viral particles production was measured by plaque assay method. Data are mean $\pm$ SEM of four independent experiments. ns: not significant, * $p < 0.05$ and ** $p < 0.01$ by two-way ANOVA.

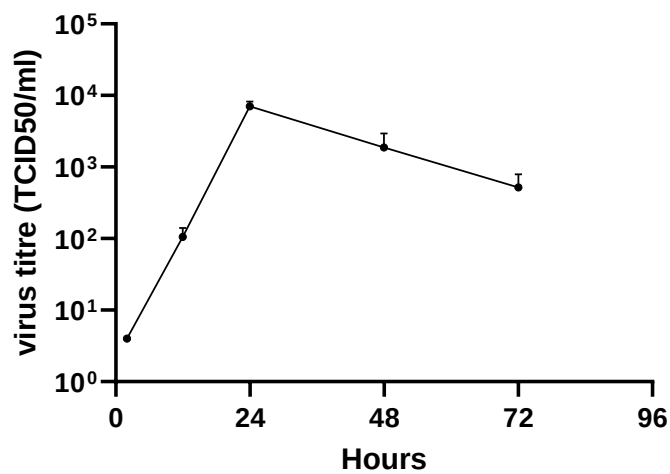


Figure 5.14: RSV replication kinetic in NHBE cells.

NHBE cells were infected with RSV A2 at MOI 1 and supernatant was collected at 2 h, 12 h, 24 h, 48 h and 72 hpi. Infectious viral particles production was measured by TCID50 method. Data are mean $\pm$ SEM of two independent experiments.

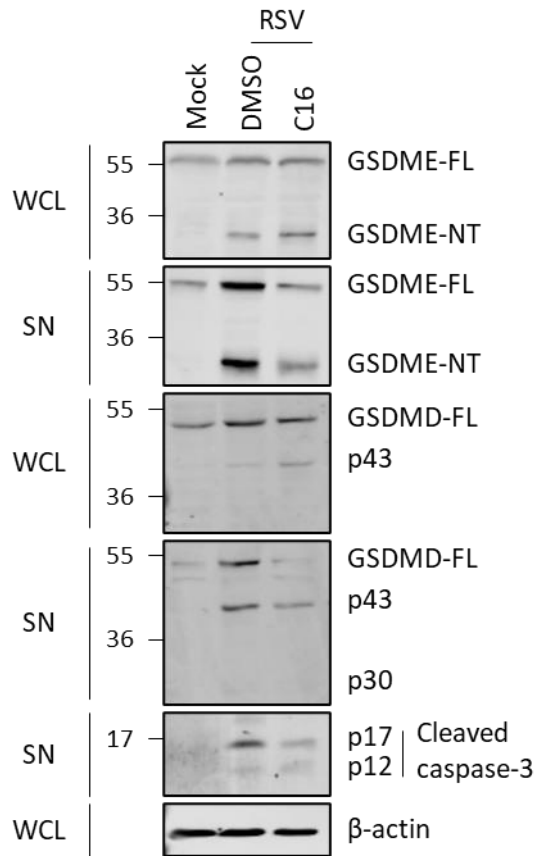


Figure 5.15: Potential contribution of PKR in GSDME cleavage following RSV infection in NHBE cells.

NHBE cells were treated with DMSO or PKR inhibitor (C16, 2 μ M) for one hour before RSV infection at MOI 10 for 30 h. Cell lysates (WCL) and supernatants (SN) were immunoblotted for the expression of full length (FL), inactivated (p43) and activated (p30) GSDMD; full length (FL) and activated (p30) GSDME; cleaved caspase-3 (p17 and p12) and β -actin. Immunoblots are representative of two independent experiments.

5.3 Discussion

In this chapter our results showed that IAV infection activated PKR that mediated caspase-3-dependent GSDME pore formation and GSDMD inactivation [Figure 5.16]. Interestingly IAV infection also triggered modest GSDMD cleavage into a pore-forming fragment that was independent of inflammasome and caspase-1 [Figure 5.16]. Additionally caspase-8 but not caspase-1 was required for lytic cell death and secretion of IL-1 β suggesting that caspase-8 could lead to GSDMD pore formation [Figure 5.16]. Importantly infection with another respiratory virus, namely RSV, showed that GSDME pore formation is a predominant hallmark in human basal respiratory epithelial cells.

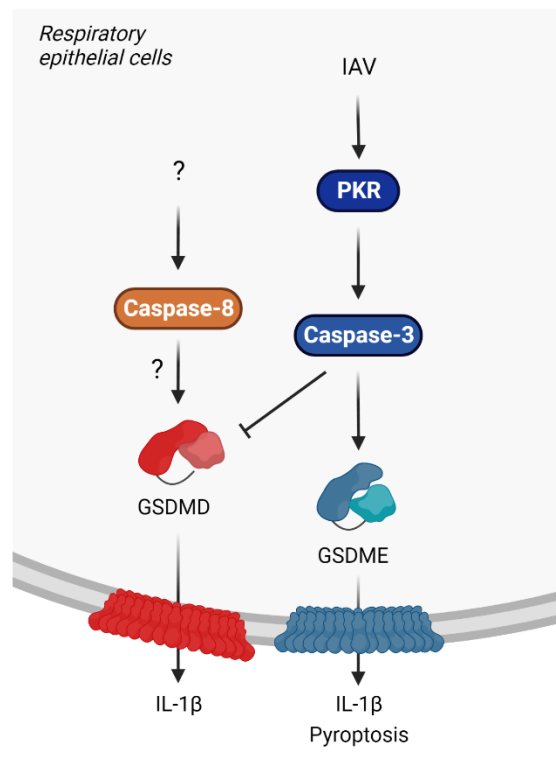


Figure 5.16: IAV infection mainly results in GSDME pore formation in NHBE cells.

IAV infection triggers strong GSDME pore formation and GSDMD inactivation, and modest GSDMD cleavage into a pore-forming fragment. Mechanistically, PKR is activated during IAV infection which results in caspase-3-dependent GSDME cleavage. Interestingly, GSDMD pore formation doesn't require inflammasome activation nor caspase-1. Since caspase-8 is involved in lytic cell death and IL-1 β secretion, caspase-8 could also cleave GSDMD into an active p30 fragment. However the activating signal upstream of caspase-8 and the relation between caspase-8 and PKR and caspase-3 are still unclear. While both GSDMD and GSDME pores contribute to IL-1 β release, only GSDME is required for lytic cell death in basal respiratory epithelial cells.

5.3.1 IAV and RSV infection triggers GSDME activation and GSDMD inactivation in NHBE cells

Both IAV WSN and RSV A2 infection led to strong GSDME pore formation and GSDMD inactivation and that IAV infection at MOI 1 also triggered modest GSDMD activation. IAV and RSV are both negative-strand RNA viruses but while IAV replicates in the nucleus, RSV replicates in the cytoplasm [3, 8]. This suggests that GSDME cleavage is a general predominant outcome during RNA respiratory virus infection in epithelial cells. This is in line with previous reports showing that another IAV strain, IAV H7N9, generated GSDME pores in lung alveolar epithelial cells [252], that SARS-CoV-2 infection induced GSDME cleavage in lung epithelial cells [145] and that VSV infection resulted in GSDME pore formation in keratinocytes [194]. In addition, IAV PR8 strain mediates GSDME activation and GSDMD inactivation in mouse BMDMs but, unlike IAV WSN, IAV PR8 also triggers strong GSDMD p30 cleavage [253]. Thereby GSDME *versus* GSDMD pore formation could be another difference between barrier epithelial cells and myeloid cells.

5.3.2 Activation of caspase-3 and caspase-8 during IAV infection in NHBE cells

Our results showed that IAV infection strongly activated caspase-3 but barely caspase-1. Using caspase inhibitors, I demonstrated that caspase-3 is involved in GSDME activation, GSDMD inactivation, lytic cell death and IL-1 β secretion during IAV infection in bronchial epithelial cells. Similar outcomes have been described during VSV infection in keratinocytes, even though IL-1 α was secreted instead of IL-1 β [194]. Interestingly our data also revealed that caspase-1 was not required for GSDMD pore-forming fragment, lytic cell death nor IL-1 β secretion during IAV infection. Although these results were puzzling at first, reports showing that caspase-8 cleaved GSDMD into an active fragment and pro-IL-1 β into its mature form at the same residues as caspase-1 during bacterial infection [212, 213, 260], provided a possible explanation. Here I confirmed that caspase-8 was necessary for IL-1 β secretion and lytic cell death following IAV infection. Given that caspase-8 is upstream of caspase-3 activation [229] one hypothesis is that caspase-8 mediates caspase-3 activation during IAV infection, as previously shown [253], in addition to GSDMD and pro-IL-1 β processing. In line with this, the detection of Zika virus genome by RIG-I in the cytoplasm of infected cells triggers the extrinsic mitochondrial pathway that activates caspase-8 which then leads to caspase-3-mediated GSDME pore formation [222]. Here I showed that MAVS-dependent signaling was not involved in GSDM cleavage but that PKR had a major role in this process. Importantly PKR can also trigger caspase-8 activation [61], thereby it is possible

that PKR is upstream of both caspase-8 and caspase-3 during IAV infection. Another explanation on how caspase-3 might be activated downstream of PKR, is that translation inhibition mediated by PKR could enhance caspase-3 activation. For instance, VSV infection suppresses protein synthesis which results in caspase-3-dependent GSDME pore formation [194]. However this mechanism was independent of caspase-8 whereas our data showed that caspase-8 triggered lytic cell death and IL-1 β secretion during IAV infection. Nevertheless it is possible that these two mechanisms coexist in the cells; PKR could suppress protein synthesis but also activate caspase-8 independently of translation inhibition, and both pathways would lead to caspase-3 activation.

5.3.3 IAV infection does not trigger inflammasome nor MAVS-dependent GSDM processing in NHBE cells

Our results demonstrated that MAVS-dependent signaling was not involved in GSDM cleavage following IAV infection in NHBE cells. However, the involvement of RLRs in GSDM cleavage shouldn't be totally excluded since RIG-I and MDA5 can remove IAV NS1 proteins bound to dsRNA via their ATPase activity which allows PKR to bind to dsRNA to promote translation inhibition [249]. Thereby RLRs could have an indirect role in this pathway in a MAVS-independent manner.

IAV infection can trigger NLRP3 inflammasome in myeloid cells [124, 125] and the IFN-dependent MxA inflammasome in respiratory epithelial cells [167]. Moreover NLRP1 inflammasome can sense Semliki Forest virus and SARS-CoV-2 infection in barrier epithelial cells [145, 153]. However our data showed that IAV didn't require an ASC-dependent inflammasome nor specifically an NLRP1 inflammasome for GSDM cleavage in NHBE cells. Several explanations and hypotheses can be ventured to explain these differences. Firstly, SARS-CoV-2 triggers NLRP1 inflammasome in differentiated NHBE cells [145] while I used non-differentiated NHBE cells in this study. Secondly, while Semliki Forest virus and SARS-CoV-2 virus are both positive-strand viruses that replicate into the cytoplasm, IAV replication occurs in the nucleus of infected cells. Thereby the nuclear membrane prevents the replicative intermediates to be released in the cytoplasm and thus to be detected by NLRP1. Finally, IAV NS1 is a potent antagonist of the host antiviral response [262] so viral NS1, or another viral protein, could counteract the activation of the NLRP1 inflammasome.

I also demonstrated that TRIF siRNA didn't impact lytic cell death nor IL-1 β secretion in IAV-infected NHBE cells. Since GSDM pores mediate cell lysis and cytokine release in NHBE cells, this suggests that TRIF is probably not required for GSDM processing during IAV infection in basal respiratory epithelial cells. Further investigation is needed to confirm this hypothesis.

5.3.4 PKR is required for GSDM cleavage in response to IAV infection in NHBE cells

Our results showed that IAV-mediated GSDME activation, GSDMD inactivation, lytic cell death and IL-1 β secretion were almost completely PKR-dependent, indicating a predominant role for PKR in bronchial epithelial cells during IAV infection. In addition our data suggested that PKR was involved in lytic cell death and potentially in GSDME cleavage during RSV infection. In Chapter 4, C16 prevented GSDME pore-forming fragment appearance in both the cell lysate and the cell supernatant at 6 h post dsRNA transfection whereas this decrease was only observed in the cell supernatant at 16 h. Thereby C16 could have a greater effect on RSV-mediated GSDME processing at an early time of infection. Thus a time course of GSDM cleavage would be useful to determine the contribution of PKR during RSV infection. Nevertheless, this indicates that PKR has a central function in sensing respiratory viral infection in lung epithelial cells. Interestingly, GSDME pore-forming fragment and GSDMD p43 fragment were not completely abrogated in the presence of PKR inhibitor in RSV-infected cells, suggesting that another pathway independent of PKR could lead to GSDM cleavage during RSV infection. Activation of PKR in epithelial cells during RSV infection has been previously demonstrated by an increase of PKR phosphorylation, a marker of activation, during infection [263]. Furthermore PKR has also an important role during IAV-mediated responses since IAV NS1 binds to PKR to prevent its activation and further downstream signaling [63, 264]. Thereby the fact that PKR strongly induced GSDME pores during IAV infection in NHBE cells was intriguing. PKR is an ISG whose expression is enhanced following IFN secretion, however I showed in the previous chapter that PKR was basally expressed in NHBE cells, thereby PKR could sense IAV infection before being targeted by viral NS1. However the mechanism of PKR activation following IAV and RSV infection is still unclear. Since IAV and RSV are both negative-strand RNA virus, replicative intermediates shouldn't be produced in sufficient amount to trigger PKR activation [265]. In addition IAV replicates in the nucleus, thereby viral dsRNA should be contained within the nucleus and not be released in the cytoplasm of the infected cells. Therefore it is possible that IAV and RSV infection causes cellular stress that releases

nuclear and mitochondrial dsRNA in the cytoplasm that is then recognised by PKR [62]. The release of nucleic acid from mitochondria has already been illustrated during IAV infection where mtDNA translocates into the cytoplasm of infected cells [21]. Additionally, PKR can also localise in the nucleus [266], so nuclear PKR might detect IAV replication. Since C16 had a greater effect on IAV-stimulated GSDM processing than on RSV-mediated GSDM cleavage, and that IAV and RSV replicate in different compartments, it is possible that cytosolic PKR is activated through cellular stress by both viruses but that in top of this, nuclear PKR also detects IAV replication but not RSV.

5.3.5 GSDM pores contribute to lytic cell death, IL-1 β secretion and constrain viral replication during IAV infection in NHBE cells

Our results indicated that while GSDME was required for lytic cell death, both GSDMD and GSDME were required for IL-1 β secretion during IAV infection. GSDMD pores are essential for IL-1 β secretion and pyroptotic cell death [174, 175] but the role of GSDME in cytokine secretion and lytic cell death, especially in the context of viral infection, has only been recently investigated. VSV infection induces GSDME-dependent pyroptotic cell death and IL-1 α secretion in keratinocytes [194]. In addition, GSDME pores can also secrete IL-1 β in macrophages [223]. The fact that GSDMD pores induce cytokine secretion but not lytic cell death during IAV infection is in accordance with previous report showing that GSDMD can secrete cytokines from living cells [182].

In addition, replication kinetic experiments showed that both GSDMD and GSDME pores were required to constrain IAV replication in NHBE cells. Consistent with this, NLRP9B inflammasome triggers GSDMD-mediated pyroptosis in mouse intestine that restricts rotavirus replication [254]. Furthermore inhibition of mitochondrial disruption decreases caspase-3-dependent GSDME pore formation that promotes VSV replication in human skin organoids, which illustrates an indirect role of GSDME-dependent pyroptosis in limiting viral replication [194]. Nevertheless GSDM pore formation has also be implicated in pathogenesis during viral infection. During IAV H7N9 infection a cytokine storm is induced that is dependent on GSDME-mediated pyroptosis in mouse alveolar epithelial cells [252]. Moreover, GSDME-mediated pyroptosis is associated with placental damages in mice infected with Zika virus [222]. Additionally, inflammasome-mediated responses can be protective for the host at an early stage of IAV infection but be harmful at a later stage [128].

Thereby, it is possible that besides constraining viral replication, GSDM pore formation might also contribute to pathogenesis through the release of pro-inflammatory cytokines.

Overall in this chapter I showed that IAV infection resulted in PKR-mediated caspase-3-dependent GSDME pore formation and GSDMD inactivation, but also to modest NLRP1- and caspase-1-independent GSDMD activation. IAV-stimulated lytic cell death and IL-1 β secretion required caspase-3- and caspase-8, but not caspase-1. Importantly, while GSDME pores were necessary for lytic cell death, both GSDMD and GSDME were important for cytokine secretion but also for constraining viral replication. Preliminary results with RSV strengthened the case that PKR-caspase-3-GSDME axis is a predominant sensing pathway following RNA virus infection in basal respiratory epithelial cells.

Chapter 6. Final discussion and future directions

6.1 Final discussion

Inflammasome activation and GSDMD pore formation have been well studied in macrophages but the role of GSDM pores, especially GSDME pores, in epithelial cells was unclear. Therefore this project aimed to investigate the mechanisms of GSDM pore formation in response to viral sensing in human respiratory epithelial cells. This study showed that both intracellular dsRNA delivery to, and respiratory virus infection of, basal respiratory epithelial cells triggered the generation of the GSDME pore-forming fragment and a GSDMD inactivated fragment. Sensing of dsRNA also caused GSDMD pore formation unlike RSV, whereas IAV only engendered slight GSDMD p30 cleavage. These results are consistent with the current literature that shows that GSDME is often activated in response to viral infection of keratinocytes and respiratory epithelial cells [145, 194]. Importantly, the kinetics of GSDM cleavage following dsRNA sensing were of interest, since GSDMD was first activated in a caspase-1-dependent manner and then inactivated in a caspase-3-dependent manner. Additionally PI uptake revealed that GSDMA also formed pores at the plasma membrane during dsRNA sensing. This illustrates the complexity of innate immune responses that needs to be considered when looking at GSDM cleavage, showing that multiple GSDMs can be cleaved in parallel in response to a given stimulus. The cleavage of GSDMD into an active fragment was subtle during IAV infection and was not detectable during RSV infection but this has only been assessed at one specific time point. Hence kinetics experiments would be useful to determine whether GSDMD pores are predominant at a certain time of the infection and whether other GSDMs such as GSDMA are involved in lytic cell death and cytokine secretion in response to live virus. Since GSDMD is important in controlling IAV replication at 48 h, it is indeed possible that GSDMD forms pores at this time point.

Even though the role of GSDME pores in lytic cell death and IL-1 release has only been recently investigated, it is now incontestable that GSDME is essential for pyroptosis and cytokine secretion [145, 194, 204, 223], which is in accordance with our findings. Nevertheless different mechanisms of GSDME cleavage for different viruses and different cells have been illustrated. During SARS-CoV-2 infection NLRP1 inflammasome is required for caspase-3-dependent GSDME pores formation in lung epithelial cells [145]. However our study indicated that PKR was involved in GSDME cleavage but not NLRP1 inflammasome following dsRNA sensing and IAV infection. In addition NLRP1 and NLRP3 inflammasomes can release IL-1 β through GSDMD pores but also through GSDME in the absence of GSDMD in macrophages [223]. Here our data showed that in

NHBE cells NLRP3 inflammasome was not activated following dsRNA sensing. Instead, NLRP1 inflammasome was required for the generation of GSDMD active p30 fragment but not for GSDME pore formation. During IAV infection, GSDM cleavage was independent of ASC and NLRP1 in NHBE cells. Another study showed that in keratinocytes, VSV infection suppressed protein translation, causing mitochondrial damage which resulted in caspase-3-dependent GSDME cleavage [194]. Since PKR is involved in GSDME cleavage during dsRNA sensing and IAV infection in NHBE cells and that PKR activation caused suppression of protein synthesis [59], it is possible that a similar pathway is activated in basal respiratory epithelial cells.

Investigation of the mechanism of GSDME pore formation and GSDMD inactivation in basal respiratory epithelial cells demonstrated that both dsRNA sensing and IAV infection mediated PKR activation that resulted in caspase-3-dependent GSDME activation. In addition PKR inhibition almost completely abrogated GSDME cleavage, suggesting that a PKR-caspase-3-GSDME axis is an essential pathway to trigger an immune response against respiratory viral infection in epithelial cells. The involvement of PKR in innate immune sensing of IAV is consistent with the fact that IAV has evolved immune evasion strategies to target PKR such as NS1 [63, 264]. However the exact mechanism of PKR activation during viral infection of epithelial cells is still unclear. PKR is a sensor of intracellular dsRNA in the cytoplasm [59] but negative single-strand RNA viruses such as RSV and IAV don't produce large amounts of replication intermediates [265] suggesting that PKR may not be activated by viral dsRNA during IAV infection. PKR could be activated through sensing of endogenous nucleic acid that are mislocated during infection. For instance cellular stress can promote the release of mtRNA into the cytoplasm which then forms dsRNA structure that can be recognised by PKR [62]. Nevertheless according to the size of mtRNA, MDA5 can also interact with these long dsRNA molecules which triggers MAVS-dependent signaling [267] yet MAVS was not involved in GSDM processing during IAV infection. PKR can also interact with non-coding RNAs released from the nuclear genome during cellular stress [62]. It is possible that such release happens during IAV nuclear replication. Another explanation could be that PKR could sense IAV replication in the nucleus through detection of cellular stress, and interestingly PKR can localise in the nucleus [266]. Nevertheless I also showed that TRIF contributed to early GSDM processing, lytic cell death and cytokine secretion during dsRNA sensing. TRIF is the adaptor protein of the endosomal TLR3 receptor. TLR3 can also recognise endogenous long dsRNA leaking into the endosome which triggers apoptotic cell death [242, 245] and this TLR3-dependent

response can be elicited in parallel of dsRNA-mediated PKR activation [242]. Thereby TRIF could sense intracellular poly(I:C) alongside PKR and NLRP1 leading to GSDME cleavage in basal respiratory epithelial cells. TLR3 signaling is also important during RSV and IAV infection to trigger NF- κ B-dependent pro-inflammatory responses [268, 269]. Since PKR-dependent signaling wasn't predominant involved in GSDME cleavage during RSV infection and that TRIF triggered GSDME pore formation during dsRNA sensing, TRIF could also contribute to GSDME activation during RSV infection in basal respiratory epithelial cells but this was not tested here. In contrast, TRIF wasn't involved in IAV-mediated cell lysis and IL-1 β secretion in basal respiratory epithelial cells, suggesting that TRIF doesn't contribute to IAV-mediated GSDM processing.

Inflammasome formation leads to activation of caspase-1 that cleaves GSDMD in its linker region to release the N-terminal pore-forming fragment from the C-terminal repressor domain [174, 175]. dsRNA sensing triggered NLRP1 inflammasome-dependent GSDMD activation whereas neither NLRP1, ASC nor caspase-1 were involved in GSDMD pore formation during IAV infection. NLRP1 has recently been described as a cytosolic receptor that recognises intracellular long dsRNA [153] which is consistent with the results of this study. However IAV is a negative single-strand RNA virus that replicates in the nucleus of infected cells, thereby it is not expected that viral replication releases significant amount of viral dsRNA into the cytoplasm [265]. Given that IAV NS1 targets NLRP3 inflammasome to prevent its activation [270], it's possible that IAV protein restrains NLRP1 inflammasome formation by targeting a key step of this signaling. Once activated, caspase-1 also cleaves pro-IL-1 β into the mature IL-1 β [197]. Here, for IAV, caspase-8 but not caspase-1 was required for virus-induced lytic cell death and IL-1 β secretion. This is in line with previous report that demonstrated that caspase-8 directly processed pro-IL-1 β into its mature form at the same residues as caspase-1 [260]. Since caspase-8 has also been shown to cleave GSDMD into a pore-forming fragment [212, 213], caspase-8 could generate GSDMD p30 fragment during IAV infection. A study reported that IAV led to apoptosis at an early stage of infection in human bronchial epithelial cells but that the secretion of IFN- β then suppressed apoptosis and caused pyroptosis which released pro-inflammatory cytokines [198]. Thereby this suggests that IAV infection triggers apoptosis via caspase-8 that directly cleaves GSDMD into a pore-forming fragment but that in parallel caspase-8 also activates caspase-3 that further elicits GSDME pore formation and GSDMD inactivation. Since both GSDMD and GSDME pores constrain viral replication and that apoptosis favours IAV replication [271], it is tempting to speculate that GSDM activation is a host mechanism to counteract virus-

induced apoptotic cell death. In contrast to IAV infection, caspase-1 mediated GSDMD activation and IL-1 β secretion during dsRNA sensing. Caspase-8 was also involved in IL-1 β release and lytic cell death following dsRNA delivery but that is explained by the fact that caspase-8 contributed to caspase-3-dependent GSDME pore formation in dsRNA-stimulated NHBE cells. It will however be interesting to determine whether caspase-8 is also involved in GSDMD activation at early times following intracellular dsRNA delivery.

I demonstrated that intracellular delivery of dsRNA activated NLRP1 inflammasome. Also, stimulation of NHBE cells with VBP triggered IL-1 secretion and lytic cell death suggesting that NLRP1 and/or CARD8 inflammasome could be activated in basal respiratory epithelial cells. Nevertheless a role for CARD8 inflammasome in dsRNA sensing can be excluded for two reasons. Firstly, CARD8 inflammasome is composed of CARD8 receptor that directly interacts with the CARD domain of pro-caspase-1, bypassing the requirement of ASC for inflammasome assembly. However our data showed that GSDM cleavage, lytic cell death and cytokine secretion were ASC-dependent in dsRNA-stimulated NHBE cells. Secondly, although NLRP1 and CARD8 have similar C-terminal domains, CARD8 is missing the NACHT and LRR domains in N-terminal position that mediates dsRNA recognition in NLRP1 [153].

RLRs have an essential role in mounting an innate immune response against viral infection by triggering MAVS-mediated type I IFN and antiviral gene transcription [43]. For instance RIG-I and MDA5 sense viral RNA such as IAV RNA and synthetic dsRNA in the cytoplasm depending on their length, leading to MAVS-dependent signaling [3, 41]. However, not much is known about the specific role of RLRs in epithelial cells, and whether in those cells they are the initial primary detectors of a viral infection. Here I established that MAVS-dependent signaling was not involved in GSDM cleavage, cytokine secretion nor lytic cell death during dsRNA sensing and IAV infection in NHBE cells. IAV NS1 binds to dsRNA to suppress an immune response [272], but RIG-I can remove viral NS1 in a MAVS-independent manner allowing dsRNA recognition by PKR [249]. Thereby, even though MAVS was not required in GSDM cleavage by IAV, RIG-I could indirectly be involved in PKR-mediated GSDM cleavage in basal respiratory epithelial cells. Interestingly, for RSV-infected cells compared to IAV-infected cells, GSDME pore-forming fragment and GSDMD p43 fragment were not completely abrogated in the presence of the PKR inhibitor. This suggests that other pathways could lead to GSDM cleavage during RSV infection. RSV transcripts are sensed by RIG-I in the cytoplasm of infected cells [273] but RLRs expression was mostly upregulated at 16 h following dsRNA delivery in a PKR and NLRP1-dependent

manner. However RSV infection could promote IFN release which would enhance RIG-I expression and therefore a MAVS-dependent pathway might be involved in GSDM cleavage during RSV infection, but this remains to be tested.

Interestingly dsRNA sensing by NLRP1 and PKR also contributed to upregulation of expression of innate proteins such as pro-IL-1 β and RLRs [Figure 4.31, 4.37, 4.45], whereas TRIF didn't have any impact. This strengthens the importance of NLRP1 and PKR in viral sensing in epithelial cells. Although both NLRP1 and PKR weren't predominant required for type I IFN release, even a modest NLRP1- or PKR-dependent induction of type I IFN could be sufficient to induce RIG-I and MDA5 upregulation. Importantly, NLRP1 triggered late IL-1 α and IL-6 secretion in an ASC-independent manner upon dsRNA delivery [Figure 4.32 compared to Figure 4.17], while IP-10 secretion was PKR-dependent but caspase-3- and GSDME-independent in IAV-infected NHBE cells [Figure 5.10 compared to Figure 5.5 and 5.12]. This suggests that NLRP1 and PKR could also have a transcriptional activity during viral infection of epithelial cells.

This difference in responses between the cell lines I initially tested compared to the NHBE cells illustrates the need to use physiologically-relevant cellular models to reveal innate immune signaling pathways of importance. Difference in lytic cell death and cytokine secretion in response to various stimuli, especially transfected poly(I:C), were observed between immortalised and cancerous epithelial cell lines and primary epithelial cells. This is in line with a paper showing that IAV induced cell death in NHBE cells but not in A549 cells [198]. Also inflammasome receptor expression can be different between epithelial cell lines and primary epithelial cells. For instance, NLRP1 is expressed in NHBE cells but not in A549 cells [145]. One limitation to the use of primary cells is that because CRISPR knockout is more difficult to implement compared to cell lines, gene silencing was implemented using siRNA, which doesn't completely abrogate expression of the target gene. However one report showed that GSDMD pores but not GSDME pores were formed in wild-type dTHP-1 cells stimulated with nigericin or VBP whereas GSDME pores were detected in GSDMD^{-/-} dTHP-1 cells [223], indicating a possible compensatory mechanism between GSDM proteins. Thereby although siRNA knockdown is not as efficient as CRISPR knockout to suppress the expression of one protein, it might prevent compensatory outcome.

Most of our knowledge of inflammasome activation and GSDM pore formation comes from studies performed in myeloid cells and mouse models. Although extremely

useful, caution should be applied when transposing these findings to human epithelial cells. For example hNLRP1 responds to ultraviolet B radiation in human keratinocytes whereas mNLRP1B in mouse keratinocytes does not [193]. Moreover, hNLRP1 is a sensor of dsRNA but not mNLRP1B. Thereby the generation of humanised mice would be necessary to study the implication of such pathways *in vivo*. Alternatively epithelial cells can be cultured at the air-liquid interface to form pseudostratified epithelium that allows the formation of ciliated cells and the production of mucin [274]. This differentiated cellular model can be used to study the response of respiratory epithelial cells in a more physiological relevant environment as it recapitulates well results shown *in vivo* [274, 275]. Ultimately, the requirement of IAV for PKR, and lack of a role for NLRP1, would need to be also tested by infecting differentiated cells with IAV.

Overall, this study aimed to investigate the mechanisms of GSDM pore formation in response to viral sensing in human basal respiratory epithelial cells. The results indicated that while dsRNA sensing triggered NLRP1 inflammasome-dependent GSDMD pore formation, IAV infection led to GSDMD activation that was probably dependent on caspase-8. Moreover, both intracellular dsRNA and IAV activated PKR that further mediated caspase-3-dependent GSDME pore formation and GSDMD inactivation. Importantly MAVS-dependent signaling was not necessary for GSDM cleavage during dsRNA sensing and IAV infection. This demonstrates a central role of NLRP1 and PKR receptors in viral sensing in human basal respiratory epithelial cells.

6.2 Future directions

Several key findings have emerged from this study. NLRP1 and PKR are the predominant pathways leading to GSDM pore formation in response to dsRNA sensing, while PKR-caspase-3-GSDME axis is a predominant pathway following dsRNA sensing and viral infection in basal respiratory epithelial cells. In addition NLRP1 and PKR are important sensors for cytosolic dsRNA unlike RLRs in basal respiratory epithelial cells.

To investigate the contribution of PKR in GSDM pore formation, I took advantage of the widely used PKR inhibitor named C16 that specifically prevents PKR-eIF2 α signaling pathway without known off-targets [276, 277]. However there is a lack of in depth *in vivo* studies [276] to completely rule out the absence of off-target effects. Recently a computational analysis showed that C16 could also inhibit the fibroblast growth factor

receptor 2 (FGFR2) kinase [278]. Thereby it will be of interest to repeat key experiments with PKR siRNA to confirm that PKR is essential for GSDME pores formation in basal respiratory epithelial cells.

Further investigation is needed to clarify the mechanism of GSDM pore formation during cytosolic dsRNA sensing and viral infection. It is indeed intriguing that PKR is strongly activated during the replication of IAV, a nuclear negative single-strand virus. Therefore evaluating whether viral or host dsRNA activates PKR during viral infection, but also the subcellular localisation of PKR during viral infection would help understand the mechanism of PKR activation. In addition, it is still unclear how caspase-3 is activated during dsRNA sensing and viral infection. It will be interesting to examine the involvement of PKR-mediated translation inhibition in this process. Since both caspase-8 and caspase-9 lead to caspase-3 activation and that these three caspases are required for GSDME pore formation in NHBE cells, looking into the contribution of PKR and TRIF in caspase-8 and caspase-9 activation is of interest. Finally our data showed that TRIF contributed to GSDME cleavage following dsRNA delivery. Hence the involvement of TRIF in GSDME pore formation during IAV and RSV infection should be examined.

Our results showed that cytosolic dsRNA sensing not only results in GSDMD and GSDME pores, but also in GSDMA pore formation over time. Evaluating the contribution of GSDMA pores in lytic cell death and cytokine secretion during dsRNA sensing and viral infection would refine our current model. Also it will be interesting to examine the mechanism of GSDMA pore formation in respiratory epithelial cells, in particular the contribution of NLRP1, PKR and TRIF in this process. Since GSDMA, GSDMD and GSDME can all localise at the plasma membrane and at the mitochondria, it is important to assess their subcellular localisation over time during dsRNA sensing and viral infection to further understand the function of these pores. Subcellular fractionation and mitochondrial isolation were technically difficult to implement with NHBE cells, however preliminary experiments using confocal microscopy showed that GSDM can be visualise with this technique in these cells.

Additionally, this study highlights that NLRP1 and PKR are involved in protein expression such as RLRs and pro-IL-1 β but also that NLRP1 contributes to cytokine secretion independently of ASC during dsRNA sensing. It will be interesting to investigate whether this is also the case during viral infection. Importantly, further work is required to

determine if NLRP1 has a transcriptional activity since a regulatory function for NLRP1 has not been revealed yet.

Finally since mouse *in vivo* models have limitations for the studies of human signaling pathways, the model used during this project could be further developed by using differentiated epithelial cells in an airway-liquid interface culture model to investigate GSDME cleavage during viral infection of that more physiologically relevant system.

This project highlights that IAV infection results in strong GSDME cleavage into a pore-forming fragment in human bronchial epithelial cells. This outcome is not specific to the strain used in these experiments, namely IAV WSN. Indeed IAV H7N5 also triggered the formation of GSDME pores in lung alveolar epithelial cells [252] suggesting that all flu infections can generate GSDME pores. This hallmark is not restricted to IAV infection as RSV infection also resulted in GSDME cleavage in human bronchial epithelial cells. In addition SARS-CoV-2 infection led to GSDME pore formation in human airway epithelial cells [145]. Together this demonstrates that GSDME pore formation is a general hallmark for respiratory viral infection in lung cells. Moreover GSDME pores can be a potential marker of COVID-19 disease severity [145]. Therefore the detection of GSDME p30 fragment could be a general marker of disease severity in patients infected with respiratory viruses. Additionally, replication kinetics experiments suggested that GSDME pores are antiviral during IAV infection. Therefore if these results are confirmed in differentiated respiratory epithelial cells, GSDME could be a potential therapeutic target. Indeed a drug that rapidly enhances GSDME cleavage without triggering IL-1 secretion will result in efficient epithelial cell death, subsequently preventing viral replication without releasing pro-inflammatory cytokines that are associated with severe diseases when uncontrolled.

Chapter 7. References

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