The role of the human cathelicidin LL-37 in rhinovirus infection

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This thesis has not been submitted for any other degree or professional qualification.

I state that this thesis is the result of my own independent work, unless otherwise noted.

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Abstract

Human rhinoviruses (HRVs) are the most common causes for symptomatic respiratory infections, and have been linked to severe respiratory conditions in children, and in immunocompromised and elderly individuals. Currently there are no specific treatments or vaccination available for HRV infections and novel antiviral therapeutics are urgently required. Cathelicidins are a well-characterized family of Host Defence Peptides (HDP) with potent antibacterial, antiviral and immunomodulatory functions. This study investigates the antiviral activity of the human cathelicidin LL-37 against human rhinovirus together with the capacity of the peptide to modulate inflammation and host cell death in rhinovirus infection. We demonstrate that LL-37 has significant antiviral activity against HRV1B when the virus is exposed to peptide prior to cell infection, and when cells are infected prior to LL-37 treatment, indicating that LL-37 exerts its effects by directly targeting the virus and/or acting on host cells reducing their susceptibility to infection. Cathelicidin-mediated inflammatory pathway modulation was measured via quantification of IL-8, IL-6 and CCL5 gene expression and protein release. Our data indicates that LL-37 can significantly reduce pro-inflammatory gene expression and protein release induced by HRV1B infection in bronchial epithelial cells, when the peptide is exposed to HRV prior to infection. However, LL-37 was shown to increase HRV-induced inflammatory gene expression and protein release by bronchial epithelial cells, when cells were infected prior to LL-37 treatment. This data indicates that the cellular microenvironment and the context of cathelicidin exposure could determine the direction of cellular response to infection. We further demonstrate that LL-37 suppresses induction of apoptotic cell death in HRV-infected cells, which may represent a novel immunomodulatory role for LL-37 in the context of this infection. Taken collectively, these data suggest that cathelicidins represent an exciting therapeutic avenue for rhinovirus infections, via targeting of virus particles and modulation of host cell responses to infection.

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Filipa Sousa

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16HBE14^{o-}	Transformed human bronchial epithelial cell line
5'UTR	5' untranslated region
A549	Adenocarcinomic human alveolar basal epithelial cells
AHR	Airway hyperresponsiveness
AIF	Apoptosis-Inducing Factor
apoA-I	Apolipoprotein A-I
ATF2	Activating Transcription Factor 2
Bcl-X_L	B-cell lymphoma-extra large
BEASB-2B	Bronchial epithelial transformed cell line
CCR1-3	C-C chemokine receptor type 1-3
CCR2	C-C chemokine receptor type 2
CD86	Cluster of Differentiation 86 (expressed on antigen-presenting cells)
CDHR3	Human Cadherin-related family member 3
cDNA	Complementary DNA
CHDP	Cationic Host Defense Peptide
COPD	Chronic obstructive pulmonary disease

COX-2	Cyclooxygenase-2
CPA	Community-Acquired Pneumonia
CryoEM	Cryo-Transmission Electron Microscopy
CXCL-10	C-X-C motif chemokine 10 (CXCL10) also known as Interferon gamma-induced protein 10 (IP-10)
CXCR-4	C-X-C chemokine receptor type 4
DAMP	Damage associated molecular pattern
DCs	Dendritic cells
DENV-2	Dengue virus type 2
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EGFR	Epidermal Growth Factor Receptor
eIF-2α	Alpha subunit of eukaryotic initiation factor 2 (eIF-2α)-specific kinase
ELISA	Enzyme-linked Immunosorbent assay
EMEA	European Medicines Agency
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorting

FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FPRL1	N-Formyl peptide receptor-like 1
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HBD	Human Beta Defensin
HBECs	Human Bronchial Epithelial Cells
hCAP18	Human Cathelicidin Antimicrobial Peptide 18kDA
HDM	House dust mite
HeLa	Human cervical epithelial cell line
HIF-1α	Hypoxia-inducible factor 1α
HIV	Human Immunodeficiency Virus
HLA-DR	Major histocompatibility complex, class II, DR alpha
HNEpCs	Human primary nasal epithelial cells
HNP	Human Neutrophil peptides
HRV	Human Rhinovirus
HSV	Herpes Simplex virus

IAP-2	Inhibitor of apoptosis protein
IAV	Influenza A virus
ICAM-1	Human intracellular adhesion molecule 1
IFNAR1 and -2	Type I IFN receptor complex
IFNβ	Interferon β
Ig	Immunoglobulin
IkBα (aka NFkBIA)	NFKB inhibitor alpha
IKKs	IKB kinases
IL-1β	Interleukin 1Beta
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-8RB	Interleukin 8 receptor Beta
IMDM	Iscove's Modified Dulbecco's Medium
IPS-1	Interferon-β promoter stimulator 1
IRF3	Interferon regulatory factor 3
ISGs	Interferon-stimulated genes
JAK-STAT	Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway

JNK	c-Jun N-terminal kinase
LDLR	Low-density lipoprotein receptor
LFA-1	Lymphocyte function-associated antigen-1
LL-37	Human Cathelicidin 37 amino acids
LPS	Lipopolysaccharides
LRP	LDLR-related protein
Mac-1	CD11b/CD18
MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemotactic Protein 1
mCRAMP	Murine Cathelicidin Related AntiMicrobial Peptide
MDA-5	Melanoma Differentiation-Associated Gene 5
MOI	Multiplicity of Infection
MyD88	Myeloid Differentiation primary response 88
NF-kB	Nuclear Factor kappa B
NFkB1	Nuclear factor kappa B subunit 1
NFkBIA	NF-kappa-B inhibitor alpha
NK	Natural-Killer cells

NO	Nitric Oxide
ORF	Open Reading Frame
p.i.	Post-infection
$P2X_7$	P2X Purinoceptor 7
p38	p38 Mitogen-Activated Protein Kinase
p48	Helix-loop-helix transcription factor p48
PBA	4-Phenylbutyrate
PBMCs	Peripheral Blood Mononuclear Cells
pDCs	Plasmacytoid Dendritic Cells
PG	Protegrin
PGE_2	Prostaglandin E2
PI	Propidium Iodide
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKR	RNA-binding protein kinase R
Poly I:C	Polyinosinic-polycytidylic acid
PR-39	Porcine antimicrobial peptide 39 amino acids
PRR	Pattern-Recognition Receptor
PS	Phosphatidylserine

RANTES (aka CCL5)	Regulated upon activation in normal T-cells, expressed and secret
RIG-I	Retinoic acid-inducible gene-I
RNA	Ribonucleic acid
RPM	Repetitions per Minute
RSV	Respiratory syncytial virus
RTD	Rhesus Teta Defensin
RT-qPCR	Real- time Polymerase chain reaction
SDS-PAGE	Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis
SMAP29	Sheep myeloid antimicrobial peptide 29 amino acids
SP-1	Transcription Factor Sp1
ssRNA	Single-stranded RNA
STAT	Signal Transducers and Activators of Transcription
STAT3	Signal Transducers and Activators of Transcription 3
TBK-1	Serine/threonine-protein kinase TBK1
$TCID_{50}$	50% Tissue Culture Infective Dose
TEM	Transmission electron microscopy

TIR	Toll/Interleukin-1 (IL-1) receptor
TLR2	Toll-Like Receptor 2
TLR3	Toll-Like Receptor 3
TLR7	Toll-Like Receptor 7
TLR8	Toll-Like Receptor 8
TNFAIP3	Tumor Necrosis Factor alpha-induced protein 3
TNF-α	Tumor- Necrosis Factor alpha
TNIP3	TNFAIP3 interacting protein 3
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing IFN-beta
Type I IFNs	Type I interferons (IFN-α/β)
Type III IFNs	Type III interferons (IFN-λ1/IL-29, IFN-λ2/IL-28A and IFN-λ3/IL-28B)
URTI	Upper Respiratory Tract Infection
UV	Ultraviolet light
VDRE	DNA binding vitamin D
vLDLR	Very-low Lipoprotein Receptor
VPs	Viral capsid proteins

CHAPTER 1

Introduction

Chapter 1. Introduction

1.1. Human Rhinovirus

Human rhinoviruses (HRVs) were first identified in 1956 by Winston H. Price, and since then have been widely reported as the most common organism for causing cold-like illnesses in both adults and children. HRV infections occur worldwide throughout the year, but tend to peak in autumn and spring seasons (Arruda et al., 1997; Monto, 2002). For a long period of time, HRVs were thought to cause relatively benign upper airway tract illnesses, however, evidence has emerged implicating HRV infections with lower respiratory tract illnesses such as exacerbations of chronic obstructive pulmonary disease and the development of asthma. Recent advances in molecular viral diagnostic techniques, such as PCR-based assays, have permitted the detection of previously undetectable respiratory viruses, and have led to a more complete understanding of the diseases caused by HRV infections. Thus HRV have now been linked to severe bronchiolitis in infants and children (Papadopoulos et al., 2002; Pitrez et al., 2005), pneumonia (Juvén et al., 2000), croup (Rihkanen et al., 2008) and otitis media (Pitkäranta et al., 1998).

1.1.1. Rhinovirus structure and replication cycle

Rhinovirus is a non-enveloped, single-stranded, positive-sense RNA (ssRNA) virus of approximately 7200 bp from the genus *Enterovirus* of the family *Picornaviridae*. The genome of HRV comprises a single gene in which the translated protein is cleaved by virally encoded proteases to produce 11 proteins (Palmenberg et al., 2010). The 5' untranslated region (UTR) of the genome includes structural and sequence elements which are required for gene translation (Figure 1). The 5'UTR region is followed by an open reading frame (ORF) which codes for the capsid proteins, VP4, VP3, VP2 and VP1, and by seven non-structural proteins which are involved in the viral genome replication and assembly. The VP1, VP2 and VP3 proteins form the major part of the viral capsid and are accountable for virus antigenic diversity, whereas VP4 protein lies at the interface between the capsid and the RNA genome, anchoring the RNA core to the capsid. The virion presents an icosahedral structure formed by the capsid which is composed of 60 copies each of the four capsid proteins.

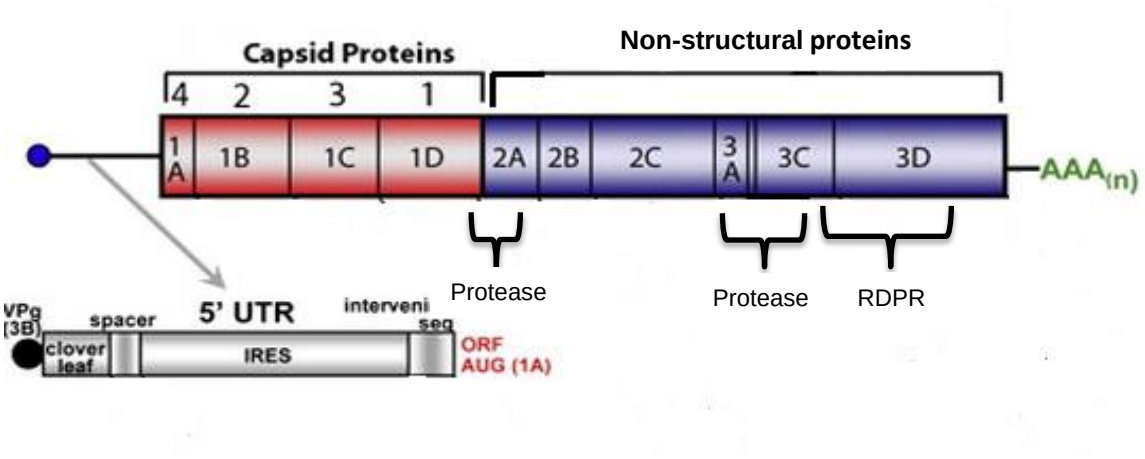


Figure 1. Genomic structure of Human rhinovirus.

The HRV genome consists of a single ORF joined to 5'UTR and a viral priming protein (VPg) which is cleaved to form 11 proteins which either form the capsid (VP4-VP1) or are necessary for viral replication. RDRP, RNA-dependent RNA polymerase; IRES, internal ribosomal entry subunit. [Image adapted from Jacobs et al., (2013)]

Initial research identified more than 100 different HRVs strains which were subsequently serotyped and divided into species HRV-A or HRV-B in accordance to their phylogeny (Conant and Hamparian, 1968). Currently, there are 77 known serotypes of HRV-A and 25 serotypes of HRV-B (Savolainen et al., 2002). With the improvement of sequencing technology, a new species has been identified termed HRV-C. Recent studies have shown that HRV-C is not a new virus, and that it has circulated in human populations for many years (Linder et al., 2013). The delay in identifying HRV-C species is attributed to the fact that they are difficult to grow in cell culture, and for this reason their discovery took longer than comparable pathogens. However, to date there are more than 60 known serotypes of HRV-C (Simmonds et al., 2010).

Human rhinoviruses replicate in epithelial cells of the upper and lower airway tract (Arruda et al., 1995; Gern et al., 1997). HRV-B and HRV-A species access the cell either via the human intracellular adhesion molecule 1 (ICAM-1) [major group of viruses] or via the low-density lipoprotein receptor (LDLR) [minor group of viruses]. The human cadherin-related family member 3 (CDHR3), an asthma susceptibility gene enconding for a transmembrane protein with unknown biological function

(Bønnelykke et al., 2014), has recently been identified has the specific cellular receptor used by HRV-C to enter cells (Bochkov et al., 2015).

Virus uptake is dependent on the receptor type and it may occur via clathrin-dependent, or independent endocytosis or through micropinocytosis (Fuchs and Blaas, 2010). The virus particles undergo conformational change forming hydrophobic subviral particles, and this is initiated by ICAM-1 and/or the low pH environment in endosomes. The viral RNA is released through an opening formed in the viral capsid and crosses the endosome membrane via a pore formed by the viral proteins or alternatively through membrane rupture allowing the RNA to enter the cytosol. The positive-sense, single stranded RNA once in the cytosol is then replicated and translated into a polyprotein by the host cell ribosome (Fuchs and Blaas, 2010). The viral replication occurs entirely in the cytosol, where ssRNA forms a dsRNA intermediate, which is known as the main form of viral RNA genome inside the host cell. The resulting polyprotein (~250 kDA) is then cleaved by viral proteases $2A^{pro}$ and $3C^{pro}$ into the 11 protein variants (4 structural and 7 non-structural proteins) (Knott et al., 1989; Sommergruber et al., 1989).

1.1.2. Transmission of rhinovirus

The major route of dissemination of HRV includes: airborne transmission of aerosolised virus which can enter the body through the airway tract; transmission from person to person via direct contact; or fomite transmission (i.e. objects that may become contaminated and serve as vehicles in transmission). The most common is through the hand-nose-hand route which is followed by self-inoculation of intranasal/conjunctival mucosa (Jennings and Dick, 1987). Studies with natural rhinovirus infected-adults provided further evidence on the transmission of rhinovirus, demonstrating that infected subjects lead to the contamination of environmental surfaces with rhinovirus, such as light switches, telephone dials and handsets, transferring the virus to the fingers of non-infected individuals (Winther et al., 2007).

1.1.3. HRV Epidemiology

In healthy individuals, rhinovirus infections are usually limited within the upper respiratory tract with little evidence of lower respiratory tract involvement (Message et al., 2008). Epidemiological studies reveal a higher incidence of HRV-induced infections in children than adults and almost all children by the age of two have experienced at least one HRV infection (Blomqvist et al., 2002). HRV is responsible for more than 80% of the common colds, with peaks of infection between April and May and between September and November which are the high prevalence seasons, nonetheless HRV is present all-year round in the community and can affect people of all ages (Turner, 1997). The HRV- induced upper airway infections include the common cold, acute otitis media and rhinosinusitis. Acute otitis media has been linked to HRV infection and complicates one third of the cold-like illnesses in young children (Winther et al., 2007).

As well as the upper respiratory tract illnesses, human rhinoviruses have been associated with lower airway infections (Hayden, 2004). A prospective 4-year study, which included adults and children hospitalized with various acute respiratory infections, identified rhinovirus infection by virus isolation from nose and throat swabs. The frequent diagnoses in young children were bronchiolitis, pneumonia and asthma (El-Sahly et al., 2000). Bronchiolitis and pneumonia are the most frequent clinical manifestations of rhinovirus in children under five years old and bronchiolitis is the most common reason for hospitalization (Jartti and Gern, 2011). HRV-associated bronchiolitis in infancy is a potential long-term risk factor for recurrent wheezing and asthma (Beigelman and Bacharier, 2013). A cohort study with very-low birth weight infants revealed that the predominant pathogen during bronchiolitis episodes was HRV (Miller *et al*., 2012). Human rhinovirus has been established as a common pathogen in children hospitalized with community-acquired pneumonia (CPA) with a rate of around 26%. Unfortunately, the clinical manifestations of CPA can be severe, particularly in children with underlying chronic diseases (García-García et al., 2012).

Rhinovirus plays a major role in triggering exacerbations of asthma and chronic obstructive pulmonary disease (COPD). A cross-sectional study of children

hospitalized with asthma exacerbations revealed that HRV was detected in 85% of the cases and the major HRV genogroup associated with this was identified as HRV-C (Mak et al., 2011). The key mechanisms in the pathogenesis of asthma and chronic obstructive pulmonary disease are associated with abnormal repair processes of the respiratory epithelium which lead to an anomalous organization and integrity of the epithelium followed by perturbations of innate immune functions (Folli et al., 2008). A study by Bossios et al. (2005) provided evidence that rhinovirus is able to delay wound healing in bronchial epithelial cells contributing to asthma exacerbations. Furthermore, children who experience wheezing as a result of rhinovirus infection are more prone of subsequently developing asthma (Gavala et al., 2011).

Rhinovirus infections represent a serious risk for elderly people, where major rhinovirus outbreaks in healthcare facilities have resulted in severe infections, as well as deaths, have been reported (Hicks et al., 2006; Louie et al., 2005). Rhinovirus infections in hospitalized elderly patients are also associated with a significantly higher mortality and longer hospitalization periods when compared to influenza virus infection (Hung et al., 2017). A study by Nicholson et al. (1996) revealed that HRV infections are the main cause of debility and lower airway infections among community-dwelling elderly.

1.1.4. Pathogenesis and host immune responses

1.1.4.1. Infection of the upper respiratory tract

HRV is responsible for most of the upper airway tract infections experienced by humans (van Kempen et al., 1999). The most common symptoms are nasal obstruction and rhinorrhea (aka “runny nose”). These symptoms are related with a neutrophilic inflammatory response which stimulates mucus hypersecretion and increases vascular permeability. Viral infections of the upper respiratory tract are frequently followed by secondary bacterial infections in the form of acute rhinosinusitis and studies have demonstrated that rhinovirus increases the expression of host cell adhesion molecules, such as, fibronectin, platelet-activating

factor receptor, in nasal epithelial cells as a possible mechanism responsible for the increase in susceptibility to bacterial infections (Wang et al., 2009).

Other respiratory viruses, such as IAV and RSV, have been shown to cause damage of airway epithelial cells (Troy and Bosco, 2016), however rhinovirus is rarely associated with cytopathic effects of the upper airway tract. Nasal biopsies from patients with natural colds showed that the epithelial cell lining and borders remained structurally intact (B. Winther et al., 1984). Nevertheless, rhinovirus was shown to disrupt the airway epithelial barrier function by dissociating the zona occludens-1, also known as tight junction protein-1, from the tight junction complex, thus enabling HRV binding, translocation, as well as facilitating secondary bacterial infection (Sajjan et al., 2008).

1.1.4.2. Infection of the lower respiratory tract

In recent years, there has been increasing evidence that supports the role of HRV as a lower airway pathogen(Gern et al., 1997). The symptoms associated with HRV-driven lower respiratory tract infections are more pronounced in patients who have underlying asthma or other chronic lung diseases and include chest tightness, wheezing, cough and shortness of breath (Folkerts et al., 1998; Las Heras and Swanson, 1983; Papadopoulos et al., 2000). There have been a number of controversies regarding the pathogenesis of HRV-induced lower respiratory tract infections which centers on the extent to which HRV can directly infect cells of the lower respiratory tract as opposed to an indirect influence related to the immune response of the upper airway infection. There are, however, various obstacles to lung infection by HRV and the temperature sensitivity of viral replication is one of them (Killington et al., 1977). Initial experiments implied that viral replication was optimal at 33°C and was significantly reduced at 37°C and 39 °C, although a study by Papadopoulos et al. (1999) demonstrated that there were minimal differences in viral replication capacities between 33°C and 37°C. In a follow-up study, Papadopoulos et al. (2000) demonstrated the potential for HRV to infect cells of the lower respiratory tract by performing *in vitro* and *in vivo* experiments. In the *in vitro* experiment, results showed that rhinovirus infection of primary human bronchial epithelial cells (HBECs) resulted in an increased production of IL-6, -8 and -16, and

RANTES as well as associated cytotoxicity. In the *in vivo* experiment, healthy volunteers were inoculated with HRV16 and bronchial biopsy samples were analysed using *in situ* hybridization method to localise rhinovirus infection which confirmed the presence of rhinovirus in the lower respiratory epithelium. Other studies have also demonstrated the presence of HRV in the lower airway tract (Gern et al., 1997; Malcolm et al., 2001). The current literature therefore supports the concept that HRV can infect cells of the lower respiratory tract and induce a pro-inflammatory response.

1.1.4.3. Innate immune responses to rhinovirus infection

In addition to the direct effects on the airway epithelial cells, the host immune responses are also implicated in the pathogenesis of HRV infections. Infection of the respiratory epithelium by rhinovirus will initially induce an innate immune response. Early innate detection of rhinovirus can depend on the ability of the host to identify and sense rhinovirus- associated molecular patterns by pathogen sensors, known as pattern recognition receptors (PRRs). These pathogen sensors recognise specific molecular patterns on the virus and elicit an inflammatory and antiviral response in order to clear the viral infection. In the initial stages of viral infection, the viral capsid is recognised by TLR2 located on the cell surface, and upon internalisation the ssRNA is recognised by TLR7 and 8 located in the endosomes (Triantafilou et al., 2011). The viral dsRNA produced during viral replication, represents an important stimulus of the host innate immune response and is recognised by four pattern recognition receptors, known as Toll-like receptor 3 (TLR3), Melanoma differentiation-associated gene 5 (MDA-5), Retinoic acid inducible gene I (RIG-I) and the cytosolic RNA-binding protein kinase R (PKR). All these receptors have been shown to be expressed in airway epithelial cells (Guillot et al., 2005; Liu et al., 2007; Sirén et al., 2006).

TLR3 is localised in the endosomal and plasma membranes. It senses dsRNA and signals through the adaptor protein toll/interleukin-1 receptor (TIR) domain-containing adaptor inducing IFN-β (TRIF) (Yamamoto et al., 2003). Poly I:C (synthetic dsRNA) upon ligation to TLR3 was shown to induce the activation of NF-kB and the production of type I interferon (Alexopoulou et al., 2001). RIG-I and MDA-5 are

homologous cytoplasmic helicases containing two N-terminal caspase activation and recruitment domains and a C-terminal DExD/H-Box RNA helicase domain (Gee et al., 2008; Kang et al., 2004) and both have been identified as intracellular receptors for dsRNA in the cytosol. RIG-I was shown to bind to dsRNA and induce antiviral responses in the host cells (Liu et al., 2007). The caspase recruitment domain found in RIG-I functions to transmit downstream signals which results in the activation of the transcription factors NF-κB and IRF3 and subsequent induction of type I interferon production (Yoneyama et al., 2004). The MDA-5 sensor is thought to function to promote IFNβ induced apoptosis and inhibit cell growth (Barral et al., 2007; Kuo et al., 2013). Both MDA-5 and RIG-I are induced by IFNβ (Kang et al., 2004). PKR is a serine/threonine kinase member of the alpha subunit of eukaryotic initiation factor 2 (eIF-2α)-specific kinase subfamily (de Haro et al., 1996). PKR is characterised by two distinct kinase activities: (1) auto-phosphorylation resulting in the activation of eIF-2α and (2) phosphorylation of eIF-2α which results in the inhibition of host cell protein synthesis (García et al., 2006). In a non-stressed environment, PKR is in a monomeric latent state, and can be activated in response to dsRNA of cellular, viral or synthetic origin. Binding of two molecules of dsRNA results in PKR dimerization into an active state (García et al., 2006), and PKR is also able to phosphorylate IκBα leading to the activation of NF-kB transcription factor (Kumar et al., 1994).

Studies have demonstrated that dsRNA upregulates the expression levels of pro-inflammatory cytokines and chemokines in BEAS2B cells and primary bronchial epithelial cells including RANTES, CXCL-10 and IL-8, through TLR3 signalling (Matsukura et al., 2006). Using short interfering RNA (siRNAs) knockdown for TLR3 in BEAS-2B cells has revealed a significant decrease in the mRNA levels of these pro-inflammatory mediators, whereas knockdown of PKR, MDA-5 and RIG-I did not significantly inhibit the poly I:C–induced cytokine and chemokine response (Matsukura et al., 2007). This indicates that TLR3 has an important role in promoting a pro-inflammatory response whereas RIG-I, PKR and MDA-5 may function, in part, by delivering an antiviral response in infected host cells. In 16HBE14^{o-} cells, TLR3 was shown to be partially required for HRV39-induced IL-8 expression (Sajjan, 2006). Nonetheless, in rhinovirus infection of human bronchial

epithelial cells, the coordination between TLR3, RIG-I and MDA-5 was essential for maximal antiviral and pro-inflammatory cytokine responses. Notably, the HRV-induced RIG-I and MDA-5 expression was shown to involve TLR3/TRIF signalling (Slater et al., 2010).

Rhinovirus infection of airway epithelial cells has been shown to induce a rapid production of several pro-inflammatory cytokines, including IL-8, IL-6, GM-CSF and CCL5 (Kim et al., 2000; Papadopoulos et al., 2001; Subauste et al., 1995). Increased levels of IL-6 (Zhu et al., 1996), IL-8 (Zhu et al., 1997) and IL-1β (Proud et al., 1994) were also detected in nasal washings taken from rhinovirus experimentally infected subjects. The levels of IL-6 and IL-8 produced during rhinovirus infection were shown to be directly linked to the severity of symptoms scores (R. B. Turner et al., 1998).

In addition to the production of pro-inflammatory chemokines and cytokines in response to rhinovirus infection, airway epithelial cells can also respond by secreting cationic host defence peptides (Hiemstra et al., 2016; Proud et al., 2004). HRV16 infection of primary bronchial epithelial cells was found to increase the expression levels of human β defensins-2 and -3 (Duits et al., 2003). Defensins are cationic host defence peptides characterised by three intramolecular disulphide bonds that determine their sub-classification into α-, or β-defensins. In addition to their broad antimicrobial activity, β-defensins have also shown potent antiviral activity against both enveloped and non-enveloped viruses (Wilson et al., 2013), and immunomodulatory properties, such as mast cell degranulation (Niyonsaba et al., 2001), and chemotactic activity for monocytes, DCs and T cells (Yang et al., 1999).

Airway epithelial cells can also respond to rhinovirus infection by invoking an antiviral response. Interferons are cytokines secreted by host cells in response to viral infection that function to limit the spread of virus by inducing the expression of many IFN-stimulated genes (ISGs) (Katze et al., 2002). The first antiviral agents to be characterised and are still regarded as essential to the early antiviral response are type I interferons (IFN-α/β). Type I IFNs are expressed by many epithelial cell types.

They bind to the type I IFN receptor complex (IFNAR1 and -2) which in turn triggers the intracellular IFN signalling pathways, mainly the JAK-STAT pathway, which eventually induce the expression hundreds of IFN-stimulated genes (Basler and García-Sastre, 2002). The most studied type I IFN-induced gene is PKR. As noted above, dsRNA can activate PKR, which can in turn, inhibit virus replication and spread. Furthermore, practically all viruses have developed strategies to block the function of PKR so that the virus replication is not compromised (Gale and Katze, 1998). A novel class of antiviral cytokines has been discovered and has been classified as type III IFNs: IFN-λ1/IL-29, IFN-λ2/IL-28A and IFN-λ3/IL-28B. Type III IFNs have similar biological activities to type I IFNs; however type III IFNs appear to be expressed specially by epithelial cells exerting host-protection primarily at epithelial surfaces (Ank and Paludan, 2009).

Rhinovirus infection of bronchial epithelial cells has been shown to strongly induce IFNβ and IFNλ (Khaitov et al., 2009). However, bronchial epithelial cells obtained from asthmatic patients have shown impaired production of IFNβ in response to HRV infection (Wark et al., 2005). Later studies subsequently revealed that rhinovirus-induced IFNβ and -λ production was not deficient in well-controlled asthma (Sykes et al., 2014). A study by Slater et al. (2010) showed that TLR3, RIG-I and MDA-5-mediated signalling was required for maximal IFN-β, -λ and pro-inflammatory cytokine gene expression by bronchial epithelial cells in response to rhinovirus infection.

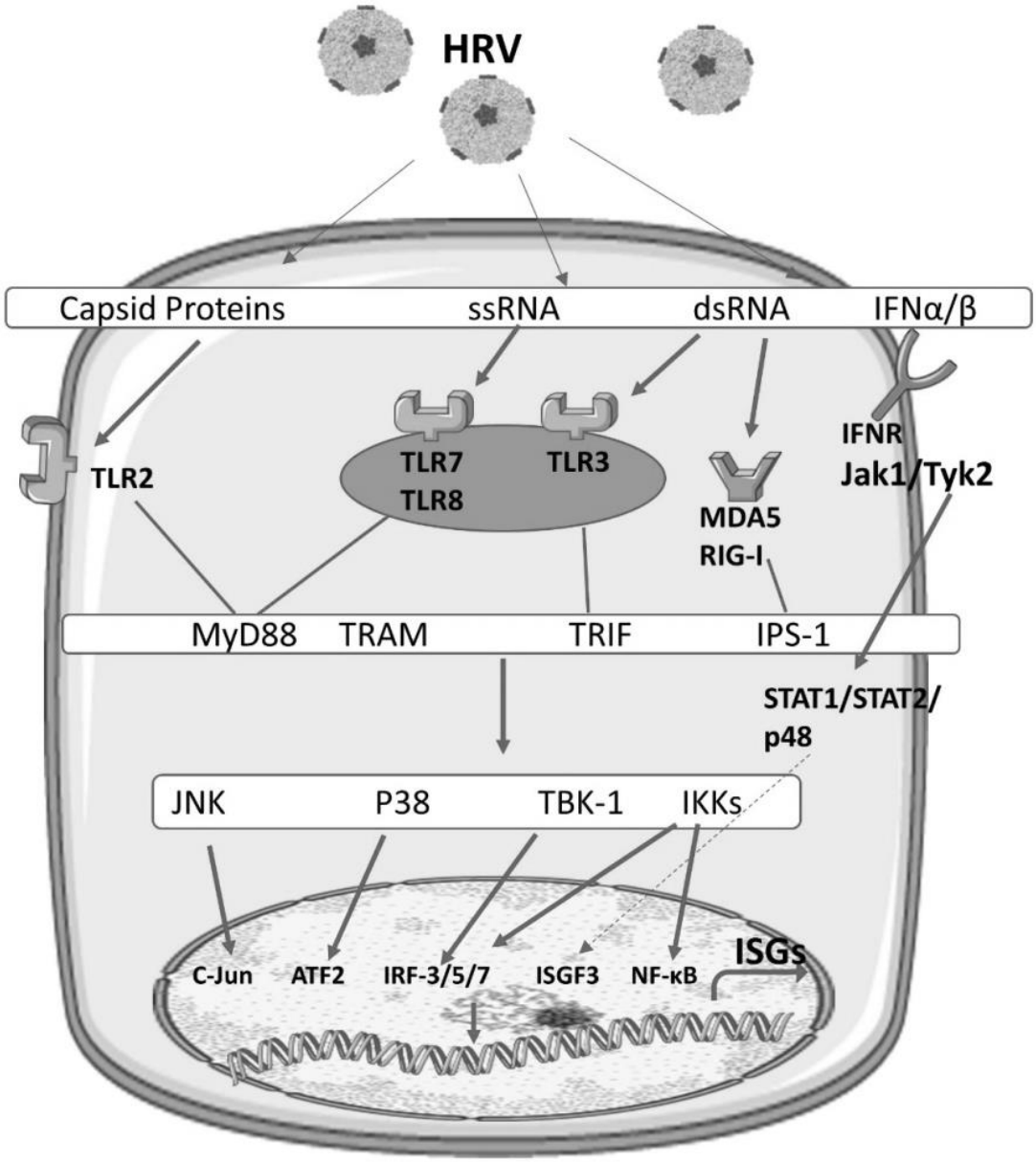


Figure 2. Signal transduction pathways activated by rhinovirus infection of airway epithelial cells.

In the endosome ssRNA and dsRNA are recognised by TLR7/8 and TLR3, respectively. Binding of dsRNA to TLR3 upregulates the expression of MDA-5 and RIG-I. In addition MDA5 and RIG-I sense newly synthesised dsRNA in the cytosol. Interaction with TLR3, RIG-I and MDA-5 leads to the upregulation of IFN gene expression as well as pro-inflammatory cytokines and chemokines, via NF-kB and IRF3 signalling. TLR7/8 and TLR2 signal through a MyD88-dependent pathway, leading to the activation of NF-κB and IRF-7.

1.1.4.4. Adaptive immune responses to rhinovirus infection

The mean incubation time after infection by rhinovirus is 2 days and the typical symptoms (common cold symptoms: rhinorrhea and nasal congestion) last on average 3 days. Viral shedding in nasal secretions can be detected already at 8-10h after exposure and reaches maximum levels between 2-7 days and in some cases will continue up to 14 days after infection (Butler et al., 1970; Couch et al., 1966). The anti-HRV secretory IgA in nasal secretions occur approximately 7 days after infection and serum IgA has shown not to increase before 6 weeks after infection. This is also the case for anti-HRV IgG response (Butler et al., 1970). The antibody response to rhinovirus does not seem to play a role in viral clearance as it appears only after the end of the illness. Nevertheless, a study by Alper et al. (1998) determined the influence of serotype-specific neutralizing serum antibodies (IgG) titers on the rate of infection after exposure of adult volunteers to HRV. Results showed that neutralizing antibodies are associated with protection against the infection as well as reduced signs and symptoms following experimental HRV exposure.

T cells, through the recognition of viral antigens, can contribute to antiviral immunity; however, the T-cell response to rhinovirus infection is not well understood. $CD4^+$ T-cells, which secrete IFNγ, have been isolated from PBMCs from subjects with previous disease (Wimalasundera et al., 1997). Furthermore, Gern et al. (1997) demonstrated that HRV-specific T cells, specifically HRV-CD4 T cells, can be activated by either serotype specific, or by shared viral epitopes. This increases the possibility of a potent T cell response due to the repeated activation of T cells by shared viral epitopes *in vivo.*

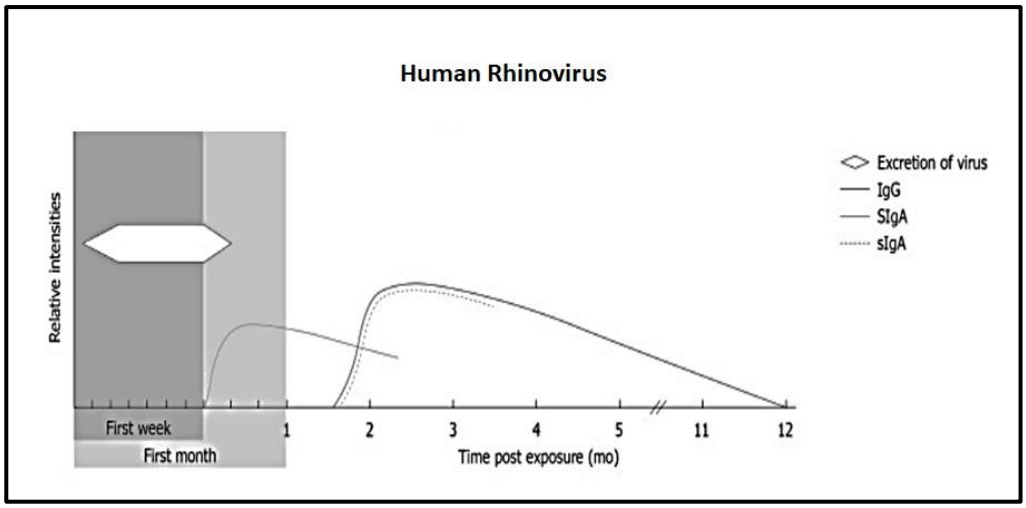


Figure 3. Antibody response to rhinovirus infection

Graph representing the relative intensities and time-course of the viral spread and antibody response against human rhinovirus. [Figure from Dotzauer and Kraemer (2012)]

1.1.5. Treatment options

There are no current specific effective treatments for HRV infections. Prevention, using vaccination, has been shown to be extremely challenging due to the fact that there are more than 150 serotypes of HRVs. The therapeutic options available are predominantly supportive measures, and include conventional symptomatic treatments (e.g. antihistamines) and dietary supplements (e.g. *Echinacea*, Zinc). These supportive measures are only applicable for mild upper respiratory infections (i.e. common cold); for serious respiratory infections, there are no efficient treatments available.

There has been a significant effort to develop anti-HRV agents to tackle different viral aspects, such as capsid binding agents, viral attachment inhibitors, or proteolytic enzyme inhibitors. None of these anti-HRV agents has yet been approved by the FDA or EMA. The capsid-binding agents bind to the hydrophobic pocket of the viral capsid, causing a conformational change which interferes with the virus capacity to interact with the host cellular receptors (Thibaut et al., 2012).

Examples of capsid-binding agents include: pleconaril which was the first compound to be submitted to FDA for regulatory approval, although its license was declined due to concerns about safety and resistance (Hayden et al., 2003a, 2002); pirodavir, an intranasal agent which demonstrated efficacy in preventing experimental colds (Andries et al., 1992); vapendavir, an orally administered drug that binds to the HRV VP1 capsid protein and prevents the release of ssRNA into the target cell (Matz, 2013). Proteolytic enzyme inhibitors are another chemotherapeutic approach. Rupintrivir is an intranasal irreversible inhibitor which has shown potent activity against all HRV serotypes and is one of the most effective to inhibit 3C protease *in vitro* (Hayden et al., 2003b).

Another approach to tackle HRV infection is to prevent viral attachment by blocking the receptors using soluble forms of ICAM-1 (Abraham and Colonno, 1984). The HRV major group use ICAM-1 receptor for attachment to host cells. Tremacamra is an example of this type of drugs and shows potential in terms of reducing the severity of the experimentally-induced infections; although currently no recent development has been reported for these agents (Turner et al., 1999). In addition, an anti-human ICAM-1 antibody has been developed, using transgenic mice over-expressing extracellular domain 1 and 2 of human ICAM-1. *In vivo* topical or systemic administration of anti-ICAM-1 prevented the entry of HRV16 and HRV14, as well as, a reduction of cellular inflammation, pro-inflammatory cytokine production and viral load (Traub et al., 2013). HRV-neutralizing antibodies have also been developed against specific epitopes found on VP proteins of the viral capsid. These anti-VP antibodies were shown to block the virus-receptor interaction, prevent virus uncoating and promote significant conformational changes on the capsid triggering the genome release (Che et al., 1998; Dong et al., 2017; Smith et al., 1996).

Due to the fact that currently there is no effective specific treatments or vaccination available for rhinovirus infections, there is a pressing need to develop novel alternative antiviral therapies.

1.2. Cationic Host Defence Peptides

Cationic host defence peptides (CHDPs; also termed antimicrobial peptides) are key components of the innate immune system, with potent microbicidal, antiviral and inflammation and immunity modulatory functions (Barlow et al., 2014; Mookherjee and Hancock, 2007; Nijnik and Hancock, 2009). CHDPs can be found in a variety of life-forms, and some of the first indications of the presence of CHDPs were in fungi and bacteria, where they were viewed as unique defence peptides in unicellular organisms (Perlman and Bodanszky, 1971). The first isolated and characterized cationic antimicrobial peptides were the cecropins from the moth *Hyalaphora cecropia* (Hultmark et al., 1980). The magainins in the skin of *Xenopus laevis* (Zasloff, 1987) were discovered later, followed by the isolation of α-defensins from mammalian neutrophils (Rice et al., 1987). To date, more than 1700 CHDPs have been described and although there is great sequence diversity, there are structural elements that are shared by the majority of CHDPs. They are typically 12-50 amino acids in length, cationic in nature (+2 to +9) and amphipathic with 40-50% hydrophobic residues. CHDPs present different secondary structures although two forms are predominant: β-sheet structures with disulphide bonds (e.g. porcine cathelicidin protegrin) and α-helical structures which are adopted by linear peptides (e.g. human cathelicidin LL-37). In mammals the two best characterized CHDP are defensins and cathelicidins and specifically in humans, the relevant and best described CHDPs include cathelicidins, defensins and histatins (De Smet and Contreras, 2005).

Relevant properties for the microbicidal activity of CHDPs include the cationic charge, which allows for the interaction of these peptides with the negatively charged microbial membranes through electrostatic forces which lead to the formation of pores and their hydrophobicity which helps the integration into the microbial cell membrane (Steinstraesser et al., 2011). It has been demonstrated that CHDPs have antimicrobial activity across a spectrum of infections, including bacterial, parasitic, and viral organisms (Bowdish et al., 2005b; Haines et al., 2003). It is believed that this is mainly through disruption of cell membranes, and that CHDP effects differ between microbe and host cell. The mechanism of action of CHDPs is best understood for bacterial targets. Bacterial cytoplasmic membranes

have high number of lipids with negatively charged phospholipids located in the outer layer of the phospholipid bilayer, whereas eukaryotic cell membranes are formed mainly by lipids with no net charge, and most of the lipids with negatively charged phospholipids are located in the inner layer which faces the cytoplasm (Matsuzaki, 1999). CHDPs preferentially bind to bacterial membranes with abundant acidic phospholipids through electrostatic interactions. Mammalian cell membranes can have a high abundance of cholesterol which makes them resistant to the activity of CHDP; this resistance may be due to interaction between the peptides and cholesterol or due to the stabilization of the lipid bilayer due to the high content of sterols (Matsuzaki, 1999).

The Shai-Matsuzaki-Huang (SMH) model attempts to explain the antimicrobial activity of most CHDP (Matsuzaki, 1999; Shai, 1999; Yang et al., 2000). The model proposes an initial interaction of the peptide with the bacteria membrane, which is followed by the shift of lipids and alteration of the membrane structure and in some cases the entry of the peptide into the target cell. In addition to this model, several other mechanisms of action have been proposed to explain how CHDP can kill microbes, and some of these include: (1) permeabilisation of cell membranes (Matsuzaki, 1999); (2) Formation of membrane pores (Yang et al., 2000); (3) Disruption of free-energy metabolism of bacteria and dissipation of the electric potential of their mitochondrial membrane (Westerhoff et al., 1989); (4) Induction of hydrolases which degrade cell walls (Bierbaum and Sahl, 1985).

1.2.1. Defensins

Defensins are small, cysteine–rich, cationic peptides with a β-sheet structure. Defensins have a broad antimicrobial activity, including antiviral properties and the capacity to modulate the host immune response. Three different subfamilies of defensins are known, and include α-, β- and Θ-defensins. The α- and β- defensins consist of a triple-stranded β-sheet with a distinctive fold, and Θ-defensins possess a cyclic structure.

The α-defensins are also known as human neutrophil peptides (HNP1, HNP2, HNP3, and HNP4) as they are primarily expressed by neutrophils (Ganz et al., 1985). These

are also found in much lower concentrations in Natural Killer cells, immature dendritic cells, monocytes, macrophages and B and γδ T-cells (Rehaume and Hancock, 2008). In contrast, human α-defensins 5 and 6 (HD5 and HD6) are constitutively expressed in Paneth cells, salivary glands and genital mucosa (Ganz et al., 1985). β-defensins are mainly expressed by epithelial cells (HBD1, -2, -3, -4, -5 and -6) (Yang et al., 2004), where HBD1 is constitutively expressed by epithelial cells, whereas HBD2 and HBD3 can be induced by pathogens, and pro-inflammatory cytokines (Duits et al., 2003; Proud et al., 2004; Sørensen et al., 2005). The Θ-defensins have been identified in leukocytes from rhesus macaques; rhesus Θ-defensins 1 (RTD1), RTD2 and RTD3 (Leonova et al., 2001). In humans, an homologous gene to rhesus Θ-defensin has been found in human bone marrow, however these transcripts contain a stop codon upstream of the signal sequence that completely prevents their translation (Nguyen et al., 2003).

Defensins provide an important link between innate and adaptive immune response, acting as both anti-and pro- inflammatory mediators. For example, HNP1-3 released by neutrophils, have been shown to induce the production of TNF-α and IFN-γ from macrophages, which in turn stimulates their phagocytotic activity enhancing the clearance of opsonised bacteria (Soehnlein et al., 2008). hBD3 was shown to activate antigen presenting cells (monocytes, dendritic cells) via TLR 1 and 2 stimulating an adaptive immune response (Funderburg et al., 2007). In addition, defensins have been shown to dampen LPS-induced pro-inflammatory cytokine response by neutralization of extracellular LPS as well as modulation of intracellular signalling pathways (Mookherjee et al., 2006; Scott et al., 2002).

In regards to their direct antiviral activity, defensins have a dual role in antiviral host defence. They are able to directly interact with the viral envelopes, possibly in a similar way they target bacterial membranes, and in addition they are able to interact with the host cell modulating the immune response to infection. The defensin-cell interactions are complex and may be mediated by interacting with cell-surface glycoproteins and/ or interacting with cell signalling pathways. Defensins have shown potent direct antiviral activity against several different viruses, including HIV, IAV, HSV, RSV and adenovirus (reviewed in Klotman and

Chang, 2006). In addition, and as mentioned previously, viral infections can induce the expression of defensins. In normal oral epithelial cells, HIV-1 was shown to induce the mRNA expression of HBD2 and HBD3, but not HBD1, and this was observed even in the absence of HIV-1 replication (Quiñones-Mateu et al., 2003). Similarly, rhinovirus infection of bronchial epithelial cells induced HBD2 and HBD3, but not HBD1 mRNA expression (Duits et al., 2003; Proud et al., 2004). In contrast to HIV-induced HBD expression, active replication of rhinovirus was required for the induction of HBD expression. Interestingly, an artificial circular peptide based on the sequence *of* θ-defensins has been created, known as retrocyclin, and this peptide has shown to have potent antiviral activity *in vitro* (Cole et al., 2002).

Due to the fact that defensins have shown potent antimicrobial and antiviral activity, as well as the ability to modulate the host immune responses to infection, this makes these peptides ideal targets for the development of potential therapeutics for a range of diseases. In fact, a number of synthetic host defence peptides (HDPs) based on defensin structure are currently in clinical trials, which not only aim to exploit the direct antimicrobial properties of these peptides, but also their ability to modulate the human immune response (Yeung et al., 2011).

1.2.2. Cathelicidins

Cathelicidins range in size from 12 to 88 amino acids and are characterized by the presence of an N-terminal signal sequence which directs the newly synthesised protein towards the secretory pathway, a conserved cathelin-like domain and variable C-terminal antimicrobial domain. The cathelin-like domain is highly conserved and shares sequence homology with the porcine cysteine protease inhibitor – cathelin. The C-terminal domain, which is highly divergent, becomes the mature functional peptide upon proteolytic cleavage. Cathelicidins have been discovered in lizards, birds, fish as well as a number of mammals and show great diversity across species (Kościuczuk et al., 2012).

In humans, only one cathelicidin has been described, which is known as Human Cationic Antimicrobial Peptide of 18kDa, or hCAP18. The cathelicidin hCAP18 is encoded by the CAMP gene on the chromosome 3p21.3. This CHDP is stored in the peroxidase-negative, or specific granules of neutrophils and is produced and secreted by macrophages, eosinophils, lymphocytes, mast cells and NK, T and B cells, as well as, expressed by epithelial cells of skin and mucosa of the respiratory, urogenital and gastrointestinal tracts (Agerberth et al., 2000; De Yang et al., 2000). hCAP18 is cleaved extracellularly by proteinase-3, a serine protease from the azurophilic granules of neutrophils, to generate LL-37 which is the dominant cleavage product (Sørensen et al., 2001). In the skin, hCAP-18 was shown to be processed into its active form LL-37 by serine proteases kallikrein 5 and -7 (Yamasaki et al., 2006).

LL-37 is a linear, 37 amino acids length peptide with two leucine residues at the N-terminal which adopts an amphipathic α-helical structure. hCAP18/LL-37 can be detected in a variety of body fluids, such as saliva, sweat, semen, milk and airway surface fluids (Bowdish et al., 2005a). In addition, three other forms of human cathelicidin, other than LL-37, have been found in sweat: KR-20, 20-aa derivative, RK-31, a 31aa-derivative; and KS-30, a 30 aa derivative. All three processed peptides show more potent antimicrobial activity compared to LL-37(Murakami et al., 2004).

The expression of LL-37 can be constitutive or inducible in response to infection, inflammatory signals, and wounding (Erdag and Morgan, 2002; Frohm et al., 1997; Nell et al., 2004; Zanetti, 2005). In addition, recent studies have demonstrated that 1,25- dihydroxyvitamin D3 induces hCAP18/LL-37 expression through a specific DNA binding vitamin D response element (VDRE) consensus located in the *CAMP* gene promoter region (T.-T.Wang et al., 2004). Further data has revealed that $1,25(OH)_2D_3$ can induce the expression of LL-37 in several cell lines, as well as in primary bronchial epithelial cells (Yim et al., 2007), keratinocytes (Weber et al., 2005), and myeloid cells (Gombart et al., 2005). A study by Hansdottir et al. (2008) provided evidence that airway epithelial cells constitutively activate vitamin D due to their high baseline levels of activating 1α-hydroxylase and low levels of inactivating 24-hydroxylase, and that viral infections lead to an increased activation of vitamin D leading to subsequent increases in the expression of cathelicidin mRNA. The capacity of 1, 25-dihydroxyvitamin D3 to increase expression of human cathelicidins *in vivo* has yet to be demonstrated. In addition to vitamin D, another exogenous approach which can regulate cathelicidin expression is through the use of butyrate and its analogues. Butyrate is a short-chain fatty acid produced in the colon by bacterial fermentation of diet fibre. A butyrate analogue, 4-phenylbutyrate (PBA), which has already been approved as a drug for use in humans, induces cathelicidin LL-37 expression in numerous epithelial cells lines, including airway epithelial cells (Steinmann et al., 2009). *In vivo* experiments also revealed the potential of PBA to induce hCAP-18 expression in the mucosal epithelial of the airway tract of a rabbit model of shigellosis (Sarker et al., 2011). It has also been shown that PBA can induce cathelicidin LL-37 expression synergistically with 1, 25-dihydroxyvitamin D3 in lung epithelial cells (Steinmann et al., 2009).

Mice express the cathelicidin mCRAMP (murine cathelin-related antimicrobial peptide) which is encoded by the *Camp* gene on chromosome 9. mCRAMP maintains high sequence identity with hCAP18 and the porcine cathelicidin PR-39 (Gallo et al., 1997). mCRAMP is proteolytically cleaved generating a 34 amino acid length active peptide with a tertiary structure formed by two amphipathic α-helices linked by a flexible region (Yu et al., 2002). In similarity with LL-37, the murine cathelicidin is stored in the granules of neutrophils and can be expressed by

epithelial cells and leukocytes (Chromek et al., 2006; Iimura et al., 2005; Nizet et al., 2001). It has shown that CRAMP-deficient mice were more susceptible to bacterial infections of the airway tract, skin, urinary tract, and gut (Chromek et al., 2006; Iimura et al., 2005; Kovach et al., 2012) than healthy mice.

Pigs express a variety of cathelicidins which differ in activity and structural motifs. The porcine cathelicidins include five different protegrins (PGs), three α-helical peptides (PMAP-23, -36,-37), two prophenins (PF-1,-2) and the PR-39 peptide. Protegrins are between 16-18 amino acids in length and are produced and stored by porcine neutrophils as inactive propeptides, but can be proteolytically cleaved into their active forms by neutrophil elastase in the extracellular environment (Panyutich et al., 1997). PGs present a two-stranded β-sheet structure in solution which is linked by a β-hairpin loop and, contain two intramolecular disulphide bonds which are required for their maximum bioactivity. A study by Wu et al. (2000) demonstrated that the expression of protegrins and PR-39 was enhanced by bacterial LPS, IL6, retinoic acid and *Salmonella* infections. It has been reported that PG-1 has the broadest spectrum of antimicrobial activity (Yasin et al., 1996) and therefore has excellent potential for the development of synthetic antimicrobial compounds.

Cathelicidins have been characterized in many other species Figure 4, such as sheep, monkeys, rabbits, horses and cows. In sheep, eight cathelin-associated peptides have been identified. The cathelicidin SMAP29 (sheep myeloid antimicrobial peptide 29) with a α-helical structure, is one of the most potent CHDP in the context of direct antimicrobial activity. The *in vitro* antimicrobial activity of SMAP-29 has been tested against a number of gram-positive and gram-negative bacteria and fungi. All bacteria strains tested were highly susceptible to SMA-29 with minimum inhibitory concentrations (MIC) in the 0.12-2 μM range of concentration, which suggest that SMAP-29 is a potent peptide with a wide spectrum of activity (Skerlavaj Barbara et al., 1999; Tomasinsig and Zanetti, 2005).

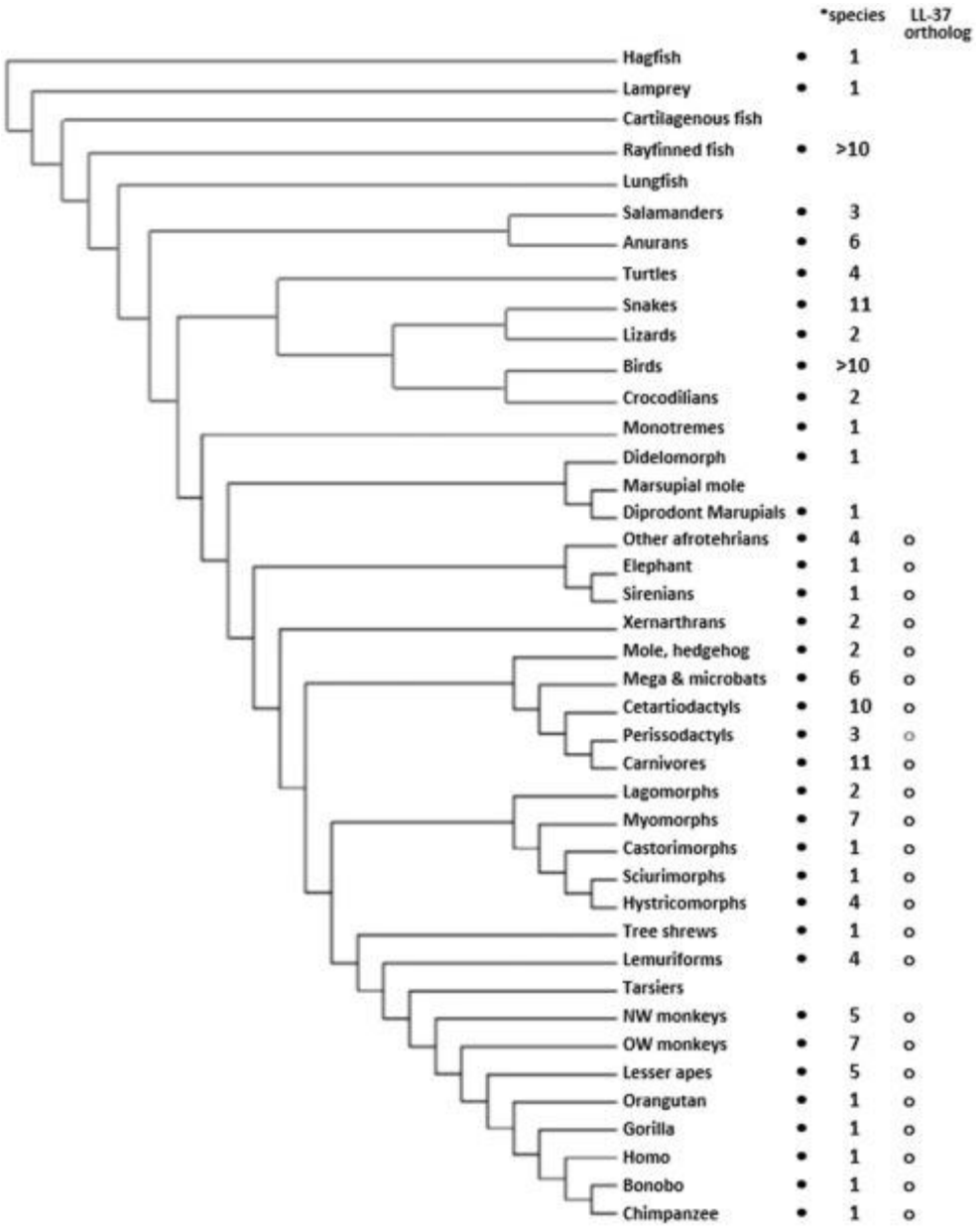


Figure 4. Phylogenetic relationships of vertebrate animal groups in which cathelicidins have been identified.

Cladogram showing the phylogenetic relationships in a reverse order from *homo.* The cathelicidin sequences have been identified for 133 species of vertebrates, 85 of which are mammalian species. ● Symbol indicates that cathelicidins have been identified and, next to it in what number of species within that group. ○ Symbol indicates the presence of a cathelicidin, that based on the c-terminal of CHDP domain, is a likely orthologue of LL-37. [image taken from Xhindoli et al.(2016)].

1.2.2.1. Antibacterial activity of cathelicidins

Cathelicidins have been shown to have antimicrobial activity against multiple gram-positive and gram-negative human pathogens. The physiological significance of the human cathelicidin LL-37 has been demonstrated in patients suffering from the rare condition known as *morbus Kostmann* disease, where neutrophils lack specific granules and are therefore cathelicidin deficient. While these patients also lack other granule contents, they display an increased susceptibility to infection, and frequently develop chronic periodontal disease (Putsep et al., 2002). Further evidence that LL-37 is important in the prevention of bacterial infections can be found by assessing hCAP18/LL-37 expression in patients with *Shigella* infections where cathelicidin expression was shown to be reduced, indicating that cathelicidin-encoding genes can be supressed by pathogens as a part of their mechanism to avoid the immune system defenses, potentially resulting in increased virulence (Islam et al., 2001). In contrast, Bals et al. (1999) demonstrated that the transfer of hCAP18/LL-37 gene into mouse airways resulted in an enhanced clearance of pulmonary *Pseudomonas aeruginosa* infection.

Extensive *in vitro* studies have shown the ability of low, physiologically relevant concentrations LL-37 to inhibit the growth of a variety of gram-negative (*P.aeruginosa*, *S. typhimurium*, *E.coli*) and gram-positive bacteria (*S.aureus, S.epidermis*, *Listeria monocytogenes* and vancomycin-resistant *enterococci*) (J. Turner et al., 1998). However, the antimicrobial activity of LL-37 has been shown to be affected by the presence of high salt conditions (≥100mM NaCl). LL-37 was shown to be highly effective against gram-positive bacteria under low-salt conditions, for instance the MIC of LL-37 against MRSA under low salt conditions was 3 μg/ml, whereas under high salt conditions the MIC of LL-37 was above 79 μg/ml.

In healthy infants and adults, hCAP-18/LL-37 has been detected in bronchoalveolar lavage fluid (BALF) at a concentration of approximately 5 μg/ml and 2 μg/ml, respectively. In newborns with pulmonary infections, the concentrations of LL-37 can reach approximately 25μg/ml and in adult patients with cystic fibrosis LL-37 concentrations reach 15 μg/ml (Chen et al., 2004; Schaller-Bals et al., 2002). The

high concentrations of LL-37 (~ 30 μg/ml) detected in BALF samples during viral pulmonary infections (Schaller-Bals et al., 2002), and specially in psoriatic skin lesions (approximately 304 μM= 1365.8 μg/ml) (Ong et al., 2002) suggest that the concentrations of LL-37 that are effective *in vitro* are compatible with those found *in vivo*. We therefore estimated an *in vitro* concentration of 10 μg/ml of LL-37 to be of relevance to a physiologically normal state, and a concentration of 30 μg/ml to be a physiologically inflammatory concentration of LL-37.

Studies using mice deficient in the murine cathelicidin (mCRAMP) have shown mice to be more susceptible to bacterial infections when lacking cathelicidin, displaying considerably more susceptibility to *Pseudomonas aeruginosa* infection and keratitis in the cornea (Huang et al., 2007) together with delayed clearance of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* from the lungs (Kovach et al., 2012). Additional studies using this model have shown decreased protection against the enteric pathogen *Citrobacter rodentium* (Iimura et al., 2005), increased susceptibility to urinary tract infections caused by *E.coli* (Chromek et al., 2006), and decreased protection against RSV infection (Currie et al., 2016).

1.2.2.2. Immunomodulatory functions of cathelicidins

In vivo, the mechanisms underlying the antimicrobial and protective role of these peptides remain to be fully elucidated, as both direct bacterial killing and the ability to modulate the host innate immune response to infection are likely key properties in terms of cathelicidins contributing to host defence. For instance, a study by Barlow et al. (2010) demonstrated the ability of LL-37 to induce apoptosis of *pseudomonas aeruginosa* –infected airway epithelial cells, suggesting that the peptide is able to modulate the hosts innate immune response to infection by inducing apoptosis of infected cells and therefore promoting the clearance of the respiratory pathogen.

Historically, the primary function of cathelicidins and other host defense peptides was first characterized to be through direct antimicrobial activity, despite in some cases, variable antimicrobial activities against pathogens (Fjell et al., 2012).

Moreover, these antimicrobial activities are frequently lost at high, but physiologically relevant, concentrations of salt and in the presence of serum/plasma (Ciornei et al., 2005; Johansson et al., 1998). However, in contrast to their antimicrobial activity, later studies have characterized the immunomodulatory activities of these peptides under physiologically relevant conditions which point towards immune modulation as being another primary role of these peptides in the host (Bowdish et al., 2006).

Cathelicidins have multiple functions in immunity and inflammation and their anti-infective properties have now been largely attributed to their capacity to mediate these immunomodulatory functions. The human cathelicidin LL-37 has shown chemotactic effects *in vitro*, promoting selective migration of different immune cells such as neutrophils, immature dendritic cells (iDC), monocytes, eosinophils, T cells to a site of infection (Agerberth et al., 2000), and the porcine cathelicidin PG-39 exhibits direct chemotactic effects for iDCs, T cells and neutrophils (J Huang et al., 1997; Tjabringa et al., 2006). Other studies have revealed that LL-37 was shown recruit neutrophils, monocytes and T cells to sites of infection through interactions with the formyl peptide receptor-like 1 (FPRL1) (De Yang et al., 2000).

At low physiologically relevant concentrations (≤1μg/ml), LL-37 is also able to promote chemotaxis of immune cells in an indirect manner, by inducing the production of chemokines, such as RANTES, MCP-1, IL-8, MIP-1β and -3α, from immune and epithelial cells (Bowdish et al., 2006; Montreekachon et al., 2011; Mookherjee et al., 2006; Scott et al., 2002). The study by Scott et al. (2002) also demonstrated that LL-37 upregulates the surface expression of chemokine receptors in macrophages, such as CXCR-4, CCR2, and IL-8RB.

The human cathelicidin was shown to chemoattract and degranulate murin, rat and human mast cells, which results in the release of histamine and prostaglandins, subsequently increasing vascular permeability via mast cell activation (Chen et al., 2006; Niyonsaba et al., 2002, 2001).

The human cathelicidin, LL-37, has previously shown to have contrasting effects on apoptotic cell death pathways in different cells (Barlow et al., 2010, 2006; Lau et al., 2006; Li et al., 2009; Suzuki et al., 2011). For example, it was shown that LL-37

preferentially induced the apoptosis of *Pseudomonas aeruginosa*-infected bronchial epithelial cells, via induction of mitochondrial membrane depolarization, cytochrome c release and activation of caspase-9 and -3 (Barlow et al., 2010). In addition, although not in the context of infection, LL-37 was shown to induce apoptosis in primary epithelial cells possibly involving the activation of $P2X_7$ receptor, which has been shown to be a partial receptor for LL-37 –host cell interaction and signalling (Barlow et al., 2006). In contrast, in keratinocytes, LL-37 was shown to supress camptothecin-mediated apoptosis via a COX-2/PGE-2 antiapoptotic pathway (Chamorro et al., 2009). A study by Barlow et al., (2006) provided evidence relative to the capacity of LL-37 to suppress neutrophil apoptosis, demonstrating the potential of cathelicidins in protecting the host against bacterial invasion by prolonging the lifetime of neutrophils. LL-37 supressed neutrophil apoptosis, signalling through P2x7 and G-protein-coupled receptors other that FPRL1, and by inducing the expression of Mcl-1, an anti-apoptotic protein, and inhibiting BID and caspase-3 activity.

At sites of inflammation, the levels of LL-37 are likely to be high (≥30 μg/ml) due to the presence of recruited neutrophils and epithelial cells which can release LL-37 into the microenvironment (Schaller-Bals et al., 2002). High concentrations of LL-37 (≥30 μg/ml) have been shown to be a potent modifier of dendritic cell (DC) differentiation, demonstrating an important link between the innate and adaptive immune responses (Davidson et al., 2004). In addition, immature DC exposed to LL-37 were shown to substantially change their phenotype, which was characterised by an increased expression of the antigen-presenting molecule HLA-DR and the co-stimulatory molecule CD86 (Bandholtz et al., 2006). The murine cathelicidin mCRAMP has also been implicated in the regulation of adaptive immune responses, as a study by Kin et al. (2011) demonstrated that mCRAMP has the ability to differentially regulate T- and B- cell functions.

Cathelicidins are known to influence several other immunomodulatory functions, such as promotion of wound healing, angiogenesis (capillary growth) and arteriogenesis (pre-existing vessels growth). LL-37 was shown to induce

neovascularization in the chorioallantoic membrane and in a rabbit model of hind-limb ischemia, as well as, increasing the proliferation and formation of vessel-like structures in endothelial cells, again via the receptor FPRL1. Angiogenesis was also shown to be induced *in vivo* and *in vitro* by the porcine cathelicidin PR39, and this was achieved through peptide-mediated inhibition of the degradation of hypoxia-inducible factor 1α (HIF-1α) by the ubiquitin-proteasome system (Li et al., 2000). HIF-1α is known to regulate the expression of various angiogenesis-related genes, such as VEGF and FLT1 (Gerber et al., 1997).

A number of *in vivo* models of infection and sepsis have shown that cathelicidins, such as LL-37 and the bovine cathelicidin BMAP-28, can modulate the host immune responses for the resolution of pathogen-induced inflammation (Cirioni et al., 2006; Giacometti et al., 2004). The anti-inflammatory activity of LL-37 has been shown to be targeted and specific. LL-37 was shown to selectively modulate inflammatory responses in LPS-stimulated THP-1 cell line and human PBMCs. The peptide was shown to significantly inhibit the expression of LPS induced-pro-inflammatory genes, including TNF-α, NFkB1 (p105/p50) and TNFAIAP2, but was shown not to inhibit, to the same extent, the LPS- induced expression of some of the known negative regulators of NFkB such as TNFAIP3, TNIP3 and NFkBIA (IkBa) (Mookherjee et al., 2006).

The gram-negative bacterial molecule LPS is a potent TLR4 agonist and engagement with this receptor can result in the translocation of the pivotal transcription factor NF-kB from the cytoplasm to the nucleus, resulting in the transcription of genes encoding for cytokines and chemokines, including TNF-α, IL-1β, IL-6 and IL-8 (Jagielo et al., 1996). The mechanism behind LL-37-mediated selective regulation of inflammatory genes is thought to be related to its capacity to supress NF-kB translocation, which can lead to the dysregulation of TLR-induced transcriptional responses (Mookherjee et al., 2006). In addition, LL-37 was shown to modulate the LPS-induced pro-inflammatory responses in macrophages both *in vivo* and *in vitro*, by suppressing LPS-induced TNFα and NO production while preserving important macrophage functions, such as ROS production, and the ability to phagocytose and kill bacteria (Brown et al., 2011). In similarity, LL-37 was shown to reduce LPS-

induced IL-8 and MCP-1 production in the A549 human lung epithelial cell line (Scott et al., 2002). However, it is notable that the role of the peptide in modulating inflammation can be paradoxical and is dependent upon the context in which a cell is exposed to both the peptide and LPS, as it has been shown that, in some instances, the peptide can modulate a pro-inflammatory response. Studies have demonstrated that LL-37 stimulation of LPS-primed monocytes can activate the inflammasome and induce the production of IL-1β and IL-18 via $P2X_7$ receptor (Elssner et al., 2004; Kahlenberg et al., 2013).

It is clear that the immunomodulatory activities of cathelicidins involve a complex array of factors, and mechanistic studies have proposed the involvement of several signalling pathways (i.e. NFkB, p38 and JNK, MAPK, and PI3K) which are activated depending on the intracellular uptake, endocytic mobilization and the interaction with a number of receptors. Intracellular uptake of LL-37 has been shown to be particularly important for the immunomodulatory activity of the peptide. LL-37 was shown to be actively taken up by A549 cells and eventually localise to the perinuclear region in a microtubules-dependent manner (Lau et al., 2005). However, the roles of specific receptors involved in the cellular uptake of LL-37 are not yet fully elucidated. Cathelicidins have been shown to interact with multiple receptors, and this interaction mediates different events depending on the cell type and exogenous stimuli.

LL-37 has been shown to be a direct chemoattractant for neutrophils, monocytes and T cells through formyl peptide receptor like-1, a G_i protein-coupled receptor (De Yang et al., 2000). It is thought that the interaction between LL-37 and FPRL1 is a low-affinity ligand-receptor interaction. The activation of the chemotactic receptor FPRL1 by LL-37 results in a G protein-mediated signalling cascade which leads to the chemotaxis of leukocytes, as well as increased adhesion, enhanced phagocytosis and increased bacterial killing (Zlotnik et al., 1999). In addition to its chemotactic activity through FPRL1, LL-37 was shown to supress neutrophil apoptosis via the activation of both FPRL1 and $P2X_7$ (Barlow et al., 2006; Nagaoka et al., 2006). Both LL-37 and mCRAMP are reported to act as agonists for the purinergic nucleotide receptor $P2X_7$, in monocytes and macrophages, inducing the activation of the inflammasome and the subsequent production of IL1β and IL-18

(Elssner et al., 2004; Kahlenberg et al., 2013). Of relevance, an intracellular receptor, GAPDH, has also been identified as a functional direct binding protein or receptor for LL-37, thus potentially contributing to peptide-mediated innate responses in monocytes and macrophages (Mookherjee et al., 2009). Furthermore, LL-37 activation of $P2X_7$ was shown to enhance COX-2 and PGE2 production via activation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) in human gingival fibroblasts (Chotjumlong et al., 2013).

LL-37 has also been proposed to transactivate the epidermal growth factor receptor, EGFR, in airway epithelial cells and keratinocytes, inducing cytokine release and cell migration (Tjabringa et al., 2003; Tokumaru et al., 2005). LL-37-induced keratinocyte migration involved STAT3 signalling and LL-37-mediated IL-8 release from airway epithelial cells has been shown to involve MAPK/ERK signalling which are both downstream of EGFR transactivation. LL-37 was also shown to be a partial agonist for insulin-like growth factor 1 receptor (IGF-1R), a receptor linked to cancer development, and this interaction is known to result in phosphorylation and ubiquitination of the receptor with downstream activation of MAPK/ERK signalling (Girnita et al., 2012). However, other specific host cell receptor interactions, and downstream mediation of immune function are not completely understood and require further characterisation.

In addition to their ability to modulate immune responses, dysregulation of LL-37 in humans has been associated with the development of pathological conditions. LL-37 has been implicated in psoriasis pathogenesis, which is a common autoimmune disease of the skin. Lande et al. (2007), showed the ability of LL-37 to bind to self-DNA forming aggregates that could be delivered to endosomes in plasmacytoid dendritic cells (pDCs), triggering TLR9 and inducing a potent interferon response. pDCs can sense viral and bacterial DNA through TLR9 receptors located in endosomes which, in turn, induce a type I response. Importantly, in healthy individuals, pDCs do not respond to self-DNA, although this pathway appears to be altered in psoriasis patients. More recently, a study by Takahashi et al., (2018) provided new insights into the mechanism by which LL-37 drives auto-inflammatory responses in the skin, such as psoriasis and rosacea. The inflammatory activity of LL-37 was shown to be mediated by a cell-surface-dependent interaction, by which LL-

37 enabled keratinocytes and macrophages to recognise self-non coding U1 RNA, released during cell death, by facilitating binding to the cell surface scavenger receptors that allow the recognition by nucleic acid pattern recognition receptors within the cell.

1.2.2.3. Antiviral activity of cathelicidins

Cationic host defence peptides have been shown to be expressed in response to viral infections, however, little is known about their expression in response to rhinovirus infection. Currently the evidence that hCAP-18/LL-37 is expressed in response to rhinovirus infection is very little. Nevertheless, a recent cohort study of hospitalised infants, which focused upon examining the role of LL-37 in bronchiolitis, revealed that infants with higher serum LL-37 levels were less likely to have RSV, but more likely to have HRV, indicating that elevated LL-37 levels were associated with rhinovirus infection (Mansbach et al., 2017). Other studies have demonstrated that HRV infection induces respiratory epithelial cell expression of both β-defensin-2 and -3 *in vitro* and *in vivo.* However, there is no evidence of their direct antiviral activity against rhinovirus. The increase in both β-defensin-2 and -3 correlates with an increase in IL-8 production; and stimulation of β-defensin-2 and -3 mRNA levels appear to be dependent on viral replication (Proud et al., 2004).

Cationic host defense peptides have primarily been described as antibacterial agents, although as has been highlighted, they have broad immunomodulatory properties and, more recently, have been shown to have a potent antiviral activity.
Cathelicidins have been shown to possess antiviral activity against a substantial number of viral pathogens including IAV (Barlow et al., 2011; Tripathi et al., 2015, 2013), HIV (Bergman et al., 2007; Levinson et al., 2009; Wong et al., 2011), Dengue Virus (Alagarasu et al., 2017; Rothan et al., 2012), RSV (Currie et al., 2013; Harcourt et al., 2016), Vaccinia virus (Dean et al., 2010; Howell et al., 2004) and Adenovirus (Gordon et al., 2005; Smith et al., 2010) (reviewed in Barlow et al., 2014).

The human cathelicin LL-37 was shown to inhibit the replication of HIV-1 in peripheral blood mononuclear cells (Bergman et al., 2007). In IAV studies, the pandemic IAV strains have been shown to be more resistant to innate inhibitors of

seasonal IAV strains, such as human and murine cathelicidins. A recent study compared the antiviral activities of LL-37 and derived fragments against seasonal and pandemic strains of IAV and revealed that the central fragment of LL-37 (GI20) showed greater activity against the pandemic IAV strain than LL-37 *in vitro* (Tripathi et al., 2015). These findings suggest the possibility of engineering new LL-37 derivatives with more potent antiviral activity.

In *in vivo* studies, murine models receiving LL-37 or mCRAMP treatment showed a significant increase in survival following IAV (Barlow et al., 2011) or RSV (Currie et al., 2016) infection compared to saline treated mice. In addition, the *Camp* $^{-/-}$ mice were shown to develop a more severe disease that the wild-type mice during RSV infection, demonstrating the important role of endogenous cathelicidins in host defense against this respiratory pathogen (Currie et al., 2016).

LL-37 has also been shown to target viral envelop proteins inhibiting virus entry into the cells. Molecular docking studies revealed LL-37 binding to the envelop E-protein of dengue virus type 2 (Alagarasu et al., 2017). The underlying antiviral mechanism of cathelicidin peptides appears to be partly due to direct effects on the virus envelope, and this has been demonstrated in a number of detailed *in vitro* studies. Both LL-37 and mCRAMP can damage the viral envelope of vaccinia virus (Dean et al., 2010), IAV (Tripathi et al., 2013), and Herpes simplex virus (Gordon et al., 2005).

Cathelicidins can also inactivate non-enveloped viruses such as adenovirus and rhinovirus (Barlow et al., 2014; Gordon et al., 2005; Sousa et al., 2017). In fact, the human cathelicidin LL-37 was shown to inhibit human adenovirus replication in A549 cells (Barlow et al., 2014). Optimal inhibition of virus replication is demonstrated when the virus is pre-incubated with the peptide prior to host cell infection, although other studies have shown that host cell pre-treatment or delayed treatment with exogenous LL-37 also inhibited RSV (Currie et al., 2013; Harcourt et al., 2016), IAV (Tripathi et al., 2013) and Adenovirus (Barlow et al., 2014) replication to an extent. *In vivo* studies have further demonstrated the ability of LL-37 to modulate inflammatory responses to viral infection by inhibiting excessive inflammation in IAV-infected mice (Barlow et al., 2011). This demonstrates that the mechanisms underpinning the antiviral activity of

cathelicidins are complex and can potentially include direct interaction with the virus particles by directly damaging the viral envelope or capsid, or by binding to specific proteins present in the envelope blocking viral entry. The interaction with host cells modulating inflammatory and innate immune responses to viral infection is also likely to be critical.

As previously mentioned there are no specific effective treatments or vaccination available for human rhinovirus infection. We will show here, in agreement with other studies, that exogenous and vitamin D-induced LL-37 has potent direct antiviral activity against HRV (Schögler et al., 2016; Sousa et al., 2017; Telcian et al., 2017). We also assess the ability of LL-37 to induce cell death in HRV-infected airway epithelial cells in the context of previous studies utilising bacteria (Barlow et al., 2010). Determining the impact of cathelicidins on rhinovirus infections may lead to the development of therapeutic strategies aimed at the prophylactic modulation of endogenous cathelicidin expression and/or the development of cathelicidin-derived analogues for the treatment of infected individuals.

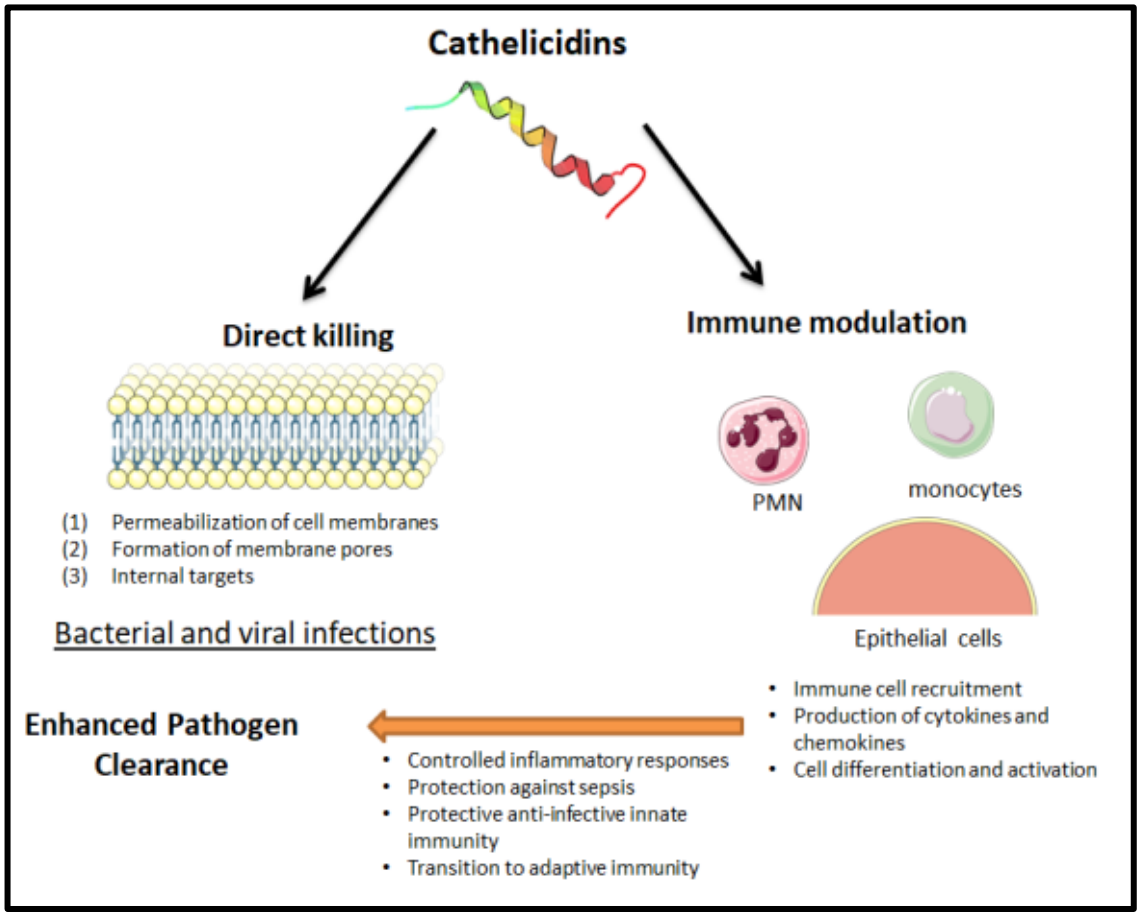


Figure 5. Functions of cathelicidins.

Cathelicidins protect against infections by directly targeting the pathogen or by modulating the host's immune responses. [Adapted from Mookherjee and Hancock, 2007)]

1.3. Aims and hypothesis

Human rhinovirus is associated with increased morbidity and mortality especially amongst individuals with underlining respiratory conditions, immunocompromised individuals, elderly and young children. Due to the lack of alternative and effective treatments or vaccination for HRV infections, novel antiviral approaches are urgently required. Cathelicidins, a key component of the innate immune system, have shown potent antiviral activity against a number of viruses. Previous work (Barlow et al., 2011) together with our initial experiments has established an antiviral role for cathelicidins in rhinovirus infection. This study aims to investigate the antiviral activity of the human cathelicidins LL-37 against HRV, via characterisation of direct antiviral activity, modulation of inflammation and cell death.

The hypotheses of this study are as follows:

(1) The antiviral activity of LL-37 against rhinovirus will likely be attributed to both direct antiviral and immunomodulatory activity, via inhibition of HRV replication, viral lysis, alterations in binding and host cell protection against HRV-induced cytopathic effects.
(2) The human cathelicidin LL-37 will modulate the pro-inflammatory cytokine and chemokine response of viral infected respiratory epithelial cells.
(3) LL-37 will affect cell death pathways of HRV-infected cells via the modulation of apoptotic pathways.
(4) LL-37 will act on several intracellular and cell-surface targets, inducing a protective antiviral state in treated airway epithelial cells.

CHAPTER 2

General Methods and Materials

Chapter 2. General Materials and Methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, Foetal bovine serum (FBS), non-essential amino acids (NEAA), Bovine Serum Albumine fraction V (BSA), Triton-X-100, Tween®20, Polyinosinic:polycytidylic acid (poly I:C), RNAzol-RT, Saponin and DNA damaging agent Camptothecin were all purchased from Sigma-Aldrich, Irvine, UK. Iscove's Modified Dubecco's Medium (IMDM) GlutaMAX™ Supplement, 0.05% trypsin-EDTA, Penicillin-Streptomycin (10,000U/ml) and DPBS were all purchased from GIBCO™, ThermoFisher Scientific, Loughborough, UK. Utroser™ G Serum Substitute was purchased from Pall Corporation, Portsmouth, UK. The human fibronectin (1 µg/ml) was purchased from Merck Millpore EMD, Watford, UK. The human LDLr APC conjugated antibody, LDLr isotype control (mouse IgG 1 APC-conjugated antibody) and Cultrex Mouse Collagen IV (0.5 µg/ml) were purchased from R&D, Abingdon, UK. The human CD54 PE conjugated antibody and ICAM-1 isotype control (PE mouseIgG1, k isotype control FC antibody) were purchased from BioLegend, London, UK. The human vLDLr FITC conjugated antibody was purchased from LSbio, Nottingham, UK. The antibodies pan enterovirus monoclonal antibody (Mouse/IgG2a, L66J), goat anti-mouse IgG, Alexa Fluor® 488 and F (ab')2 anti-Mouse IgG PE (A10543) were all purchased from ThermoFisher Scientific, Loughborough, UK. The anti-cleaved caspase 3 antibody was purchased from Cell signalling Technology, Hitchin, UK. The goat BV421-anti-rabbit was purchased from BD Horizon™, BD Biosciences, UK. Vectashield Hardset mounting medium with 49, 6-diamidino- 2-phenylindole (DAPI) was supplied by Vector Laboratories, Peterborough, UK. RT-PCR primers were ordered from Eurofins (Ebersberg, Germany) unless otherwise stated. The RNeasy mini kit was supplied by Quiagen, Manchester, UK. CytoTox 96 Non-Radioactive Cytotoxicity Assay (LDH) was supplied by Promega, Southampton, UK. FITC Annexin V Apoptosis Detection Kit I was purchased from BD Pharmingen™, Oxford, UK.). ELISA kits were purchased from R&D (Abingdon, UK), unless otherwise stated. Reconstituted peptide masses were characterised by MALDI-TOF analysis (Proteomics Facility Moredun Research Institute, UK).

2.2. Peptide synthesis

The peptides were assembled using the Fmoc/tBu solid-phase peptide synthesis approach (Zughaier et al., 2010) using either model 433A (Applied Biosystems, CA, USA) or model Liberty (CEM Corporation, NC, USA) automated peptide synthesizers followed by cleavage in the trifluoroacetic acid (TFA)/phenol/thioanisole/ethanedithiol/water (10:0.75:0.5:0.25:0.5, w/w) mixture at 25 C for 90 minutes followed by precipitation with cold diethyl ether. The crude peptides were purified by preparative reversed-phase high-pressure liquid chromatography (RP-HPLC). The peptide purity (>98%) was confirmed by analytical RP-HPLC, and the masses were confirmed by mass spectrometry. Following lyophilization, the purified peptides were obtained in the form of their TFA salts; namely: LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES), LL-37 analog having "scrambled" sequence (RSLEGTDRFPFVRLKNSRKLEFKDIKGIKREQFVKIL), termed sLL-37 (control peptide). All peptides were dissolved in endotoxin-free ultrapure water (Sigma-Aldrich, Irvine, UK) and stored at −80 C until use.

2.3. Respiratory epithelial cell culture

A549 (adenocarcinoma human alveolar basal epithelial cells) and HeLa (adenocarcinoma human cervix epithelial cells) were sourced from the European Collection of Authenticated Cell Cultures (Public Health England, Salisbury, UK). The 16HBE14^{o-} transformed human bronchial epithelial cell line was a kind gift from former Professor Dieter Gruenert (University of California, San Francisco, USA). A549 and HeLa cells were cultured in Dulbecco's modified eagle medium high glucose (DMEM, 4.5 g/L glucose), supplemented with 1% Penicillin-Streptomycin, and 10% fetal bovine serum (FBS). 16HBE14^{o-} cells were cultured in Iscove's modified Dulbecco's medium (IMDM), GlutaMAX™ supplemented with 1% streptomycin-penicillin and 10% FBS. For 16HBE14^{o-} cell culture, flasks were coated with a basement layer of Cultrex mouse collagen IV (0.5 µg/ml), human fibronectin (1 µg/ml) and bovine serum albumin fraction V (100 µg/ml), before seeding cells, for cell adhesion, in order to allow the formation of tight junctions in this cell line. All cell lines were grown at 37^{o}C in a humidified incubator with 5% CO_2. Cells were detached with 0.05% Trypsin-EDTA and seeded at 5x10^{4}cells/ml (16HBE14^{o-} cells) or

$1x10^5$cells/ml (A549 cells) for 12-well plate and allowed to grow for 24h prior to experiments. The passage number was controlled and all experiments were performed with low passage number.

2.4. Viral propagation and infection

HRV1B viral stocks (Public Health England Virus Collection, Salisbury, UK) were propagated in HeLa cells grown at 33^{o}C/ 5% CO_2. HeLa cells were previously tested for LDLr and ICAM-1 surface expression by flow cytometry and exhibited high basal levels of these receptors. Briefly, HRV1B stocks were exposed to confluent monolayers of HeLa cells in serum free DMEM for 2h at 33^{o}C. Cells were then washed and left in DMEM supplemented with 5%FBS. After 5 days, HRV infected or non-infected cells (HeLa lysates control) were subjected to three freeze-thaw cycles, harvested and spun to remove cellular debris (3000xg for 0.5h at 4^{o}C), and then aliquoted and frozen at -80^{o}C.

2.5. RNA isolation

Total RNA was extracted using RNAzol® RT (Sigma-Aldrich, Irvine, UK) or RNeasy mini kit (Quiagen, Manchester, UK) according to manufacturer's protocol. RNA concentrations were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Loughborough, UK). RNA integrity was determined using an Agilent Bioanalyser (Agilent, Craven Arms, UK), where RIN≥8 was used as a quality filter for further downstream analyses.

2.6. qPCR

Total RNA (0.5-1µg) was transcribed to cDNA using Precision RT all-in-one mix kit (PrimerDesign Ltd, Camberley UK) as per manufacturer's instructions. qPCR reactions included cDNA (25 ng), 250 nM specific primers and SYBR green mastermix (PrimerDesign Ltd, Camberley UK) in total volume of 20µl. qPCR was performed using the StepOnePlus instrument (Applied Biosystems, Warrington, UK). A panel of six human reference genes were evaluated using a geNorm kit (Primerdesign Camberley,UK) and qbase^{+} (Biogazelle Zwijnaarde, Belgium) software. The geometric means of *ACTB* and *GAPDH* genes were selected as the most stable

combination for normalization in 16HBE14^{o-} cells. The $2^{\Delta\Delta Ct}$ method was used and data is represented as fold changes over untreated cells (Pfaffl MW, 2004).

ΔΔCT= (CT(TARGET, UNTREATED - CT(REF, UNTREATED))-(CT(TARGET, TREATED)-CT(REF, TREATED))

2.7. ELISAs

Supernatants from experiments were analysed for the release of IL-8, IL-6, CCL5 and IFN1β by ELISA, as per the manufacturer's instructions. The human IL-8 DuoSet ELISA DY208-05, Human IL-6 Duoset ELISA DY206-05 and Human CCL5/RANTES DuoSet ELISA DY278-05 were purchased from R&D, Abingdon, UK and the human IFN β ELISA kit was purchased pbl Assay Science, (Piscataway Township, USA).

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 6.0. (GraphPad Inc, San Diego, CA, USA). Data are presented as means ± SEM. Data were analysed using the appropriate statistical test and post-test as stated in the figure legends. A p-value ≤0.05 was considered statistically significant.

CHAPTER 3

Characterisation of airway epithelial cell models of rhinovirus infection

Chapter 3. Characterisation of airway epithelial cell models of rhinovirus infection

3.1. Introduction

3.1.1. Rhinovirus Infection of the airway epithelium

Human rhinoviruses replicate in epithelial cells of the upper and lower airway tract. HRV-B and HRV-A species access the cell either via ICAM-1, known as the major group of viruses (Greve et al., 1989) or via LDLr or vLDLr, known as the minor group of viruses (Hofer et al., 1994; Nicodemou et al., 2005). The minor group of virus do not access airway cells via ICAM-1, although studies have provided evidence on their ability to upregulate surface ICAM-1 expression in airway cells indicating that the induction of ICAM-1 is not receptor- or serotype restricted (Papi and Johnston, 1999; Terajima et al., 1997). The minor group of virus bind to LDLr or vLDLr, which are typically endocytic-recycling receptors and endocytosis of the virus is thought to occur in a clathrin-dependent manner (Snyers et al., 2003), although some virus can enter cells in clathrin-independent mediated endocytosis. The virus-receptor complex is delivered to early endosomes and later progresses to late endosomes and lysosomes (Schober et al., 1998). The low pH leads to the uncoating of the virus and release of viral RNA into the cytoplasm. In the cytoplasm, the positive RNA stand is used for viral replication and viral protein synthesis. The virus particles are assembled and new, infectious virions are released to infect other neighbouring cells (Figure 6).

The virus is able to reside and replicate within the airway epithelial cell without compromising the host cell viability, as shown by their inability to alter the morphology or integrity of the airway epithelium (Birgit Winther et al., 1984). Instead, the epithelial cells respond to rhinovirus infection by inducing an antiviral and pro-inflammatory response, which contributes to the recruitment of and activation of inflammatory cells, such as neutrophils, eosinophils and T cells (Sanders et al., 1998; Subauste et al., 1995; Wang et al., 1996). HRV infection of airway epithelial cells has been shown to induce a rapid production of several pro-inflammatory cytokines, including IL-8, IL-6, GM-CSF and CCL5 (Kim et al., 2000;

Papadopoulos et al., 2001; Subauste et al., 1995). Increased levels of IL-6 (Zhu et al., 1996), IL-8 (Zhu et al., 1997) and IL-1β (Proud et al., 1994) were also detected in nasal washings taken from rhinovirus experimentally infected subjects. In addition to the production of pro-inflammatory mediators, airway epithelial cells also respond to HRV infection by activating an antiviral signalling pathway. Interferons are an important component of the innate immune response, being able to directly interfere with viral replication thereby limiting the spread of infection (Parronchi et al., 1992). Rhinovirus infection of primary human bronchial epithelial cells has been shown to induce IFN1β and IFN-λ production (Wark et al., 2005).

An important factor which until recently has been neglected are strain specific effects, current *in vivo* and *in vitro* studies have provided an insight on the importance of strain differences for HRV pathogenesis. A study by Rajan et al. (2013), using a co-culture system with airway epithelial cells and PBMCs, demonstrated that both differences in the HRV strains and the source of PBMCs contributed to changes in the expression of several cytokines and chemokines. Interestingly, clinical studies have linked minor group of HRVs to increase rates of asthma exacerbations (Denlinger et al., 2011). *In vitro* studies of airway cell response to rhinovirus infection have been extensively studied; however, the focus of the studies was mainly on HRV-A species (Y. Chen et al., 2006; Proud et al., 2008; Subauste et al., 1995).

In this chapter, we investigated the ability of minor group HRV1B to replicate in alveolar and bronchial epithelial cell lines as well as characterising the antiviral and pro-inflammatory response of these cell models to viral infection. We assessed the direct airway epithelial cell response to HVR infection by investigating the gene expression of pro-inflammatory mediators, IL-8, IL-6 and CCL5, as well as antiviral mediators, IFN1β, induced at different stages of rhinovirus 1B infection in alveolar and bronchial epithelial cells.

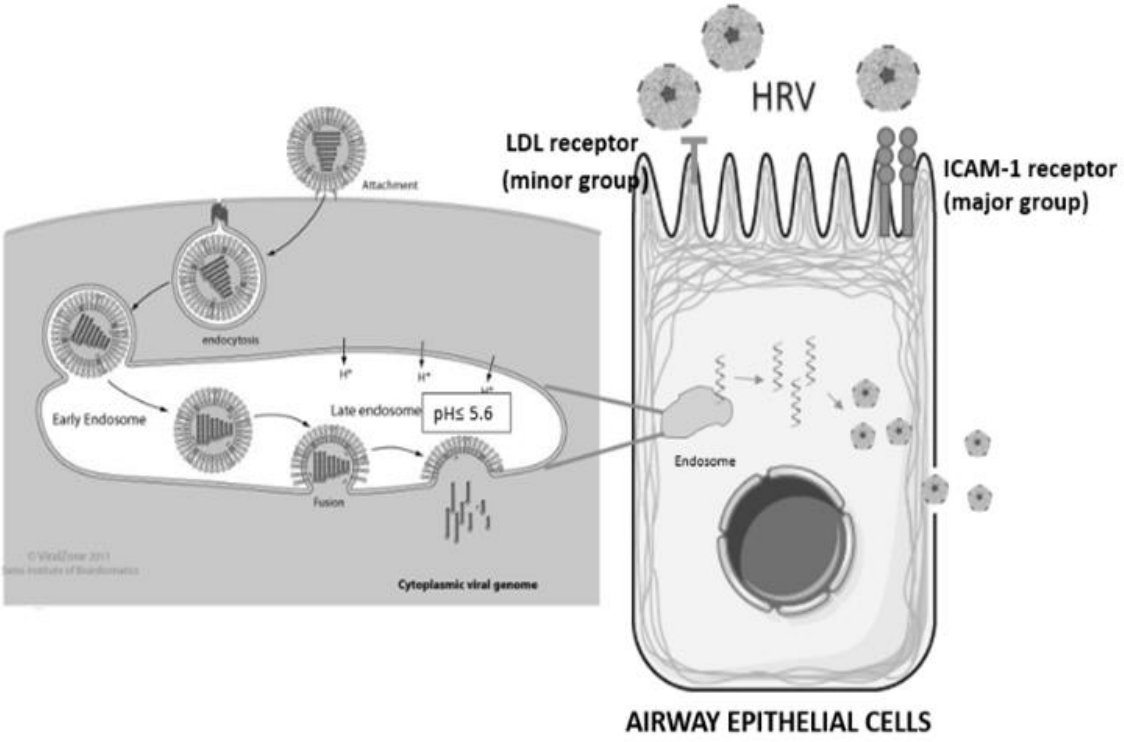


Figure 6. Rhinovirus replication in airway epithelial cells.

HRV bind to ICAM-1 or LDLr, and enter cells by clarthin dependent or independent endocytosis, the virus-receptor complex are delivered to early endosomes and later move to late endosomes and lysosomes. The acidic pH leads to the dissociation from the receptors and to the uncoating of the virus. Viral RNA is released into the cytoplasma and used for viral replication and translation of viral proteins. Image adapted from Swiss Institute of bioinformatics.

3.2. Materials and Methods

3.2.1. Cell culture

A549 (adenocarcinoma human alveolar basal epithelial cells) and HeLa (adenocarcinoma human cervix epithelial cells) were sourced from the European Collection of Authenticated Cell Cultures (Public Health England, Salisbury, UK). The 16HBE14o$^-$ transformed human bronchial epithelial cell line was a kind gift from former Professor Dieter Gruenert (University of California, San Francisco, USA). A549 and HeLa cells were cultured in Dulbecco's modified eagle medium high glucose (DMEM, 4.5 g/L glucose), supplemented with 1% Penicillin-Streptomycin, and 10% fetal bovine serum (FBS). 16HBE14o$^-$ cells were cultured in Iscove's modified Dulbecco's medium (IMDM), GlutaMAX™ supplemented with 1% streptomycin-penicillin and 10% FBS. For 16HBE14o$^-$ cell culture, flasks were coated with a basement layer of Cultrex Mouse Collagen IV (0.5 µg/ml), human fibronectin (1 µg/ml) and Bovine Serum Albumine fraction V (100 µg/ml), before seeding cells. All cell lines were grown at 37^{o}C in a humidified incubator with 5% CO_2. Cells were detached with 0.05% Trypsin-EDTA and seeded at 5x10^{4}cells/ml (16HBE14^{o-} cells) or 1x10^{5}cells/ml (A549 cells) for 12-well plate and allowed to grow for 24h prior to experiments.

3.2.2. Viral propagation and infection

HRV1B viral stocks (Public Health England Virus Collection, Salisbury, UK) were propagated in HeLa cells grown at 33^{o}C/ 5% CO_2. HeLa cells were previously tested for LDLr and ICAM-1 surface expression by flow cytometry and exhibited high basal levels of these receptors. Briefly, HRV1B stocks were exposed to confluent monolayers of HeLa cells in serum free DMEM for 2h at 33^{o}C. Cells were then washed and left in DMEM supplemented with 5%FBS. After 5 days, HRV infected or non-infected cells (HeLa lysates control) were subjected to three freeze-thaw cycles, harvested and spun to remove cellular debris (3000xg for 0.5h at 4^{o}C), and then aliquoted and frozen at -80^{o}C.

3.2.3. RNA isolation and qPCR

Total RNA was extracted using RNAzol® RT (Sigma-Aldrich, Irvine, UK) according to manufacturer's protocol. Briefly, A549 cells or 16HBE14^{o-} cells were seeded at 1x10^5 or 5x10^4 cells/well in a 12-well plate, respectively, and incubated at 37^{o}C overnight. The following day, different doses of HRV1B (MOI 0.1, 1, 5) were used to infect cells for 2h at 33^{o}C in serum-free IMDM media. Cells were then washed and re-immersed in fresh media supplemented with 5% FBS for different time-points (ranging from 2h up to 72h). After each time-point, cell supernatants were aspirated and stored at -80^{o}C until further use, and ice-cold 500µl RNAzol® RT was added to each well. Cells were scrapped of the surface of the plate with a cell-scrapper. Cell lysates were stored at -80^{o}C until extraction. Total RNA was isolated using RNAzol® RT according to manufacturer's protocol. RNA concentrations were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Loughborough, UK). RNA integrity was determined using an Agilent Bioanalyser (Agilent, UK), where RIN≥8 was used as a quality filter for further downstream analyses.

For the molecular quantification of viral infectivity, viral RNA copies isolated from cell lysates or cell supernatants were determined using the Genesig Human Rhinovirus (all subtypes) qPCR kit (PrimerDesign, Camberley, UK) according to manufacturer's protocols.

For the determination of IL-8, IL-6, CCL5 and IFN1β gene expression, total RNA (0.5-1µg) was transcribed to cDNA using Precision RT all-in-one mix kit (PrimerDesign Ltd, Camberley, UK) as per manufacturer's instructions. qPCR reactions included cDNA (25 ng), 250 nM specific primers (Table 1) and SYBR green mastermix (PrimerDesign Ltd, Camberley, UK) in total volume of 20µl. qPCR was performed using the StepOnePlus instrument (Applied Biosystems). A panel of six human reference genes were evaluated using a geNorm kit (Primerdesign) and qbase$^+$ (Biogazelle) software. The geometric means of *ACTB* and *GAPDH* genes were selected as the most stable combination for normalization in both 16HBE and A549 cell lines. The $2^{\Delta\Delta Ct}$ method was used and data is represented as fold changes over untreated cells.

Table 1. qPCR primer sequences

Target	Forward	Reverse	Reference
GAPDH	AAGCTCATTTCCTGGTATGACA	TCTTACTCCTTGGAGGCCATGT	(Wang et al., 2012)
ACTB	GGACTTCGAGCAAGAGATGG	AGGAAGGAAGGCTGGAAGAG	(Tarrats et al., 2011)
IL-8	ACTGAGAGTGATTGAGAGTGGAC	AACCCTCTGCACCCAGTTTTC	(Tsai et al., 2009)
IL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC	(Keller et al., 2003)
CCL5	CAGTCGTCTTTGTCACCCGA	CGGGTGGGGTAGGATAGTGA	Designed
IFN1β	TCTGGCACAACAGGTAGTAGGC	GAGAAGCACAACAGGAGAGCAA	(Warnking et al., 2015)

3.2.4. IL-8 ELISA

Supernatants from the different infection time points of 16HBE14o$^-$ and A549 cells were analysed by ELISA for IL-8 release, as per the manufacturer's instructions (human IL-8 DuoSet ELISA DY208-05, R&D, UK).

3.3. Results

3.3.1. Assessment of rhinovirus replication in airway epithelial cell models

In order to determine the ability of HRV1B to replicate in alveolar epithelial cells, the number of viral RNA copies was determined in samples collected from A549 cell lysates at indicated time points. As shown in Figure 7 a higher multiplicity of infection used to infected cells corresponded to a greater viral load. The viral RNA copy number is substantial increased by 2h post infection indicating that the virus is rapidly up-taken by A549 cells. Peak viral loads were observed at 18h post-infection and maintained up to 48h after infection followed by a steady decrease at 72h post infection. This pattern was seen for a MOI 1 and 5, a MOI 0.1 used to infect cells was too low to generate a high viral copy number.

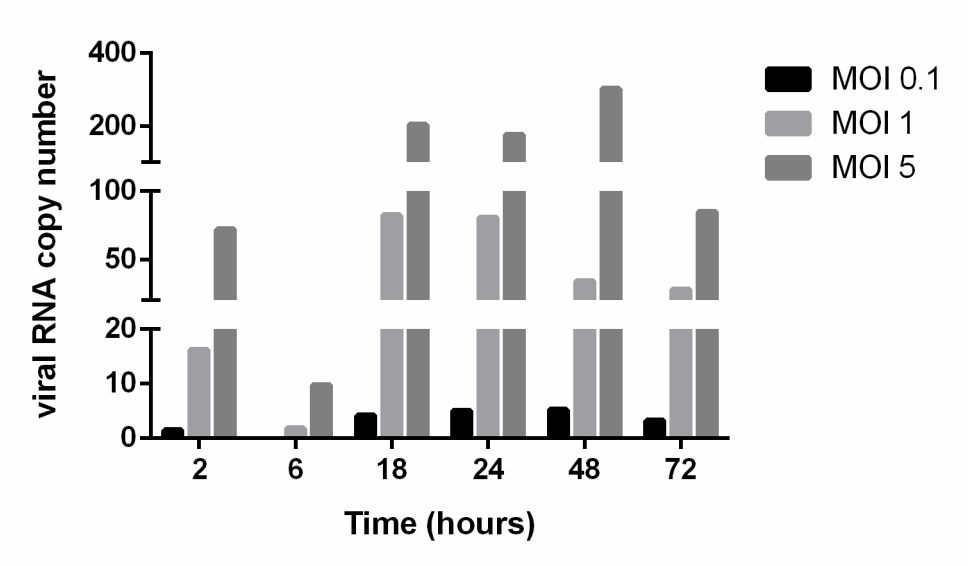


Figure 7.Rhinovirus 1B rapidly enters and actively replicates in A549 cell model of infection.

A549 cells were infected with different MOIs of HRV1B (0.1, 1 and 5) for 2h in serum-free media and washed prior to re-immersing cells in fresh DMEM supplemented with 5% FBS for 2, 6, 18, 24, 48 or 72h at 33^{o}C. Total RNA was isolated from cell lysates and viral RNA copies were determined by qPCR at indicated time points.

To ensure that the observed increases in intracellular viral RNA isolated from A549 cell lysates were associated with an active intracellular replication and adequate assembly of viral replicative units and release into the supernatants, we determined the viral RNA copies in A549 cell supernatants at indicated time points. Figure 8

shows the viral RNA copy number in A549 cell lysates **(A)** and in A549 cell supernatants **(B)** from the same experiment. Results show that at 2h post-infection the viral RNA copy number in A549 cell supernatants was 2.4 times higher compared to intracellular viral RNA copy number. No viral RNA copies were detected intracellularly at 6h post-viral infection (Figure 8A). No infective virus was detectable in cell supernatants within 6h and 18h post inoculation (Figure 8B). In cell lysates peak titers were observed within 48h and declined by 72h after infection (Figure 8A), whereas in cell supernatants viral RNA copies increased from 24 to 72h post-infection (Figure 8B).

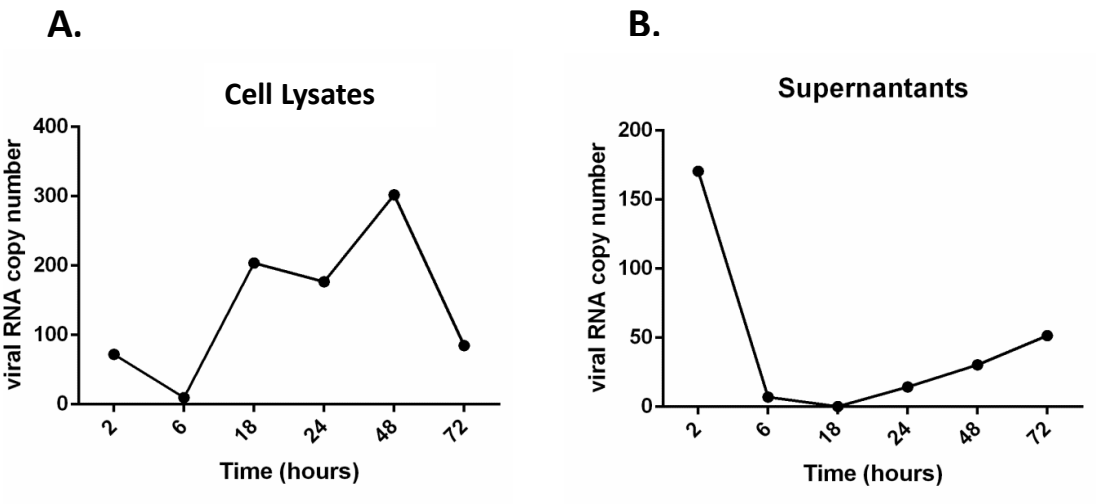


Figure 8.Rhinovirus 1B actively replicates in A549 cell model.

A549 cells were infected with different MOI of HRV1B (0.1, 1 and 5) for 2h in serum- free media and washed prior to re-immersing cells in fresh DMEM supplemented with 5% FBS for 2, 6, 18, 24, 48 or 72h at 33^{o}C. Total RNA was isolated from cell lysates and supernatants and viral RNA copies were determined by qPCR at indicated time points.

In order to determine the ability of HRV1B to replicate in bronchial epithelial cells, the number of viral RNA copies was determined in samples collected from 16HBE14^{o-} cell lysates at indicated time points. As shown in Figure 9 a higher multiplicity of infection corresponds to a greater viral load. A marked increase in viral RNA copies in HRV1B- infected 16HBE14^{o-} cell lysates was observed within 18 and 24h followed by a steady decrease at 48-72h. All multiplicity of infection used to infect 16HBE14^{o-} cells followed the same pattern of replication.

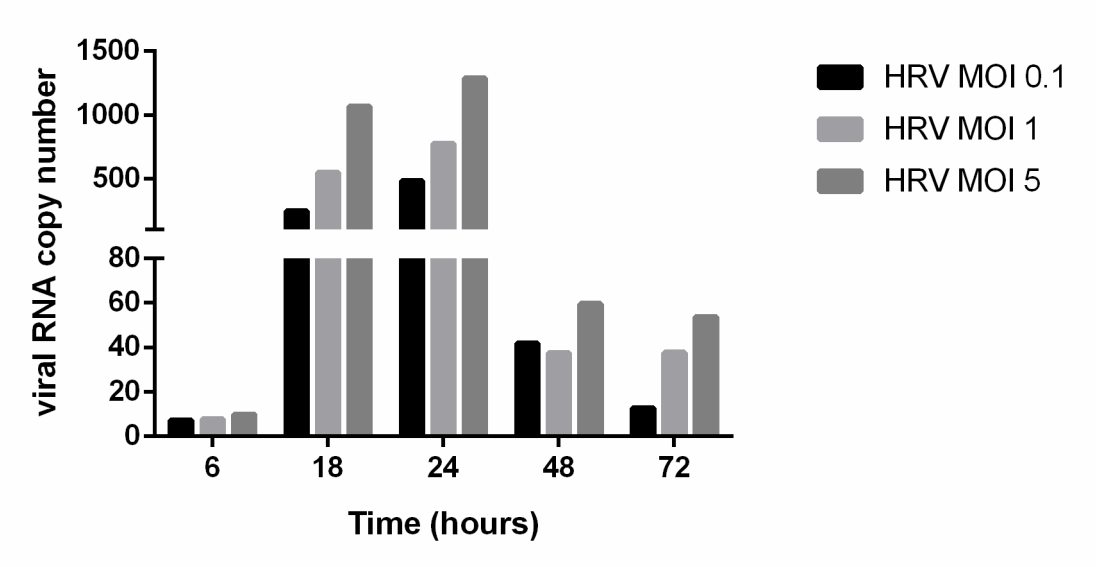


Figure 9. Rhinovirus 1B replicates in bronchial epithelial cells

Bronchial epithelial cells (16HBE14^{o-}) were infected with different MOI of HRV1B (0.1, 1 and 5) for 2h in serum- free media and washed prior to re-immersing cells in fresh IMDM supplemented with 5% FBS for 4, 16, 22, 46 or 70h at 33^{o}C. Total RNA was isolated from cell lysates and supernatants and viral RNA copies were determined by qPCR at indicated time points.

3.3.2. Determination of basal levels of host cell-surface receptors used by HRV to access airway epithelial cells

ICAM-1, LDLr and vLRDL are known to be found in the cell surface of airway epithelial cells and are important receptors in rhinovirus binding, uptake and signal transduction processes. The transcriptional and translational basal levels of ICAM-1, vLDLr and LDLr were investigated in alveolar and bronchial epithelial cells and compared to levels expressed on HeLa cells. Results indicated that 16HBE14^{o-} cells expressed high levels of surface ICAM-1 and LDLr proteins, displaying 95.1% positive cells for both ICAM-1 and LDLR staining. HeLa cells displayed 99.3% positive cells for both ICAM-1 and LDLr staining. A549 cells exhibited 28.4% positive cells for both ICAM-1 and LDLr staining and 71.3% positive cells for LDLr staining (Figure 10A). mRNA levels indicated that all cells lines highly expressed *LDLr* and *vLDLR* genes, whereas the *ICAM-1* gene was highly expressed in 16HBE14^{o-}and HeLa cells, but not in the A549 cell line (Figure 10 B).

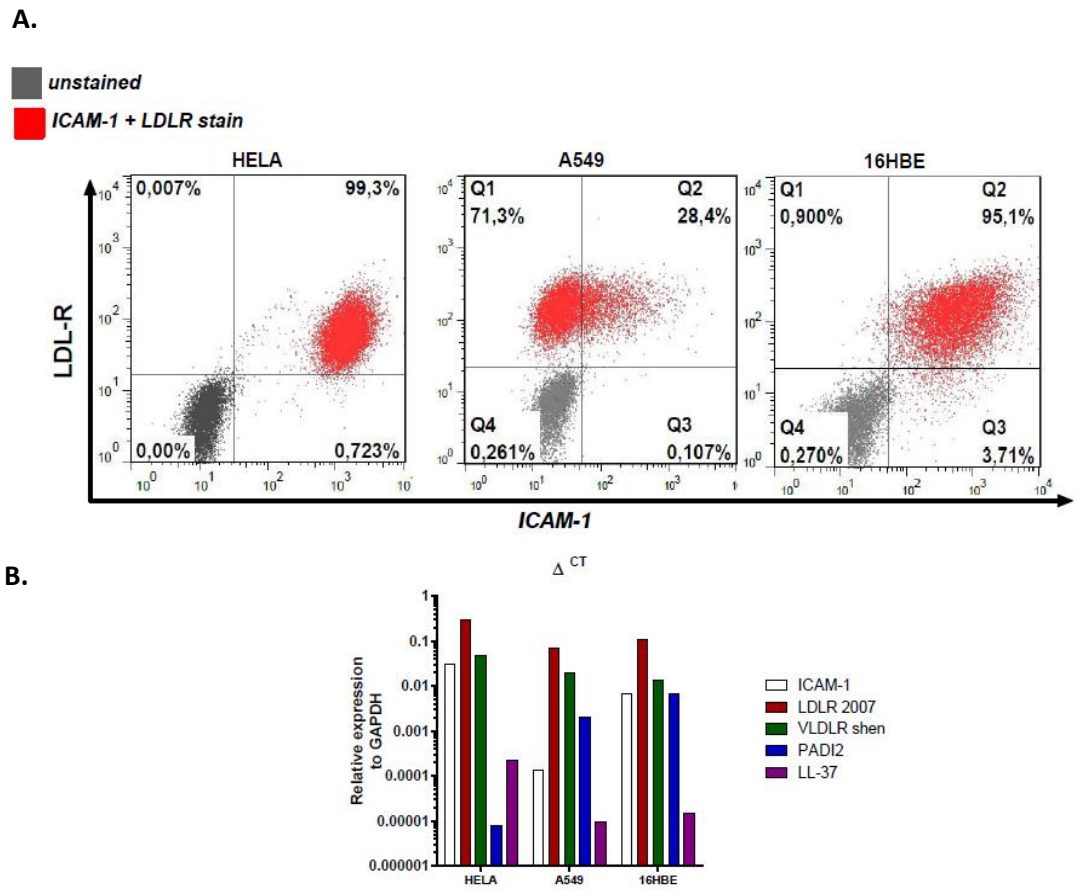


Figure 10. Alveolar and bronchial epithelial cells express high levels of HRV-target host cell receptors.

(A) Surface ICAM-1 and LDLr were measured by flow cytometry on HeLa, A549 and 16HBE cells. All cells were stained for ICAM-1 and LDLr using conjugated antibodies and unstained cells were used as a control to set the negative population. Representative flow cytometric histogram indicates surface expression of ICAM-1 and LDLR in HeLa, A549 and 16HBE14^{o-} cells. (B) ICAM-1, LDLr and vLDLR gene expression were determined in HeLa, A549 and 16HBE cells by qPCR. Relative expression of ICAM-1, LDLr and vLDLr was normalised to GAPDH. Results represent one independent experiment. Results kindly provided by Dr Victor Casanova.

3.3.3. Characterisation of HRV-mediated pro-inflammatory and antiviral response in alveolar and bronchial epithelial cells

In order to determine the antiviral and pro-inflammatory responses of airway epithelial cells to rhinovirus 1B infection, we assessed IFN1β, IL-8, IL-6 and CCL5 gene expression changes as well as IL-8 protein release across different time points in alveolar and bronchial epithelial cell models of infection.

As shown in Figure 11 a greater upregulation of IL-8, IL-6, CCL5 and IFN1β gene expression corresponds with a higher MOI, indicating that a higher viral titer is associated with an increase antiviral and pro-inflammatory response in A549 cells. HRV infection markedly induced IL-8 mRNA expression in a MOI and time-dependent manner (Figure 11A). HRV induced a greater increase in IL-8 mRNA expression at later stages of infection (5.9-fold at 72h, MOI 1 and 7.4–fold at 48h, MOI 5). IL-6 mRNA levels were markedly induced at later stages of infection (i.e. 72h), in which a higher MOI resulted in a 5.6-fold increase in comparison to uninfected A549 cells (Figure 11 B). Early stages (i.e. 6-24h) of HRV infection of A549 cells did not strongly upregulate CCL5 mRNA expression. The maximum upregulation of CCL5 mRNA expression was seen with a MOI 1 at later stages of infection – 72h- reaching 3.77-fold increase in comparison to uninfected cells (Figure 11C). We also observed increased levels of mRNA encoding for IFN1β at later stages of infection, reaching 5.4-fold increase compared to uninfected cells at 48h with a MOI 5 (Figure 11 D). Since the virus inoculum was a crude preparation, we used HeLa lysates as a control, and observed, using a threshold of 2, no upregulation of IL-8, and IL-6, CCL5 or IFN1β expression at any of the indicated time points and doses (Appendix 12).

In addition to determining mRNA fold-changes we assessed the viral copy numbers for the indicated time-points and doses of HRV used for infection. We previously reported this data although the viral load was determined at 2, 6, 18, 24, 48 and 72 post-infection, whereas in this case the viral load is determined at 4, 16, 22, 46 and 70h post-infection (Figure 11E). As shown in Figure 11 a marked increase in viral RNA copies was seen at 18h of infection and was relatively maintained up to 72h of infection. A greater viral load was directly associated with the dose of HRV used to infect cells.

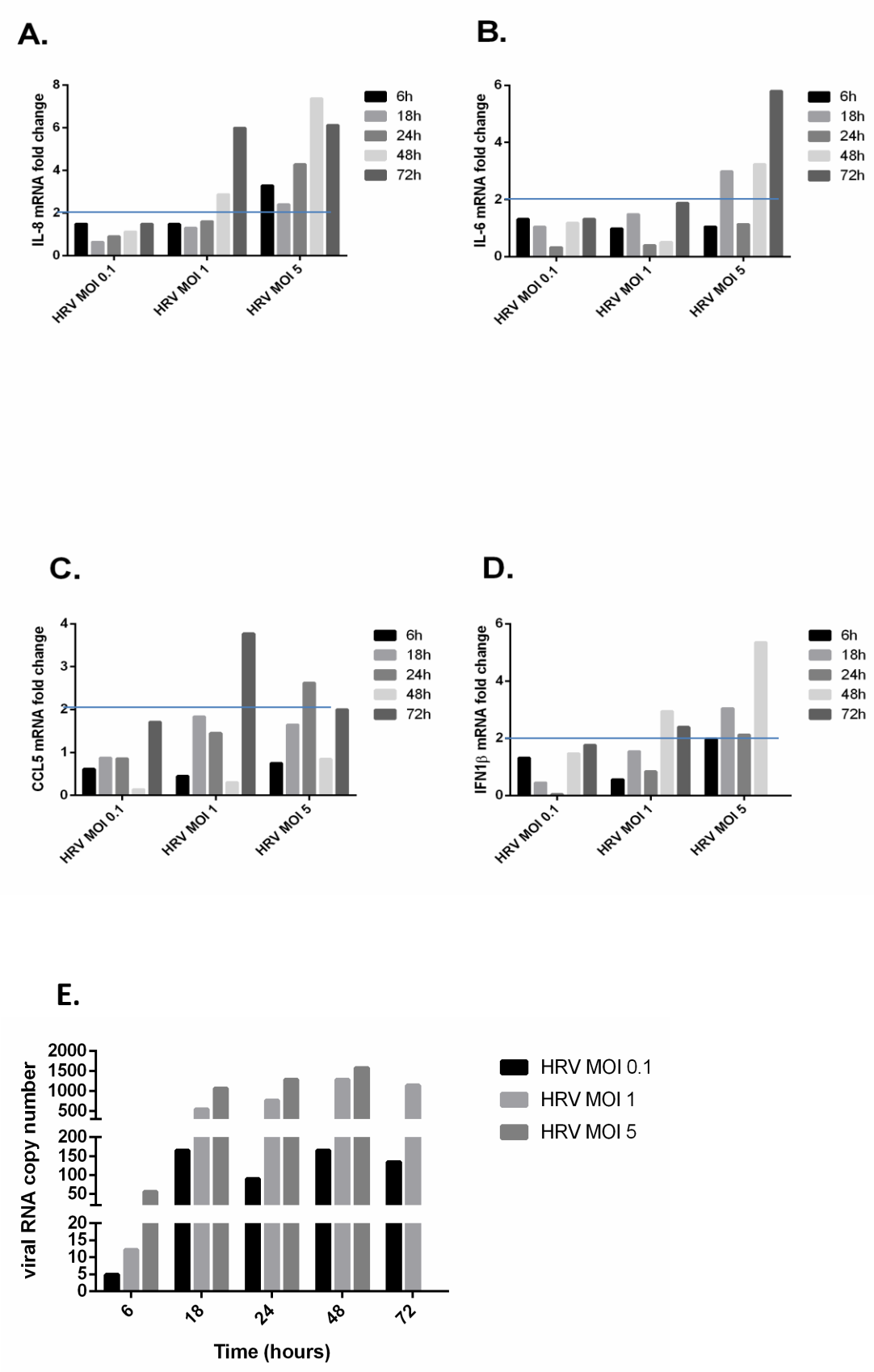


Figure 11.HRV1B infection upregulates IL-8, IL-6, CCL5 and IFNβ gene expression in a MOI and time-dependent manner in A549 cell model.

A549 cells were infected with different MOIs of HRV1B (0.1, 1 and 5) for 2h in serum- free media and washed prior to re-immersing cells in fresh DMEM supplemented with 5% FBS for 4, 16, 22, 46 or 70h at 33^{o}C. Total RNA was extracted from A549 cell lysates and qPCR

was performed to determine viral RNA copies as well as the relative expression of IL-8 (A), IL-6 (B), CCL5 (C) and IFN1β (D). The GAPDH and actin genes were used as reference genes. Results represent one independent experiment.

To confirm changes in mRNA transcript level were translated to changes at the protein level, we assessed cell supernatants for IL-8 protein release at indicated time-points (Figure 12). In agreement with the mRNA transcript levels, HRV infection of A549 cells induced IL-8 secretion in a time-dependent manner. We observed no apparent differences in IL-8 concentration using different MOI to infect cells (i.e. MOI1 and 5). Lower MOI 0.1 did not induce a marked IL-8 release above uninfected cells at any of the time-points indicated. Early stages of infection (i.e. 6-18h – MOI 1 and 5) did not result in a marked increase in IL-8 release compared to uninfected cells. Later stages of HRV infection induced a marked increase in IL-8 secretion compared to uninfected cells (109.2 pg/ml MOI 1 and 107.7 pg/ml MOI 5 compared to 54.2 pg/ml for uninfected at 48h time point and 177.2 pg/ml for MOI 1 and 162.3 pg/ml MOI 5 compared to 96.4 pg/ml for uninfected cells at 72h time point). Poly I:C was used as a positive control and as expected strongly induced IL-8 release in a time-dependent manner.

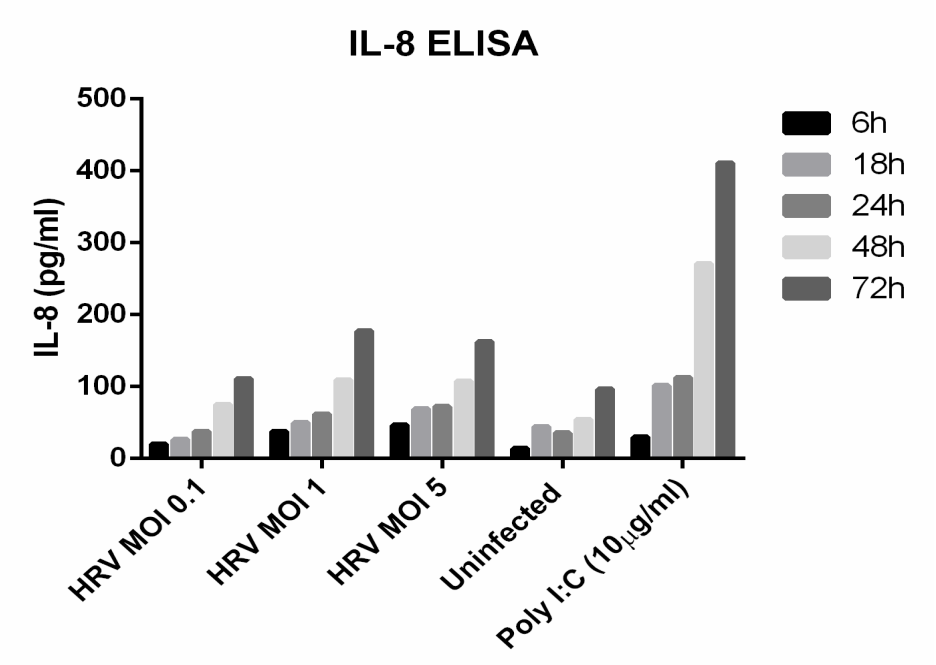


Figure 12.HRV infection induces IL-8 secretion in A549 cell model.

A549 cells were infected with different MOIs of HRV1B (0.1, 1 and 5) for 2h in serum- free media and washed prior to re-immersing cells in fresh DMEM supplemented with 5% FBS

for 4, 16, 22, 46 or 70h at 33^{o}C. A549 cells were exposed to poly I:C (10 μg/ml) for 6, 18, 24, 48 or 72h in DMEM supplemented with 5%FBS. IL-8 protein levels released into cell supernatants were assessed by ELISA at the indicated time points. Results represent one independent experiment.

We determined the antiviral and pro-inflammatory response to HRV infection in bronchial epithelial cells, where 16HBE14^{o-} cells were infected with different multiplicity of infection of HRV and IL-8, IL-6, CCL5 and IFN1β gene expression and IL-8 release were determined at indicated time points (Figure 13).

As shown in Figure 13, mRNA transcript levels of IL-8, IL-6, CCL5 and IFN1β genes follow a similar pattern, where a time-dependent induction occurs at early stages of infection peaking between 18-24h and a steady decrease is observed at later stages of infection, between 48-72h. Infection with HRV induced a marked increase in IL-8 mRNA in a non-MOI and time-dependent manner, in which the highest upregulation was seen at 48h (15.1-fold MOI 0.1, 14.8-fold at MOI 1 and 14.3-fold MOI 5 over uninfected) [Figure 13 A]. IL-6 gene expression was strongly induced at 18 and 24h of infection exhibiting greater changes at MOI 5 (7.4-fold at 18h and 5-fold at 24h over uninfected) [Figure 13 B]. Infection with HRV strongly induced CCL5 mRNA levels. Highest induction of CCL5 mRNA was observed at 24h of infection (33-fold MOI 0.1, 23.7-fold MOI 1 and 67.8-fold MOI 5 over uninfected cells) [Figure 13C]. A similar pattern was seen for IFN1β mRNA, where highest induction was observed after 24h of infection (3653.3-fold MOI 0.1, 2941.6-fold MOI 1 and 8807.8-fold MOI 5 over uninfected), followed by a steady decrease at 48h and at 72h [Figure 13 D]. Since the virus inoculum was a crude preparation, we used HeLa lysates as a control, and observed, using a threshold of 2, no upregulation of IL-8, and IL-6, CCL5 or IFN1β expression at any of the indicated time points and doses (Appendix 13).

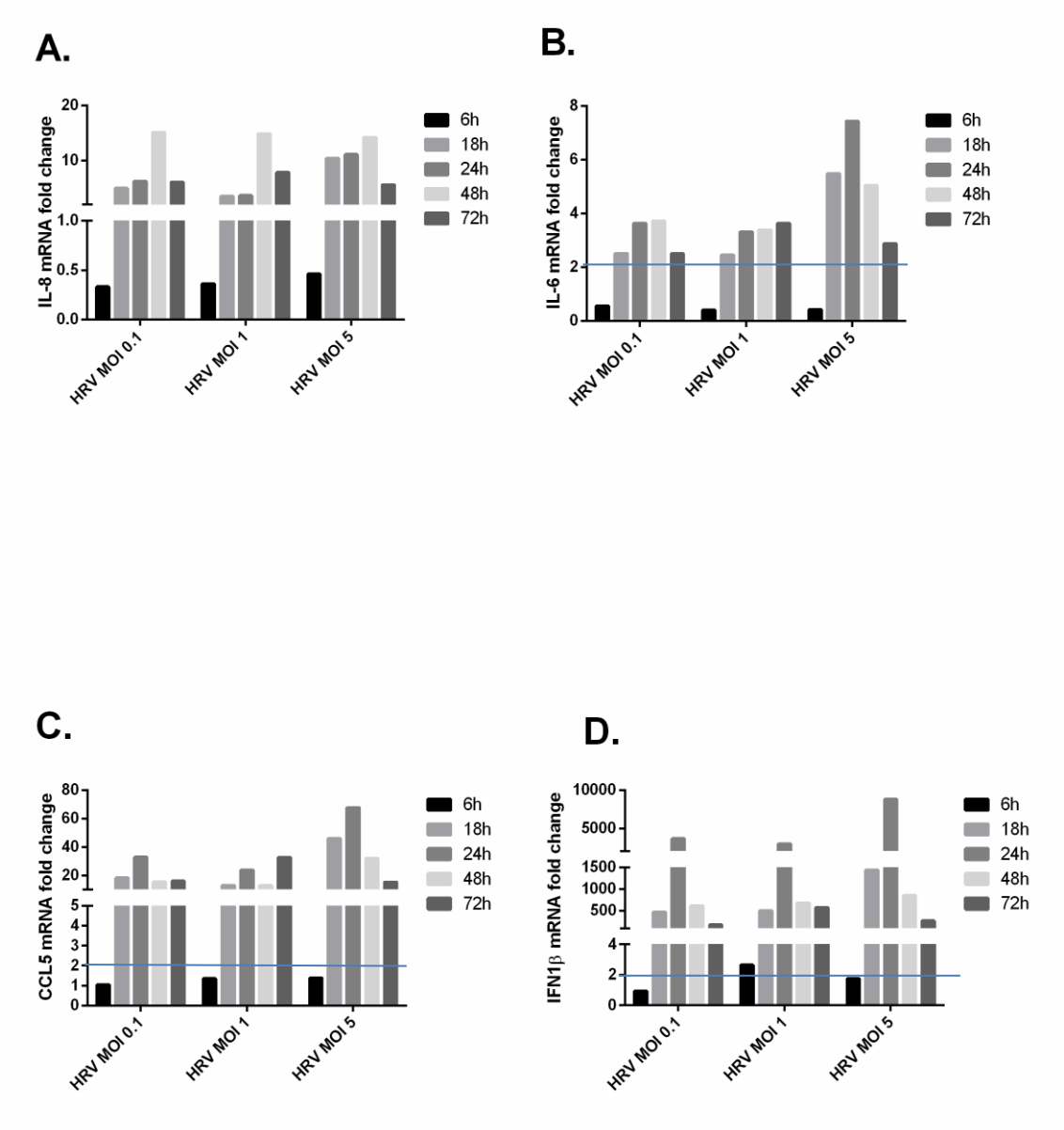


Figure 13. HRV1B infection strongly induces IL-8, IL-6, CCL5 and IFNβ gene expression in 16HBE14^{o-} cell model.

16HBE14^{o-} cells were infected with differing MOI of HRV1B (0.1, 1 and 5) for 2h in serum-free media and washed prior to re-immersing cells in fresh IMDM supplemented with 5% FBS for 4, 16, 22, 46 or 70h at 33^{o}C. Total RNA was extracted from 16HBE cell lysates and qPCR was performed to determine the relative expression of IL-8 (A), IL-6 (B), CCL5 (C) and IFN1β (D). The GAPDH and β-actin genes were used as reference genes. Results represent one independent experiment.

To confirm changes in mRNA transcript level were translated to changes at the protein level, we assessed cell supernatants for IL-8 protein release at the indicated time-points (Figure 14). In contrast to mRNA transcript data, induction of IL-8 release was only observed at later stages of HRV infection where concentrations of IL-8

were dramatically increased compared to uninfected cells (2103.7pg/ml MOI 0.1, 1969.7pg/ml MOI 1 and 2054.9pg/ml MOI 5 compared to 244.5pg/ml for uninfected cells at 72h). In agreement with IL-8 mRNA expression data, induction of IL-8 release was not dependent on the MOI utilised to infect 16HBE cells. Poly I:C was used as a positive control and as expected strongly induced IL-8 release in a time-dependent manner.

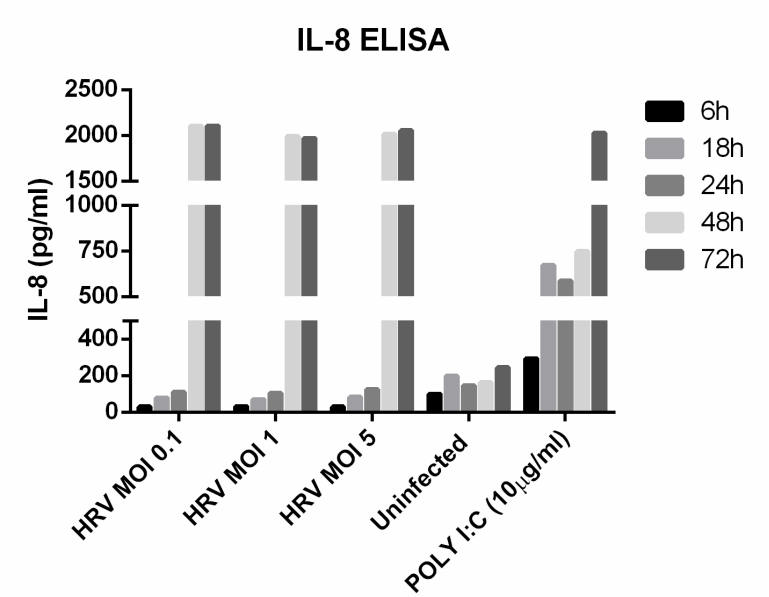


Figure 14. HRV infection induces IL-8 secretion in 16HBE14^{o-} cell model.

16HBE14^{o-} cells were infected with different MOIs of HRV1B (0.1, 1 and 5) for 2h in serum-free media and washed prior to re-immersing cells in fresh IMDM supplemented with 5% FBS for 4, 16, 22, 46 or 70h at 33^{o}C. 16HBE cells were exposed to poly I:C (10 μg/ml) for 6, 18, 24, 48 or 72h in IMDM supplemented with 5%FBS. IL-8 protein levels released into cell supernatants were assessed by ELISA at the indicated time points.

3.3. Discussion

The data in this supplementary chapter provides an analysis of rhinovirus 1B replication cycle and host cell response to viral infection based on a time course using two different cell models of the lower respiratory tract; adenocarcinoma human alveolar epithelial cells (A549 cells) and SV40-immortalized human bronchial epithelial cells (16HBE14^{o-} cells). The respiratory epithelium is the primary site of rhinovirus infection and replication (Arruda et al., 1995). With the development of novel diagnostic techniques, such as PCR based assays, rhinovirus was shown to infect cells of the lower airway tract (Gern et al., 1997; Mosser et al., 2002). Several *in vitro* (Bochkov et al., 2010; Y. Chen et al., 2006) and *in vivo* (Proud et al., 2008) studies have provided a comprehensive notion of the host response to HRV infection, focusing on the gene expression patterns induced by rhinovirus infection of the lower respiratory tract. However, majority of the studies focused on a single species of HRV-A. Therefore, in this chapter we aimed to characterise HRV 1B infection in both alveolar and bronchial epithelial cell lines.

Several different cell lines have been utilised to investigate rhinovirus, including HeLa cells (Amineva et al., 2011), A549 cells (Jang, 2006), 16HBE14^{o-} cells (Xatzipsalti and Papadopoulos, 2007) and BEAS-2B (Hudy et al., 2010). However much of what is known about rhinovirus live cycle was generated in HeLa cell models. A study by Amineva et al. (2011) compared rhinovirus infection in human primary epithelial cells and HeLa cells and revealed that the replication patterns in both cell models were remarkably similar. These cells are also commonly used to propagate sufficient quantities of virus (Lee et al., 2015). Although, of notice, HeLa cells are not airway cells making them a poor cell model of HRV infection. For the purpose of this thesis, HeLa cells were only used to propagate HRV1B.

A549 and 16HBE14^{o-}cell models both originated from airway epithelium, where A549 cell line was initiated from lung carcinomatous tissue (Giard et al., 1973) and 16HBE14^{o-}cell line was isolated from normal human bronchial epithelium and then transformed with the recombinant retrovirus LXSN16E6E7 containing the human HPV E6E7 gene and cloned (Tsao et al., 1996). 16HBE14^{o-} cells maintain many of the characteristics of differentiated primary cells, such as the presence of tight

junctions and cilia (Cozens et al., 1994). Both A549 and 16HBE14^{o-} cell models can therefore be determined to be adequate for the detailed investigation of respiratory virus-epithelial cell interactions.

In order to investigate the ability of HRV1B to replicate in A549 and 16HBE14^{o-} cells, HRV1B was exposed to airway cells for 2h at 33^{o}C in serum-free medium. The two-hour incubation period was required for the virus to attach to, and enter, epithelial cells. Cells were washed with saline to remove any unattached virus and re-immersed in fresh media supplemented with 5% FBS. Viral RNA copies were determined in samples collected from A549 and 16HBE14^{o-} cell lysates and cell supernatants at the indicated time points.

HRV infection of alveolar epithelial cells resulted in a MOI and time-dependent increase in viral RNA copies isolated from A549 cell lysates, with a peak viral load at 18h and at 24h post-infection, followed by a steady decrease at 48h and 72h post-infection. Although, when the highest MOI used to infect cells, peak viral load was observed at 48h post-infection. To ensure that the increases in viral RNA copies extracted from lysates of A549 cell pellets were due to the ability of rhinovirus to assemble functional virus capable of infecting neighbouring cells, we investigated the viral RNA copies in A549 cells supernatants. Results showed that at 2h post-viral inoculation the viral RNA copies were detectable both intracellularly and in cell supernatants, where the number of viral RNA copies was 2.4x higher in cell supernatants compared to cell lysates. This initial quantification of copies of viral RNA collected from cell lysates may be in fact the detection of viral RNA from the inoculum most likely without active intracellular replication yet, indicating that rhinovirus 1B is rapidly taken-up by A549 cells. In the cell supernatants, the presence of such a high number of viral RNA copies may be in fact be attributed to detection of copies from the inoculum used to initially infect cells, which was not completely removed during the wash steps. At 6h after infection, no viral RNA was detectable intracellularly or in the extracellular environment. HRV at this stage has normally initiated replication but would not have completed a full replicative cycle not yet being released into cell supernatants. During viral replication there are two detectable RNA strands, the genomic positive RNA strand and the replicative negative RNA strand (Johnston et al., 1998). It is possible that at 6h post-virus

inoculation, rhinovirus has initiated replication and there is an abundance of one of the strands, most likely the replicative negative RNA strand. For the quantification of viral RNA copies, we utilised a specific kit from Primerdesign®, which provides HRV specific primers. The nucleotide sequence of these primers is unknown due to commercial reasons. Therefore, a possible explanation for the lack of intracellular viral RNA copies at 6h post-viral inoculation may be that these primers target the genomic positive RNA strand. Viral RNA copies were detected both intracellularly and in cell supernatants up to 72h post-viral inoculation. According to the data acquired from experiments analysing both intracellular and extracellular viral RNA copies, it is apparent that HRV1B achieved higher viral loads approximately at 48h post infection. Viral RNA copies were also determined at 4, 16, 22, 46 and 70h post-infection, and followed a similar replication pattern, although a striking difference was the greater number in viral RNA copies compared to the previous time-points utilised. This discrepancy may be due to the differences in viral stocks used.

HRV1B infection of 16HBE14^{o-} cells was found to occur most easily when the virus was exposed to sub-confluent monolayers of cells. The reasons behind this are unclear. 16HBE14^{o-} cells maintain many of the characteristics of differentiated cells, such as the presence of tight junctions (Wan et al., 2000). Confluent monolayers of 16HBE14^{o-} cells can form tight junctions, which can act as a barrier to virus entry and spread throughout the airway monolayer (Vareille et al., 2011). Differentiated human airway epithelial cells were shown to be more resistant to rhinovirus infection compared to poorly differentiated cells (Lopez-Souza et al., 2004). Although 16HBE14^{o-} cells are relatively undifferentiated, they form tight junctions in confluent monolayers, which could explain the decreased susceptibility to rhinovirus infection. This aligns with other work, as A549 cells, which fail to form a tight epithelial barrier, exhibit increased susceptibility to rhinovirus infection when the virus is exposed to a confluent monolayer of cells (Grek et al., 2009).

HRV infection of bronchial epithelial cells resulted in a MOI and time-dependent increase in intracellular viral RNA copies, with a peak viral load at 16h and at 22h, followed by a steady decrease at 46h and 70h post-infection. In accordance with our data, both A549 and 16HBE cell lines are permissive to HRV1B. The replication cycle of HRV is similar in both alveolar and bronchial epithelial cells, where viral

replication occurs rapidly and viral synthesis peaks within 48h and declines by 72h after infection.

To further characterise alveolar and bronchial epithelial cells as relevant cell models for HRV1B infection, we analysed the mRNA levels and surface receptor expression of ICAM-1, LDLr and vLDLr on these cell lines. The cellular receptors for HRV1B are LDLr and vLDLr, and although HRV1B does not require ICAM-1 to enter airway cells, this cellular receptor has been shown to be induced during minor group HRV infection, which indicates that induction of ICAM-1 is not receptor- or serotype restricted. IL-8, TNFα and IL1β, cytokines known to be released following rhinovirus infection, were shown to increase ICAM-1 expression on uninfected cells (Sethi et al., 1997). ICAM-1 is therefore indirectly induced by the minor group of viruses possibly through inflammatory mediators produced after viral infection (Matsukura et al., 1996).

These receptors, found in cell surface of epithelial cells, are important for rhinovirus binding, uptake and signal transduction processes (Kim and Schleimer, 2009). Results indicated that ICAM-1 and LDLr gene and protein basal levels were highly expressed in 16HBE14o$^-$ cells, similar to basal levels observed on HeLa cells. A549 cells highly expressed LDLr mRNA and surface protein levels; however, ICAM-1 expression was considerably lower compared to 16HBE14o$^-$ and HeLa cells. vLDLr basal mRNA levels were high in all cell lines investigated. A study by Suzuki et al. (2001) showed that using an antibody against LDL receptor inhibited the RV2 (minor group) infection without affecting RV14 (major group) infection. This suggests that LDL receptor is specific for minor group of rhinoviruses. LDL receptor is shown to be highly expressed in A549 and 16HBE14^{o-} cells, which makes these cells highly permissive to HRV1B infection, and relevant cell models of infection for the minor group of rhinovirus.

To assess the HRV-induced pro-inflammatory and antiviral responses by alveolar and bronchial epithelial cells we determined the gene expression induced at different stages of rhinovirus infection by qPCR analysis. In addition, we assessed

cell supernatants for IL-8 protein release at the different time-points during HRV infection.

Human rhinovirus infection can stimulate airway epithelial cell pro-inflammatory chemokines and cytokine production. We assessed IL-8, IL-6 and CCL5 gene expression induced at different stages of rhinovirus infection, as these pro-inflammatory mediators have been shown to be rapidly and strongly induced during rhinovirus infection *in vitro* (Subauste et al., 1995) and *in vivo* (Zhu et al., 1997, 1996). These inflammatory mediators have been extensively studied and associated with pathogenesis of rhinovirus infection (Chun et al., 2013; Grünberg et al., 1997).

HRV1B infection of alveolar A549 epithelial cells induced IL-8 mRNA within 24h after infection (MOI 5) with marked accumulation of IL-8 in supernatants within 48-72h after infection. This is agreement with previous earlier studies demonstrating that infection of epithelial cells with rhinovirus (Proud et al., 2008; Subauste et al., 1995) also induce IL-8 production within this time-frame. IL-8 mRNA levels tended to decrease at subsequent time points; however IL-8 secretion increased with later time points. This is in agreement with a study by Chun et al. (2013) demonstrating an increase IL-8 release from A549 cells in a time-dependent manner. Although at later time point after infection (48-72h), it is difficult to distinguish between changes induced by direct viral induction compared to those induced by feedback action of other cellular products induced by viral signalling. IL-6 and CCL5 gene expression induction was seen within 48h and increased at subsequent time points. This was also reported in RSV studies, in which increased levels of IL-6 in supernatants were only detected at later time points (Noah and Becker, 1993). Of interest, HRV infection induced IL-8, IL-6 and CCL5 mRNA expression in a dose-dependent manner, which is indicative of a direct correlation between the viral load and pro-inflammatory induction. Previous studies have demonstrated a direct correlation between virus titer and occurrence, along with severity of infection (Douglas et al., 1966).

HRV1B infection strongly induced IL-8, IL-6 and CCL5 mRNA expression in bronchial epithelial cells. IL-8, IL-6 and CCL5 genes followed a similar pattern of expression, where enhanced mRNA expression was seen within 18h of infection followed by a

decrease at 48h and 72h of infection; this coincided with an increase viral titer seen at 18h and at 24h followed by a drop at later stages of the infection. Previous studies have reported HRV ability to induce mRNA expression of several different genes at early stages of infection, revealing a significant difference between HRV-infected and uninfected alveolar epithelial cells at 6h post-infection (Reza Etemadi et al., 2017) although this contrasts with other studies which have reported no upregulation of differentially expressed genes at early stages of infection and were in agreement with our study showing increased upregulation within 24h of infection (Y. Chen et al., 2006; Chun et al., 2013). Cell supernatants were assessed for IL-8 release and results indicated a marked increase in IL-8 release at 48h and at 72h of infection.

IL-8, IL-6 and CCL5 mRNA expression were highly expressed by bronchial epithelial cells compared to alveolar epithelial cells following rhinovirus infection. A549 and 16HBE cells are both permissive to HRV1B and relatively similar viral loads were observed in these two cell lines. It is unclear why rhinovirus infection induces a much higher pro-inflammatory response in 16HBE14^{o-}cell line compared to A549 cell line. ICAM-1 expression was shown to be enhanced by both major and minor group of viruses (Papi and Johnston, 1999). Pro-inflammatory mediators produced following viral infection can lead to an increase in ICAM-1 expression (Papi and Johnston, 1999; Sethi et al., 1997). We compared basal ICAM-1 expression on alveolar epithelial cells and bronchial epithelial cells and observed a marked difference in expression. The basal expression of ICAM-1 on A549 cells is much lower compared to 16HBE14^{o-} cells. ICAM-1 mediates not only viral entry but also the activation of signalling pathways that lead to an enhanced inflammatory response to rhinovirus infection by airway epithelial cells. Syk, an important immunoregulatory protein tyrosine kinase, has been shown to be downstream of ICAM-1 mediating the inflammatory response of the airway epithelium via activation of p38 MAPK pathway. However, syk-mediated inflammatory response occurs as a result of ICAM-1 engagement with HRV rather than viral replication (Wang et al., 2006). This signals that viral replication of the minor group of virus would not suffice for an ICAM-1-enhanced inflammatory response, as HRV1B does not directly engage with ICAM-1. Nevertheless, ICAM-1 was shown to initiate NF-kB

activation upon viral entry in bronchial epithelial cells exposed to influenza virus (Othumpangat et al., 2016). NF-kB pathway plays an essential role in the regulation of inflammatory responses (Ghosh et al., 1998) and in airway epithelial cells, HRV-induced IL-8 and IL-6 production is mediated, in great extent, by an NFkB-dependent transcription activation process (Zhu et al., 1996 and Zhu et al., 1997). Therefore, it is possible that HRV1B replication upregulates ICAM-1 which in turn activates NF-kB signalling pathway leading to an enhanced inflammatory response. Due to the fact that A549 cells do not express high basal levels of ICAM-1, this could indeed explain the defective pro-inflammatory response to HRV1B infection compared with infection of 16HBE14^{o-} cells.

Induction of type I interferon is a common phenomenon in HRV infection of airway epithelial cells. Rhinovirus infection of tracheobronchial and bronchial epithelial cells has been shown to result in a significant induction of IFNβ (Y. Chen et al., 2006; Wark et al., 2005). In this chapter, we showed that HRV1B infection of alveolar epithelial cells did not result in a significant induction of IFN 1β mRNA expression. IFN1β mRNA expression was induced at later stages- 72h of infection- at the highest dose of HRV used to infect cells. Similar studies have reported that HRV14 and HRV72 infection failed to induce high levels of IFN1β mRNA in A549 cells (Kotla et al., 2008; Reza Etemadi et al., 2017). Kotla et al. (2008) revealed that the low levels of IFN1β could be explained by the impaired activation of IRF3 in the presence of the activated NF-kB and ATF-2. IRF-3 is essential for the activation of the type I interferon response following viral infection (Hiscott, 2007). In contrast, IFN 1β mRNA expression was strongly induced in 16HBE14^{o-} cells, with the highest fold-changes occurring at 24h post infection. Similar results were observed by Chen et al. (2006) using tracheobronchial cells.

This chapter shows a detail analysis of HRV1B-epitheial cell interaction using alveolar and bronchial epithelial cells with the aim of determining the most suited cell model for rhinovirus 1B infection in order to study the modulatory effects of the human cathelicidin, LL-37, on the host response to viral infection. The findings based on these models led us to conclude that the most relevant cell model is 16HBE14^{o-} cells derived from normal human bronchial epithelium. 16HBE14^{o-} cells were permissive to HRV1B exhibiting high basal levels of surface LDLr, vLDLr and

ICAM-1, as well as, responding to infection with a strong and rapid production of antiviral and inflammatory mediators. Additionally, 16HBE14^{o-} cells maintain many of the characteristics of primary differentiated cells, such as the presence of tight junctions (Cozens et al., 1994), better mimicking the differentiated airway epithelium in comparison to A549 cell line. Furthermore, 16HBE14^{o-} cells are easily maintained, and avoid several of the difficulties including high cost and reproducibility associated with primary cell culture methods (Ehrhardt et al., 2002).

CHAPTER 4

Investigating the antiviral activity of LL-37 against rhinovirus

Chapter 4. Investigating the antiviral activity of LL-37 against rhinovirus

4.1. Introduction

The underpinning mechanism for cathelicidin-mediated antiviral activity remains largely indeterminate. The capacity of LL-37 to reduce viral infection has been associated with the ability of the peptide to directly target the virus particles (Alagarasu et al., 2017; Barlow et al., 2011; Currie et al., 2013; Harcourt et al., 2016), as well as targeting the host cell (Currie et al., 2013; Harcourt et al., 2016).

In this chapter we aim to characterise the antiviral activity of LL-37 against rhinovirus 1B in alveolar and bronchial epithelial cells in the context of a number of LL-37 concentrations and exposure regimens.

4.1.1. Rhinovirus targeted- airway epithelial cell receptors

Human rhinoviruses are one of the most common causes for airway infections and exacerbations of COPD and bronchial asthma. Around 90% of HRV serotypes use ICAM-1 to infect target cells, and these serotypes are known as the major group of rhinovirus (Greve et al., 1989). The remaining serotypes, known as the minor group of virus, can enter host cells via the LDL receptor family (Uncapher et al., 1991).

ICAM-1 (CD54) is a cell surface glycoprotein that belongs to the immunoglobulin superfamily, and contains a cytoplasmic tail, a transmembrane region and five extracellular domains for binding to leukocytes (Simmons et al., 1988). ICAM-1 has been proposed to be ligand for both lymphocyte function-associated antigen-1 (LFA-1) [Rothlein et al., 1986] and Mac-1 (CD11a/CD18) [Diamond et al., 1990], which are members of the β_2 subfamily of integrins. The interactions between ICAM-1 expressed in epithelial and endothelial cells, and the β_2-integrins expressed in leukocytes are essential for the complex cascade of adhesive events, which

include leukocyte rolling, adhesion and trans-(endo)epithelium migration to the sites of inflammation (Springer, 1990).

ICAM-1 has been shown to modulate eosinophil and lymphocyte infiltration. There is substantial evidence that ICAM-1 plays an important role in the activation of these inflammatory cells contributing to the pathogenesis of asthma (Manolitsas et al., 1994). Epithelial ICAM-1 expression is increased by allergen challenge (Ciprandi et al., 1994), upon stimulation with TNF-α and IFNγ (Chang et al., 2002; Krunkosky et al., 2000), or other stimuli, such as LPS (Fakler et al., 2000; Madjdpour et al., 2000), and exposure to pathogens (Avadhanula et al., 2006; Papi and Johnston, 1999). Notably, while ICAM-1 acts as the cellular receptor for the major group of rhinovirus, it has been demonstrated that rhinovirus can induce a marked increase in ICAM-1 expression, in both primary bronchial epithelial cells and in A549 cell line (Papi and Johnston, 1999). HRV infection of airway epithelial cells mediates increased surface ICAM-1 expression via NF-B-mediated transcriptional upregulation. The minor group of rhinovirus is also able to upregulate surface ICAM-1 expression in airway cells, which indicates that the induction of ICAM-1 is not receptor- or serotype restricted (Papi and Johnston, 1999; Terajima et al., 1997). Therefore, rhinovirus infection may cause both a direct increase in ICAM-1 in upper and lower respiratory tract epithelial sites, but also an indirect increase via other factors that are produced as a result of the viral infection.

LDL receptors are important in lipid metabolism through facilitating the transport of cholesterol into cells via receptor-mediated endocytosis. It is known that these receptors are expressed by airway epithelial cells and can be used by the minor group of rhinoviruses to access and infect these cells. In the airway epithelium, LDLr has been shown to negatively regulate house dust mite (HDM) - induced airway hyper-responsiveness (AHR) by binding to apolipoprotein E. Thus, the activation of the apolipoprotein E-low-density lipoprotein receptor pathway may be a novel target for asthma therapy (Yao et al., 2010). The LDLr, VLDLr and LDLR-related protein (LRP) are known to bind to, and internalise, apolipoprotein E, which is expressed by lung macrophages, functionally attenuating airway inflammation. The vLDLr is a multi-ligand receptor, which is known to mediate pleiotropic biological processes (Fredriksson et al., 2014). However, there is a relative paucity of

information on the involvement of vLDLr in inflammatory or immune responses. Interestingly, a study by Baitsch et al. (2011) revealed that the apolipoprotein E-very-low-density lipoprotein receptor pathway promoted the conversion of a pro-inflammatory M1 macrophage phenotype to the anti-inflammatory M2 macrophage phenotype. A recent study has also identified a novel role for the vLDLr receptor in which they supress DC-mediated Th2 adaptive immune responses in response to HDM- induced airway hyperresponsiveness (Fredriksson et al., 2014). Taken together, these studies point towards the involvement of LDL receptor family members in limiting excessive HDM-induced airway inflammation by binding to apolipoprotein-E, and this may represent a novel target for the development of treatment approaches for asthma.

The minor group HRV2 is known to infect primary airway cells via binding to the LDL receptor expressed on these cells. It has been demonstrated that HRV2 infection can result in an increased production of IL-1β, IL-8, IL-6 and TNF-α and upregulation of cell surface LDL receptor expression and mRNA expression in human tracheal epithelial cells. It is thought that increased LDL receptor expression by HRV2 infection may be mediated through the activation the transcriptional factor SP-1, which was shown to be upregulated by HRV2 infection in human tracheal epithelial cells. It was also shown that both minor and major groups induced LDL receptor expression in human airway cells. An interesting finding showed that blocking LDLr inhibited HRV2 infection and the production of cytokines in response to viral infection, but had no effect on HRV14 infection (Suzuki et al., 2001).

The modulation of cell surface receptors has been proposed as a possible HDP-mediated antiviral mechanism targeting the host cell. The β-defensins HBD2 and HBD3, but not HBD1, were shown to reduce cell surface expression of CXCR4, but not CCR5, during HIV-1 infection (Feng et al., 2006; Quiñones-Mateu et al., 2003). The chemokine receptors CXCR4 and CCR5 function as co-receptors for HIV-1 entry into $CD4^+$ T cells and peripheral blood mononuclear cells (PBMCs) (Bleul et al., 1997). Studies further confirmed that CXCR4 was down-modulated in PBMCs by HBD2 and HNP1 (Seidel et al., 2010). As previously noted, HRV enters respiratory epithelia cells only via ICAM-1 (major group) or LDLr (minor group). We propose

that LL-37 may modulate the HRV-targeted receptor expression on bronchial epithelial cells as a mechanism to prevent virus entry and dissemination.

4.2. Materials and Methods

4.2.1. Virus purification

HRV1B virus stock was transferred into Vivaspin protein concentrator spin columns (100.00MW filter size column, GE Healthcare Life Sciences, Chalfont Saint Giles, UK) and centrifuged at 775 RCF (Eppendorf centrifuge 5804 series rotor S-4-72, Stevenage, UK) for 2h at 4^{o}C to concentrate the virus. A 30% sucrose gradient was prepared in DPBS (30% sucrose w/v, pH 71.-7.2). Using a syringe, sucrose was transferred into an ultracentrifuge tube (Quick-Seal®, Polypropylene, 8 mL, Beckman coulter, High Wycombe, UK) and the concentrated virus was gently layered on top of the sucrose gradient. The sucrose gradient with layered virus was then ultracentrifuged at 76 790 RCF for 3h at 16^{o}C in a SW32.1Ti Swinging Bucket Rotor (Beckman coulter, High Wycombe ,UK). The sucrose was discarded and the pellet was gently washed with PBS and re-suspended in fresh PBS. The concentrated virus was aliquoted and was stored at -80^{o}C.

4.2.2. Virus purification for transmission electron microscopy analysis

HeLa cells were grown at 37^{o}C/5% CO_2 in T175 flasks and infected with HRV1B when they had reached a confluency of 60-70%. HRV1B was propagated in HeLa cells by infecting cells in serum-free DMEM for 2h at 33^{o}C with an MOI 10. The inoculum was then removed and cells were incubated for 2-3 days in low serum media (5% FBS supplemented DMEM). Cells were then subjected to three cycles of freeze/thaw before harvesting the supernatant containing released virus. The supernatant was collected and stored at -80^{o}C in BD Falcon tubes. The virus stock was thawed at room temperature and centrifuged at 3500 RCF for 30 minutes at 4^{o}C. The supernatant was recovered and cell pellet was disposed. An 8% PEG solution was added to supernatants and left overnight at 4^{o}C in order to precipitate the virus. The supernatant was centrifuged at 3500 RFC for 0.5h at 4^{o}C in order to pellet the virus. The supernatant was then discarded and the pellet was re-suspended in HEPES buffer (50mM Hepes, 200 mM NaCl, pH=8). The solution was centrifuged at 3500 RCF for 0.5h at 4^{o}C and the supernatant was transferred to an ultra-clear™ centrifuge tube (Beckman Coulter, High Wycombe UK). A solution of

30% sucrose in HEPES buffer (2ml) was added to the bottom of the tube to create a gradient allowing the virus to be positioned above the sucrose. Tubes were centrifuged at 77,140 RCF for 3h at 4^{o}C (SW41 rotor, Beckman Coulter, High Wycombe, UK). After centrifugation, the supernatants were discarded and 500µl of HEPES buffer was added to the pellet and left overnight at 4^{o}C. The pellet was carefully re-suspended in the buffer and centrifuged at 3500xg for 1h at 4^{o}C. Supernatant was recovered and kept on ice; HEPES buffer (200µl) was added to the pellet and centrifuged at 3,500 RCF for 1h at 4^{o}C. A sucrose gradient (15-45%) was constructed with the help of a gradient master (Biocomp, York, UK). The supernatant was placed on top of the gradient and ultracentrifuged at 77,140 RCF for 3h at 4^{o}C (SW42 rotor, Beckman coulter, High Wycombe UK). Using a white light under the ultraclear tube, the blue band corresponding to the virus and other cellular components was visualised. Desalting columns (Thermo Scientific™ Zeba Spin 7K MWCO Desalting Columns, Loughborough, UK) were subsequently used to remove sucrose. The virus fractions were added to desalting columns and centrifuged for 2 minutes at 1000 RCF. The virus was collected and transferred into Vivaspin Protein concentrator spin columns (100.00MW filter size column, GE Healthcare Life Sciences, Chalfont Saint Giles, UK) and centrifuged at 3,500 RCF for 10 minutes. Concentrated virus was stored at -80^{o}C until further use.

4.2.3. SDS-PAGE

Each fraction obtained from the ultracentrifuged sucrose gradient was mixed with loading buffer (NuPAGE™ LDS sample buffer 4x, Thermo Fisher Scientific, Paisley, UK) and heated in a heating block for 2 minutes at 95^{o}C. The samples and protein ladder (PageRuler™ prestained protein ladder, 10 to 180kDA, Thermo Fisher Scientific, Loughborough, UK) were loaded into the wells of a precast polyacrylamide gel (4-20% Mini-PROTEAN® TGX ™ Precast protein gel, Bio-Rad, Watford, UK). The gel was run for 1h at 120V and the proteins were detected by staining the gel with InstantBlue™ (Expedeon, Cambridge, UK) for 15mins.

4.2.4. Transmission Electron Microscopy

For negative stain transmission electron microscopy a concentration of 73 µg/ml of purified rhinovirus 1B was exposed to 50µM of LL-37 or endotoxin-free water and incubated for 2h on ice. A volume of 1µl of sample was applied to carbon coated

copper grids (agar scientific, Stansted, UK). The stained layer was dried and imaged by TEM Technai T12 operated at 120kEV.

For cryo-transmission electron microscopy a concentration of 136 µg/ml of purified rhinovirus 1B was exposed to 50µM of LL-37 or endotoxin-free water and incubated for 5 minutes on ice. A volume of 1µl of sample was applied to cryoEM C-flat™ holey carbon and copper grids CF-2/1-2C (Protochips, USA) and vitrified with a FEI Vitrobot IV (FEI, Hillsboro, OR). Electron micrographs were collected on a FEI Technai Polara microscope operated at 300kEV with the grids at liquid nitrogen temperature. Micrographs were semi-automatically acquired with EM-tools (TVIPS software). The absolute magnification of the digital micrographs collected on a Gatan ultrascan 4000 UHS CCD camera was 37,037x corresponding to a pixel size of 1.35 Å on a molecular scale.

4.2.5. Molecular quantification of viral infectivity

To determine rhinovirus infectivity, A549 and 16HBE14^{o-} cells were used as *in vitro* models of respiratory infection and a number of treatment approaches were employed. Briefly, A549 or 16HBE14^{o-} cells were seeded in a 12-well plate and incubated in a heated humidified incubator at 37^{o}C/5% CO_2, and were subsequently subjected to two different treatment approaches. For the "virus-peptide" approach, HRV1B was exposed to varying concentrations of LL-37 for 2h at 33^{o}C in serum-free media. Cells were exposed to the mixture for 1h, washed and re-immersed in low-serum (5% FBS) supplemented media (DMEM or IMDM) for 23h at 33^{o}C (Figure 15).

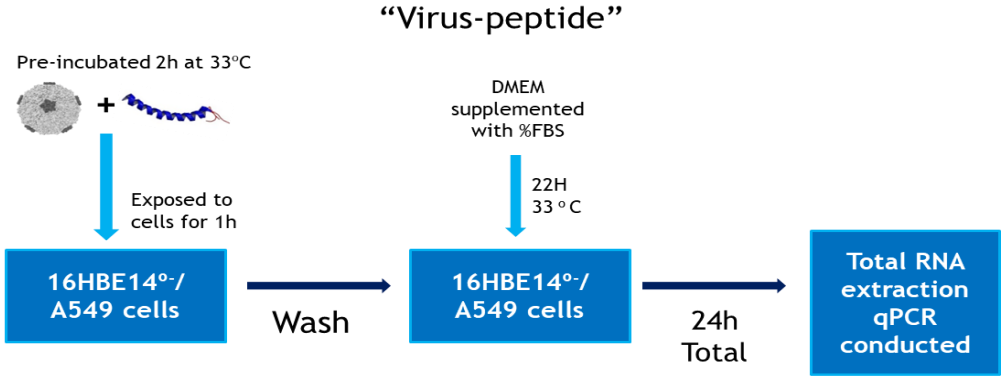


Figure 15. "Virus-peptide" experimental regimen.

HRV1B was exposed to varying concentrations of LL-37 (10 µg/ml, 30 µg/ml or scramble LL-37 30 µg/ml) for 2h at 33°C in serum-free media. Cells (A549 or 16HBE14^{o-}) were exposed to the mixture for 1h, washed and re-immersed in low-serum (5% FBS) supplemented media (DMEM or IMDM) for 23h at 33°C.

For the "cell-peptide" approach, A549 or 16HBE14^{o-} cells were infected with HRV1B for 2h at 33°C in serum-free media, washed with saline and exposed to LL-37 for 22h at 33°C in DMEM or IMDM supplemented with 1% (v/v) Ultroser G (Figure 16).

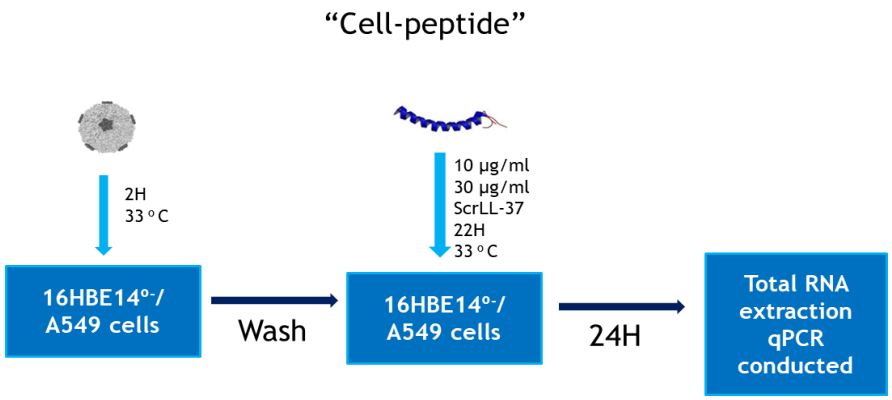


Figure 16."Cell-peptide" experimental regimen.

Airway epithelial cells (A549 or 16HBE14^{o-} cells) were infected with HRV1B for 2h at 33°C in serum-free media, washed and exposed to LL-37 37 (10 µg/ml, 30 µg/ml or scramble LL-37 30 µg/ml) for 22h at 33°C in DMEM or IMDM supplemented with 1% (v/v) Ultroser G.

A549 and 16HBE14^{o-} cells were pre-treated with LL-37 prior to HRV infection using different LL-37 exposure times. In brief, for the "2h treatment" A549 or 16HBE14^{o-} cells were exposed to LL-37 (0-30µg/ml) for 2h at 33°C in DMEM or IMDM supplemented with 1% (v/v) Ultroser G. Cells were washed and infected with HRV1B for 2h in serum-free media, followed by washing and re-immersing cells in 5% FBS supplemented media for 20h at 33°C (Figure 17).

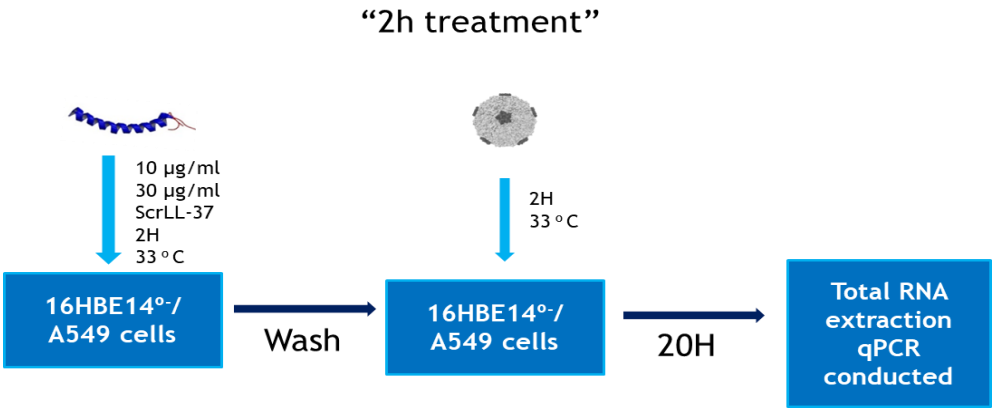


Figure 17. The 2h treatment" experimental regimen.

Airway epithelial cells (A549 or 16HBE14^{o-} cells) were exposed to LL-37 (10 µg/ml, 30 µg/ml or scramble LL-37 30 µg/ml) for 2h at 33^{o}C in DMEM or IMDM supplemented with 1% (v/v) Ultroser G. Cells were washed and infected with HRV1B for 2h in serum-free media, followed by washing and re-immersing cells in 5% FBS supplemented media for 20h at 33^{o}C.

For the "24h treatment" regimen, A549 cells were exposed to LL-37 (0-30 µg/ml) for 24h at 33^{o}C in DMEM supplemented with 1% (v/v) Ultroser G. Cells were washed and exposed to HRV1B for 2h in serum-free media, followed by washing cells with saline and re-immersing them in 5% FBS supplemented DMEM for 20h at 33^{o}C (Figure 18).

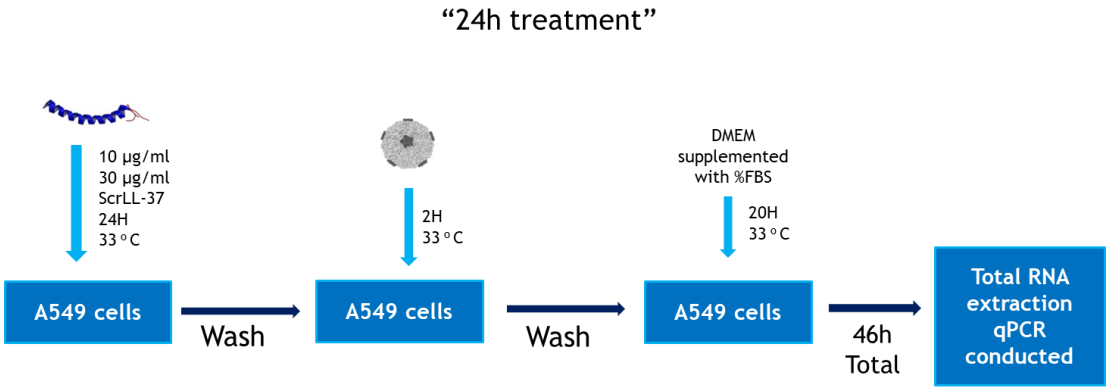


Figure 18. The "24h treatment" experimental regimen.

A549 cells were exposed to LL-37 (10 µg/ml, 30 µg/ml or scramble LL-37 30 µg/ml) for 24h at 33^{o}C in DMEM supplemented with 1% (v/v) Ultroser G. Cells were washed and exposed to HRV1B for 2h in serum-free media, followed by washing cells with saline and re-immersing them in 5% FBS supplemented DMEM for 20h at 33^{o}C.

For the "24h + wash treatment" regimen, A549 cells were exposed to LL-37 for 2h in DMEM supplemented with 1% (v/v) Ultroser G, washed and left in contact with 5% FBS supplemented DMEM for 22h at 33°C prior to HRV1B infection for 2h in serum-free DMEM. A549 cells were washed and re-immersed in 5% FBS supplemented DMEM for 20h at 33°C (Figure 19).

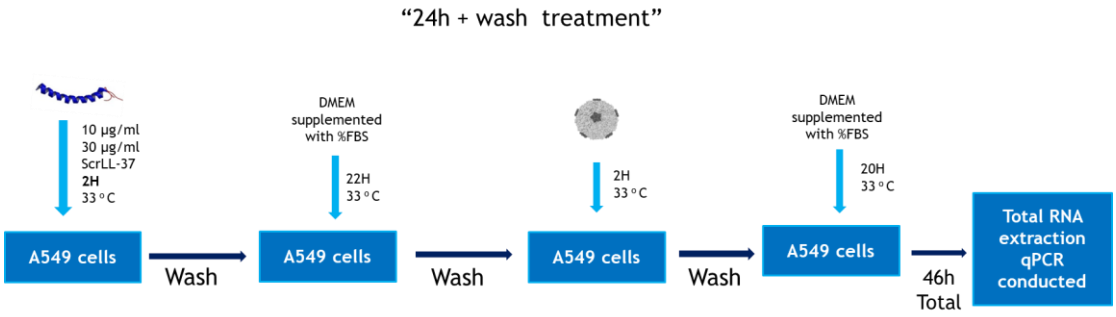


Figure 19. The "24h + wash treatment" experimental regimen.

A549 cells were exposed to LL-37 (10 µg/ml, 30 µg/ml or scramble LL-37 30 µg/ml) for 2h in DMEM supplemented with 1% (v/v) Ultroser G, washed and left in contact with 5% FBS supplemented DMEM for 22h at 33°C prior to HRV1B infection for 2h in serum-free DMEM. A549 cells were washed and re-immersed in 5% FBS supplemented DMEM for 20h at 33°C.

After each treatment, media was aspirated and 300µl RLT buffer from RNeasy® mini kits (Qiagen, Crawley, UK) was added to each well and cells were scrapped of the surface of the plate with a cell-scrapper. Cell lysates were stored at -80°C until extraction. Total RNA isolation was performed using the RNeasy® mini kit system following manufacturer's protocols. RNA integrity was determined using an Agilent Bioanalyser (Agilent, UK), where RIN≥8 was used as a quality filter for further downstream analyses. Viral RNA was quantified using the Genesig Human Rhinovirus (all subtypes) qPCR kit (PrimerDesign, Camberley, UK) according to manufacturer's protocols.

4.2.6. qPCR to determine mRNA levels of IFN1β, PKR and IL-8

Total RNA (0.5-1µg) was transcribed to cDNA using Precision RT all-in-one mix kit (PrimerDesign Ltd, Camberley, UK) as per manufacturer's instructions. qPCR reactions included cDNA (25 ng), 250 nM specific primers (Table 2) and SYBR green

mastermix (PrimerDesign LTtd, Camberley,UK) in total volume of 20µl. qPCR was performed using the StepOnePlus instrument (Applied Biosystems,Thermo Fisher Scientific, Loughborough, UK). A panel of six human reference genes were evaluated using a geNorm kit (Primerdesign, Camberley, UK) and qbase^{+} (Biogazelle, Zwijnaarde, Belgium) software. The geometric means of *ACTB* and *GAPDH* genes were selected as the most stable combination for normalization in both 16HBE and A549 cell lines. The $2^{\Delta\Delta Ct}$ method was used and data is represented as fold change over untreated cells (Pfaffl MW, 2004). Statistical analysis was performed on the Δct values, unless otherwise stated in figure legend. We applied a threshold of two-fold above unstimulated control to consider upregulation of the expression of the genes of interest.

Table 2. qPCR primer sequences

Target	Forward	Reverse	Reference
GAPDH	AAGCTCATTTCCTGGTATGACA	TCTTACTCCTTGGAGGCCATGT	(Wang et al., 2012)
ACTB	GGACTTCGAGCAAGAGATGG	AGGAAGGAAGGCTGGAAGAG	(Tarrats et al., 2011)
IL-8	ACTGAGAGTGATTGAGAGTGGAC	AACCCTCTGCACCCAGTTTTC	(Tsai et al., 2009)
IFN1β	TCTGGCACAACAGGTAGTAGGC	GAGAAGCACAACAGGAGAGCAA	(Warnking et al., 2015)
PKR	CAGTTTGCCTTCCTGGATTTGTA	CTTCCCGTATCCTGGTTGGAA	Designed

4.2.7. Quantification of IFN1β release by 16HBE14^{o-} cells

Briefly, HRV1B was exposed to varying concentrations of LL-37 for 2h at 33^{o}C in serum-free IMDM media. 16HBE14^{o-} cells were exposed to the mixture for 1h, washed and re-immersed in low-serum (5% FBS) supplemented media IMDM for

23h at 33^{o}C. Supernatants from each well were collected and stored at -80^{o}C until further use. Supernatants were analysed for the release of IFN1β by ELISA, as per the manufacturer's instructions (Human IFN beta ELISA kit, pbl Assay Science, Piscataway Township, USA).

4.2.8. Viral titer assessment ($TCID_{50}$/ml)

The infectious titer of rhinovirus was determined by $TCID_{50}$ assay. For experimental assessment of the antiviral effects of LL-37, WI-38 cells were seeded at 2x10^{4} per well in a 96-well plate and cultured at 37^{o}C/5% CO_2 overnight. Supernatants from experiments in which HRV1B (MOI 5) was exposed LL-37 prior to cell infection ("virus-peptide") or 16HBE14^{o-} cells which were first infected with HRV 1B (MOI5) prior to LL-37 treatment ("cell-peptide") were collected and used for $TCID_{50}$ assay. Briefly, these supernatants were 10-fold diluted in serum-free media and subsequently added to WI-38 cells, which were then incubated for 1h at 33^{o}C. Following incubation, the inoculum was removed and cells were cultured in 5% FBS supplemented DMEM for a period of 5 days before assessment of cytopathic effects. $TCID_{50}$ assay was determined using the Reed-Muench method (Reed and Muench, 1938).

4.2.9. Flow cytometric analysis

16HBE14^{o-} cells were seeded at 1x10^{5} per well in a 12-well plate and cultured overnight at 37^{o}C / 5% CO_2. HRV1B (MOI 5) was exposed to varying concentrations of LL-37 (0-30μg/ml) for 2h at 33^{o}C in serum-free IMDM. 16HBE cells were exposed to HRV+LL-37 mixture for 1h and then washed and exposed to 5% FBS supplemented IMDM for a further 23h or 47h according to treatment regimen. Cell media was aspirated and stored for further analysis and cells were washed with saline before incubation with 2x Trypsin/EDTA solution for 5 minutes to detach cells from the surface of the plate. Complete culture media was added to neutralise trypsin and cells were transferred into BD Falcon 5 ml polystyrene tubes and centrifuged at 230×*g* for 5minutes. Supernatant was discarded and cells were washed with 10% FBS in PBS and centrifuged at 230×*g* for 5 minutes. Supernatant

was discarded leaving approximately 200µl of 10% FBS in PBS in contact with cells for 20 minutes at room temperature for further blocking. Without removal of the blocking solution, 2.5µl of each antibody was added to each tube (human LDLr APC conjugated antibody, R&D Abingdon, UK; human CD54 PE conjugated antibody, BioLegend, London, UK; human vLDLr FITC conjugated antibody, LSbio Nottingham, UK). Isotype controls for ICAM-1 (PE mouse IgG1, k isotype control FC antibody, Biolegend, London, UK) and LDLr (mouse IgG 1 APC-conjugated antibody, R&D Abingdon, UK) were also added to respective tubes. Unstained cells were retained as a negative control. Cells were incubated with antibodies for 25 minutes in the dark at 4^{o}C and subsequently washed twice with 2 ml of PBS. Cells were then re-suspended in 200µl of PBS before analysis by flow cytometry (FACS Calibur, BD biosciences, Heidelberg, Germany).

4.2.10. Confocal microscopy

16HBE14^{o-} cells were seeded at 2.5x10^{4} per well in an 8-well chamber slide (Thermo Scientific™, Loughborough, UK) and cultured overnight in a heated humidified incubator at 37^{o}C / 5%CO^{2}. A purified stock of HRV1B (MOI 5) was exposed to varying concentrations of LL-37 (0-30µg/ml) for 2h at 33^{o}C in serum-free IMDM. 16HBE14^{o-} cells were exposed to the HRV+LL-37 mixture for 1h and then washed and exposed to 5% FBS supplemented IMDM for further 23h. Supernatants were removed and cells were washed with warm PBS. Cells were fixed for 20 minutes at room temperature using 4% paraformaldehyde. Cells were washed 3x times with PBS and permeabilised with 0.1% Triton-X in PBS for 5 minutes. Cells were washed with PBS to remove Triton-X and subsequently blocked with 5% goat serum in PBS for 1h at room temperature. The blocking solution was removed and cells were incubated with a pan enterovirus monoclonal antibody (mouse IgG2a, L66J,Invitrogen, ThermoFisher Scientific, Loughborough, UK) diluted in 5% goat serum in PBS (1:200) overnight at 4^{o}C. The primary antibody was removed and cells were washed 4 times with 0.05% Tween20 in PBS. Goat anti-mouse IgG, Alexa Fluor® 488 (ThermoFisher Scientific, Loughborough, UK) was diluted in 5% goat serum in PBS (1:1000) and incubated with cells for 1h, in the dark, at room temperature. Cells were washed 3 times with 0.05% Tween20 in PBS followed by 2

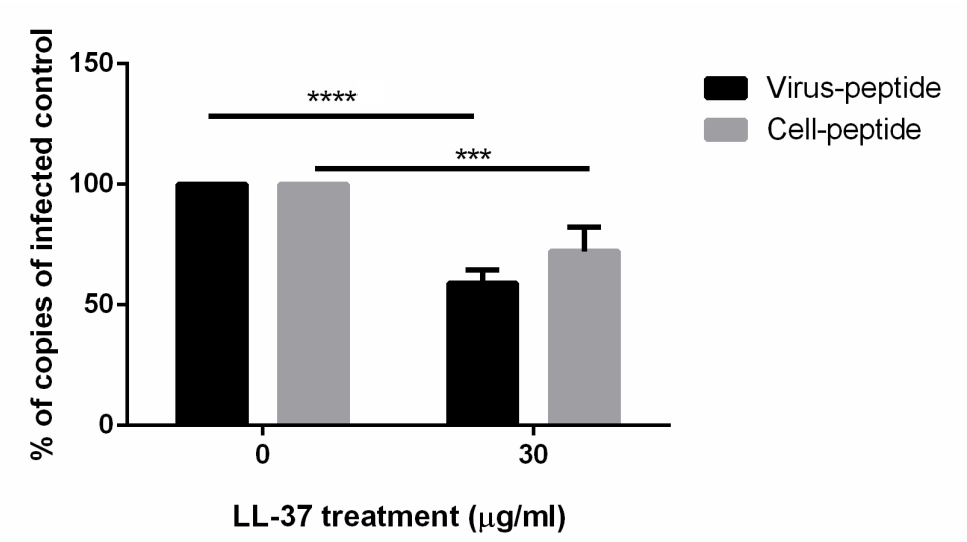
more washes with PBS. DAPI (1mg/ml) was diluted 1:1000 in PBS and incubated with cells for 10 minutes. Cells were washed 3 times with PBS before adding mounting media (Vectashield, Vector Laboratories, Peterborough, UK) and removing the plastic structure. Slide was covered with a glass coverslip and edges were sealed with nail polish. Slides were kept in the fridge protected from direct light. Stained cells were assessed using a Zeiss LSM 880 AxioObserver Z1 Confocal fluorescent microscope (wavelengths: 405 nm, 488 nm, 594 nm, and laser power set at 2%).

4.3. Results

4.3.1. Assessment of the antiviral activity of LL-37 against rhinovirus

In order to assess the antiviral potential of the human cathelicidin, LL-37, against human rhinovirus, the A549 airway epithelial cell line was used as cell model of infection. To mimic interactions that could occur *in vivo* between LL-37 and HRV in the airway surface liquid before infection of airway cells, the virus was incubated with LL-37 prior to A549 cell infection and the number of viral RNA copies were assessed by qPCR after 24hours. The incubation of HRV1B with LL-37 peptide resulted in a significant reduction in viral RNA copies (****p<0.0001) compared to virus alone (Figure 20A). A scrambled LL-37 peptide exhibited no antiviral effects against HRV1B (Figure 20B). In addition, infection of A549 cells for 2h prior to LL-37 treatment also resulted in a significant reduction of detectable viral RNA copies (***p<0.001) compared to virus alone (Figure 20A). The scrambled LL-37 control showed no antiviral activity (Figure 20B).

A.



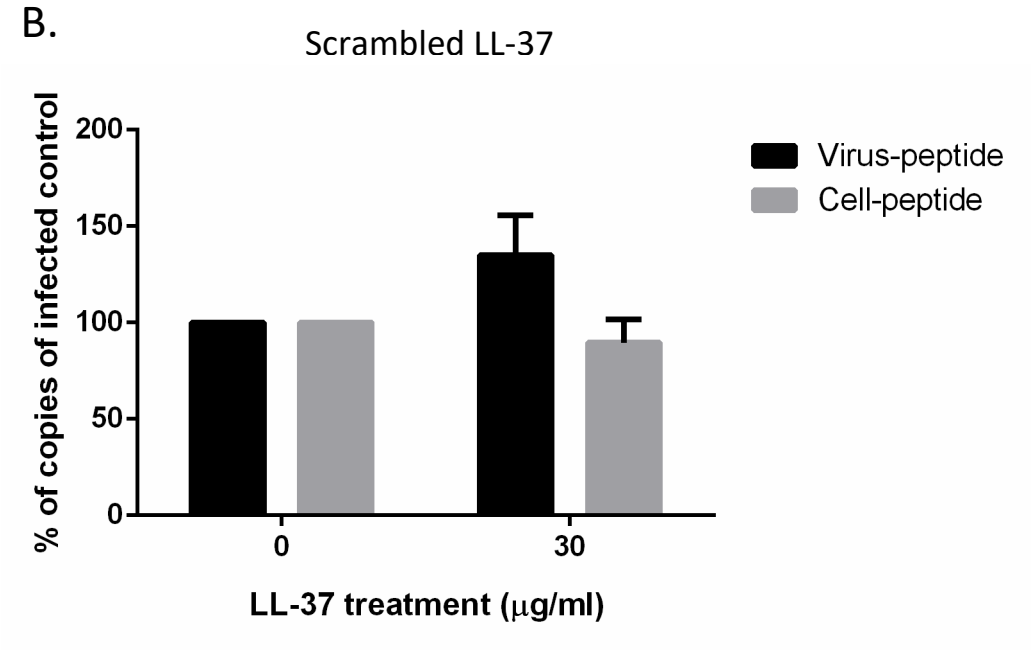


Figure 20. LL-37 displays direct and cell based antiviral activity against rhinovirus in an A549 cell model

(A) Human rhinovirus was incubated with LL-37 for 2h prior to cell infection ("virus-peptide") or A549 cells were infected with HRV1B (MOI 1) for 2h prior to LL-37 treatment ("cell-peptide"). (B) Human rhinovirus was incubated with a scrambled LL-37 peptide for 2 h prior to cell infection ("virus-peptide") or A549 cells were infected with HRV1B (MOI 1) for 2h prior to scrambled LL-37 treatment("cell-peptide"). All treatments were then incubated for either 22h ("cell-peptide") or 23h ("virus-peptide") at 33^{o}C. Total RNA was extracted from cell lysates and qPCR was performed to determine the number of viral RNA copies. Figures represent at least five independent experiments showing mean values ± SEM. The percentage data was converted into arcsin values and statistical analysis was performed on these values using an unpaired t-test to compare virus only (control) to peptide treatment (30μg/ml) (*** p< 0.001; ****p<0.0001).

The antiviral effects of LL-37 against HRV1B were also investigated in the 16HBE14^{o-} bronchial epithelial cells and assessed by $TCID_{50}$ assay and qPCR for quantification of viral RNA. The pre-incubation of HRV1B with LL-37 peptide resulted in a significant reduction in viral RNA copies at ≥10 μg/ml of LL-37 (****p<0.0001) compared to virus alone (Figure 21A) and this was reflected in a reduction in the measurable virus titer determined by $TCID_{50}$ assay (p>0.05), although, there was a trend no statistical significance was found (Figure 21C). Interestingly, LL-37 did not elicit a reduction in detectable viral RNA copy number when 16HBE cells were infected for 2h prior to LL-37 treatment (Figure 21A). However, infective titer assessment by $TCID_{50}$/ml assay revealed a statistically significant reduction of virus titer at both

doses of LL-37 (*p<0.05 at 10 µg/ml and 30 µg/ml) compared to virus alone (Figure 21C). The scrambled LL-37 peptide did not induce a reduction in measureable viral RNA copy number (Figure 21B) or a reduction in the infective virus titer compared to virus alone (Figure 21D) with either treatment regimen.

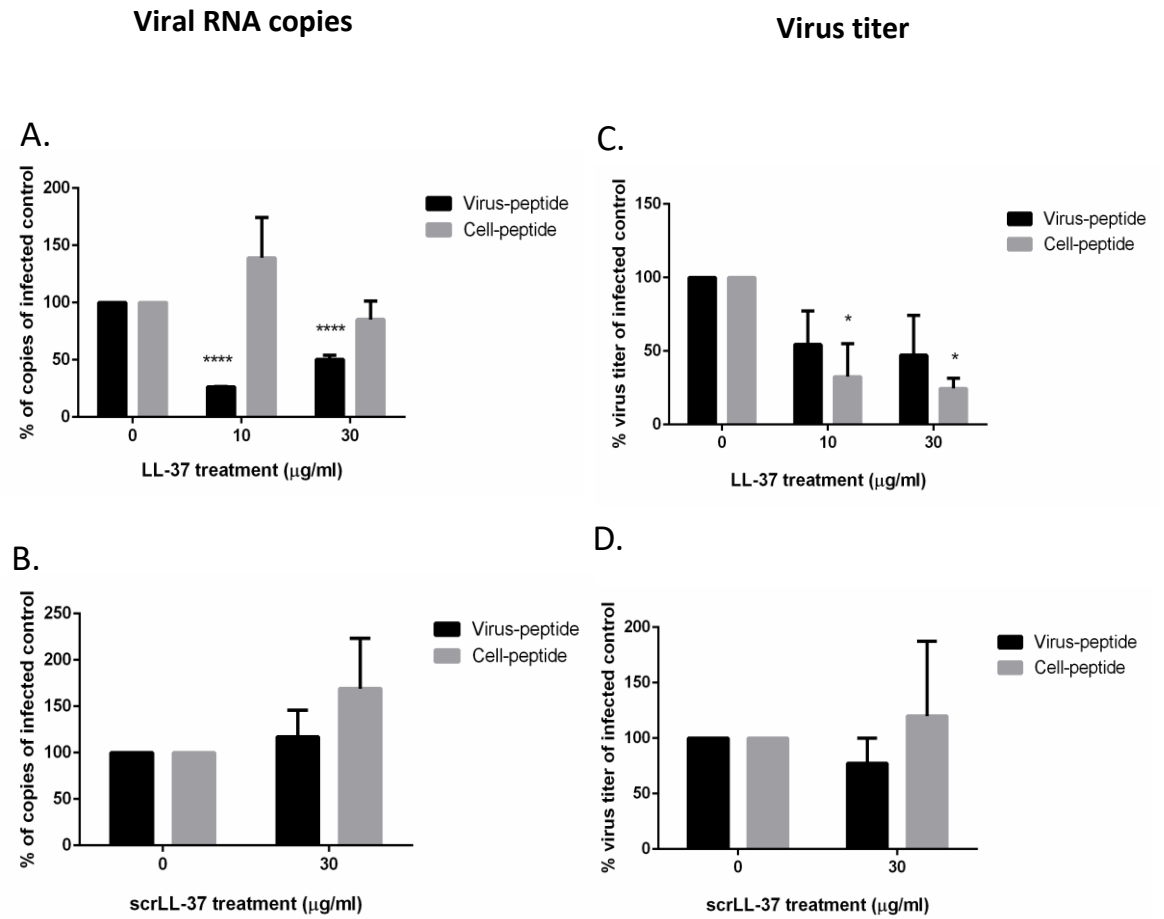


Figure 21. LL-37 displays direct and cell based antiviral activity against rhinovirus in a 16HBE14^{o-} cell model

Human rhinovirus was incubated with LL-37 (A&C) or Scrambled LL-37 (B&D) for 2h prior to cell infection or 16HBE14^{o-} cells were infected with HRV1B (MOI 5) for 2h prior to LL-37 (A&C) or Scrambled LL-37 (B&D) treatment for 22h. Total RNA was extracted from cell lysates and qPCR was performed to determine the number of viral RNA copies (A&B) and $TCID_{50}$ assay was performed to assess infective virus titer in cell supernatants 4 days after infection (C&D). Figure represents at least 4 independent experiments and displays mean values ± SEM. The percentage data was converted into arcsin values and statistical analysis was performed on these values using one-way ANOVA with Tukey´s multiple comparisons post-test to compare virus only (control) to LL-37 treatment (*p<0.5 **** p<0.0001).

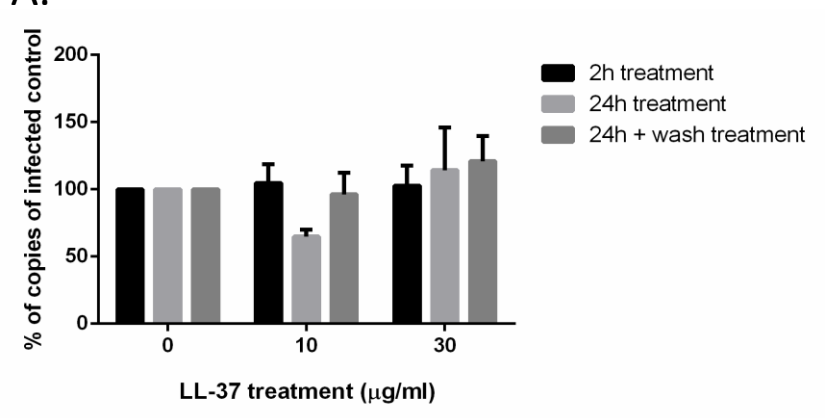
4.3.2. Assessment of the potential for LL-37 to enhance host cell resistance to rhinovirus infection

We sought to investigate the antiviral potential of LL-37 against rhinovirus when epithelial cells were briefly exposed to the peptide prior to infection. To assess this, A549 cells were treated with LL-37 for 2h prior to HRV infection and assessment of viral RNA copy number was performed 24h after infection ("2h treatment"). Exposure of A549 cells for 2h prior to infection did not result in a reduction viral copy number in comparison to untreated control (Figure 22A). We further assessed the effects of a 24h LL-37 treatment prior to HRV infection ("24h treatment"). Our results indicated that there was a reduction of viral RNA copies when 10µg/ml of LL-37 was utilised, however this reduction was not statistically significant there was a trend observed. No reduction in viral RNA copies was observed at 30 µg/ml of LL-37 (Figure 22A).

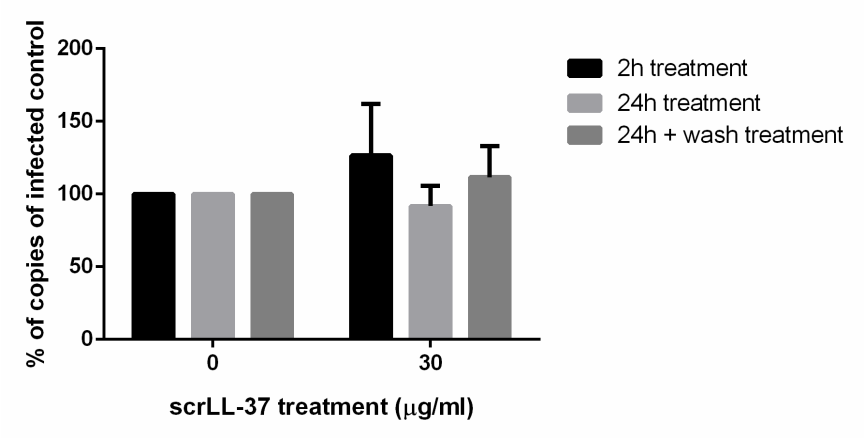
In contrast, we observed treatment of A549 cells with LL-37 for 2h followed by a wash step and the addition of fresh maintenance media for up to 24h prior to HRV infection ("24h + wash treatment"), resulted in a loss of the antiviral activity of the peptide (Figure 22A). No antiviral effects were observed using a scrambled LL-37 under the same experimental conditions (Figure 22B).

In order to establish if LL-37 was internalised by A549 cells, cells were exposed to DANSYL-labelled LL-37 for 30 minutes, washed and then imaged by confocal microscopy. The DANSYL-labelled LL-37 peptide was observed to be associated with A549 cells (Figure 22C). The intracellular localization of the peptide was relatively diffuse throughout the cell. A z-stack analysis further confirmed that the localization of LL-37 was intracellular (Appendix 1).

A.



B.



C.

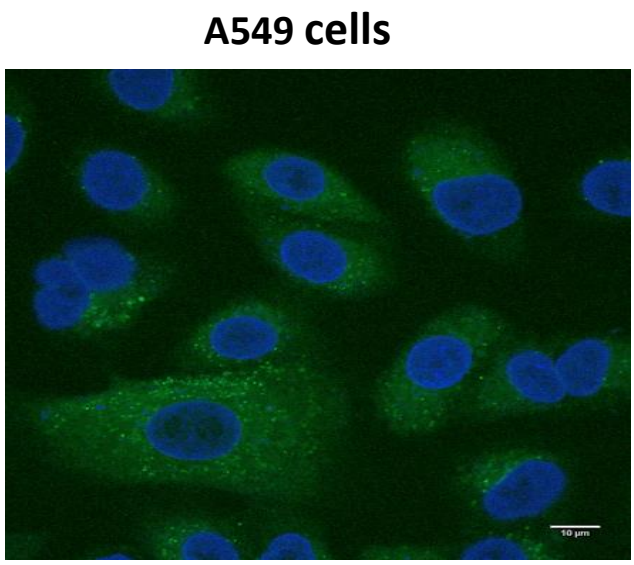
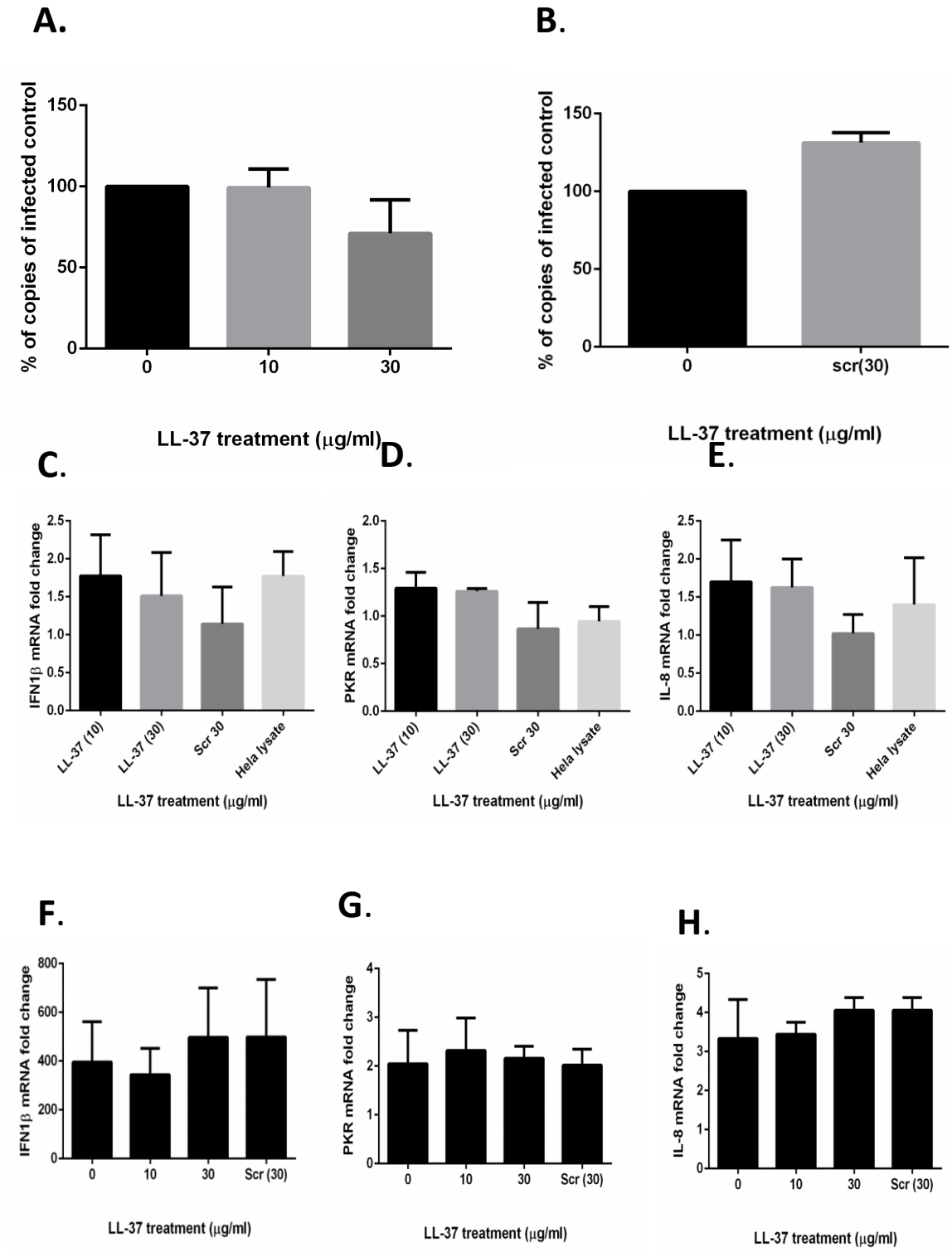


Figure 22. Prolonged LL-37 treatment can enhance host cell resistance to rhinovirus infection

A549 cells were treated with LL-37 (A) or scrLL-37 (B) for 2h ("2h treatment), or 24h ("24h continuous treatment") or 2h followed by wash step and 22h in contact with 5%FBS in DMEM ("24h wash+treatment"). After each corresponding treatment A549 cells were washed and infected with HRV1B (MOI 1) for 2h in serum-free media, washed and left in contact with 5% FBS in DMEM for 20h at 33^{o}C. Total RNA was extracted from cell lysates to determine the number of viral RNA copies (A&B). Figure represents at least 4 independent experiments and shows mean values ± SEM. The percentage data was converted into arcsin values and statistical analysis was performed on these values using one-way ANOVA with Tukey´s multiple comparison post-test to compare virus only (control) to LL-37 treatment ($p>0.05$). (C) A549 cells were exposed to DANSYL-labelled LL-37 for 30 minutes, washed, fixed and mounted with DAPI prior to confocal microscopic analysis (63 x magnification). This representative image combines the wavelength of DANSYL-tagged LL-37 in green (cadaverin 488/516 nm) and nuclei in blue (DAPI 405/454 nm).

We subsequently investigated the antiviral protective effects of LL-37 in bronchial epithelial cells, whereby 16HBE14^{o-} cells were treated with LL-37 for 2h prior to HRV1B infection. The number of viral RNA copies was determined by qPCR after a period of 24h. Results showed a moderate reduction in RNA viral copies at 30 µg/ml of LL-37 ($p>0.05$) compared to virus alone (Figure 23A), however there was a trend no statistical significance was found. Scrambled LL-37 did not alter the host cell response (Figure 23 B). To confirm the internalization and retention of LL-37 by 16HBE14^{o-} cells, cells were exposed to DANSYL-labelled LL-37 for 30 minutes, washed and then imaged by confocal microscopy (Figure 23 I). As with A549 cells, DANSYL-labelled LL-37 was observed to be associated with 16HBE14^{o-} cells (Figure 23 I), localizing around the perinuclear region. A confocal z-stack analysis indicated that the LL-37 was predominantly intracellular (Appendix 2).

Using this cell model, we investigated the ability of LL-37 to enhance the host cell antiviral response by modulating the expression of type I interferon and the viral sensor PKR as well as pro-inflammatory mediator IL-8 in healthy and infected 16HBE14^{o-} cells. Our data indicated that LL-37 did not upregulate IFN1β (Figure 23C), PKR (Figure 23 D), or IL-8 (Figure 23 E) gene expression in comparison to control in healthy non-infected cells. In addition, in 16HBE14^{o-} cells treated with LL-37 prior to HRV1B infection, our data also indicated that there was no statistically significant difference in *IFN1β* (Figure 23 F,) *PKR* (Figure 23 G) and *IL-8* (Figure 23 H) gene expression between LL-37 treatment and virus alone. Control cell (HeLa) lysates also had no effect.


A.
% of copies of infected control
150
100
50
0
0
10
30
LL-37 treatment (μg/ml)
B.
% of copies of infected control
150
100
50
0
0
scr(30)
LL-37 treatment (μg/ml)
C.
IFN1β mRNA fold change
2.5
2.0
1.5
1.0
0.5
0.0
LL-37 (10)
LL-37 (30)
Scr 30
Hela lysate
LL-37 treatment (μg/ml)
D.
PKR mRNA fold change
2.0
1.5
1.0
0.5
0.0
LL-37 (10)
LL-37 (30)
Scr 30
Hela lysate
LL-37 treatment (μg/ml)
E.
IL-8 mRNA fold change
2.5
2.0
1.5
1.0
0.5
0.0
LL-37 (10)
LL-37 (30)
Scr 30
Hela lysate
LL-37 treatment (μg/ml)
F.
IFN1β mRNA fold change
800
600
400
200
0
0
10
30
Scr (30)
LL-37 treatment (μg/ml)
G.
PKR mRNA fold change
4
3
2
1
0
0
10
30
Scr (30)
LL-37 treatment (μg/ml)
H.
IL-8 mRNA fold change
5
4
3
2
1
0
0
10
30
Scr (30)
LL-37 treatment (μg/ml)

I.

16HBE cells

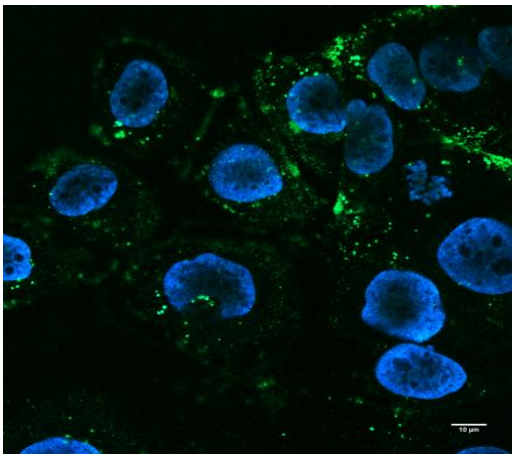


Figure 23. LL-37 can rapidly enter 16HBE14^{o-} cells but does not alter the type I interferon, PKR or IL-8 response of the host cell during rhinovirus infection.

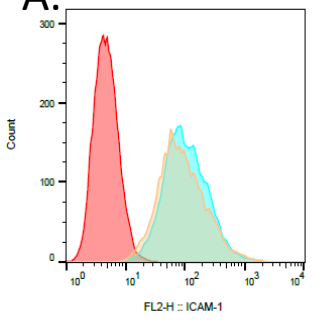
16HBE14^{o-} cells were treated with LL-37 for 2h, washed and infected with HRV1B (MOI 5) for 2h. Cells were washed and left in contact with 5%FBS in IMDM for 20h at 33^{o}C. (A-B) Total RNA was extracted from 16HBE14^{o-} cell lysates and qPCR was performed to determine viral RNA copy number. Each figure represents three independent experiments and shows mean values ± SEM. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-test to compare virus only (control) to LL-37 treatment. (C-H) qPCR was performed to determine IFN1β gene expression in healthy (C) and infected cells (F) treated with LL-37, PKR gene expression in healthy (D) and infected cells (G) treated with LL-37, and IL-8 gene expression in healthy (E) and infected cells (H) treated with LL-37. The GAPDH and β-actin genes were used as reference genes and each figure represents three independent experiments (mean values ± SEM).). (I) 16HBE14^{o-} cells were exposed to DANSYL-labelled LL-37 peptide for 30 minutes. Cells were subsequently washed, fixed and mounted with DAPI prior to confocal microscopic analysis (63 x magnifications). The image combines the wavelength of DANSYL-tagged LL-37 in green (Cadaverin 488/516 nm) and nuclei in blue (DAPI 405/454 nm).

3.3.3. Assessment of HRV and LL-37 mediated alteration of HRV-targeted receptor expression on host cells

In order to establish if LL-37 was able to modulate the expression of cell surface receptors utilised by rhinovirus for infection, we first assessed the ability of rhinovirus 1B to induce ICAM-1, LDLr and vLDLR cell surface protein expression in 16HBE14^{o-} cells during replication. Cell surface expression of ICAM-1, LDLr and vLDLR were determined by flow cytometry at 18, 24, 48 and 72h post-HRV1B infection. Our data indicated that rhinovirus infection increased surface ICAM-1 expression after 24h (Figure 24D) and that the increase in expression was enhanced at 48h post infection (Figure 24G) and 72h (Figure 24J). LDLr surface protein was considerably increased at 48h (Figure 24H) and 72h (Figure 24K) post infection. vLDLr surface protein was expressed at an extremely low level in 16HBE14^{o-} cells, and HRV1B infection did not appear to alter expression of this receptor in comparison to unstimulated cells (Figure 24 C, F, I, L).

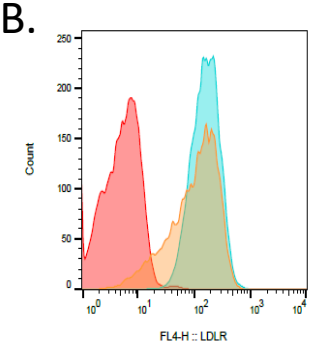
18 h time point

A.



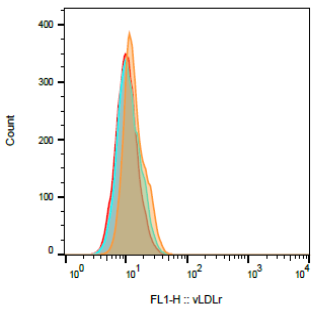
Sample Name	Subset Name	Geometric Mean : FL2-H
HRV MOI 5 ALL.015	16hb	91.5
untreated ALL.016	16hb	104
unstained.011	16hb	4.87

B.



Sample Name	Subset Name	Geometric Mean : FL4-H
HRV MOI 5 ALL.015	16hb	94.7
untreated ALL.016	16hb	157
unstained.011	16hb	5.15

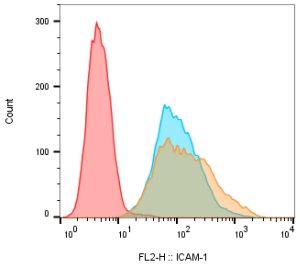
C.



Sample Name	Subset Name	Geometric Mean : FL1-H
HRV MOI 5 ALL.015	16hb	13.9
untreated ALL.016	16hb	11.4
unstained.011	16hb	10.4

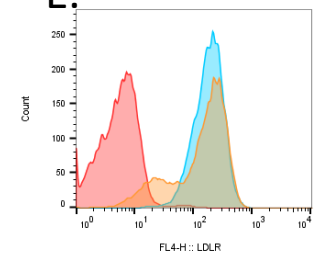
24h time point

D.



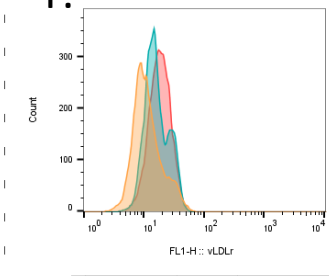
Sample Name	Subset Name	Geometric Mean : FL2-H
hrv moi 5 ALL.014	16hbe	135
UNTREATED ALL.015	16hbe	99.3
UNSTAINED.016	16hbe	4.78

E.



Sample Name	Subset Name	Geometric Mean : FL4-H
hrv moi 5 ALL.014	16hbe	119
UNTREATED ALL.015	16hbe	178
UNSTAINED.016	16hbe	5.38

F.



Sample Name	Subset Name	Geometric Mean : FL1-H
hrv moi 5 ALL.014	16hbe	11.7
UNTREATED ALL.015	16hbe	16.4
UNSTAINED.016	16hbe	18.3

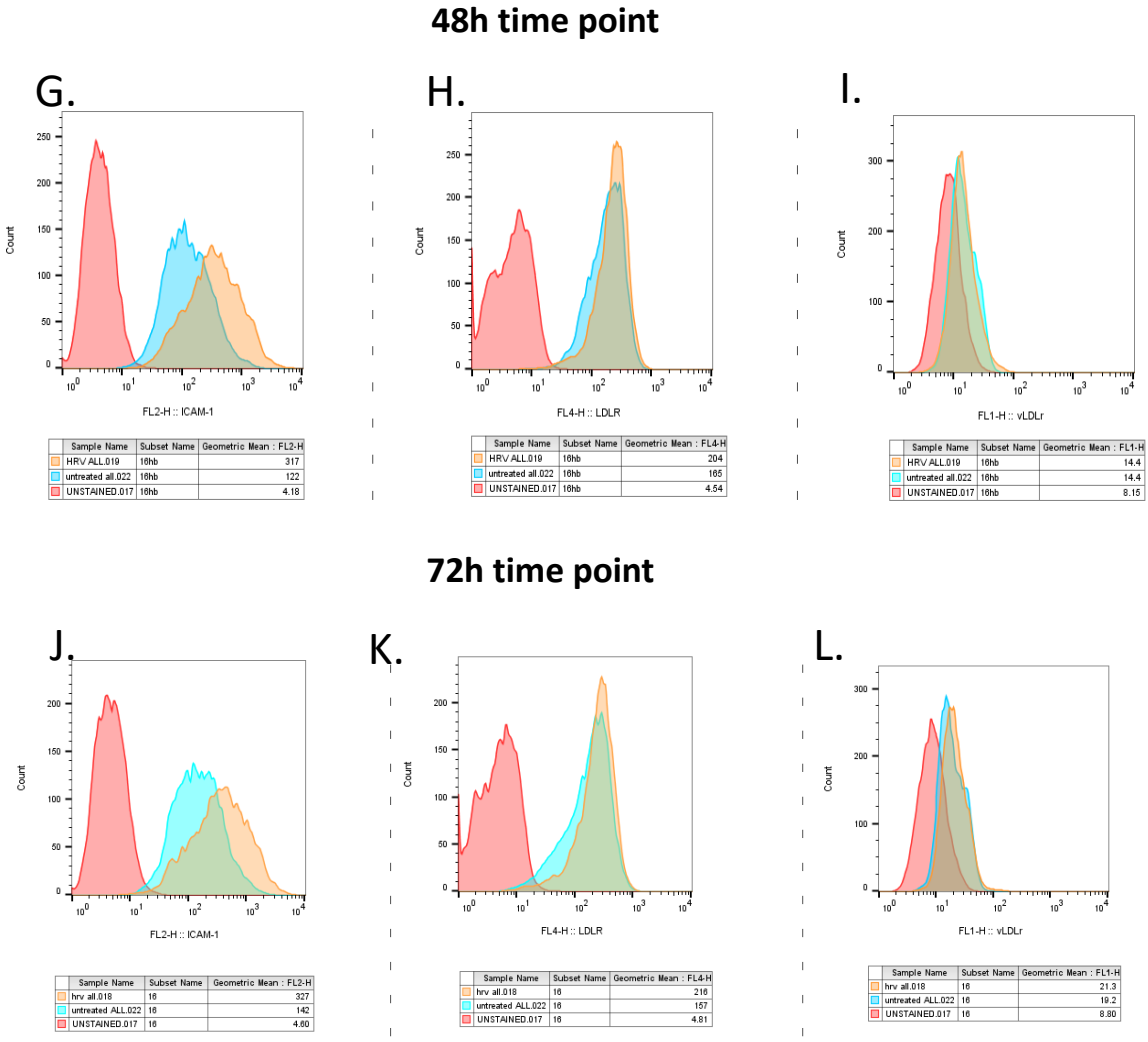


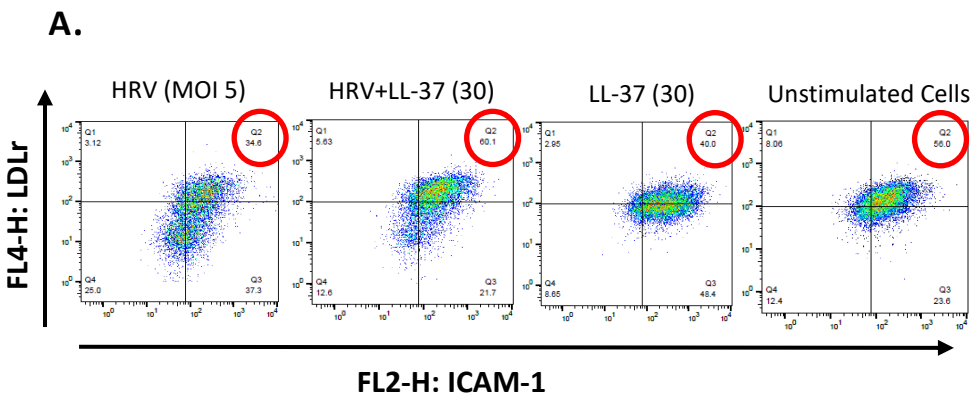
Figure 24. Time course of rhinovirus-induced ICAM-1 and LDLr surface expression on 16HBE epithelial cells.

Surface LDLr, vLDLr and ICAM-1 were measured by flow cytometry on healthy or RV1B-infected 16HBE cells (MOI 5; 2h). Cells were washed and incubated in maintenance media for 18, 24, 48 and 72h. All cells were stained for ICAM-1, LDLr and vLDLR using conjugated antibodies with unstained cells as a control for background autofluorescence to set the negative population. Representative flow cytometric histograms for each time point indicating surface expression of ICAM-1, LDLr and vLDLr in HRV1B –infected and uninfected 16HBE cells are displayed. Geometric mean values are shown for infected, non-infected and unstained cells for each fluorescence channel.

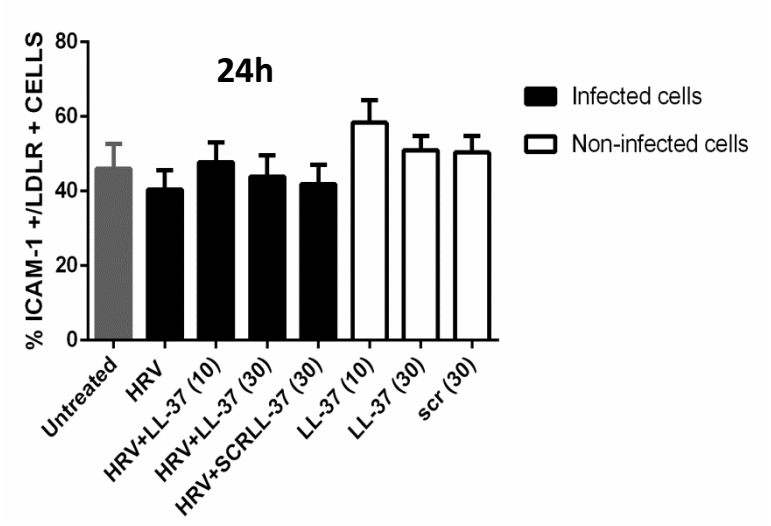
We assessed the ability of LL-37 to modulate HRV-target receptors. Rhinovirus was incubated with LL-37 prior to infection of 16HBE14^{o-} cells and cell surface expression of ICAM-1 and LDLr were determined by flow cytometry at 24 and 48h.

At 24h, HRV replication was shown to induce the expression of ICAM-1 and at 48h, HRV greatly increased the expression of ICAM-1 as well as LDLr. Therefore these two time points were selected to further study the modulatory properties of LL-37 on HRV-target receptors. In addition several other parameters were measured at these time points throughout this project, such as viral RNA copies, cytokine and chemokine expression and production, which together can help to fully characterise the peptides antiviral properties. The percentage of positive events for both ICAM-1 and LDLr surface expression was determined for each condition at 24 and 48h. Our data indicated that at 24h, rhinovirus infection alone did not increase expression of cells positive for both ICAM-1 and LDLr compared to untreated cells, and at this timepoint LL-37 did also not alter ICAM-1 and LDLr surface expression in both infected and uninfected 16HBE cells (Figure 25B).

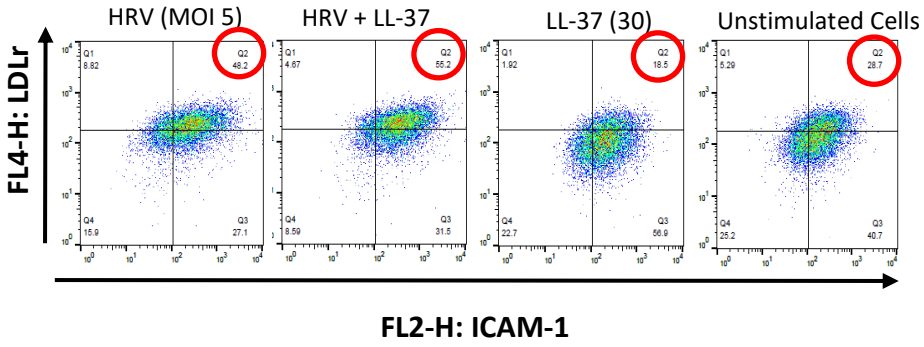
However, while rhinovirus infection significantly increased the percentage of ICAM-1 and LDLr positive cells compared to uninfected cells (*$p<0.05$) at 48h, LL-37 treatment inhibited the HRV-mediated increase in receptor expression (Figure 25D). The scrambled control peptide treatment of both infected and uninfected cells, together with LL-37 treatment of healthy cells, did not induce any changes in surface receptor expression (Figure 25D).



B.



C.



D.

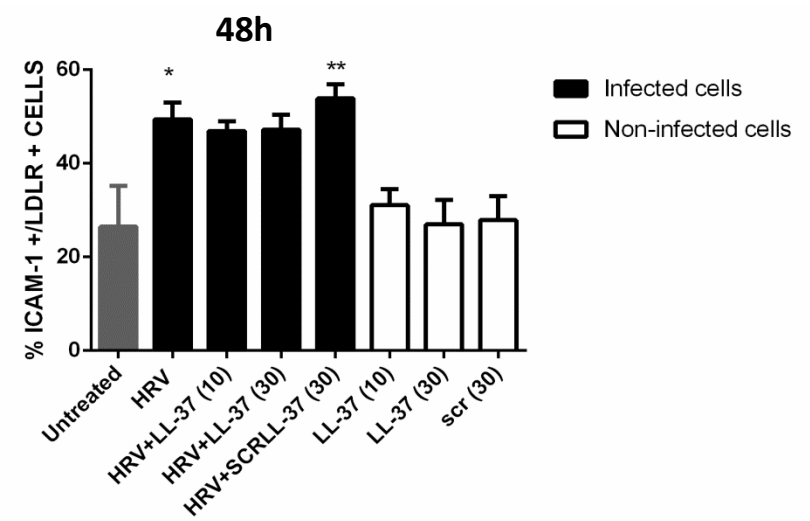


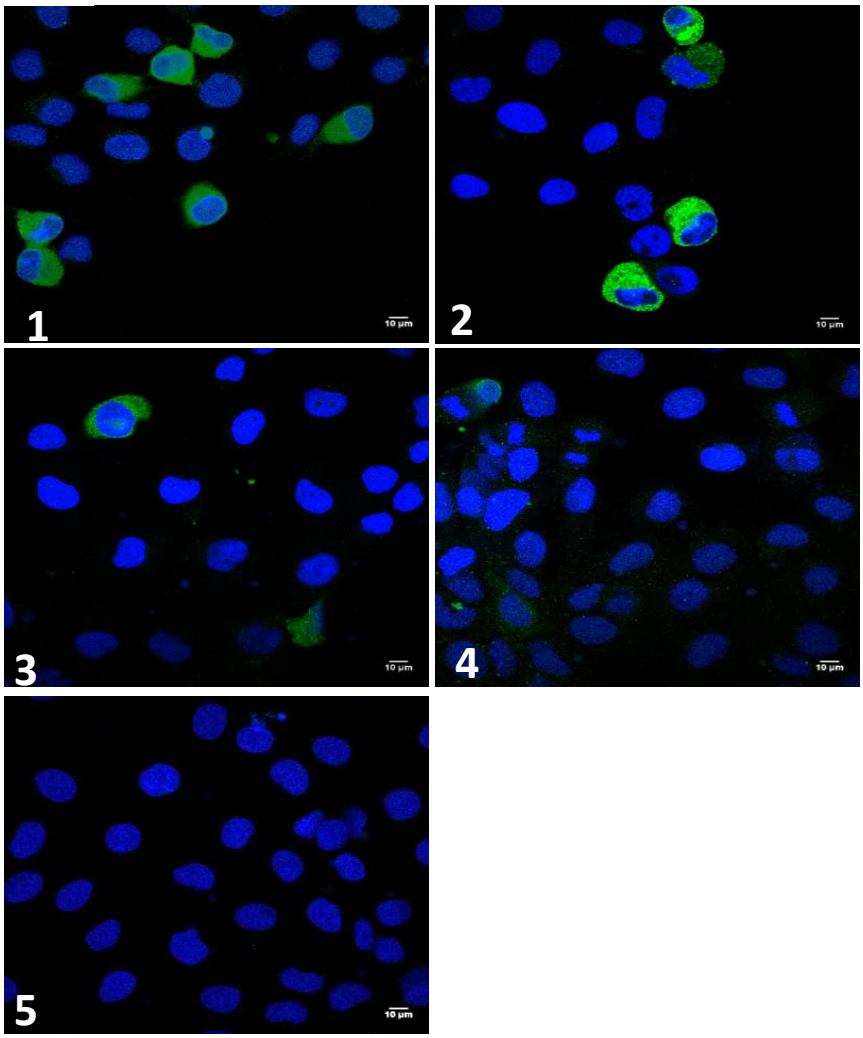
Figure 25. LL-37 modulates ICAM-1 and LDLr cell surface expression in response to rhinovirus infection.

Purified rhinovirus 1B (MOI 5) was incubated with LL-37 in serum-free media for 2h at 33^{o}C prior to 16HBE14^{o-} cell infection. Cells were subsequently incubated for a total of 24h (A&B) or 48h (C&D). Cell surface ICAM-1 and LDLr were measured by flow cytometry and data shown are representative scatter dot plots showing the percentage of positive events for both ICAM-1 and LDLr surface protein expression at 24h (A) and 48h (C). Graphical representation (mean values ± SEM) of percentage of positive events for both ICAM-1 and LDLr surface proteins are shown at 24h (B) and 48h (D). Figures represent at least four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey´s multiple comparisons post-test to compare infected and un-infected cells treated with LL-37 to untreated cells(* $p<0.05$; **$p< 0.01$).

4.3.4. Characterisation of LL-37 mediated alterations in HRV infectivity

Our data has shown that incubating rhinovirus with LL-37 prior to 16HBE14^{o-} cell infection led to a significant reduction in viral RNA copies compared to virus alone (Figure 21). For additional characterisation of this observation and to assess the effects of LL-37 on viral infectivity, a pan-enterovirus monoclonal antibody was used to detect rhinovirus by confocal microscopic analysis. Quantification of the fluorescence detected shows a dose-dependent decrease in viral infectivity in airway cells infected with HRV exposed to LL-37 prior to infection (Figure 26 A and B). Scrambled LL-37 did not induce a reduction in the number of infected cells compared to virus alone (Figure 26 A and B).

A.



1. HRV1B (MOI 1)	**2**. HRV1B (MOI 1) + scrLL-37 (30μg/ml)
3. HRV1B (MOI 1) + LL-37 (10μg/ml)	**4.** HRV1B (MOI 1) + LL-37 (30μg/ml)
5. Uninfected (primary ab+ secondary ab)	

B.

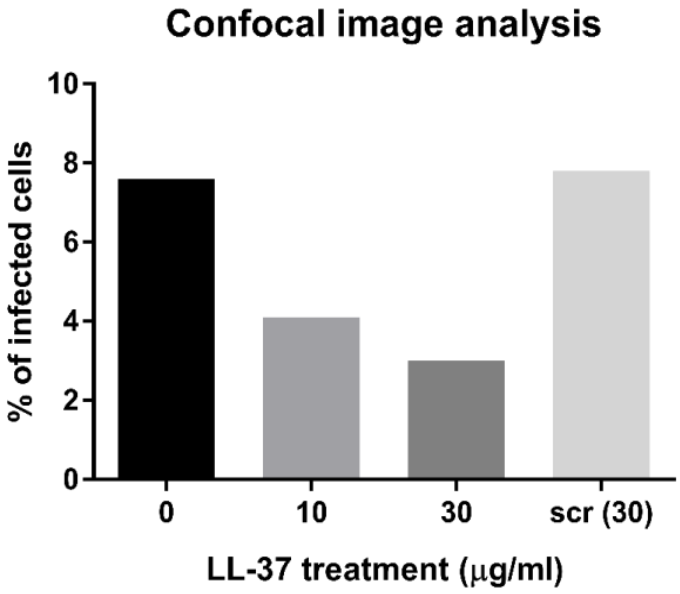


Figure 26. LL-37 exposure affects rhinovirus infectivity.

Human rhinovirus 1B (purified stock) was incubated with LL-37 for 2h at 33^{o}C prior to 16HBE14^{o-} cell infection (MOI 1). 16HBE14^{o-} cells, previously seeded in chamber slides, were exposed to peptide/virus mixture for 1h, washed and cultured for further 23h in maintenance media at 33^{o}C. Cells were washed, fixed and permeabilised with 0.1% Triton-X. A pan-enterovirus monoclonal antibody (raised in mouse) was used to detect rhinovirus VP3 and imaged by confocal microscopy. (A) A goat anti-mouse IgG (Alexa Fluor 488) was used as a secondary antibody for primary pan-enterovirus antibody; Virus appear in green and nuclei were stained with DAPI and appear blue. Images taken at 63x objective. (B) Representative graph of the % infected cells counted from at least 3 fields of view with more than 40 cells per field of view. Images taken at 40x objective.

To further determine if LL-37 binding to HRV resulted in destabilization of the viral particle structure, we assessed viral capsid integrity by transmission electron microscopy in the presence and absence of LL-37. In order to visualise the HRV virions, a number of purification steps were undertaken to ensure high purity prior to imaging. A sucrose gradient ultracentrifugation was performed to concentrate/purify the virus particles according to their density, thereby separating them from other cellular material. SDS-PAGE was performed to identify the presence of viral proteins (VP1-VP3 ~ 20-30 kDA) [Appendix 4]. We exposed HRV1B to LL-37 for 2h at 4^{o}C before imaging by negative staining in transmission electron microscopy (Figure 27). Imaging data showed a dramatic reduction in the number of HRV1B virions when exposed to LL-37, compared to untreated, purified HRV1B (Figure 27). A second sucrose gradient ultracentrifugation of HRV1B samples was conducted to obtain virus preparations of a higher concentration and purity to image with cryotransmission electron microscopy. Following analysis by SDS-PAGE, the second gradient purification resolved in a clear band indicating that the sample was highly pure (Appendix 5). We subsequently exposed the purified HRV1B to 50 µM LL-37 for 5 minutes at 4^{o}C, or endotoxin-free water as a negative control, before imaging of the samples by cryoEM. In accordance with the negative staining obtained through transmission electron microscopy imaging (Figure 27), the cryoEM micrographs also revealed a dramatic reduction in virions in LL-37 exposed HRV1B compared to control HRV1B (Figure 27). However, in both untreated and LL-37-exposed HRV1B samples, the virions demonstrated similar morphology and appearance, indicating no perceptible alterations on the viral capsid and no free capsid content.

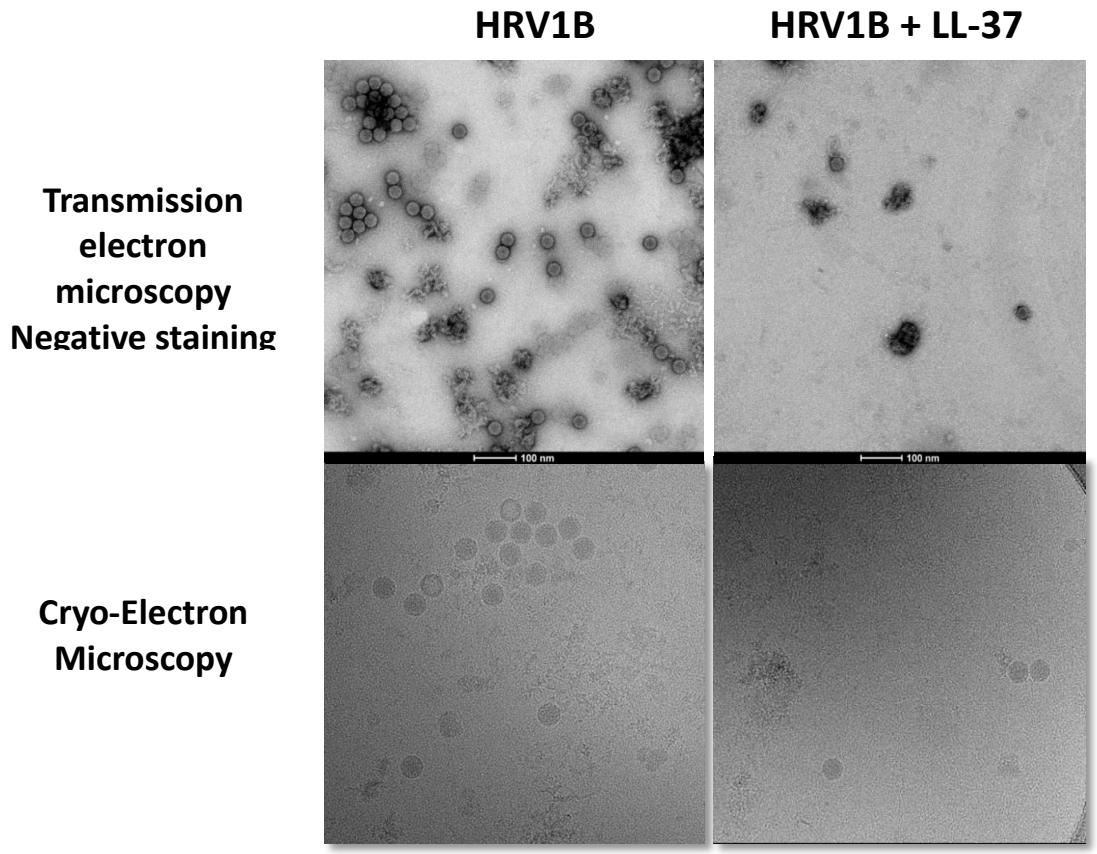


Figure 27. LL-37 reduces viable rhinovirus concentrations but does not appear to alter virus ultrastructure.

Negative staining electron microscopy and cryo-transmission electron microscopy micrographs of control HRV and HRV exposed to LL-37. HRV1B was exposed to LL-37 or endotoxin-free water for 2h (TEM) or 5 minutes (cryoEM) on ice. For negative stain, samples were added to carbon coated copper grids. The stained layer was dried and imaged by TEM Technai T12 running at 120keV. For cryoEM, samples were added to C-flat™ holey carbon coated copper grid and vitrified with a FEI Vitrobot IV. Electron micrographs were collected on a FEI Technai Polara microscope operated at 300 keV. The absolute magnification was 37,037x corresponding to a pixel size of 1.35 Å on a molecular scale. Micrographs represent observations from a minimum of two grids analysed per condition.

4.3.5. Assessment of the impact of LL-37 on the bronchial epithelial cell response to HRV infection

We previously showed that treating HRV with LL-37 prior to cell infection leads to a reduction in viral infectivity (Figure 21) and cryoEM studies further revealed the ability of the peptide to directly target the virus reducing the number of virions (Figure 27). To then assess the impact of LL-37 on bronchial epithelial cell response to HRV infection, we investigated the mRNA transcript levels of type I interferon genes-IFN1β and interferon inducible gene PKR as well as IFN1β protein levels.

HRV infection strongly induced IFN1β transcription (301-fold above unstimulated cells) and only mildly induced PKR transcription (3.4-fold above unstimulated cells) although in LL-37 treated cells transcription levels were significantly lower (Figure 28A&B). Addition of LL-37 reduced virus induced IFN1β gene expression to 54.7-fold at 10 μg/ml (*$p<0.05$) and 44.8-fold at 30 μg/ml ($p<0.05$) [Figure 28A], and PKR gene expression to 1.8-fold at 10μg/ml and 1.6-fold at 30 μg/ml (Figure 28b). Scramble LL-37 did not alter IFB1β and PKR mRNA expression in comparison to infected cells (Figure 28 A&B). LL-37 exposure to uninfected cells did not induce IFN1β or PKR gene expression above uninfected cells (Figure 28 A&B).

To confirm that the mRNA transcript levels were translated to changes at the protein level, we assessed cell supernatants for IFN1β protein release. In accordance to the gene expression data, HRV infection induced an increase in IFN1β secretion (42.9 pg/ml) and the addition of LL-37 lead to a significant dose dependent decrease in IFN1β secretion (13.89 pg/ml for 10μg/ml and 10.63 pg/ml at 30μg/ml, *$p<0.05$) [C]. The IFN1β protein concentration in uninfected control was too low to detect. Scramble LL-37 did not alter IFN1β secretion compared to HRV-infected cells (Figure 28C).

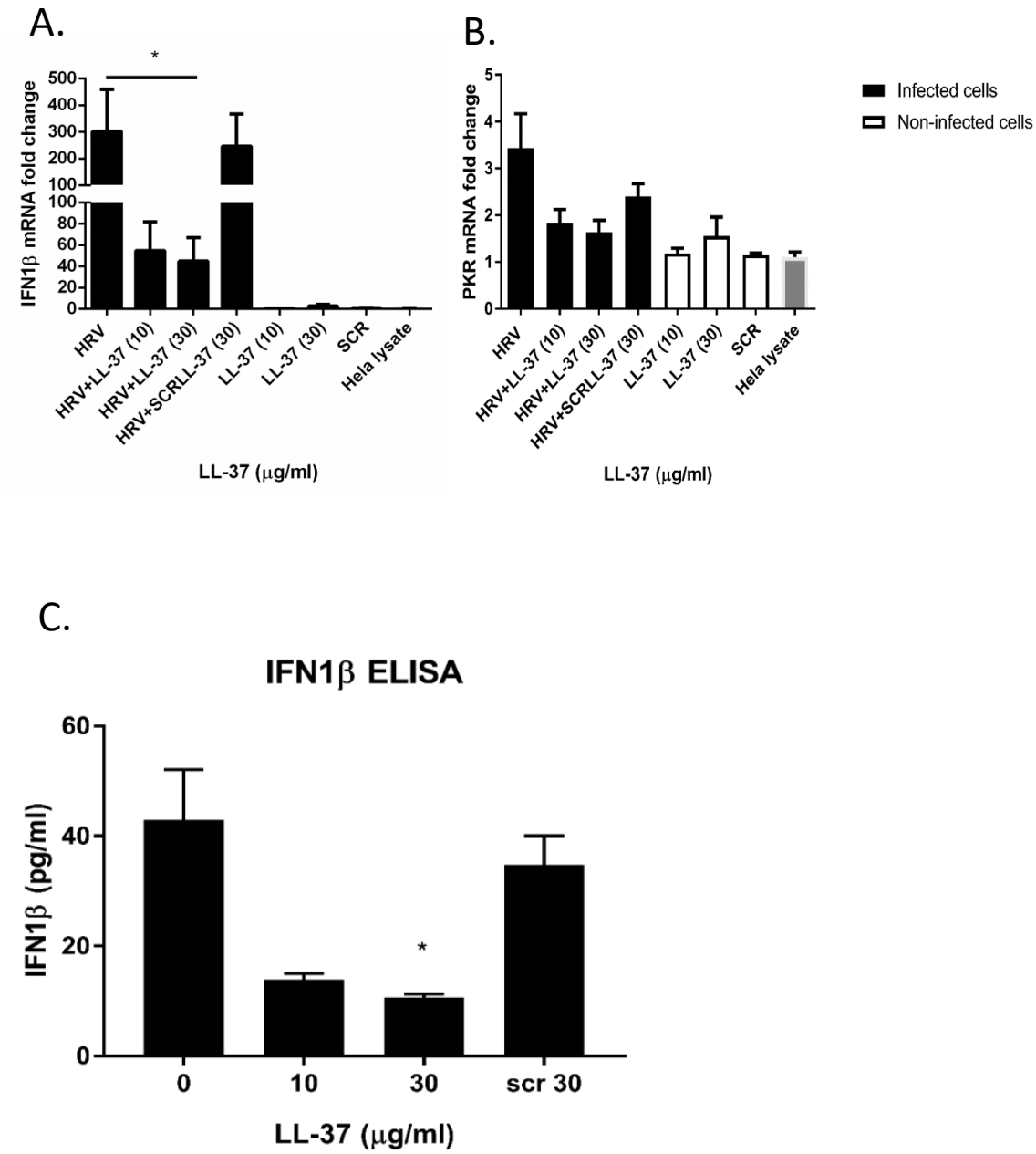


Figure 28. LL-37 treatment reduces the antiviral response associated with HRV1B infection.

HRV1B (MOI 5) was pre-incubated with LL-37 in serum-free media for 2h prior to cell infection. 16HBE14^{o-} cells were exposed to mixture for 1h, washed and cultured for further 23h in maintenance media at 33^{o}C. Total RNA was extracted from 16HBE14^{o-} cell lysates and qPCR was performed to determine levels of *IFN1β* (A) and *PKR* (B) mRNA transcripts. Values represent fold-changes over uninfected cells. The *GAPDH* and *β-Actin* genes were used as reference genes. IFN1β protein levels released into the supernatants were determined by IFNβ ELISA (C). Each figure represents three independent experiments. Figures show mean values ± SEM. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons post-test to compare virus only to LL-37 treatment (* $p<0.05$).

4.4. Discussion

The sole human cathelicidin, LL-37, has been shown to possess antiviral activity against a number of viruses (reviewed in Barlow et al., 2014). hCAP18/LL-37 is produced primarily by neutrophils and airway epithelial cells and can be upregulated in response to infection and inflammation in the airway environment. In this study, we aimed to characterise the antiviral activity of LL-37 against rhinovirus in the context of a number of concentrations and exposure regimens.

In healthy infants and adults, hCAP-18/LL-37 has been detected in bronchoalveolar lavage fluid (BALF) at a concentration of approximately 5µg/ml and 2µg/ml, respectively (Schaller-Bals et al., 2002). Higher concentrations of the peptide have been detected during infection. In newborns with pulmonary infections, concentrations of LL-37 can reach approximately ~25 µg/ml in the lungs, and in adult patients with cystic fibrosis, LL-37 concentrations are estimated to be ~15µg/ml (Chen et al., 2004; Schaller-Bals et al., 2002). In the literature, and in this current study, an *in vitro* concentration of 10 µg/ml of LL-37 has been considered to be of relevance to a physiologically normal state, and a concentration of 30 µg/ml to be a physiologically inflammatory level of LL-37. Furthermore, LL-37 has been shown to be significantly inhibited by the presence of human serum (Ciornei et al., 2005) and Wang et al. (1998) reported that apolipoprotein A-I (apoA-I) present in human plasma can bind and scavenge LL-37, thus inhibiting its activity. Thus, to appropriately represent the serum-free environment of the lung, all treatments were conducted either in serum-free media or in the presence of ultroSer™ G synthetic serum substitute.

In this chapter we show that the human cathelicidin, LL-37, has potent antiviral activity against HRV1B in *vitro*. The antiviral effects of LL-37 were assessed in relevant pulmonary cell models of rhinovirus infection, A549 and 16HBE14^{o-} cells. In A549 cells, we determined that the antiviral effects were most visible when the virus was exposed to peptide prior to infection, as well as when cells were infected with virus prior to peptide treatment.

In 16HBE14^{o-} cells, LL-37 showed potent antiviral activity at both physiologically normal (10μg/ml) and physiologically inflammatory (30μg/ml) concentrations, decreasing the measurable viral RNA copies in infected cells by approximately 50-70%, when the virus was exposed to the peptide prior to cell infection (****$p \leq 0.0001$). This was confirmed by $TCID_{50}$ assay, which indicated that LL-37 led to a dose-dependent decrease in virus titer compared to virus alone when HRV is treated with LL-37 prior to cell infection ("virus-peptide", $p>0.05$), however this reduction was not statistically significant a trend was observed. In contrast to data obtained using A549 cells, treating infected 16HBE14^{o-} cells with LL-37 did not lead to a reduction in viral RNA copy number. However, analysis by $TCID_{50}$ assay revealed that LL-37 did lead to a statistically significant reduction of virus titer in this cell model. Previous studies have reported similar findings investigating the antiviral effects of LL-37 against viral airway pathogens. Currie *et al*., (2013) assessed the effects of LL-37 against respiratory syncytial virus (RSV) revealing that LL-37 has the potential to directly target RSV and/or host airway epithelial cells, and a maximal antiviral effect was achieved when exposing RSV to the peptide prior to cell infection. Similar results have also been reported in studies using Influenza A virus (Barlow et al., 2011; Tripathi et al., 2013). In the context of this study, a lack of reduction in viral RNA copies, but a measurable decrease in virus titer measured by $TCID_{50}$ assay may indicate that LL-37 directly affects the virus extracellularly, or indeed alters the host cell response, inhibiting virus entry or exit, in turn inhibiting infection of neighboring cells. Indeed, in the context of other HDP, HBD2 was shown to block viral cellular entry of RSV through its ability to destabilize the RSV viral envelope (Kota et al., 2008). In the non-enveloped adenovirus, HBD5 was shown to induce AdV aggregation (Gounder et al., 2012), which could impact viral infectivity by impeding cell binding.

Alternatively, we hypothesised that LL-37 could target the host cell by directly modulating host cell surface receptors important for HRV entry. Support for this hypothesis came from studies that have shown that HBD2 and HBD3 were able to suppress the expression of CXCR4 in PBMC and T cell lines during HIV-1 infection, thereby reducing HIV infectivity of these cells (Quiñones-Mateu et al., 2003). In addition, an important factor to consider in this experimental context is the

prolonged exposure of 16HBE14^{o-} cells to LL-37. Studies have shown that LL-37 can exhibit cytotoxicity activity toward eukaryotic cells (Johansson et al., 1998); the peptide was shown to cause cell death in human nasal epithelial cells through the pro-inflammatory necrotic and/or pyroptotic pathways rather than apoptosis (Thomas et al., 2017). Thus, in Chapter 5 we explored the relationship between decreased viral infectivity induced by inflammatory concentrations of LL-37 in tandem with cytotoxicity, as a potential mechanism of action.

To determine if the antiviral effects of LL-37 were sequence specific, we tested the antiviral activity of a scrambled LL-37 peptide with identical amino-acid sequence at inflammatory concentrations (30μg/ml). Our data indicated that the control scrambled LL-37 peptide did not exhibit any anti-HRV activity. This is consistent with previous studies investigating the antiviral activity of LL-37 against IAV (Barlow et al., 2011), RSV (Harcourt et al., 2016; Tripathi et al., 2013) and dengue virus (Alagarasu et al., 2017).

Human rhinoviruses infect the lower respiratory tract (LRT), with a major site of infection occurring in bronchial epithelial cells (Papadopoulos et al., 2000). Several studies have provided evidence of rhinovirus ability to replicate and infect alveolar epithelial cells, further supporting the implication of the virus in lower respiratory tract infections (Johnston et al., 1998; Kennedy et al., 2012; Reza Etemadi et al., 2017). Due to the fact that HRV has showed the ability to replicate in different locations of the lower respiratory tract, we have chosen two different cell models representing the bronchial (16HBE14^{o-}) and alveolar epithelium (A549), which we previously shown in chapter 3 to be relevant cells models of HRV infection.
A549 cells are derived from a human alveolar carcinoma cells with properties of type II alveolar epithelial cells. Type II cells secrete pulmonary surfactant and their morphology is granular and roughly cuboidal (French, 2009). 16HBE14^{o-} are a human bronchial epithelial cell line isolated from a 1 year old male patient and immortalised with the origin of replication (ORF-) defective SV40 plasmid (pSVori-). This cell line maintains the characteristic features of normal differentiated bronchial epithelial cells such as the cobblestone morphology, cytokeratin expression, the ability to form tight junctions and directional ion transport (Cozens et al., 1994;

Wan et al., 2000). To fully elucidate the ability of the virus to infect the LRT and to investigate the ability of LL-37 to inhibit HRV replication, studies were carried out in both bronchial and alveolar cell line, which possess different functions and morphologies in the LRT.

Our data showed that physiologically normal concentrations of LL-37 (10µg/ml) significantly reduced the ability of A549 cells to be permissive to HRV infection when cells were pre-treated with peptide for 24h and washed before HRV infection. We also found that exposing A549 cells to LL-37 for 2h prior to HVR infection did not result in a reduction of viral infectivity, and indeed, similar results were seen when cells were treated with LL-37 for 2h, washed and left in maintenance media for up to 24h prior to infection with the virus. This suggests that a prolonged exposure to LL-37 may be required for an anti-HRV effect on the host epithelial cell. Other studies have reported similar results, in which cells were pre-treated with LL-37 and washed prior to viral infection, observing reductions in RSV viral load (Currie et al., 2013) and IAV infectivity (Tripathi et al., 2013). The human cathelicidin LL-37 inhibits influenza A viruses through a mechanism distinct from that of surfactant protein D or defensins (Tripathi et al., 2013). We propose that rhinovirus may interact with cell-associated LL-37 which was not removed during the wash step or that LL-37 is internalised by host cells and modulates the host response to HRV infection. However, given that we did not see a substantial loss of measureable virus with this treatment regimen, it is likely that other factors are involved.

We investigated the internalisation of LL-37 by airway epithelial cells by exposing A549 and 16HBE14^{o-} cells to DANSYL-labelled LL-37 and imaging by confocal microscopy. Our data demonstrated that LL-37 treatment prior to viral infection resulted in rapid peptide internalisation and retention. Using fluorescent LL-37, we also assessed LL-37 degradation over time after cell internalization and found no alteration in mean DANSYL intensity values after a period of 16h (Appendix 3). However, this does not exclude the possibility of peptide degradation, as the fluorochrome attached to LL-37 may remain intact even after peptide degradation. Previous studies have demonstrated that LL-37 can interact with epithelial cells, being actively taken-up by A549 epithelial cells and trafficked to the perinuclear

region in a microtubule-dependent manner (Lau *et al*., 2005), but fully characterising intracellular peptide stability requires further study.

Viral infections are known to induce a rapid and strong type I interferon response which has a potent ability to alter viral replication. Previous studies have investigated the ability of LL-37 to modulate a type I interferon response in different systems, inducing IFN-1β expression in plasmacytoid dendritic cells (pDC) and in normal epidermal keratinocytes via TLR9 or TLR3, respectively (Lande *et al*., 2007; Takiguchi *et al*., 2014). We therefore investigated whether the internalised peptide could induce an antiviral response which would protect cells from subsequent infection. Our data suggested that, in 16HBE14^{o-} cells, LL-37 did not induce IFN-1β or PKR gene expression in healthy cells, and in HRV-infected cells there was no enhancement of IFN 1β or PKR gene expression above that observed in infected cells. This data indicated that internalised LL-37 could mediate moderate protection against HRV infection in A549 and 16HBE cells, although the mechanism behind the activity of the peptide did not appear to involve the modulation of type I interferon production.

Human rhinoviruses replicate efficiently in epithelial cells of the upper and lower airway tract. The major group of this virus accesses airway cells via the ICAM-1 receptor (Greve et al., 1989) and the minor group of the virus accesses airway cells via LDLr or vLDLr (Hofer et al., 1994; Nicodemou et al., 2005). Our initial studies demonstrated that rhinovirus 1B infection of 16HBE14^{o-} cells increased the cell surface expression of ICAM-1 and LDLr protein. ICAM-1 expression was induced at 24h post-infection and expression increased throughout time peaking at 72h. LDLr expression was induced after 48h and remained elevated above unstimulated cells for up to 72h post-infection. Similar results have also been shown in A549 cells during HRV16 infection, a major group of virus, although peak expression of surface ICAM-1 was seen at 8h post-infection. ICAM-1 expression was still significantly increased compared to unstimulated cells at 72h p.i (Papi and Johnston, 1999). The minor group of virus, HRV2, was also shown to upregulate ICAM-1 expression at 8h p.i in A549 cells. In human tracheal epithelial cells, HRV2 (minor group) was shown to induce surface expression of LDLr three days after infection and HRV14 (major

group) was shown to induce LDLr surface expression five days after infection. An antibody against LDLr inhibited HRV2 infection and cytokine production in response to infection but had no effect on HRV14 infection or cytokine production (Suzuki et al., 2001). This indicates that rhinovirus-induction of ICAM-1 and LDLr is not virus-strain specific. This further signifies that both ICAM-1 and LDLr cell receptors can be upregulated in an indirect form due to viral infection. Rhinovirus-induced ICAM-1 expression is dependent upon upregulation of NFkB, and LDLr expression is dependent in the upregulation of SP-1 transcriptional factor, both of which have been shown to be upregulated by both minor and major group of virus during infection of airway cells (Papi and Johnston, 1999; Suzuki et al., 2001).

Having found that HRV1B enhanced ICAM-1 and LDLr surface expression in 16HBE14^{o-} cells, we sought to investigate the ability of LL-37 to modulate the expression of these receptors during HRV infection. 16HBE14^{o-} cells were infected with HRV1B that had been pre-treated with LL-37, and receptor expression was assessed after 24 and 48h by flow cytometry. Results indicated that rhinovirus infection did not increase the expression of either ICAM-1 or LDLr in 16HBE14^{o-} cells above untreated cells after a period of 24h. LL-37 did also not alter the expression of both receptors in infected cells and healthy cells. At 48 h, HRV infection significantly increased the expression of both receptors on 16HBE14^{o-} cells above unstimulated cells, and this was also seen in 16HBE14^{o-} cells infected with HRV pre-treated with scrambled LL-37. However we did observe that at this time-point, in 16HBE14^{o-} cells infected with HRV pre-treated with LL-37, there was not a significant increase in the expression of ICAM-1 and LDLr compared to unstimulated 16HBE cells, indicating that the peptide appeared to suppress virus mediated expression of these receptors. These results suggest that LL-37 can down-modulate rhinovirus target receptor expression in bronchial epithelial cells either through a direct effect on the virus or on the host cell. For example, the modulatory effects of LL-37 on HRV-target receptors may be in fact due to the direct effects of the peptide on the virus, inhibiting viral replication and consequently resulting in a lower expression ICAM-1 and LDLr surface proteins.

In similarity to our own results, HBD2 and HBD3 have shown the ability to downregulate CXCR4 expression in PBMCs, which is a chemokine receptor that functions as a co-receptor for HRV-1 entry into CD4 positive cells, blocking HIV-1 replication (Quiñones-Mateu et al., 2003). Consequently our results suggest that LL-37 could inhibit HRV1B replication both via direct interaction with the virion, and potentially through the down-modulation of ICAM-1 and LDLr receptor expression in bronchial epithelial cells. However, further investigation will be required to elucidate the exact mechanism of action, and whether any effect is likely to be temporal or cell type specific.

A number of studies have attempted to provide insights on the direct mechanism of action of LL-37 against a number of viruses. Currie et al., (2016) demonstrated that LL-37 mediates an antiviral effect on RSV by directly damaging the viral envelope, disrupting viral particles and reducing virus binding to, and infection of, human airway epithelial cells. This was also observed in IAV, in which LL-37 was found to cause disruption in viral membranes (Tripathi et al., 2013). LL-37 has also been shown to target DENV-2, by binding to the E protein inhibiting virus cell entry (Alagarasu et al., 2017). However, the mechanism of action of LL-37 against non-enveloped virus, such as HRV, is not yet well understood. A study by Smith et al. (2010) did investigate the mechanism of HD5-mediated neutralization of adenovirus, with CryoEM structural studies demonstrating critical sites on the adenovirus capsid which HD5 binds to prevent subsequent steps of the virus uncoating. We suggest that this would be a valuable way forward in the context of this study.

Our confocal microscopy analysis revealed a dose-dependent reduction of infection in cells infected with HRV pre-treated with LL-37. Utilising this method, we observed a substantial reduction in infection at inflammatory concentrations of LL-37. Our initial data obtained by electron microscopy also showed a substantial reduction in the number of virions in LL-37-treated HRV sample in comparison to control HRV, but with no obvious alteration of the viral capsid structure, and no increase in the number of empty capsids in LL-37-treated samples. This data in combination with our results showing a reduction in infectivity when HRV is pre-treated with LL-37,

further confirms the ability of LL-37 to target HRV potentially disrupting or preventing viral replication.

The immunomodulatory properties of LL-37 involve the capacity to alter pattern recognition receptor signalling, in which the peptide was shown to facilitate the recognition of dsRNA in a TLR3 dependent manner in rhinovirus infected bronchial epithelial and promote cytokine release (Lai et al. 2011) as well as the capacity to induce IFN1β in keratinocytes in the presence of poly I: C (Takiguchi et al., 2014). The pattern recognition receptor, TLR3, directly contributes to the immune response of airway cells to rhinovirus infection and Poly I:C (Lai et al., 2011; Matsukura et al., 2007). The activation of TLR3 leads to the upregulation of inflammatory cytokines and chemokines in airway cells including RANTES, IL-8, IL-6 as well as activating an antiviral response upregulating IFN1β (Guillot et al., 2005). It is thought that MDA5 and TLR3 are each necessary for maximal HRV-induced IFN responses (Q. Wang et al., 2009). In addition, studies have reported dsRNA-PKR pathway as a major signalling pathway induced by rhinovirus infection responsible for the activation of IFN production (Y. Chen et al., 2006). We generated the hypothesis that treating rhinovirus with LL-37 prior to cell infection could facilitate the recognition of the viral infection through this system resulting in an enhanced antiviral type I interferon response. In contrast to our initial hypothesis, our data demonstrated that treating HRV1B with LL-37 prior to 16HBE cell infection led to a significantly lower production of type I interferon as well as PKR gene expression by bronchial epithelial cells *in vitro*. Together with the gene transcription data IFN1β protein levels were also reduced in the presence of LL-37.

Taken together, we now propose that direct exposure of rhinovirus to LL-37, but not scrambled LL-37, prior to epithelial cell infection, results in a reduction of infectivity that is most likely due to direct interaction with the viral capsid. This interaction subsequently inhibits replication, and consequently, reduces the host cell response to the viral infection.

CHAPTER 5

Investigating the role of LL-37 on the host inflammatory response to rhinovirus infection

Chapter 5. Investigating the role of LL-37 on the host inflammatory response to rhinovirus infection

5.1. Introduction

5.1.1. Pro-inflammatory responses of bronchial epithelial cells to rhinovirus infection

The airway epithelium is intimately involved in the front line of defence against invading microorganisms, and expresses a variety of pattern-recognition receptors (PRRs), that allow cells to sense and respond to pathogens. Respiratory epithelial cells can produce and secrete the cytokines and chemokines responsible for initiating and tightly regulating lung inflammation and immune responses. Rhinovirus infection of the airway epithelium can initially lead to the induction of an innate immune response, which will include the production of pro-inflammatory chemokines and cytokines. Some of the most important determinants of the clinical outcome of HRV infection comprise growth factors, such as GM-CSF (Subauste et al., 1995), chemokines, such as IL-8 (Grünberg et al., 1997), RANTES (Schroth et al., 1999), CXCL10 (Spurrell et al., 2005), and cytokines, such as IL-6 (Zhu et al., 1996), IL-11 (Einarsson et al., 1996) and IL-1β (Terajima et al., 1997).

IL-8, a potent neutrophil chemoattractant and activator, was shown to be increased in nasal secretions from rhinovirus experimentally infected symptomatic subjects compared to those infected asymptomatic or sham-challenged subjects, and the severity of symptoms coincided with the change of IL-8 concentrations from baseline (Turner at al 1998). These findings therefore implicate IL-8 in the pathogenesis of rhinovirus infections. Furthermore, IL-6 was shown to be present in exaggerated quantities in nasal washings of rhinovirus experimentally infected symptomatic subjects (Zhu et al. 1996) and this correlates with previous studies in naturally occurring URTI in children (Noah et al 1995). IL-6 is a cytokine responsible for the production of acute phase proteins, B- cell differentiation, T –cell activation, as well as changing the nature of the leukocyte infiltrate (Gaby et al. 2006). The increased presence of IL-6 in rhinovirus infection is therefore thought to correlate with severity of infection and possibly reflective of an acute-phase inflammatory response. The T-cell and eosinophil-attracting chemokine CCL5, also known as

RANTES, has also been shown to be upregulated in primary bronchial epithelial cells upon rhinovirus infection (Schroth et al. 1999) and to be increased in nasal secretions of children with natural virus-induced asthma exacerbations (Teran et al. 1999). CCL5 is not only a potent chemoattractant for eosinophils and is capable inducing eosinophil degranulation, but it is also a potent chemoattractant for monocytes and T cells, acting via receptors CCR1, CCR3 and CCR5 (Schall et al., 1990).

In addition to the production of pro-inflammatory mediators in response to rhinovirus infection, airway epithelial cells also respond by secreting cationic host defense peptides (Hiemstra et al., 2016; Proud et al., 2004). The sole human cathelicidin, LL-37, is known to be produced by airway epithelial cells and secreted into the airway surfactant (Bals et al., 1998). LL-37 has been shown to modulate chemokine and cytokine responses to pathogen-associated stimuli, eliciting the production of several chemokines and cytokines, particularly IL-8 and IL-6, in response to poly I:C (Filewod et al., 2009; Lai et al., 2011), LPS (Filewod et al., 2009; Pistolic et al., 2009), flagellin (Pistolic et al., 2009), and HRV infection (Lai et al., 2011), as well as enhancing responses to IL-1β (Filewod et al., 2009; Pistolic et al., 2009; Yu et al., 2007). In contrast, LL-37 has been shown to significantly inhibit the pro-inflammatory cytokine responses to LPS (Mookherjee et al., 2006; Scott et al., 2002), *mycobacterium tuberculosis* infection (Torres-Juarez et al., 2015), IAV infection (Barlow et al., 2011) and RSV infection (Harcourt et al., 2016). Various factors have shown to be involved in LL-37 capacity to enhance or inhibit pro-inflammatory stimuli, such as the timing and context within cells that are exposed to peptide and stimuli, as well as, the cell type and the microenvironment (Lai et al., 2011; Scott et al., 2002).

As IL-8, IL-6 and RANTES have been shown to be critical in the pathogenesis of rhinovirus infection; in this chapter we present our findings in relation to the characterisation of the ability of LL-37 to modulate these inflammatory cytokine and chemokine responses to rhinovirus infection in bronchial epithelial cells.

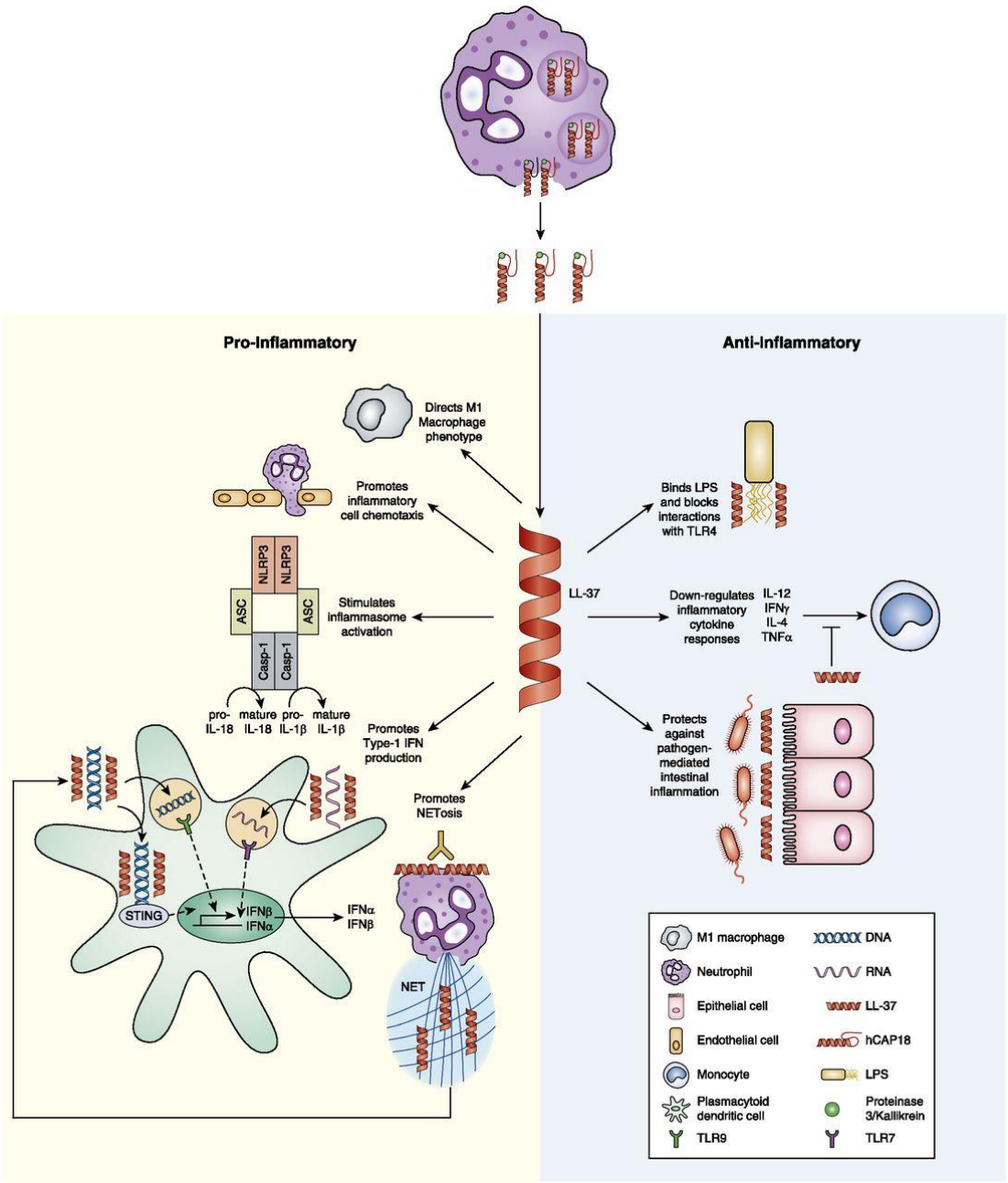


Figure 29. The numerous immunomodulatory functions of LL-37.

This diagram by Kahlenberg and Kaplan, (2013) fully depicts the numerous immunomodulatory fuctions of LL-37 which are dependent on the environment and the cellular context. The exposure to LL-37 results in the recruitment of inflammatory cells, induction of M1 macrophages, and stimulation of inflammatory responses such as type I IFN production and inflammasome activation. Nevertheless, LL-37 has also been shown to have a strong anti-inflammatory activity, including the the ability to neutralise TLR4 activation by LPS, downregulation of pro-inflammatory cytokine responses, and preventing invasion and inflammatory responses to pathogenic bacteria.

5.2. Materials and Methods

5.2.1. Reagents

Polyinosinic:polycytidylic acid, low molecular weight (LMW poly I:C) was purchased from Sigma Aldrich (Irvine, UK) and supplied in lyophilized powder form. The product was dissolved in ultrapure water to a stock concentration of 10mg/ml and aliquots (10µl) were prepared and stored at -20°C until further use.

5.2.3. UV inactivation of HRV

HRV1B virus stock (1ml) was exposed to UV light for 15 minutes at 100mJ/cm^2 using a UV crosslinker linker XL-1000 Series (Spectrolinker™ Series, Burton, USA) [Chattoraj et al. 2011]. A $TCID_{50}$ assay was used to confirm that the procedure disrupted the ability of rhinovirus to replicate (Appendix 6).

5.2.2. RNA isolation

Total RNA was extracted using RNeasy mini kit (Quiagen, Crawley, UK) or RNAzol® RT (Sigma Aldiritch, Irvine UK) according to manufacturer's protocol. Briefly, 16HBE14^{o-} cells were seeded at 5x10^4 cell/well in a 12-well plate and incubated at 37°C overnight. The following day, HRV1B (MOI 5) was exposed to varying concentrations of LL-37 (10 and 30µg/ml) for 2h at 33°C in serum-free IMDM media. 16HBE14^{o-} cells were exposed to the mixture for 1h, washed and re-immersed in low-serum (5% FBS) supplemented IMDM media for 23h at 33°C (Figure 30).

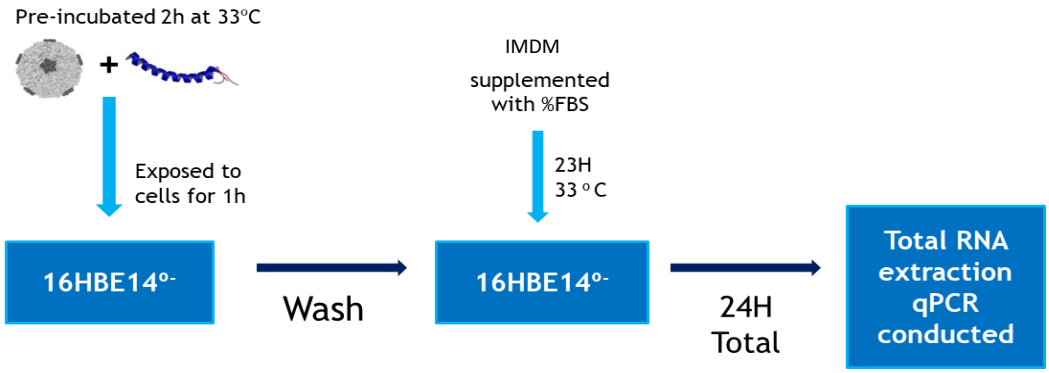


Figure 30. "Prophylactic treatment" experimental regimen.

HRV1B was exposed to varying concentrations of LL-37 (10µg/ml, 30µg/ml or scramble LL-37 30µg/ml) for 2h at 33°C in serum-free media. 16HBE14^{o-} cells were exposed to the mixture for 1h, washed and re-immersed in low-serum (5% FBS) supplemented media (IMDM) for 23h at 33°C.

After each treatment, media was aspirated and stored at -80°C until further use, and 300µl RLT buffer from RNeasy® mini kits (Qiagen, Crawley, UK) was added to each well. Cells were scraped from the surface of the plate with a sterile cell-scraper. Cell lysates were stored at -80°C until extraction. Total RNA isolation was performed using the RNeasy® mini kit system following manufacturer's protocols. For the following experiments, RNAzol RT was used to isolate RNA. Briefly, 16HBE14^{o-} cells were seeded at 5x10^{4}cells/well in a 12-well plate and incubated at 37°C overnight and subsequently subjected to different treatment approaches. (1) Cells were exposed to purified stock of HRV1B (MOI 5) or UV-inactivated (MOI 5) or Poly I:C (10µg/ml) for 2h in serum-free IMDM, washed with saline, and incubated with LL-37 (10 and 30µg/ml) for 4h (poly I:C) or 22h in IMDM supplemented with 1% (v/v) Ultroser G (Figure 31);

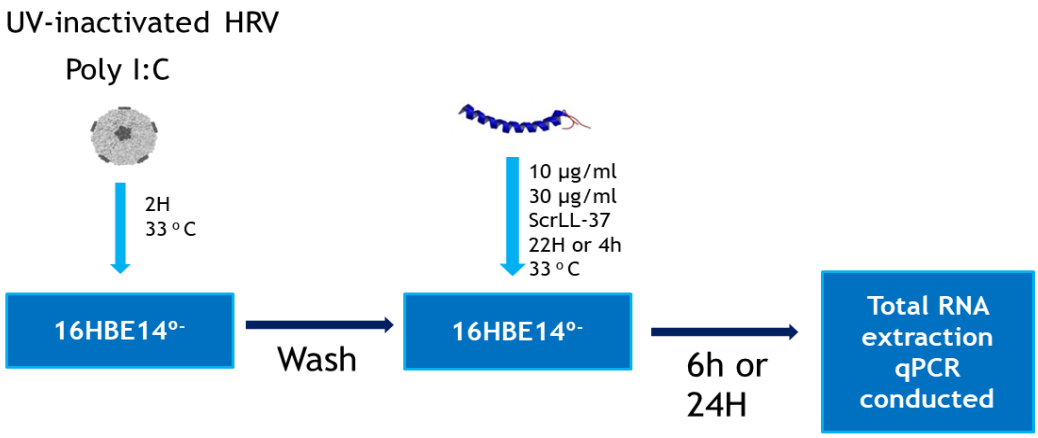


Figure 31. "Therapeutic treatment" experimental regimen.

Bronchial 16HBE14^{o-} cells were exposed to HRV1B (MOI 5) or UV-inactivated (MOI 5) or Poly I:C (10µg/ml) for 2h in serum-free IMDM, washed with saline, and incubated with LL-37 (10 and 30µg/ml) for 22h or (poly I:C) or 4h in IMDM supplemented with 1% (v/v) Ultroser G.

(2) 16HBE cells were infected with HRV1B (MOI1) for 2h, washed and re-immersed in 5% FBS supplemented IMDM media for 22h at 33°C. After 24h, cells were washed and exposed to LL-37 (10 and 30µg/ml) for 2h in IMDM supplemented with 1% (v/v) Ultraser G, followed by a wash step and re-immersion in 5% FBS supplemented IMDM for 22h at 33°C (Figure 32).

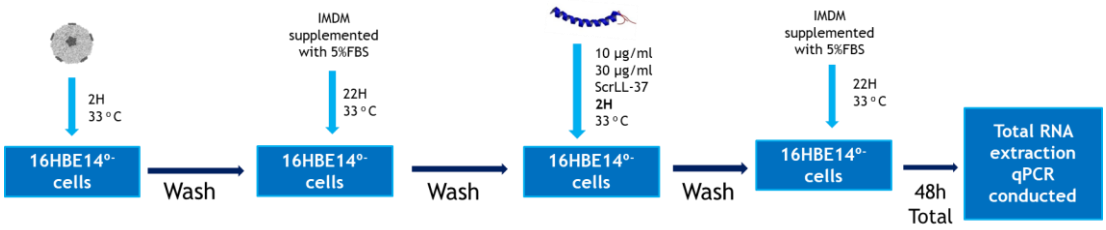


Figure 32. The "24h post-infection treatment" experimental regimen

Bronchial 16HBE14^{o-} cells were infected with HRV1B (MOI1) for 2h, washed and re-immersed in 5% FBS supplemented IMDM media for 22h at 33^{o}C. After 24h, cells were washed and exposed to LL-37 (10 and 30µg/ml) for 2h in IMDM supplemented with 1% (v/v) ultraser G, followed by a wash step and re-immersion in 5% FBS supplemented IMDM for 22h at 33^{o}C

(3) 16HBE14^{o-} cells were infected with purified stock HRV1B (MOI 1) for 2h, washed and re-immersed in 5% FBS supplemented IMDM media for 22h at 33^{o}C. After 24h, cells were washed and exposed to LL-37 (1 and 10µg/ml) for 24h in IMDM supplemented with 1% (v/v) Ultroser G at 33^{o}C (Figure 33).

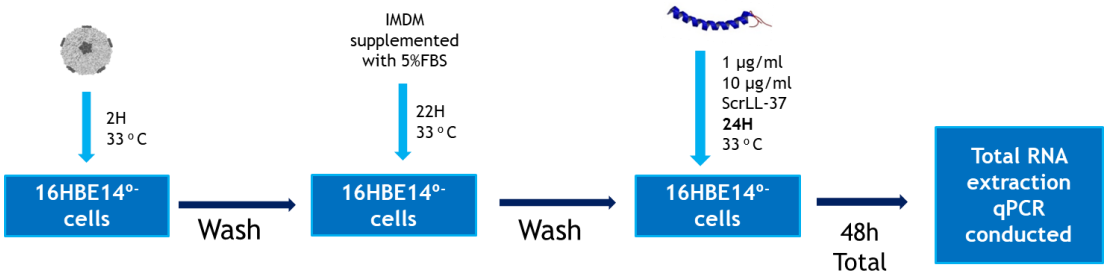


Figure 33. The "24h post-infection treatment with lower and physiological concentrations of LL-37" experimental regimen.

Bronchial 16HBE14^{o-} cells were infected with purified stock HRV1B (MOI 1) for 2h, washed and re-immersed in 5% FBS supplemented IMDM media for 22h at 33^{o}C. After 24h, cells were washed and exposed to LL-37 (1 and 10µg/ml) for 24h in IMDM supplemented with 1% (v/v) Ultroser G at 33^{o}C.

After each treatment, media was aspirated and stored at -80^{o}C until further use, and ice-cold 500µl RNAzol® RT was added to each well. Cells were scraped from the surface of the plate with a cell-scrapper. Cell lysates were stored at -80^{o}C until extraction. Total RNA isolation was performed using RNAzol® RT according to manufacturer's protocol. RNA concentrations were determined using a NanoDrop

1000 spectrophotometer (Thermo Fisher Scientific, Loughborough, UK). RNA integrity was determined using an Agilent Bioanalyser (Agilent, UK), where RIN≥8 was used as a quality filter for further downstream analyses.

5.2.4. qPCR to determine mRNA levels of IL-8, IL-6 and CCL5

Total RNA (0.5-1µg) was transcribed to cDNA using a Precision RT all-in-one mix kit (PrimerDesign Ltd, UK) as per manufacturer's instructions. qPCR reactions included cDNA (25 ng), 250 nM specific primers (Table 3) and SYBR green mastermix (PrimerDesign Ltd, UK) in total volume of 20µl. qPCR was performed using the StepOnePlus instrument (Applied Biosystems). A panel of six human reference genes were evaluated using a geNorm kit (Primerdesign) and qbase^{+} (Biogazelle) software. The geometric means of *ACTB* and *GAPDH* genes were selected as the most stable combination for normalization in 16HBE14^{o-} cells. The $2^{\Delta\Delta Ct}$ method was used and data is represented as fold changes over untreated cells (Pfaffl MW, 2004). We applied a threshold of two-fold above unstimulated control to consider upregulation of the expression of the genes of interest.

Table 3. qPCR primer sequences

Target	Forward	Reverse	Reference
GAPDH	AAGCTCATTTCCTGGTATGACA	TCTTACTCCTTGGAGGCCATGT	(Wang et al., 2012)
ACTB	GGACTTCGAGCAAGAGATGG	AGGAAGGAAGGCTGGAAGAG	(Tarrats et al., 2011)
IL-8	ACTGAGAGTGATTGAGAGTGGAC	AACCCTCTGCACCCAGTTTTC	(Tsai et al., 2009)
IL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC	(Keller et al., 2003)
CCL5	CAGTCGTCTTTGTCACCCGA	CGGGTGGGGTAGGATAGTGA	Designed

5.2.5. Quantification of cytokine/chemokine release by 16HBE14^{o-} cells

Supernatants from experiments described in section 4.2.2 were analysed for the presence of IL-8, IL-6 and CCL5 by ELISA, as per the manufacturer's instructions (human IL-8 DuoSet ELISA DY208-05, R&D; Human IL-6 DuoSet ELISA DY206-05, R&D; Human CCL5/RANTES DuoSet ELISA DY278-05, R&D, Abingdon, UK).

5.2.6. Statistical analysis

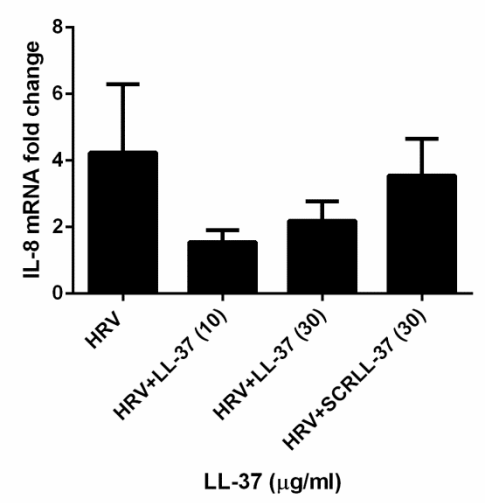
Statistical analysis was performed using GraphPad Prism Version 6.0. (GraphPad Inc, San Diego, CA, USA). Data are presented as means ± SEM. Data were analysed using the appropriate statistical test and post-test as stated in the figure legends. A p-value ≤0.05 was considered statistically significant. For the statistical analysis of the qPCR data the Δct values were utilised to determine statistical significance, unless otherwise stated in figure legend.

5.3. Results

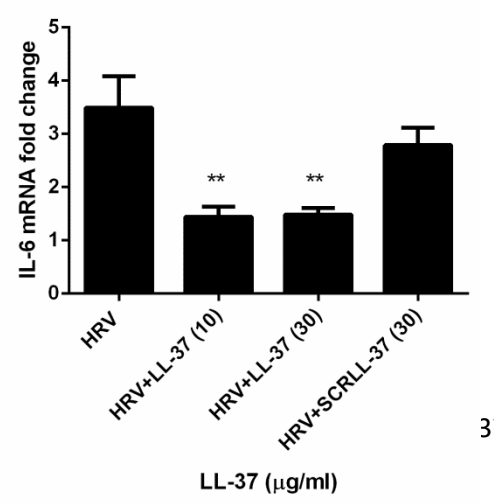
5.3.1. Assessment of the capacity of LL-37 to alter HRV-induced inflammatory cytokine expression in bronchial epithelial cells

Rhinovirus infection of airway epithelial cells induces the release of inflammatory cytokines and chemokines. We sought to investigate the ability of LL-37 to modulate HRV-induced pro-inflammatory cytokine responses using a lung epithelial cell model of infection (Figure 34 & Figure 35). Bronchial epithelial cells (16HBE14^{o-}) were infected with HRV that had been previously exposed to LL-37 and mRNA expression of IL-8 (Figure 34A), IL-6 (Figure 34B), and CCL5 (Figure 34C) was determined by qPCR after a period of 24h. Infection with HRV1B that had not been treated with peptide increased IL-8 (4.2- fold above uninfected cells), IL-6 (3.49- fold above uninfected cells) and CCL5 (7.8-fold above uninfected cells) mRNA levels. Notably the addition of LL-37 to virus prior to infection reduced HRV-induced mRNA expression of IL-8 (to 1.6 –fold at 10µg/ml of LL-37 and 2.2-fold at 30µg/ml of LL-37), IL-6 (to 1.4-fold at 10µg/ml of LL-37, **p <0.01, and 1.5-fold at 30 µg/ml of LL-37, **p<0.01), and CCL5 (to 2.8-fold at 10 µg/ml of LL-37 and 4.1-fold at 30 µg/ml of LL-37). A scrambled LL-37 peptide did not alter HRV-induced pro-inflammatory cytokine expression (Figure 34 A, B and C). LL-37 treatment of uninfected cells did not induce IL-8, IL-6 or CCL5 mRNA expression above unstimulated cells (Figure 34 D, E and F). However, higher concentrations of LL-37 (30µg/ml) induced the expression of CCL5 in untreated cells (Figure 34 F). HeLa lysates control did not upregulate IL-8, IL-6, or CCL5 gene expression above unstimulated cells (Figure 34 D, E and F).

A.



B.



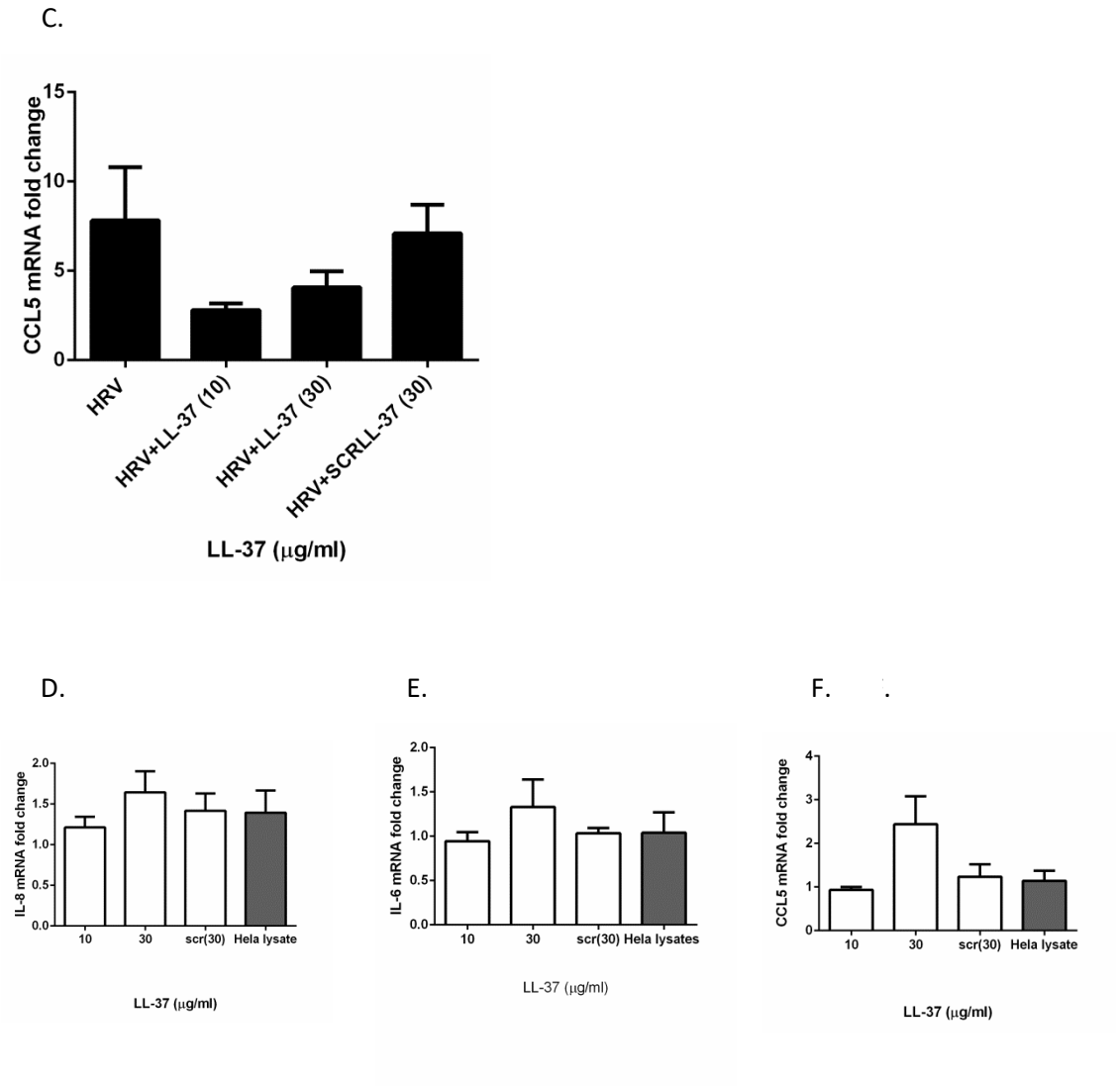


Figure 34. LL-37 treatment of HRV reduces viral mediated inflammatory cytokine gene expression in bronchial epithelial cells.

HRV1B (MOI 5) was incubated with LL-37 in serum-free media for 2h prior to cell infection. 16HBE14^{o-} cells were exposed to the mixture for 1h, washed and cultured for further 23h in maintenance media at 33^{o}C. qPCR was performed to determine levels of IL-8 (A), IL-6 (B) and CCL5 (C) mRNA transcripts. Values represent fold-change over uninfected cells. The GAPDH and β-Actin genes were used as reference genes Results are represented as the means +/- SEM from three independent experiments. Statistical analysis was performed on ΔΔct values using one-way ANOVA with Tukey's multiple comparisons post-test (**p<0.01).

To confirm that the mRNA transcript levels were translated to changes at the protein level, we assessed cell supernatants for IL-8 (Figure 35A) and CCL5 (Figure 35B) protein release by ELISA. Rhinovirus infection induced a strong IL-8 response in the host cells compared to untreated cells and in accordance with the mRNA expression data, the addition of LL-37 to the virus prior to infection resulted in lower IL-8 secretion (Figure 35A). A similar trend was observed for CCL5 secretion, as infection with rhinovirus induced a strong CCL5 response in the host cells compared to untreated cells and the addition of LL-37 to the virus prior to infection resulted in a statistically significant reduction in CCL5 release at both concentrations used (10µg/ml of LL-37, **p<0.01 and 30µg/ml of LL-37, ****p<0.0001). Scrambled LL-37 exposure did not alter the ability of HRV to induce IL-8 and CCL5 secretion (Figure 35A&B). Notably, exposure of uninfected cells to physiologically inflammatory concentrations of LL-37 (30µg/ml) did result in increased IL-8 secretion from 194.9pg/ml in untreated cells to 436.8pg/ml (Figure 35A) and CCL5 secretion from 7.23 pg/ml in untreated cells to 12.24pg/ml (Figure 35B) although this effect was not statistically significant.

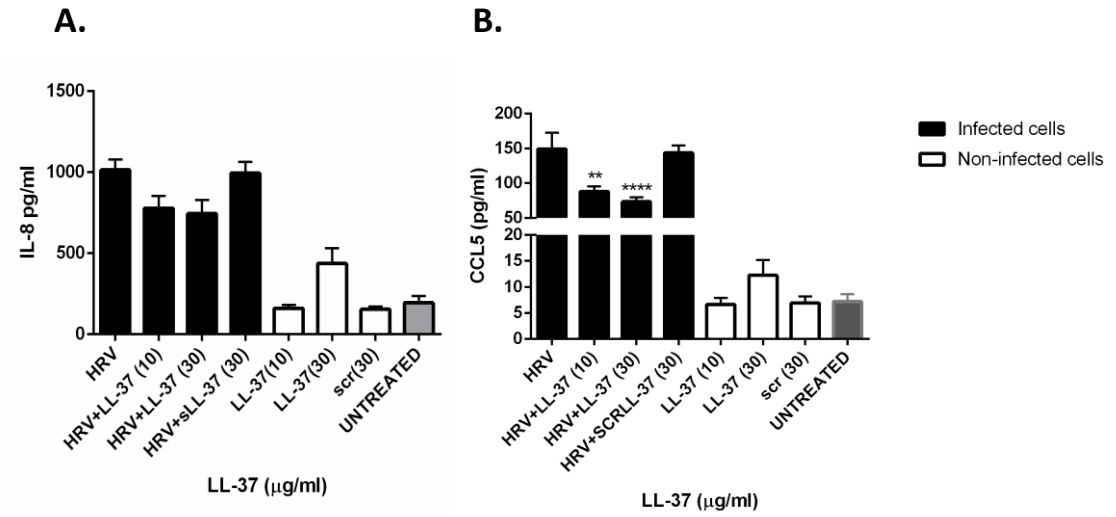


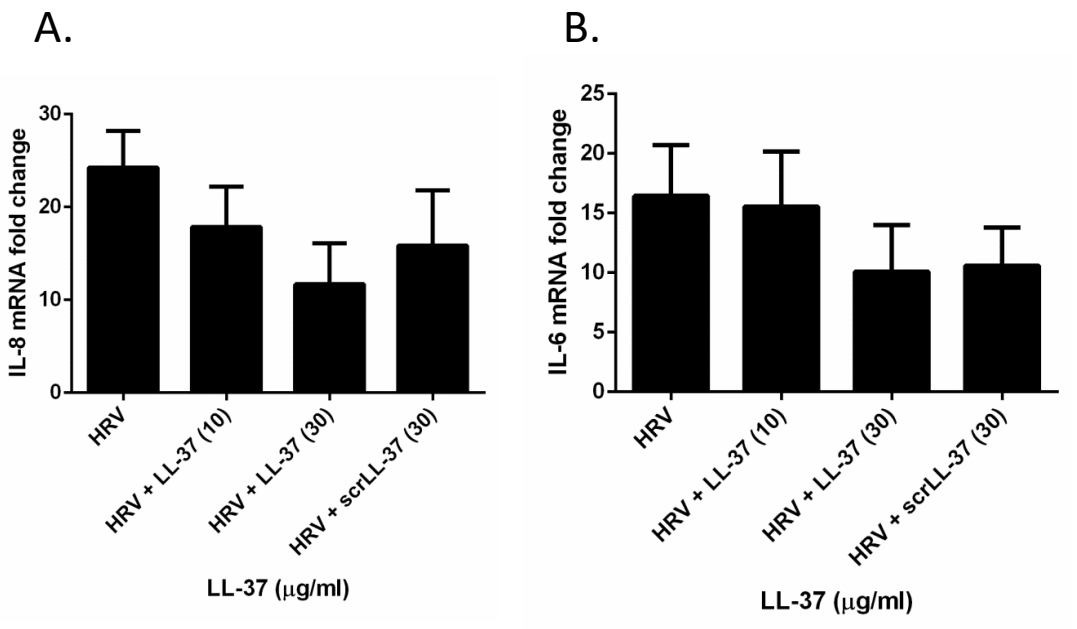
Figure 35. LL-37 reduces pro-inflammatory cytokine release induced by HRV1B infection in bronchial epithelial cells.

Purified HRV1B viral particles (MOI 5) were pre-incubated with LL-37 in serum-free media for 2h prior to cell infection. 16HBE14^{o-} cells were incubated with the peptide/virus mixture for 1h, washed and incubated for further 23h in maintenance media at 33^{o}C. IL-8 (A) and CCL5 (B) protein concentrations released into cell supernatants were quantified by ELISA. Results are represented as the means +/- SEM from four independent experiments.

Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparisons post-test to compare virus only to LL-37 treatment (** p<0.01, ****p<0.0001).

5.3.2. Assessment of the effects of host cell exposure to LL-37 on the inflammatory cytokine response during HRV- infection

To determine the ability of LL-37 to modulate the pro-inflammatory response in rhinovirus-infected bronchial epithelial cells, 16HBE14^{o-} cells were infected with HRV for 2h prior to LL-37 treatment for 22h at 33^{o}C. IL-8 (Figure 36A), IL-6 (Figure 36B) and CCL5 (Figure 36C) and mRNA transcript levels were assessed by qPCR after a period of 24h. Rhinovirus infection induced a strong IL-8 (24.3-fold above uninfected cells), IL-6 (16.4-fold above uninfected cells) and CCL5 (49.3 fold above uninfected cells) response in the context of gene expression. Addition of LL-37 did not significantly alter HRV-induced IL-8 (to 17.9-fold at 10µg/ml of LL-37 and 11.7-fold at 30µg/ml of LL-37), IL-6 (to 10.1-fold at 30µg/ml of LL-37) and CCL5 (to 23-fold at 30µg/ml of LL-37) gene expression. LL-37 (10 µg/ml) did not reduce the expression of IL-6 or CCL5 genes in comparison to HRV alone, although unusually, scrambled LL-37 reduced cytokine gene expression (Figure 36A, B and C). LL-37 treatment of uninfected cells, together with the Hela lysate control did not upregulate IL-8, IL-6 or CCL5 gene expression above that of untreated cells (Figure 36 A, B and C).



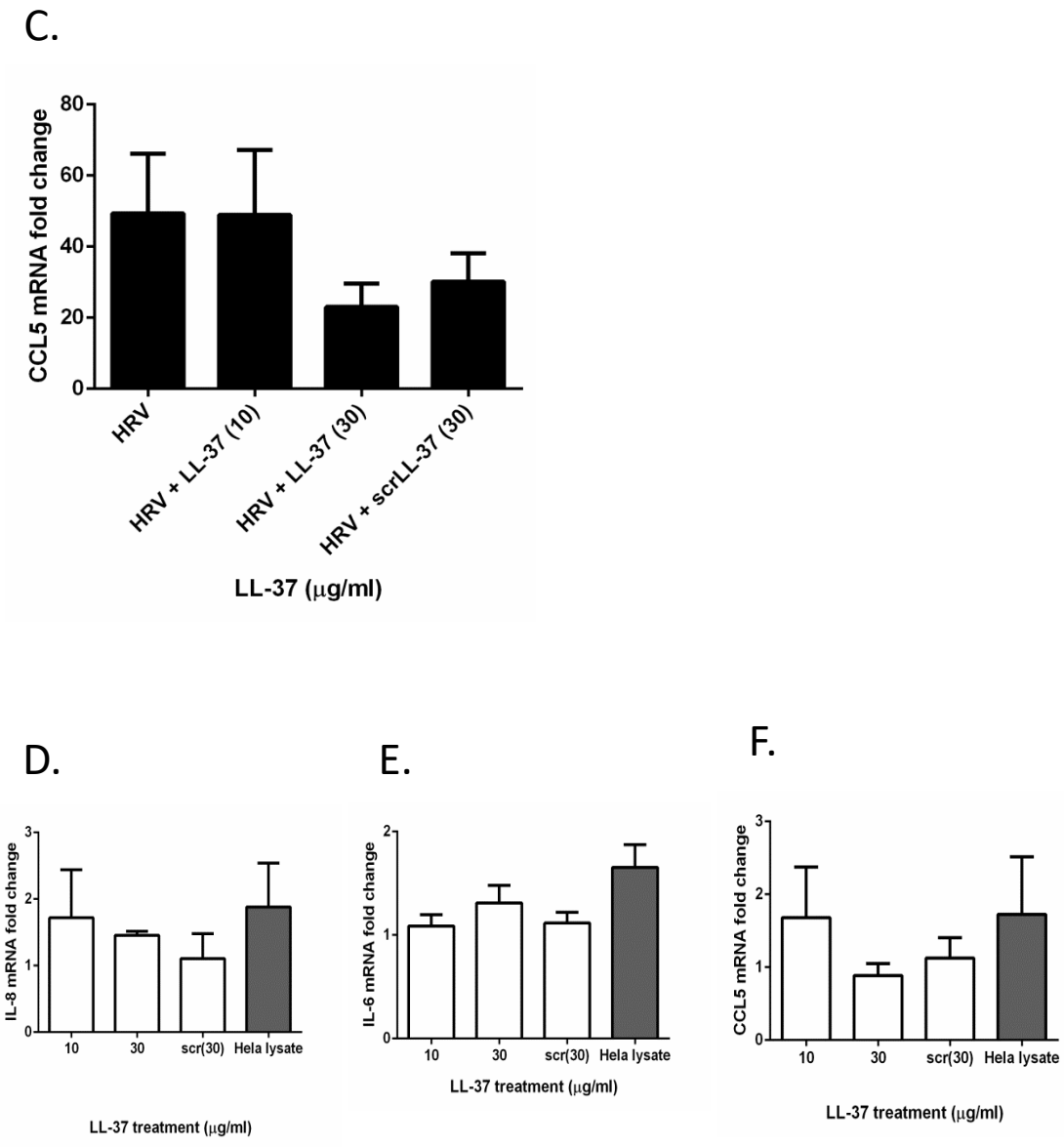


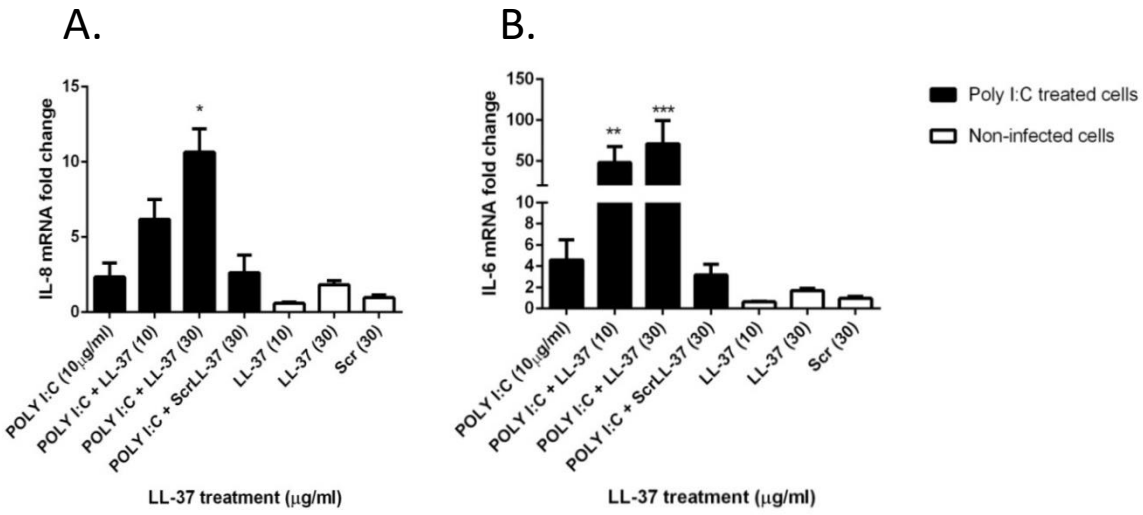
Figure 36. LL-37 treatment does not alter the pro-inflammatory cytokine gene expression in response to HRV infection in bronchial epithelial cells.

16HBE14^{o-} cells were left unstimulated (control) or were exposed to purified stock of HRV (MOI 5) for 2 hours at 33^{o}C in serum-free media, washed with saline before incubating with LL-37 at concentrations indicated for 22 hours at 33 ^{o} C. qPCR was performed to determine mRNA expression levels of IL-8 (A), IL-6 (B) and CCL5 (C). The *GAPDH* gene was used as a reference gene. Results are represented as the means +/- SEM from four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-test.

5.3.3. LL-37 enhances Poly I:C mediated pro-inflammatory cytokine release

The capacity for LL-37 to modulate the expression of pro-inflammatory cytokines during rhinovirus infection can be, in part, through direct antiviral effects but also through peptide-mediated effects on host cells. In order to further understand the effects of the peptide on bronchial epithelial cells during host cell response to infection, we exposed cells to the TLR3 ligand poly I:C, which is a synthetic analog of double-stranded RNA that mimics viral infection, and to UV-inactivated HRV, which has previously been shown to be taken up by cells but lacks the ability to replicate (Newcomb et al., 2008) [Figure 37 and Figure 38].

Bronchial epithelial cells (16HBE14^{o-}) were exposed to poly I:C for 2h and washed prior to LL-37 treatment for 4h. IL-8 (Figure 37 A), IL-6 (Figure 37 B), and CCL5 (Figure 37 C) mRNA expression was determined by qPCR after a total period of 6h. Poly I:C stimulation increased mRNA transcript levels of IL-8, IL-6 and CCL5. Addition of LL-37 was shown to enhance poly I:C induced cytokine gene expression. Scrambled LL-37 did not alter poly I:C-induced IL-8, IL-6 or CCL5 mRNA expression when compared to poly I:C alone (Figure 37 A, B and C). LL-37 exposure of untreated cells did not enhance IL-8, IL-6 or CCL5 gene expression (Figure 37 A, B and C).



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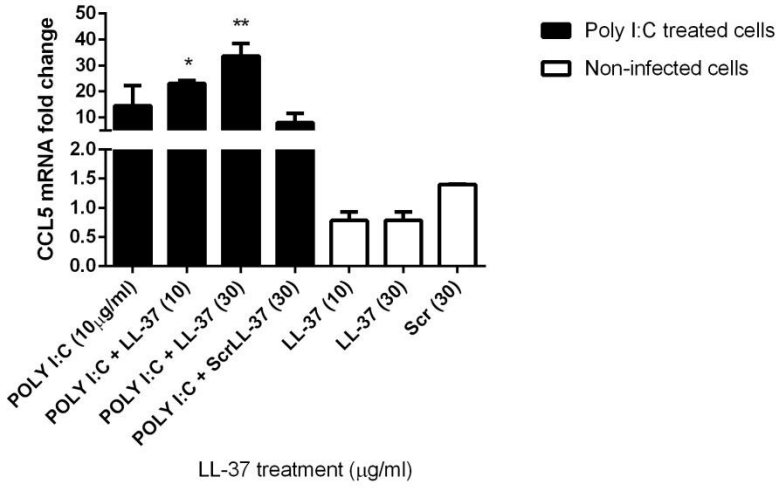


Figure 37. LL-37 enhanced dsRNA –induced pro-inflammatory cytokine response in bronchial epithelial cells.

Bronchial epithelial cells (16HBE14^{o-}) were either left unstimulated (control) or were exposed to poly I:C (10 μg/ml) for 2 hours in serum-free media, washed with saline before incubating with LL-37 at concentrations indicated for 4 hours at 33^{o}C. qPCR was conducted to determine levels of IL-8 (A), IL-6 (B) and CCL5 (C) mRNA transcripts. The GAPDH gene was used as a reference gene. Results are represented as the means +/- SEM from four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-test (* $p<0.05$; ** $p<0.01$; *** $p< 0.001$).

Bronchial epithelial cells (16HBE14^{o-}) were exposed to UV-inactivated HRV1B for 2h and washed before treating cells with LL-37 for 22h at 33^{o}C. IL-8 (Figure 38 A), IL-6 (Figure 38 B), and CCL5 (Figure 38 C) mRNA expression were determined by qPCR after a period of 24h. UV-inactivated virus did not induce IL-8, IL-6 or CCL5 mRNA expression above that of unstimulated cells. We observed no upregulation of IL-8, IL-6 and CCL5 mRNA expression by LL-37 treatment, although 30 μg/ml of LL-37 did induce a 2.7 –fold increase in IL-8 mRNA levels (A). Scrambled LL-37 had no effect on IL-8, IL-6 or CCL5 gene expression.

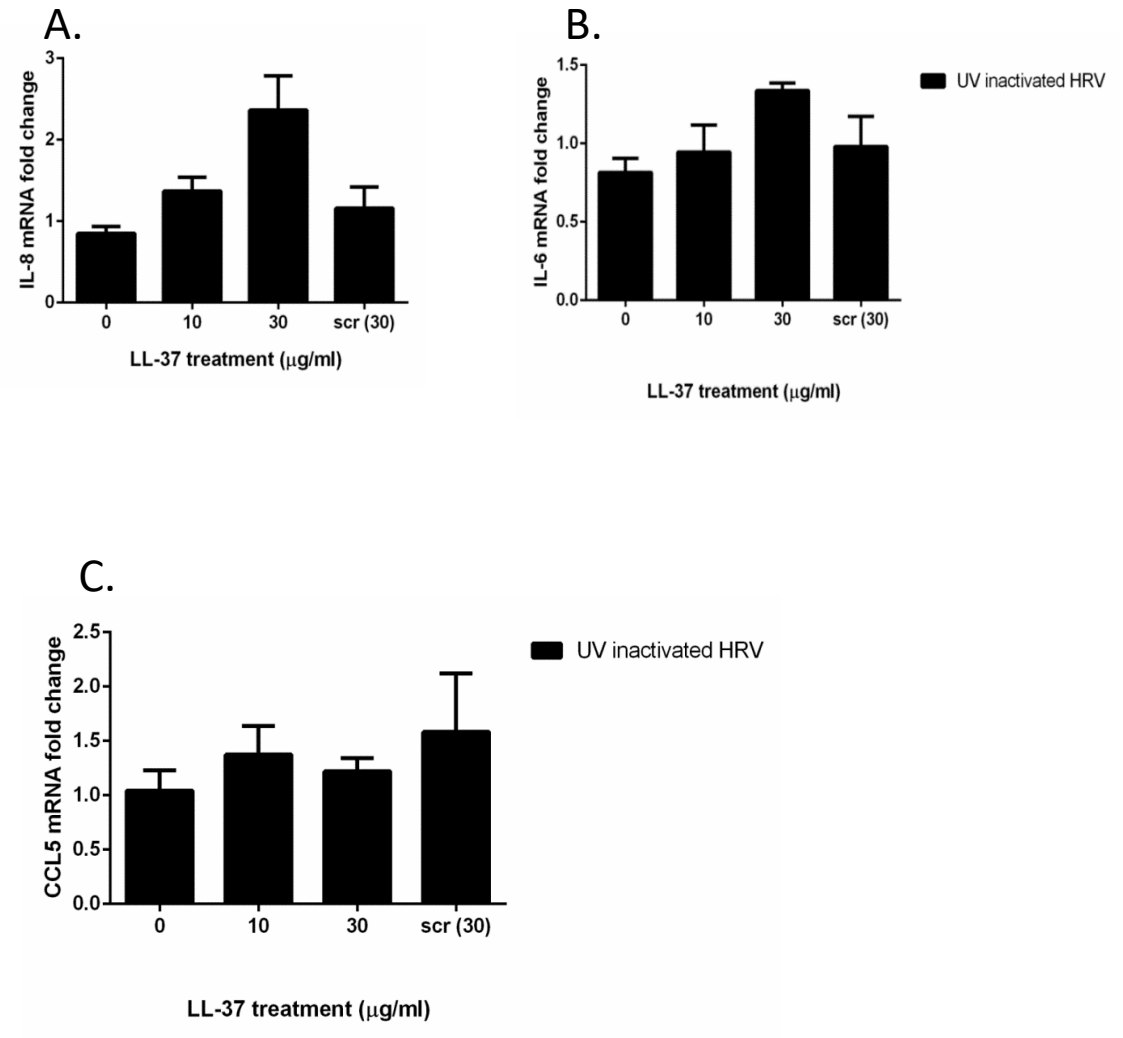
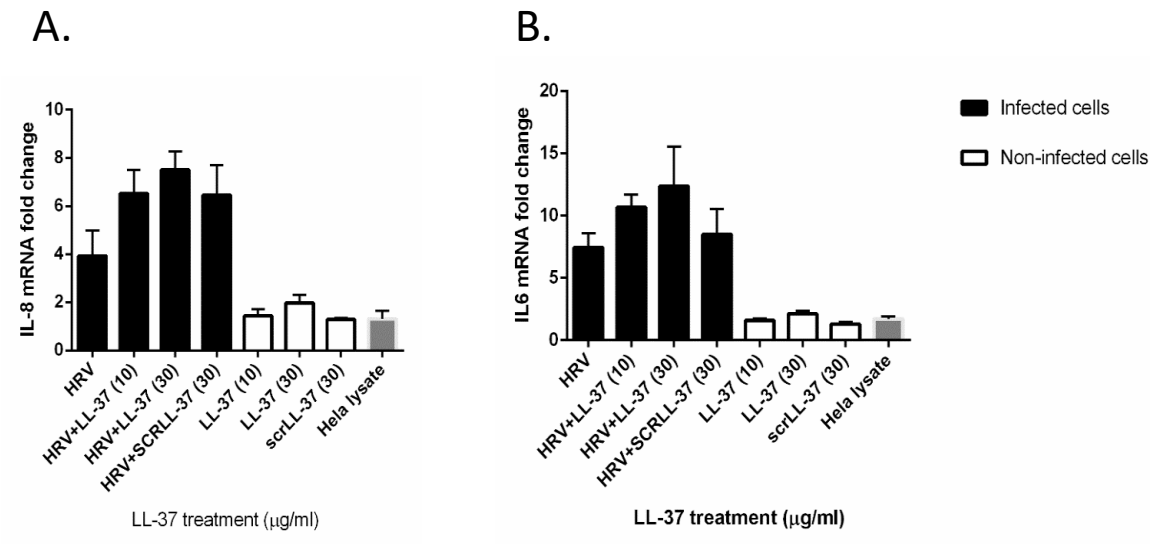


Figure 38. LL-37 treatment does not alter cytokine gene expression following host cell exposure to UV –inactivated HRV

Bronchial epithelial cells (16HBE14^{o-}) cells were exposed to UV-inactivated HRV1B (MOI 5) for 2H in serum-free media, washed with saline before incubating with LL-37 at concentrations indicated for 22 hours at 33^{o}C. qPCR was conducted to determine levels of IL-8 (A), IL-6 (B) and CCL5 (C) mRNA transcripts. The GAPDH gene was used as a reference gene. Results are represented as the means +/- SEM from at least four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-test.

5.3.4. Assessment of the inflammomodulatory effects of delayed LL-37 treatment in HRV-infected bronchial epithelial cells

In order to determine the inflammomodulatory effects of LL-37 on later stages of HRV replication, 16HBE14^{o-} cells were infected with HRV for 2h, washed and left in contact with maintenance media for 22h prior to LL-37 treatment, which consisted of 2h treatment, a wash step to remove non-internalised peptide and re-immersion of cells in fresh maintenance media for further 22h at 33^{o}C. The mRNA transcript levels of IL-8 (Figure 39A), IL-6 (Figure 39B), and CCL5 (Figure 39C) were determined by qPCR after a period of 48h. Infection with rhinovirus induced the expression of genes for IL-8, IL-6 and CCL5. Addition of LL-37 to cells did not alter the expression of IL-8, IL-6 and CCL5 genes. Scrambled LL-37 enhanced HRV-induced IL-8 (to 6.5-fold above unstimulated cells), but did not alter IL-6 or CCL5 mRNA levels compared to virus alone (Figure 39). LL-37 treatment of uninfected cells or Hela lysate treatment did not induce expression of IL-8, IL-6 or CCL5 above 2-fold (Figure 39).



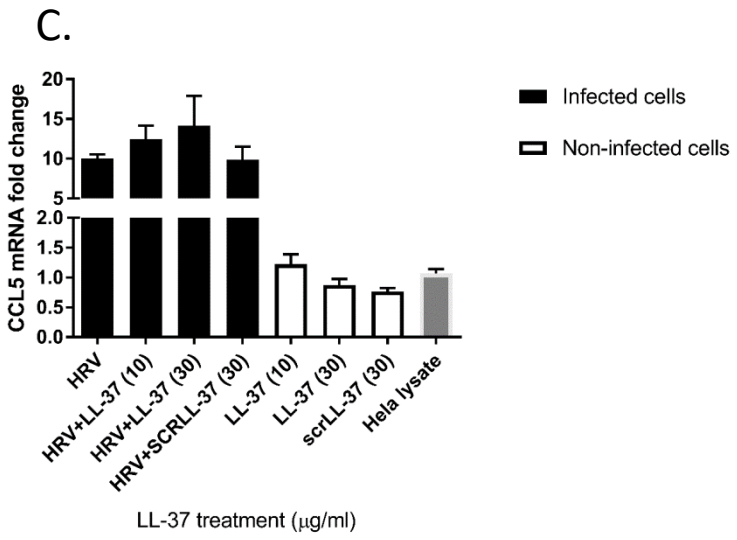


Figure 39. LL-37 treatment did not alter the HRV-induced pro-inflammatory cytokine response in bronchial epithelial cells at later stages of infection.

Bronchial epithelial cells (16HBE14^{o-}) were infected with HRV1B (MOI 1) or left unstimulated in serum-free media for 2H, washed and re-immersed in fresh maintenance media for further 22h. Cells were treated with LL-37 for 2h, washed and re-immersed in fresh maintenance media for further 22h at 33^{o}C.qPCR was conducted to determine IL-8 (A), IL-6 (B) and CCL5 (C) gene expression. The GAPDH gene was used as a reference gene. Results are represented as the means +/- SEM from three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-test.

To confirm that the mRNA transcript levels were translated to changes at the protein level, we assessed cells supernatants for IL-8 protein release using ELISA (Figure 40). In agreement with mRNA data, rhinovirus infection considerably induced IL-8 release compared to uninfected cells (from 34.2pg/ml to 109.4pg/ml). Addition of LL-37 enhanced HRV-induced LL-37 (to 147.9pg/ml at 10μg/ml of LL-37 and 230.6pg/ml at 30μg/ml of LL-37, *$p<0.05$). It is noteworthy that inflammatory concentrations of LL-37 (30μg/ml) increased IL-8 release in uninfected cells compared to negative control; however this was not statistically significant.

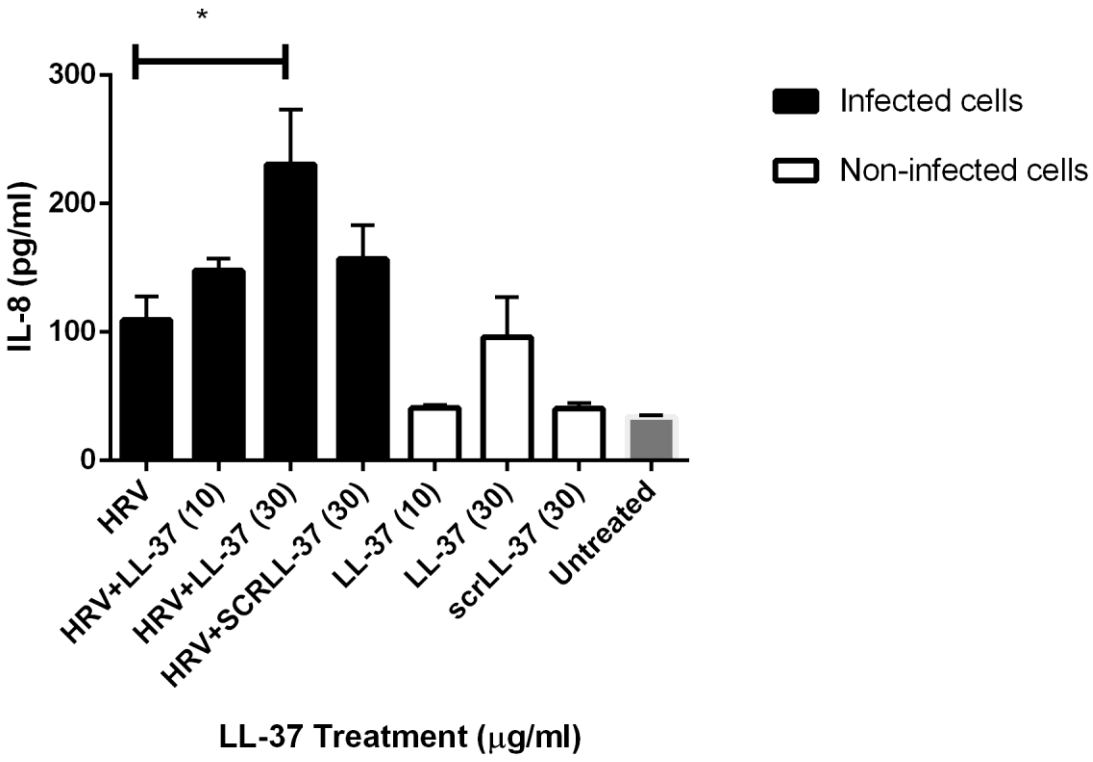
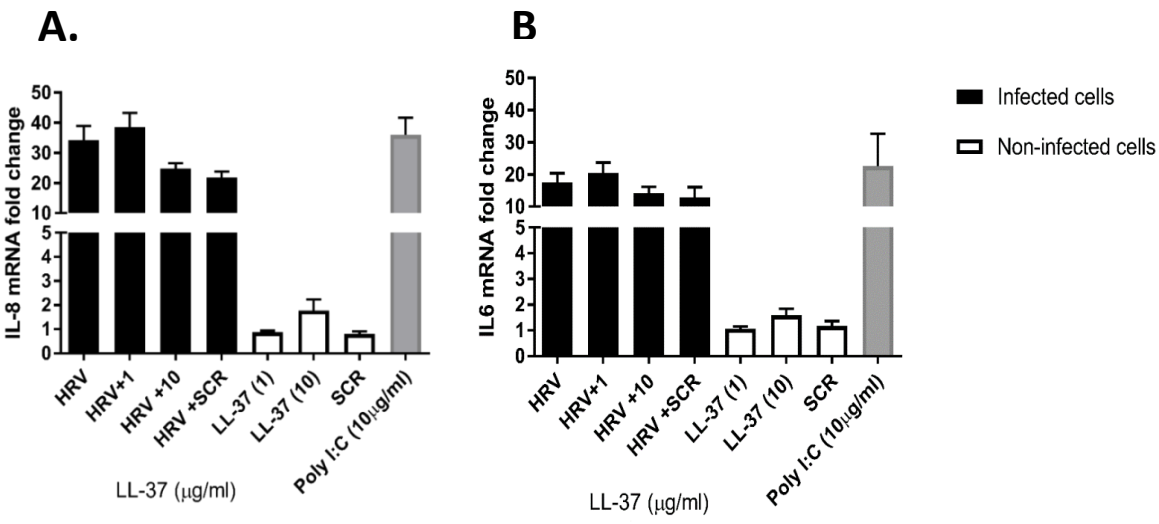


Figure 40. LL-37 enhances the HRV-induced inflammatory response in bronchial epithelial cells at later stages of viral infection.

Bronchial epithelial cells (16HBE14^{o-}) cells were infected with HRV1B (MOI 1) or left unstimulated in serum-free media for 2H, washed and re-immersed in fresh maintenance media for further 22h. Cells were treated with LL-37 for 2h, washed and re-immersed in fresh maintenance media for further 22h at 33^{o}C. IL-8 protein levels were assessed by ELISA. Results are represented as the means +/- SEM from three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-test to compare virus only to LL-37 treatment (* $p<0.05$).

5.3.5. Assessing the effects of low concentrations of LL-37 (1-10µg/ml) on the host cell inflammatory response to rhinovirus infection

To determine the inflammomodulatory effects of low concentrations of LL-37 during later stages of the rhinovirus replication cycle, 16HBE14^{o-} cells were infected with HRV for 2h, washed and re-immersed in fresh maintenance media for 22h prior to LL-37 treatment for 24h at 33^{o}C. The mRNA transcript levels of IL-8 (Figure 41A), IL-6 (Figure 41B), and CCL5 (Figure 41C) were determined by qPCR after a period of 48h. Rhinovirus infection of 16HBE14^{o-} cells strongly induced gene expression of all cytokines. The addition of LL-37 did not significantly alter the expression of HRV-induced pro-inflammatory cytokines at any concentration tested. LL-37 treatment of uninfected cells did not induce the expression of pro-inflammatory cytokines above unstimulated cells. Poly I:C was used as a positive control and induced IL-8 and IL-6 gene expression to a similar extent as HRV infection and strongly induced CCL5 above HRV infection (up to 1000-fold above unstimulated cells).



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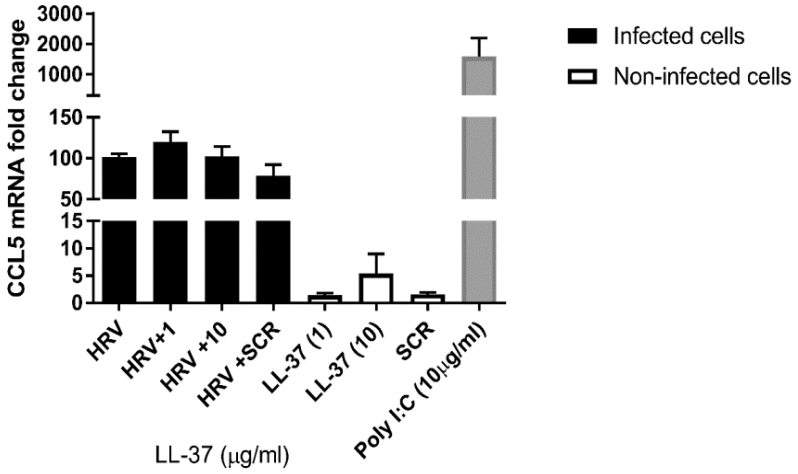


Figure 41. Low concentrations of LL-37 do not alter the pro-inflammatory cytokine response in HRV infection of bronchial epithelial cells.

Bronchial epithelial cells (16HBE14^{o-}) cells were infected with purified HRV1B (MOI 1) or left unstimulated in serum-free media for 2H, washed and re-immersed in fresh maintenance media for further 22h. Cells were washed and treated with LL-37 for 24h at 33^{o}C. Poly I:C was used as a positive control for pro-inflammatory response and was incubated with 16HBE cells for 48h in 5% FBS in IMDM. qPCR was conducted to determine IL-8 (A), IL-6 (B) and CCL5 (C) gene expression after 48h. The *GAPDH* and *βActin* genes were used as reference genes. Results are represented as the means +/- SEM from three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-test.

Again, to confirm that the mRNA transcript levels were correlated with protein release, we assessed cells supernatants for IL-8 (Figure 42A), IL-6 (Figure 42B) and CCL5 (Figure 42 C) protein release using ELISA assay. In agreement with mRNA data, infection with rhinovirus strongly induced all cytokines in comparison to uninfected cells, but addition of LL-37 did not significantly alter the release of HRV-induced pro-inflammatory cytokines in comparison to virus alone.

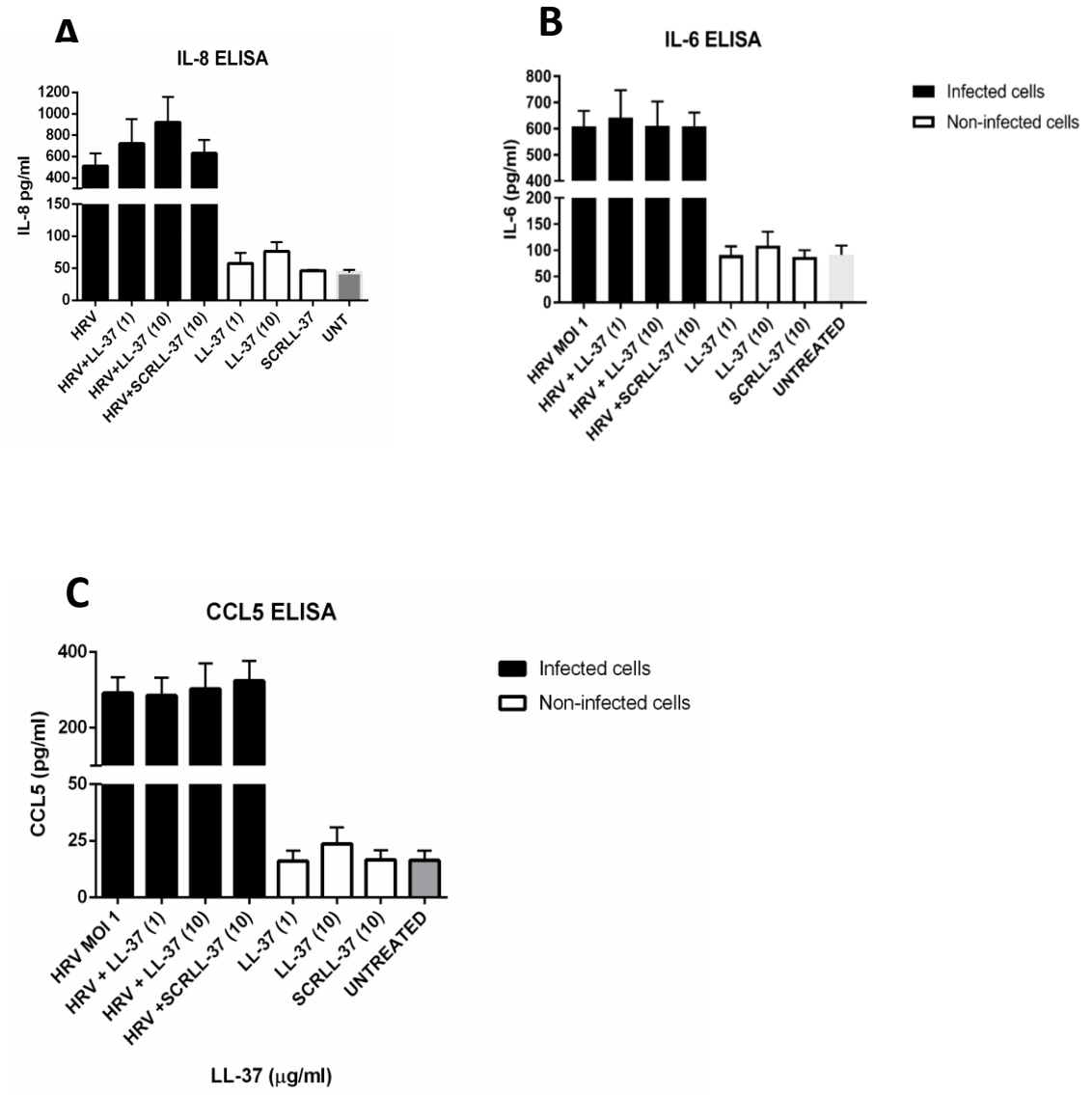


Figure 42. Lower and physiologically relevant concentrations of LL-37 do not alter the pro-inflammatory cytokine response in HRV infection of bronchial epithelial cells.

Bronchial epithelial cells (16HBE14^{o-}) cells were infected with purified HRV1B (MOI 1) or left unstimulated in serum-free media for 2H, washed and re-immersed in fresh maintenance media for further 22h. Cells were washed and treated with LL-37 at concentrations indicated for 24h at 33^{o}C. IL-8 (A), IL-6 (B) and CCL5 (C) protein levels were assessed by ELISA. Results are represented as the means +/- SEM from three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-test.

5.4. Discussion

The immunomodulatory activities of the sole human cathelicidin, LL-37, have been the subject of a number of studies demonstrating that the peptide can modulate immune and inflammatory pathways that may contribute to the resolution of viral infections. In this chapter, we investigated the ability of LL-37 to modulate the host inflammatory response to rhinovirus infection in the context of a range of LL-37 concentrations and exposure regimens. hCAP18/ LL-37 is frequently expressed by cells in direct contact with the environment, and in the airways it is expressed in surface epithelia as well as serous and mucous cells of the bronchial airway (Bals et al. 1998). The expression of LL-37 in these cells is primarily controlled by inflammatory pathways, as well as by ER stress (Park et al. 2011) and 1, 25-dihydroxyvitamin D3 concentrations (Gombart et al., 2005; Schauber and Gallo, 2008). However, the involvement of host defence peptides in rhinovirus infection remains unclear, as only recently has it been identified that HDP are expressed/released during the innate response to this pathogen. A study by Rohde et al., (2014) analysed the endogenous expression levels of antimicrobial peptides in BAL fluid of subjects with HRV-induced experimental asthma exacerbations and demonstrated an increase in the levels of HNP1-3, however, no significant increase was seen in endogenous levels of LL-37. Nevertheless, a recent cohort study of hospitalised infants, which focused upon examining the role of LL-37 in bronchiolitis, revealed that infants with higher serum LL-37 levels were less likely to have RSV, but more likely to have HRV, indicating that elevated LL-37 levels were associated with rhinovirus infection (Mansbach et al., 2017).

Infection of human airway epithelial cells with rhinovirus has been shown to induce the rapid production of several pro-inflammatory cytokines, including IL-8 (Kim et al, 2000; Subauste et al., 1995), IL-6 (Kim et al, 2000; Subauste et al., 1995), GM-CSF (Kim et al, 2000; Subauste et al., 1995) and CCL5 (Papadopoulos et al 2001). Increased levels of IL-6 (Zhu et al.1996), IL-8 (Zhu et al., 1997) and IL-1β (Proud et al. 1994) were also detected in nasal washings taken from rhinovirus experimentally infected subjects. In this chapter, we focussed on the ability of LL-37 to modulate HRV-induced IL-8, IL6, and CCL5. These pro-inflammatory markers were chosen as

they are rapidly and strongly induced by rhinovirus infection both *in vitro* and *in vivo*, and have been implicated in the pathogenesis of rhinovirus infection. As markers of inflammation they have been extensively studied and associated with the severity of symptoms related to respiratory viral infections (Jacobs et al., 2013).

To determine the impact of LL-37 treatment on rhinovirus-induced cytokine and chemokine responses in bronchial epithelial cells, HRV1B was pre-incubated with LL-37 prior to 16HBE14^{o-} cell infection ("prophylactic treatment") and expression levels of IL-8, IL-6 and CCL5 were determined after a total period of 24h. Our results indicated that LL-37 treatment in this exposure regimen was associated with reduced cytokine levels. Similarly, in RSV infection, the incubation of RSV with LL-37 prior to infection of Calu-3 cells cultured at an air-liquid interface (ALI) resulted in a significant decrease in expression levels of IL-6, RANTES, CXCL10 and G-CSF (Harcourt et al., 2016). Similarly, in IAV-infected mice treated with LL-37 prior to, and during an IAV infection, the levels of IL-1β, GM-CSF, KC and RANTES determined in the BAL fluid were all significantly lower (Barlow et al., 2011). In Chapter 3, we demonstrate that incubation of HRV1B with LL-37 resulted in a significant drop in viral titer, indicating that the reduction in cytokine expression levels may be partly due to the reduced amount of viral RNA.

We further evaluated our hypothesis by infecting cells for 2h prior to LL-37 treatment for 22h at 33^{o}C ("therapeutic treatment") and determining the expression levels of the same cytokines after a total period of 24h. Exogenous LL-37 treatment of infected cells did not alter IL-8, IL-6 and CCL5 gene expression. However, studies have shown that LL-37 used to therapeutically treat RSV-infected ALI calu-3 cells or *Mycobacterium tuberculosis*- infected macrophages resulted in a significant reduction in the expression levels of CCL5, IL-6, CXCL10 and G-CSF in RSV infection *(Harcourt et al., 2016)*, and TNF-α and IL-17 in *M. tuberculosis* infection (Torres-Juarez et al., 2015). The differences observed between these studies and our own observations using similar treatment approaches, may be explained the use of different cells models and pathogens.

In this experimental regimen, rhinovirus infection induced IL-8, IL-6 and CCL5 gene expression to a greater extent when compared to the "prophylactic treatment" exposure regimen. The differences in gene expression levels observed between both exposures regimens could be explained by the use of different viral stocks. For the "therapeutic treatment" exposure regimen we used a purified virus stock and for the "prophylactic treatment" exposure regimen a crude virus stock. The use of a purified virus stock could potentially lead to an enhanced pro-inflammatory response in bronchial epithelial cells.

In addition, our data indicates that LL-37 can be rapidly internalised and retained by 16HBE14^{o-} cells. Taken together this data indicates that LL-37 may be effective in part through the direct interaction with rhinovirus, and in part through uptake and interaction with16HBE14^{o-} cells. In airway epithelial cells, HRV-induced IL-8 and IL-6 production is primarily mediated by an NFkB-dependent transcription activation process (Zhu et al., 1997, 1996). The mechanism by which LL-37 modulates cell responses to infection is still unclear. Several cellular receptors have been identified, such as FPRL1 (De Yang et al., 2000), EGFR (Tjabringa et al., 2003), $P2X_7$ (Elssner et al., 2004), and GAPDH (Mookherjee et al., 2009). LL-37 has been shown to activate airway epithelial cells by transactivation of the EGFR via metalloproteinase-dependent processing of EGFR ligands *(Tjabringa et al., 2003)*. Activation of these receptors by LL-37 may lead to downstream effects on inflammatory pathways. The ability of LL-37 to limit HRV-induced pro-inflammatory cytokine expression by bronchial epithelial cells may therefore also be partly due peptide-mediated effects on host cells. Previous studies showed that exogenous LL-37 and mCRAMP inhibited NF-kB activity and TNF-α expression in response to C*lostridium difficile* toxin A and B in monocytes and mouse macrophages, respectively (Hing et al., 2013). It is therefore plausible that LL-37 reduces HRV-associated inflammation by inhibiting NF-kB activity in response to rhinovirus infection of bronchial epithelial cells, however further studies are required to investigate this hypothesis.

In previous studies utilising a BEAS-2B epithelial cell model, LL-37 was shown co-localise with TLR3 in the endosome and the co-localization was increased in the presence of dsRNA – an intermediate in the replication cycle of rhinovirus (Singh et

al., 2013*)*. LL-37 was also shown to enhance TLR3-mediated signalling and IL-6, CXCL10 and MCP-1 cytokine production in response to rhinovirus infection (Lai et al., 2011). This contrasts with our own data, in which LL-37 treatment was associated with reduced expression levels of IL-8, IL-6 and CCL5 in response to rhinovirus infection. TLR3 is an endosomal sensor involved in the recognition of rhinovirus infection in human airway epithelial cells and is responsible for modulating inflammatory responses to HRV infection (Slater et al., 2010*)*. Previous studies have demonstrated that TLR3 is required for HRV-induced IL-8, CCL5 and CXCL10 (Slater et al., 2010*)*, and while TLR3 has been associated with increased levels of pro-inflammatory mediators in response to rhinovirus infection, we believe the lower viral loads observed in LL-37 treated 16HBE14^{o-} cells, may partly be responsible for the reduction in cytokine expression in this study.

In order to further understand peptide-mediated effects on the bronchial epithelial cells during virus infection, we exposed cells to the TLR3 agonist, poly I:C, which mimics viral infection, and also to UV-inactivated HRV, which is able to enter cells but unable to replicate. Poly I:C is known to activate the antiviral pattern recognition receptors TLR3, RIG-I/MDA5 and PKR thus inducing signalling via multiple inflammatory pathways and triggering a strong inflammatory response in airway epithelial cells (Ritter et al., 2005; Slater et al., 2010). Bronchial epithelial cells were exposed to poly I:C for 2h, washed to remove non-internalised poly I:C, and treated with LL-37 for 4h. This experimental approach was designed to mimic rhinovirus infection in the context of the therapeutic treatment. A time-course experiment indicated that the expression levels of IFN1β and PKR induced by poly I:C peaked at 6h (Appendix 7), thus this time-point was chosen to investigate the poly I:C- induced pro-inflammatory cytokine response. Our observations revealed that poly I:C upregulated IL-8, IL-6 and CCL5 and the addition of LL-37, but not scrambled LL-37, significantly enhanced the poly I:C-induced IL-8, IL-6 and CCL5 mRNA expression in a dose-dependent manner.

In accordance with our own data, LL-37 was previously shown to enhance the poly I:C mediated induction of IL-6 and IL-8 cytokine production by BEAS2B cells (Lai et al., 2011). Due to its high affinity for nucleic acids, LL-37 has been shown to bind to DNA and facilitate its delivery across membranes (Zhang et al., 2010), and in

plasmacytoid dendritic cells, the LL-37-DNA/RNA complexes modulate the TLR9/7 activation (Ganguly et al., 2009; Lande et al., 2007). Interestingly, studies have reported that poly I:C alone and poly I:C-LL-37 complexes can enter cells using a different endocytic mechanism, further demonstrating that trafficking of the poly I:C-LL-37 complex into endosomes was dependent on FPRL1 receptor in BEAS-2B cells (Singh et al., 2013). The experimental approach used in these reports (Lai et al., 2011; Singh et al., 2013) consisted of the LL-37/poly I:C co-stimulation of BEAS-2B cells, which facilitated LL-37 binding to poly I:C forming LL-37-poly I:C complex before trafficking to the endosome. Our experimental approach exposed 16HBE14^{o-} cells to poly I: C prior to LL-37 treatment. The differences in regimens used, and the similar results obtained in this study and those in the literature indicate that the formation of the complex prior to trafficking into the endosome may not be necessary for the enhanced TLR-3 signalling. However, the increased pro-inflammatory response in the presence of LL-37 could be related to increased cytotoxicity, as poly I:C alone has been shown to be cytotoxic to bronchial epithelial cells and the combination of LL-37 was shown to increase the observed cytotoxic effects (Filewod et al., 2009). While the capacity of LL-37 to mediate TLR3-signalling during viral infections has been well documented, it would be interesting to further investigate the modulatory effects of the peptide on MDA-5 and RIG-I-signalling following rhinovirus infection.

Our results showed that at 24h, UV-inactivated HRV infection of 16HBE14^{o-} cells did not induce IL-8, IL-6 and CCL5 expression levels above uninfected cells. Previous studies have reported the ability of UV-inactivated rhinovirus to induce a significant IL-8 expression indicating that viral replication is not required for the IL-8 response (Newcomb et al., 2008). Early events before viral replication, such as viral attachment and internalisation may be sufficient for rhinovirus to induce an epithelial cell response (Newcomb et al., 2008). It is known that UV irradiation may partially inhibit viral attachment (Nuanualsuwan and Cliver, 2003), and therefore the lack of cellular response to UV-inactivated rhinovirus could be explained by an underestimation of the sufficient amount of virus to induce a host response, as well as, the 24h time-point chosen to perform the experiment. Our data also indicated that LL-37 treatment did not alter the expression levels of IL-8, IL-6 and CCL5

compared to UV-inactivated HRV alone, suggesting that when cells are infected with UV-inactivated rhinovirus, LL-37 did not exert measurable immunomodulatory effects. This suggests active viral replication is required to observe LL-37 mediated alteration of pro-inflammatory signalling although further studies will be needed to fully understand the mechanism through which LL-37 downregulates HRV-induced pro-inflammatory cytokine and chemokine production. Our data indicates that LL-37 may be effective in part through the direct interaction with rhinovirus, and in part through uptake and interaction with airway epithelial cells. Previous studies showed that exogenous LL-37 and mCRAMP inhibited NF-kB activity and TNF-α expression in response to C*lostridium difficile* toxin A and B in monocytes and mouse macrophages, respectively (Hing et al., 2013). It is therefore plausible that LL-37 reduces HRV-associated inflammation by inhibiting NF-kB activity in response to rhinovirus infection of bronchial epithelial cells, however further studies are required to investigate this hypothesis.

We investigated the effects of LL-37 on cytokine and chemokine expression induced in response to later stages of rhinovirus replicative cycle. Our data indicated that LL-37 treatment at this point in the viral replication cycle enhanced the cytokine and chemokine responses of the cells. Statistical analysis showed that only LL-37 (30μg/ml) + HRV co-stimulation exhibited a statistically significant increase in IL-8 production in comparison to infected cells only (*$p<0.05$), indicating that co-stimulation treatment synergistically increased IL-8 production in 16HBE14^{o-} cells. The induction of an inflammatory cellular response by LL-37 at this stage may have a protective effect against an increased number of viral particles. LL-37 was shown to co-localise with TLR3 in the endosome and co-localization of LL-37 was increased in the presence of dsRNA (Singh et al., 2013). In 16HBE cells, TLR3 is primarily located in the endosomes, not at the cell surface, and is required for HRV29-induced IL-8 expression in 16HBE airway cell (Sajjan, 2006). The airway epithelial cell line BEAS-2B displayed an increased expression of IL-6 when co-stimulated with HRV and LL-37 compared to HRV infection, and this was shown to be due to the activation of TLR3 (Lai et al., 2011). TLR3 and MDA-5, but not RIG-I, have been shown to be essential for maximal sensing of HRV dsRNA in BEAS-2B human bronchial epithelial cells. Both TLR3 and MDA5 signal through a common signalling

intermediate - IRF3 (Q. Wang et al., 2009). It appears that LL-37 may act to enhance the pro-inflammatory response of airway epithelial cells during viral infection by increasing the production of cytokines and chemokines. This could increase the number of recruited immune cells to the site of infection, ultimately leading to a more rapid and efficient clearance of the viral infection.

In the respiratory tract, cathelicidin over-expression studies have shown improved clearance of pulmonary pathogens, which is consistent with the hypothesis that LL-37 is an effector molecule of the airway immune responses (Bals et al., 1999a, 1999b). In *in vitro* studies, LL-37 has been shown to induce IL-8 and IL-6 production in a dose- and time-dependent manner and to promote the activation of NF-kB, mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase 1/ 2 (ERK1/2) and p38 in bronchial epithelial cells (Bowdish et al., 2004; Pistolic et al., 2009; Tjabringa et al., 2003). In the context of the data in this study, LL-37 may act cooperatively to enhance the innate response to rhinovirus infection in bronchial epithelial cells in later stages of the infection.

Finally, our data suggests that low concentrations of LL-37 did not alter the pro-inflammatory response of airway epithelial cells to rhinovirus infection, although in contrast to our findings, previous studies have shown that the simultaneous exposure of bronchial epithelial cells to 3μg/ml of LL-37 and poly I:C or flagellin resulted in an increase production of IL-8 (Filewod et al., 2009). We believe, therefore, that in the context of an active replicating pathogen, higher concentrations of LL-37 may be required to elicit an effect.

In summary, our data indicates that the capacity of LL-37 to modulate the pro-inflammatory response of bronchial epithelial cells to HRV could have a strong influence on host cell responses to infection. In this chapter, we have shown that at early stages of rhinovirus infection, LL-37 reduces the HRV-induced expression of IL-8, IL-6 and CCL5, but at later stages of infection, LL-37 enhances the HRV-induced expression of IL-8, IL-6 and CCL5. We suggest that at earliest stages of infection, LL-37 modulates inflammation primarily by direct anti-viral mechanisms, as indicated by the reduction in viral load. However, at later stages of infection, LL-37 may act as stimulatory agent in order to increase the sensitivity to rhinovirus infection.

However, it is clear that further studies are required to determine the exact mechanisms and signalling pathways by which LL-37 modulates inflammatory responses to rhinovirus infection.

CHAPTER 6

Investigating LL-37 ability to modulate cell death pathways in rhinovirus-infected airway epithelial cells

Chapter 6. Investigating LL-37 ability to modulate cell death pathways in rhinovirus-infected airway epithelial cells

6.1. Introduction

6.1.1. Apoptotic and necrotic cell death

Apoptosis is a genetically programmed and tightly regulated form of cell death which is characterised by distinctive morphological changes including cell shrinkage, blebbing of the plasma membrane, chromatin condensation and nuclear fragmentation (Kerr et al., 1972). These morphological apoptotic characteristics are a strategic mechanism to contain damage associated molecular patterns (DAMPs) and avoid undesirable immune responses (Green et al., 2009). Apoptosis is induced by two main pathways: the **extrinsic pathway** which is initiated by binding of death ligands, such as TNF or Fas, to extracellular death receptors, resulting in the activation of caspase-8 followed by downstream caspase-7 and -3 activation; and the **intrinsic pathway**, which is initiated by internal signalling, resulting in mitochondrial outer membrane permeabilization with cytochrome c release into cytoplasm. This is followed by apoptosome formation, activation of caspase-9 and downstream activation of caspase-3 and caspase-7 (Shiozaki et al., 2002). Caspases are a family of cysteine proteinases which coordinate apoptotic cell death. They are generated by proteolytic cleavage of their inactive pro-forms, and are grouped into initiator caspases (caspase-2, -8, -9), executioner caspases (caspase-3, -6 and -7) and inflammatory caspases (caspase-1, -4, and -5).

Necrosis has historically been considered a passive form of cell death induced by accidental injury of tissue with no activation of any specific cellular pathway. The typical morphological characteristics that define necrotic cells include early loss of plasma membrane integrity and cell swelling which culminate in the cell bursting and releasing the intracellular contents (Kögel and Prehn, 2013). However, in addition to passive necrosis, several studies have described that cells can undergo programmed necrosis (aka necroptosis), which occurs in the absence of chromatin condensation and caspase activation (Cho et al., 2009; Oberst et al., 2011 and Rodriguez et al., 2016).

In this chapter we assessed apoptosis and necrosis by flow cytometry quantification of FITC-labelled annexin V and propidium iodide (PI) staining. In healthy cells the plasma membrane is composed of lipids asymmetrically distributed in the inner and outer leaflets of the plasma membrane and one of these lipids, known as phosphatidylserine (PS), is located in the inner leaflet of the plasma membrane. Early events of apoptosis include the translocation of PS from the inner leaflet to the outer leaflet of the plasma membrane. AnnexinV is a 36 kDA Ca^{2+}dependent phospholipid-binding protein with high affinity to PS, and can be fluorescently labelled in order to detect PS that is present in the surface of apoptotic cells by flow cytometry. Later stages of apoptosis and necrosis are accompanied by loss of membrane integrity, which can be stained with Annexin V used in combination with PI. Healthy cells with intact plasma membranes exclude PI; however, in damaged and dead cells the plasma membrane becomes permeable to PI. Consequently, healthy cells are negative for both annexin V and PI staining, early apoptotic cells are positive for annexin V and negative for PI staining, and late apoptotic or necrotic cells are positive for both annexin V and PI staining (Crowley et al., 2016). In addition we further assessed apoptosis by detecting caspase-3 activation through flow cytometry quantification of cleaved caspase-3.

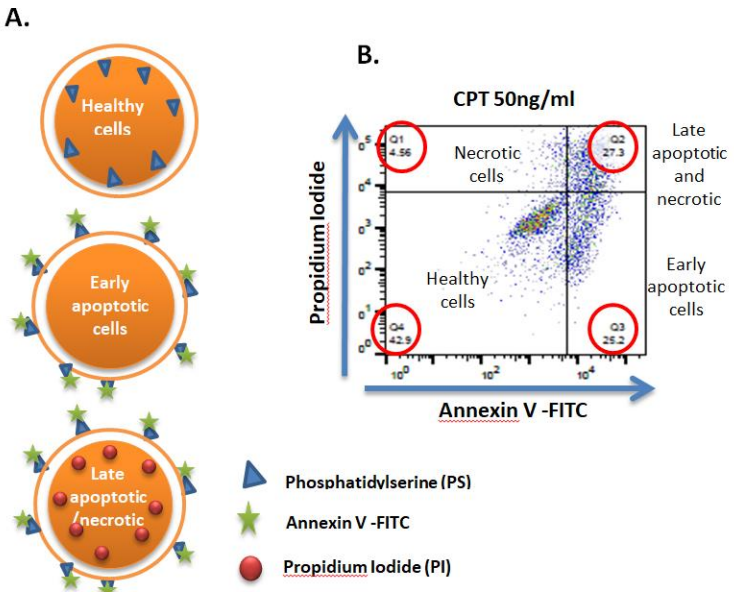


Figure 43.Representation of apoptotic and necrotic cell death.

(A) Illustration showing cell death markers used for detection of apoptotic and necrotic cells death. (B) Representative dot blot of $16HBE14^{o-}$ treated with camptothecin CPT (50 ng/ml) for 48h at 33°C.

The modulation of cell death pathways has been proposed as an important component of the pathogenesis of viral infections (Thomson, 2001) and most picornaviruses have been shown to induce cytopathic effects on host cells. Several studies have demonstrated, under certain conditions, that some of these viruses induce apoptotic cell death pathways (Belov et al., 2003; Carthy et al., 2003; Girard et al., 1999).

The dysregulation of apoptosis have been associated with the development of a wide diversity of diseases in humans, including cancer, autoimmune diseases, and neurodegenerative disorders (Bellamy et al., 1995). Nevertheless, apoptosis may be an important host defense mechanism for the clearance of viral infections. However, certain viruses have evolved to induce apoptosis as a mechanism of dissemination and to avoid the detection of the immune system. Poliovirus (PV) is an enterovirus part of the family *Picornaviridae* and is the etiological agent of paralytic poliomyelitis. The cytopathic effects of PV on the central nervous system (CNS) were shown to be associated with the induction of apoptosis during paralytic poliomyelitis. *In vitro* studies showed that PV infection activated an apoptotic pathway, involving mitochondrial damage, cytochrome c release, followed by activation of caspase-9 and caspase-3, which suggests that the major apoptotic pathway induced by PV infection is mitochondrial mediated (Belov et al., 2003). *In vivo* studies using transgenic mice expressing the human PV receptor demonstrated that PV infection induced apoptosis by displaying DNA fragmentation in CNS tissue from paralysed mice (Girard et al., 1999). Rhinoviruses have also been associated with apoptosis, a study by Taimen et al. (2004) demonstrated that HRV1B and HRV9 infection of HeLa cells induced apoptosis. However, this study did not fully characterise the different apoptotic pathways induced by rhinovirus. A recent study revealed that following HRV14 infection, apoptotic cell death was observed in both HeLa cells and 16HBE14^{o-} cells and this was accompanied by the release of cytochrome c and activation of caspase-9 and -3, but not caspase-8. This indicates that HRV14 induces apoptosis predominantly by the intrinsic pathway. The use of general caspase inhibitors resulted in the reduction of released virus, which indicates that apoptosis may be a key pathway for the release of newly synthesised viral particles (Deszcz, 2005).

The human cathelicidin, LL-37, has previously shown to have contrasting effects on apoptotic cell death pathways in different cells (Barlow et al., 2010, 2006; Lau et al., 2006; Li et al., 2009; Suzuki et al., 2011). For example, it was shown that LL-37 preferentially induced the apoptosis of *Pseudomonas aeruginosa*-infected bronchial epithelial cells, via induction of mitochondrial membrane depolarization, cytochrome c release and activation of caspase-9 and -3 (Barlow et al., 2010). In addition, although not in the context of infection, LL-37 was shown to induce apoptosis in primary epithelial cells possibly involving the activation of $P2X_7$ receptor, which has been shown to be a partial receptor for LL-37 –host cell interaction and signalling (Barlow et al., 2006). In contrast, in keratinocytes, LL-37 was shown to supress camptothecin-mediated apoptosis via a COX-2/PGE-2 antiapoptotic pathway (Chamorro et al., 2009).

In this chapter we aim to investigate LL-37 ability to modulate apoptotic cell death in the context of rhinovirus infection of bronchial epithelial cells using different exposure regimens and different experimental methods to assess apoptotic and necrotic cell death.

6.2. Materials and Methods

6.2.1. Annexin V/PI detection by flow cytometry

To assess cell death pathways in infected cells, A549 and 16HBE14^{o-} cells were used in *in vitro* models of infection and a number of treatment approaches were employed. Briefly, A549 and 16HBE14^{o-} cells, were seeded at $1x10^5$ and $5x10^4$ in a 12-well plate, respectively, and incubated at 37^{o}C / 5% CO_2. After 24h, A549 and 16HBE14^{o-}cells were infected with HRV (MOI 1 and MOI 5) for 2h in serum-free media, washed and treated with LL-37 (0-30µg/ml) for 22h in DMEM or IMDM supplemented with 1% (v/v) Utroser™ G Serum at 33^{o}C, respectively (Figure 16). In other experimental approaches with longer infection periods, 16HBE14^{o-} cells were infected with purified HRV (MOI 1) for 2 h in serum-free media, washed and re-immersed in IMDM supplemented with 5% FBS for 22h at 33^{o}C. Cells were washed prior to two different LL-37 exposure regimens which included (1) cells treated with LL-37 (10 and 30µg/ml) for 2h washed and re-immersed in IMDM supplemented with 5%FBS for 22h at 33^{o}C (Figure 32) or (2) cells treated with low concentrations of LL-37 (1 and 10µg/ml) for 24h in IMDM supplemented with 1% (v/v) Utroser™ G Serum (Figure 33). Positive controls for apoptosis and necrosis were cells treated with camptothecin and scraped cells, respectively. After each treatment, media was collected; cells were washed and detached with 0.05% trypsin-EDTA. Trypsin was subsequently neutralised by the addition of cell media and cells were then placed in flow cytometry tubes and centrifuged at 230 x g for 5 minutes. Supernatant was discarded and cells were re-suspended in 2 ml of annexin V binding buffer (1x) and the wash step was repeated twice. Supernatant was discarded, leaving approximately 200µl in which 2.5 µl FITC-labeled annexin V (BD Pharmigen, Oxford, UK) was diluted. Cells were incubated for 20 minutes at room temperature in the dark, and once again centrifuged to remove FITC-Annexin V, and subsequently re-suspended in binding buffer. Immediately prior to analysis by flow cytometry, 2.5 µl of propidium iodide (BD Pharmigen™, Oxford, UK) was added to cells.

6.2.2. Detection of cleaved caspase-3 and HRV1B by flow cytometry

In order to detect HRV and activation of caspase-3 during viral replication, a pan-enterovirus monoclonal antibody was used for HRV VP3 detection and anti-cleaved caspase 3 antibody was used to detect cleaved caspase-3. Briefly, 16HBE14^{o-} cells were infected with purified HRV (MOI 1) for 2h in serum-free media, washed and re-immersed in IMDM supplemented with 5%FBS for 4, 16, 22 and 46h at 33^{o}C. Cells treated with camptothecin (6µM) were used as a positive control for caspase-3 activation. After each time-point, supernatants were collected and cells were detached with 0.05% trypsin-EDTA (GIBCO, UK). Cells and supernatants were collected into a FACS tube and centrifuged at 300 x g for 5 minutes. Supernatants were discarded and re-suspended in 4% PFA for 15minutes at room temperature. PBS was added to dilute PFA and cells were centrifuged at 300 x g for 5 minutes. Supernatants were discarded and cells were permeabilised by adding ice cold 90% methanol while gently vortexing. Cells were incubated for 30 minutes on ice. Methanol was removed by washing cells with PBS and centrifuging cells at 400 x g for 4 minutes. Cells were re-suspended in 100µl of diluted primary antibodies in 0.5% BSA in PBS (pan-enterovirus monoclonal antibody, mouse, 1:200 and cleaved caspase-3, rabbit, 1:800) and respective isotype controls (mouse and rabbit used at the same concentrations as the primary antibodies). Cells were incubated for 1h at room temperature and then washed with PBS before re-suspending cells in 100 µl of diluted goat PE anti-mouse (1:1000) and goat BV421-anti-rabbit (1:1000) in 0.5% BSA in PBS. Cells were incubated for 30 minutes at room temperature, and washed twice in 0.5% BSA in PBS before flow cytometry analysis in a BD FACS Celesta instrument (BD Biosciences, UK). In order to determine if LL-37 modulates caspase-3 activation in HRV-infected cells, 16HBE14^{o-} cells were infected with purified HRV (MOI1) for 2h in serum-free media, washed and re-immersed in IMDM supplemented with 5% FBS prior to treatment with LL-37 (1 and 10µg/ml) for 24h in IMDM supplemented with 1% v/v Ultroser G at 33^{o}C (Figure 33). Cells treated with camptothecin (6µM) were used as a positive control for caspase-3 activation. Supernatants and trypsinised cells from each condition were collected in flow cytometry tubes and centrifuged at 300 x g for 5 minutes. Cells were washed with PBS and supernatants were discarded before incubation with 4% PFA for 15 minutes at room temperature. Cells were washed with PBS at 300 x g for 5 minutes and

supernatants were discarded. Cells were re-suspended in 0.05% saponin in PBS and centrifuged immediately at 400 x g for 4 minutes. Blocking was performed with 10% goat serum in 0.05% saponin in PBS for 20 minutes at room temperature and cells were subsequently centrifuged at 400 x g for 4 minutes. Cells were then re-suspended in 100µl primary antibody (anti-cleaved caspase 3, rabbit, 1:800) and isotype control (rabbit isotype control, 1:800) diluted in 10% goat serum in 0.05% saponin in PBS and incubated overnight at 4^{o}C. Cells were washed with PBS and re-suspended in 100µl secondary antibody (BV421-anti-rabbit, 1:1000) diluted in 10% goat serum in 0.05% saponin in PBS. Cells were washed twice with PBS before flow cytometry analysis in a FACS Celesta instrument.

6.2.2. Lactate dehydrogenase (LDH) quantification

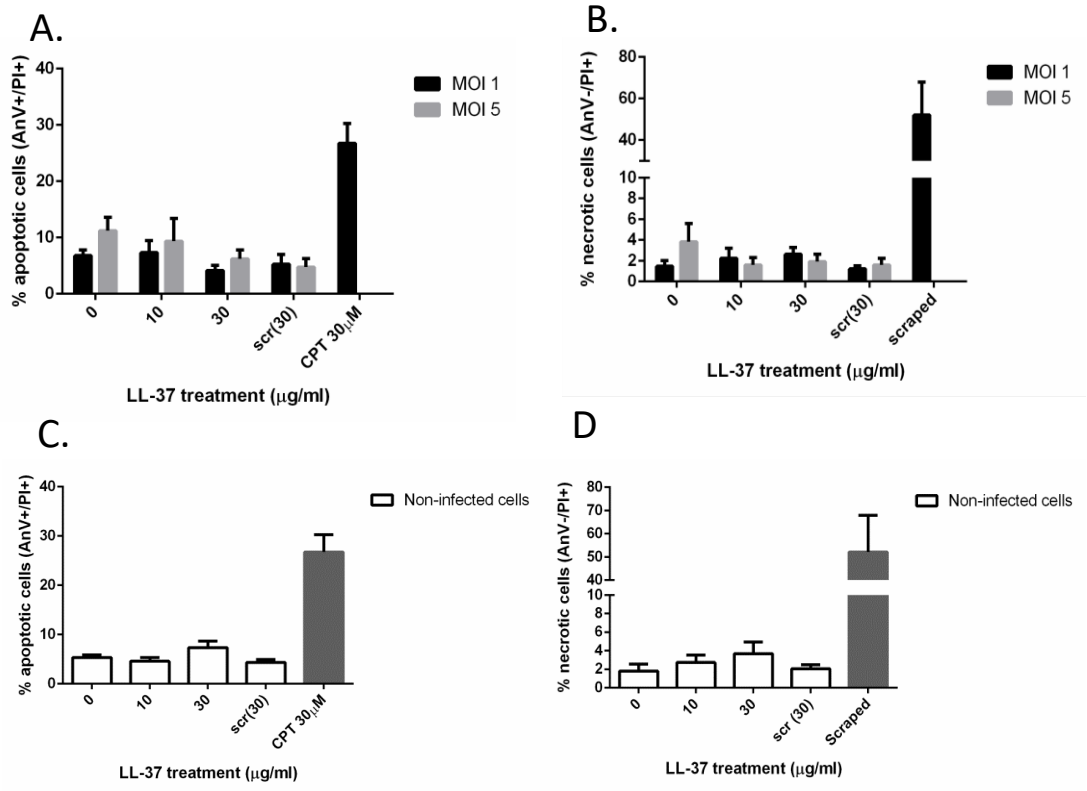
16HBE14^{o-} cells were infected with HRV (MOI 5) for 2h in serum-free media, washed and treated with LL-37 (0-30µg/ml) in IMDM supplemented with 1% (v/v) Ultroser G for 22h at 33^{o}C. After 24h, cell supernatants were aspirated and LDH activity was measured using LDH cytotoxicity detection kit (Promega, UK) according to manufacturer's instructions. Controls included untreated cells as a negative control, and cells treated with lysis buffer (1x) 45 minutes before end of each treatment, as a positive control for maximum LDH release. The formula used to calculate the percent LDH release was:

$$\%\ \text{LDH release (\% cytotocity)} = 100 \times \frac{\text{Experimental LDH Release (OD492)}}{\text{Maximum LDH Release (OD492)}}$$

6.3. Results

6.3.1. Assessment of the capacity of LL-37 to modulate cell death of HRV-infected airway epithelial cells

In order to determine whether LL-37 modulated host cell apoptosis or necrosis of HRV-infected airway epithelium, the human alveolar epithelial cell line A549 was infected with HRV1B (MOI 1 or 5) for 2h prior to treatment with LL-37 (10 and 30 µg/ml) for further 22h, or treated with camptothecin (30 µM) as a positive control for apoptosis. These cells were examined for apoptotic and necrotic cell death by flow cytometry. HRV alone did not induce cell death at this time-point (Figure 44. LL-37 does not modulate apoptotic or necrotic cell death pathways in A549 cells infected with HRV1B. A, B), and this was also the case with LL-37 alone (Figure 44. LL-37 does not modulate apoptotic or necrotic cell death pathways in A549 cells infected with HRV1B. C, D). LL-37 treatment of infected cells did not increase induction of apoptosis or necrosis in this cell line (Figure 44. LL-37 does not modulate apoptotic or necrotic cell death pathways in A549 cells infected with HRV1B. A, B), and a scrambled LL-37 peptide also had no effect on apoptosis or necrosis (Figure 44A, B).



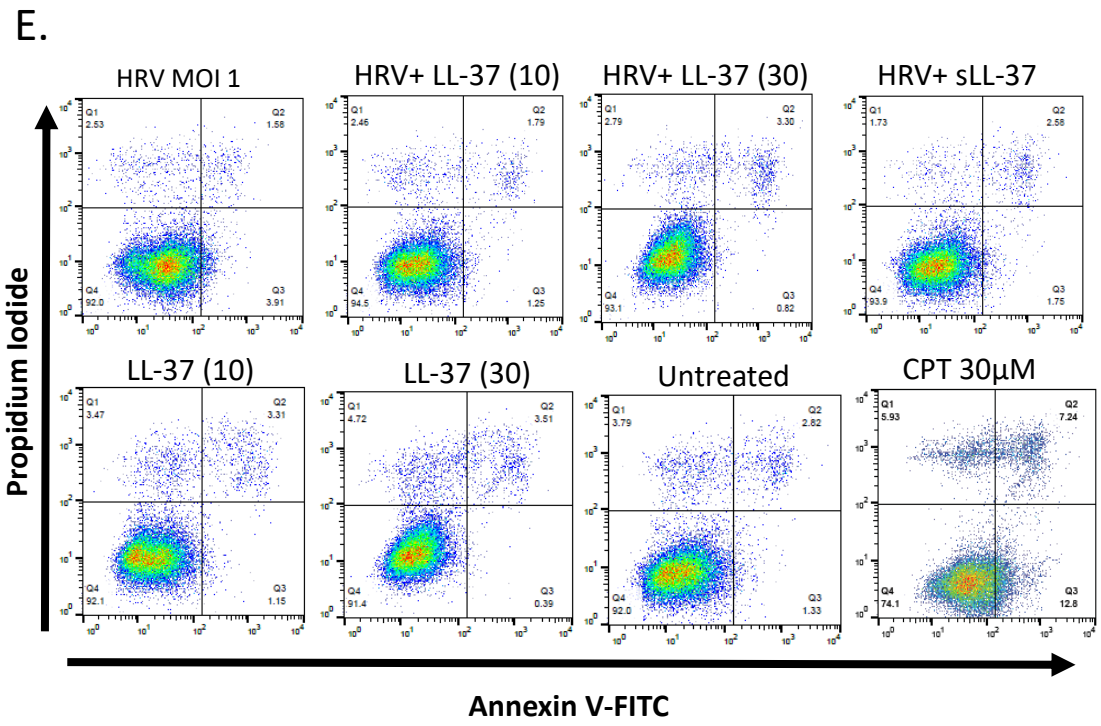
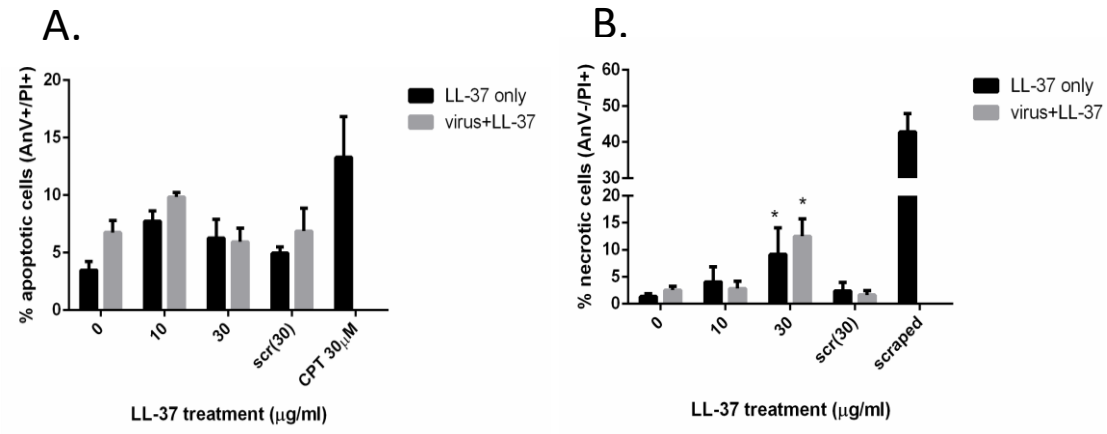


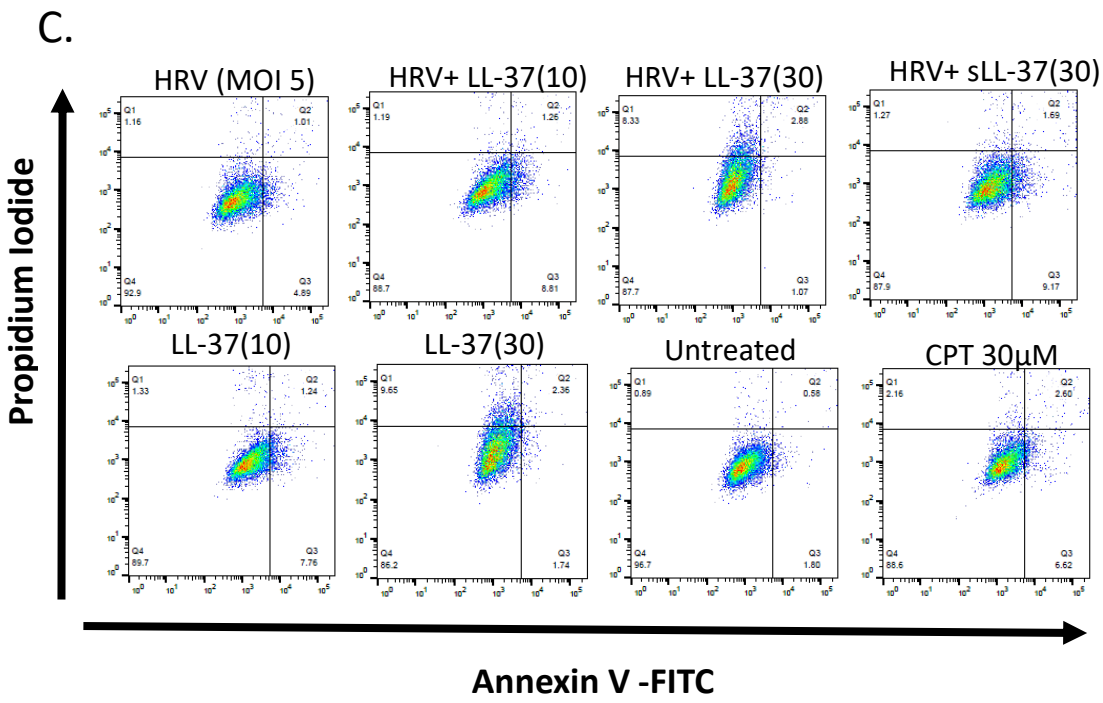
Figure 44. LL-37 does not modulate apoptotic or necrotic cell death pathways in A549 cells infected with HRV1B.

A549 cells were left untreated or infected with HRV1B (MOI 1 or 5) for 2h in serum-free media at 33°C. Cells were washed and treated with LL-37 (10 and 30 µg/ml) for 22h at 33°C. After a period of 24h, cells were stained with Annexin V / PI and assessed by flow cytometry. (A, C) Graph of percentage of cell population that was AV+ / PI– (early apoptosis) and AV+ / PI+ (late apoptosis). (B, D) Graph of percentage of cell population that was AV– / PI+ (necrosis). (E) Representative scatter plot for each treatment. Results are represented as the means ± SEM from three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-test.

The capacity of LL-37 to induce or suppress cell death in HRV-infected airway epithelial cells was also investigated in the 16HBE14^{o-} bronchial epithelial cell line. 16HBE14^{o-} cells were infected with HRV1B (MOI 5) for 2h prior to treatment with LL-37 (10 and 30 µg/ml) for further 22h, or with camptothecin as a positive control (30µM). Apoptotic and necrotic cell death was assessed by flow cytometry. In accordance with our observations in 16HBE14^{o-} cells, HRV1B alone did not induce apoptotic or necrotic cell death in this cell line (Figure 45 A, B). However, LL-37 peptide alone did induce necrosis at the highest treatment concentration (30 µg/ml, *p<0.05) compared to untreated cells (Figure 45B). LL-37 treatment of cells infected

with HRV did not have any effect on the basal rate of apoptosis in this cell line, however LL-37 treatment of infected cells also resulted in a significant increase in necrotic cell death at 30 µg/ml (*p<0.05) compared to infected control cells (Figure 45 A,B). Assessment of the release of cytosolic lactate dehydrogenase indicated that LL-37 (30µg/ml) treatment of HRV infected cells resulted in a statistically significant increase in LDH detected in the supernatant(*p < 0.05), but that this did not occur when healthy cells were treated with LL-37 (Figure 45 D). A scrambled LL-37 peptide had no effect on cell death under any conditions (Figure 45 A,B,C and D).





D.

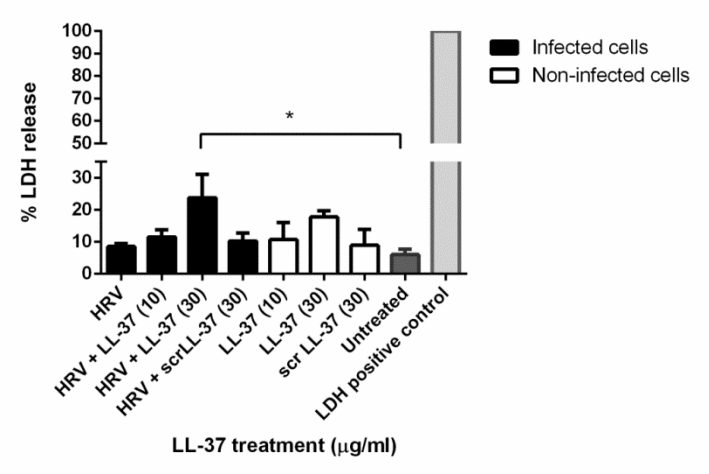


Figure 45. Inflammatory concentrations of LL-37 induce necrotic cell death in HRV-infected bronchial epithelial cells.

16HBE14^{o-} cells were left untreated or infected with HRV1B (MOI 5) for 2h in serum-free media at 33^{o}C. Cells were washed and treated with LL-37 (10 and 30μg/ml) for 22h at 33^{o}C. After a period of 24h, cells were stained with Annexin V /PI and cell death was assessed by flow cytometry. (A) Graph of percentage of cell population that was AV+ / PI– (early apoptosis) and AV+ / PI+ (late apoptosis). (B) Graph of percentage of cell population that was AV– / PI+ (necrosis). (C) Representative scatter plot for each treatment. (D) Cytosolic release of LDH assessed by LDH assay. Results are represented as the means ± SEM from three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-test (* $P \leq 0.05$).

6.3.2. Assessment of LL-37 mediated modulation of cell death at later stages of HRV infection in bronchial epithelial cells

In order to determine the ability of LL-37 to modulate cell death pathways at later stages of the HRV replication cycle, 16HBE14^{o-} cells were infected with HRV1B (MOI 1) for 2h, washed and re-immersed in fresh media for 22h prior to treatment with LL-37 (10 and 30μg/ml) for 2h before re-immersing cells in fresh media for further 22h. Cells treated with camptothecin (50ng/ml) were used as a positive control for apoptosis and apoptotic and necrotic cell death was assessed by flow cytometry. At 48h, cells infected with HRV showed significantly increased rates of apoptosis in comparison to uninfected cells (**p<0.01 and *p<0.05, respectively) [Figure 46A], but necrotic cell death rates were unaltered (Figure 46B). LL-37 treatment of infected cells reduced the rate of apoptosis in cells to levels that were no longer

significantly different from the negative control. The scrambled LL-37 peptide had no effect on the rate of HRV-mediated apoptosis (Figure 46 A). LL-37 alone had no effect on the basal levels of apoptosis and necrosis in this cell line (Figure 46A,B). LL-37 treatment of HRV-infected cells did induce a small increase in levels of necrosis of infected cells but this effect was not statistically significant (Figure 46B).

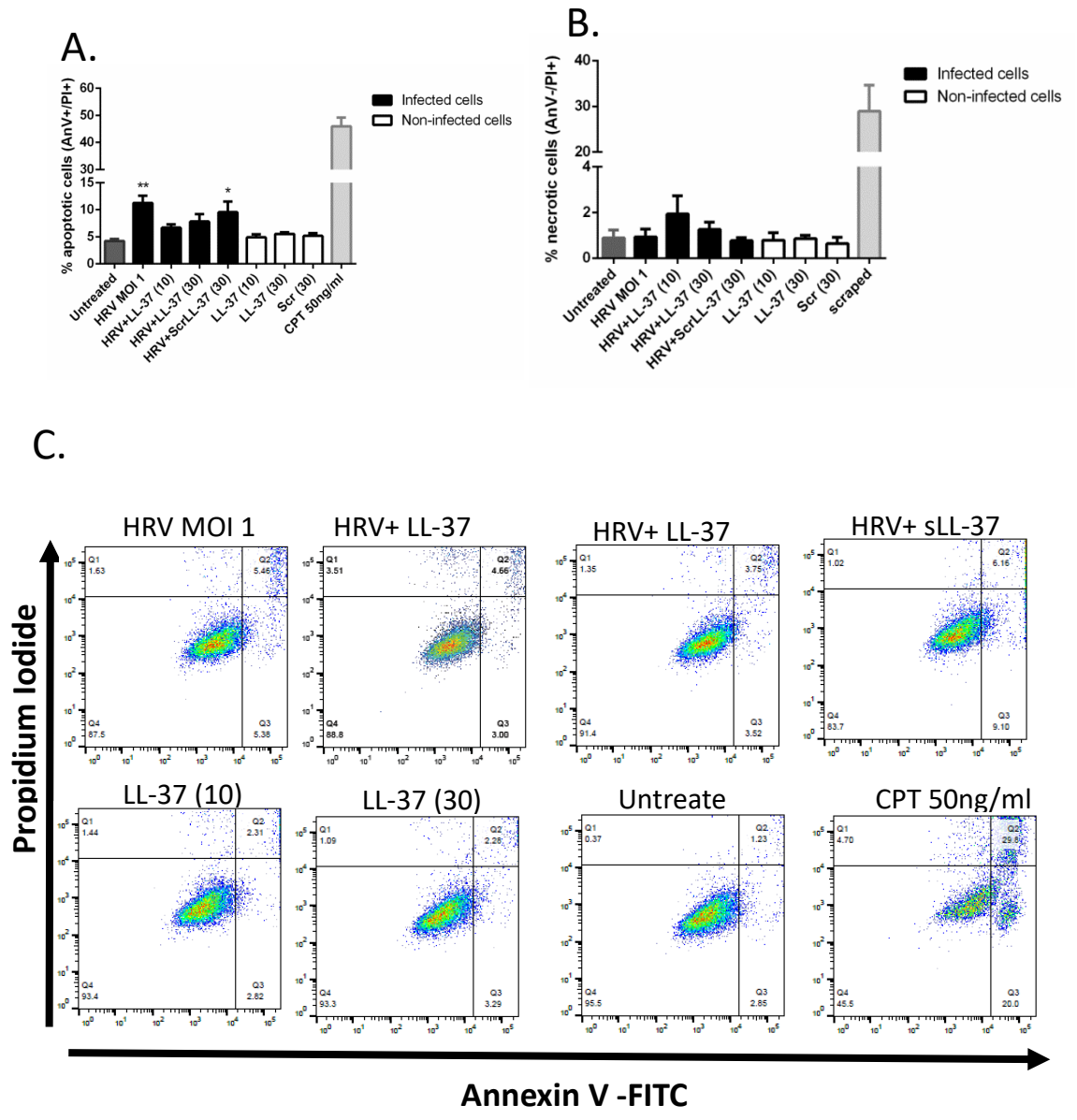
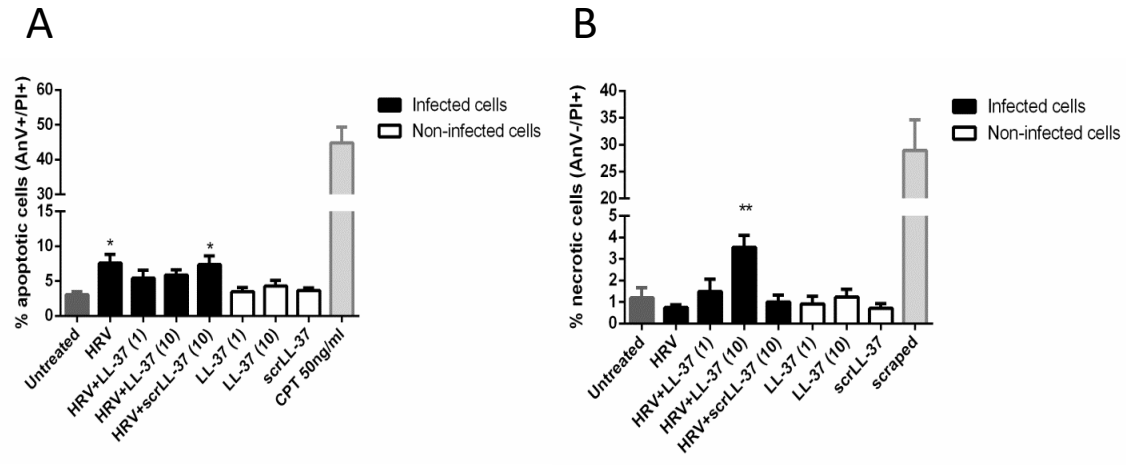


Figure 46. LL-37 treatment of infected cells reduced the rate of apoptosis in cells to levels that were no longer significantly different from the negative control.

Bronchial epithelial cells (16HBE14^{o-}) were left untreated or infected with purified HRV1B (MOI 1) for 2h in serum-free media, washed and re-immersed in media supplemented with 5% FBS for 22h at 33^{o}C. Cells were washed and exposed to LL-37 for 2h, washed and re-immersed in media supplemented with 5% FBS for 22h. After a total period of 48h, 16HBE14^{o-} cells were stained with Annexin V / PI and cell death was assessed by flow cytometry. (A) Graph of percentage of cell population that was AV+ / PI– (early apoptosis)

and AV+ / PI+ (late apoptosis). (B) Graph of percentage of cell population that was AV– / PI+ (necrosis). (C) Representative scatter plot for each treatment. Results are represented as the means ± SEM from four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-test (* $P \leq 0.05$, ** $P \leq 0.01$).

Respiratory epithelial cells, typically the first cells types to be infected, have an important role in limiting the virus and activating the immune system to respond to infection. Viral infection triggers the production and release of host defense peptides, such as cathelicidins, which initiate the host innate and adaptive immune responses. To determine the capacity of lower, physiologically relevant concentrations of LL-37 to modulate cell death in infected airway epithelial cells, the human bronchial epithelial cell line, 16HBE14^{o-}, was infected for 2h with HRV 1B (MOI 1), washed and re-immersed in fresh media for 22h prior to treatment with LL-37 (1 and 10µg/ml) for 24h. Cells treated with camptothecin (50 ng/ml) were used as a positive control for apoptosis, and apoptotic and necrotic cell death was assessed by flow cytometry. Infected cells showed significant levels of apoptosis in comparison to uninfected cells (*P<0.05) [Figure 47A], but necrotic cell death was not observed (Figure 47B). Similarly, infected cells treated with scrambled LL-37 also showed increased rates of apoptosis (Figure 47A). LL-37 treatment of healthy cells did not alter apoptosis and necrosis (Figure 47A,B). Interestingly, LL-37 treatment of infected cells reduced the rate of apoptosis in cells to levels that were no longer significantly different from the negative control (Figure 47A). In addition, treatment of infected cells with LL-37 (10µg/ml) induced a significant increase in necrosis compared to uninfected cells at this time point, although only 3.5% of cells were found to be necrotic (**p<0.01) [Figure 47B].



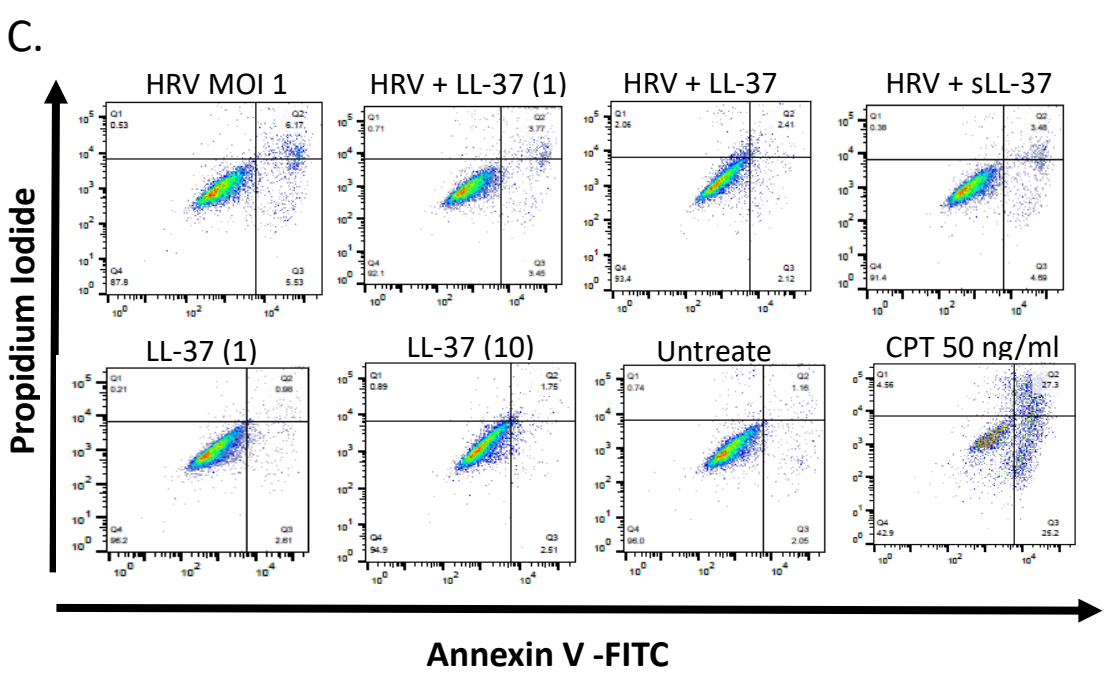
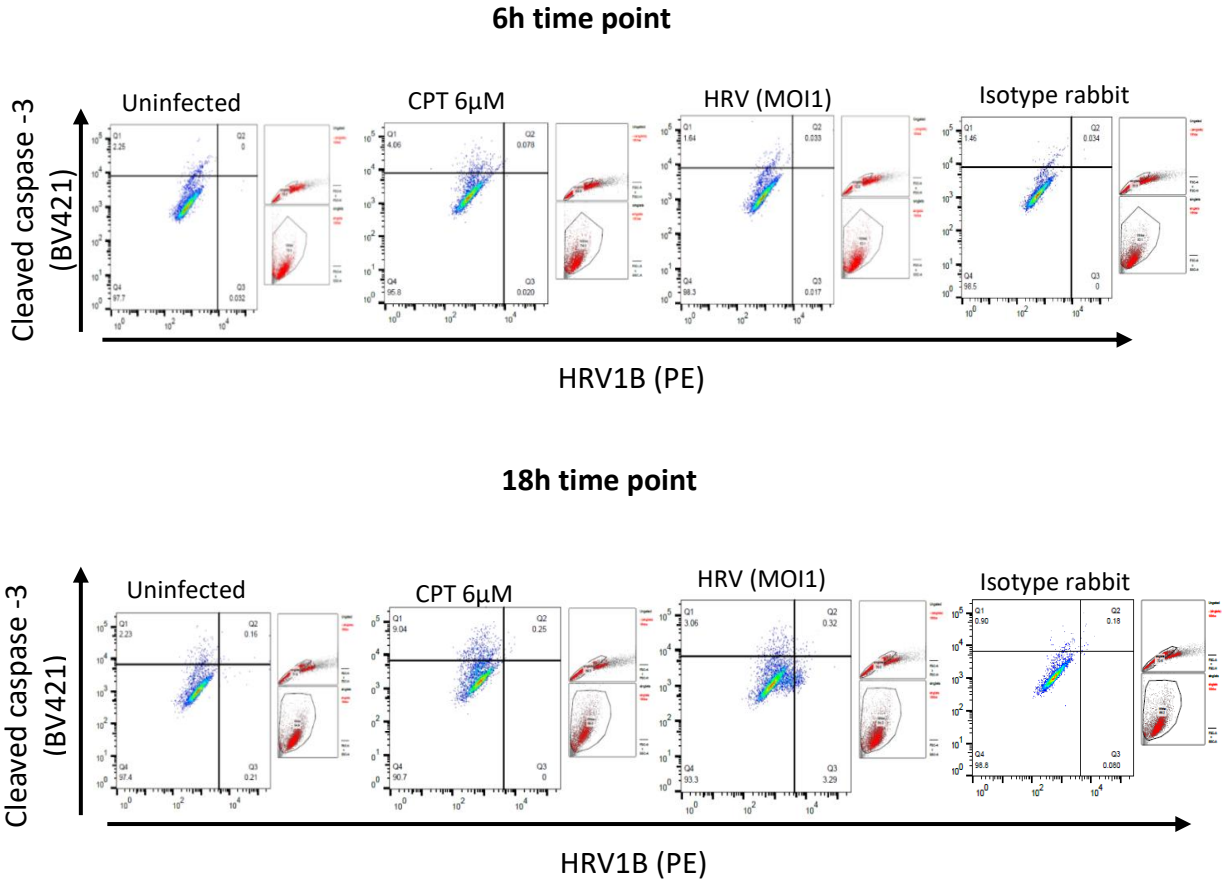


Figure 47. Lower and physiologically relevant concentrations of LL-37 suppress HRV-induced apoptosis, but enhance necrosis in bronchial epithelial cells

Bronchial epithelial cells (16HBE14^{o-}) cells were left untreated or infected with purified HRV1B (MOI 1) for 2h in serum-free media, washed and re-immersed in media supplemented with 5% FBS for 22h at 33^{o}C. Cells were washed and treated with LL-37 (1 and 10µg/ml) for 24h in media supplemented with 1% Ultroser G. After a total period of 48h. 16HBE14^{o} cells were stained with Annexin V / PI and cell death was assessed by flow cytometry. (A) Graph of percentage of cell population that was AV+ / PI– (early apoptosis) and AV+ / PI+ (late apoptosis). (B) Graph of percentage of cell population that was AV– / PI+ (necrosis) (C) Representative scatter plot for each treatment. Results are represented as the means ± SEM from five independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-test (* p≤0.05, **P≤0.01).

6.3.3. Assessment of HRV and LL-37 mediated effects on activation of caspase 3

To establish if LL-37 modulated apoptosis in rhinovirus-infected cells, we assessed caspase-3 activation by flow cytometry. Our initial approach was to assess the ability of HRV1B to induce caspase-3 activation in 16HBE14^{o-} cells during viral replication. A pan-enterovirus monoclonal antibody was used to detect rhinovirus and anti-cleaved caspase-3 was used to detect cleaved caspase-3 by flow cytometry at 6, 18, 24 and 48h post-HRV1B infection (Figure 48). Camptothecin was used a positive control. The data showed a substantial increase in cleaved caspase-3 at 24h post-infection compared to uninfected cells (from 4.8% to 13.94%). From those 13.94% cells positive for cleaved caspase-3, human rhinovirus was detected in 2.54% of this population. Similar results were seen at 48h after infection (12.13% positive cells for cleaved caspase-3); however, there was a reduction in the number of cells positive for HRV (from 2.54% at 24h to 1.53% at 48h) [Figure 48].



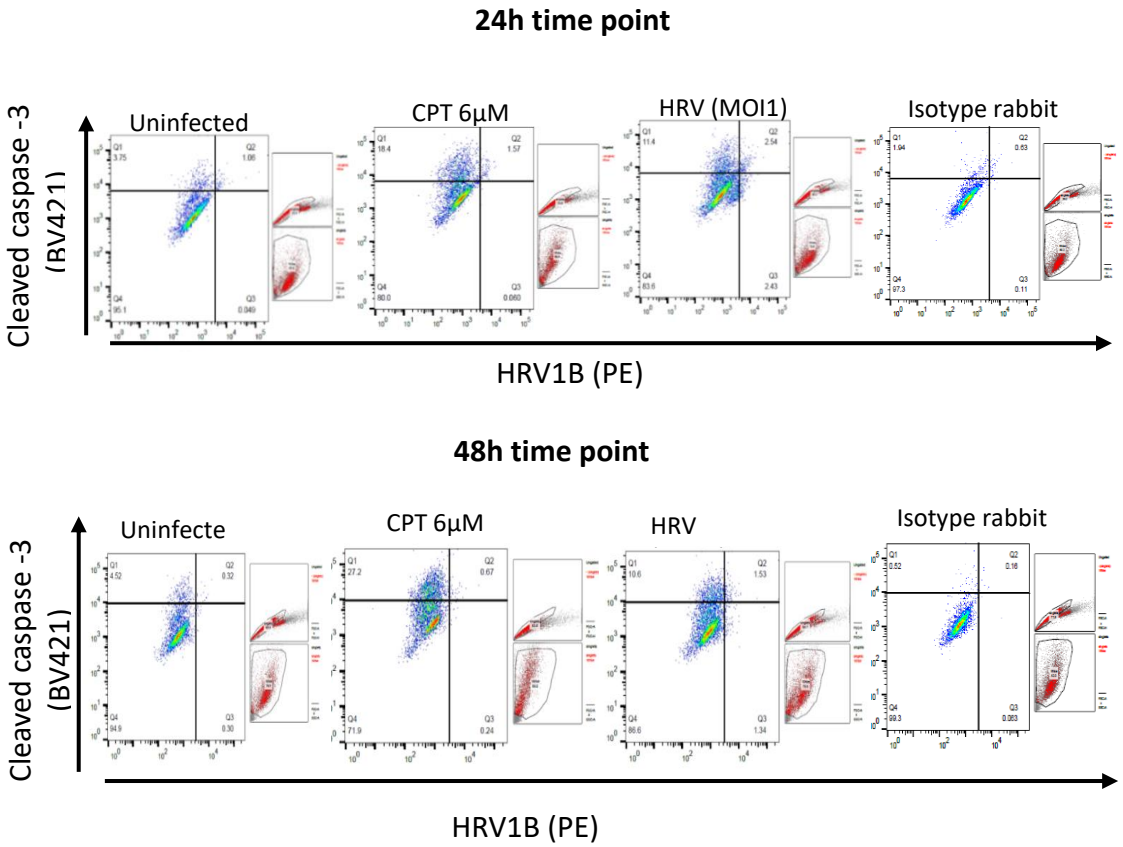


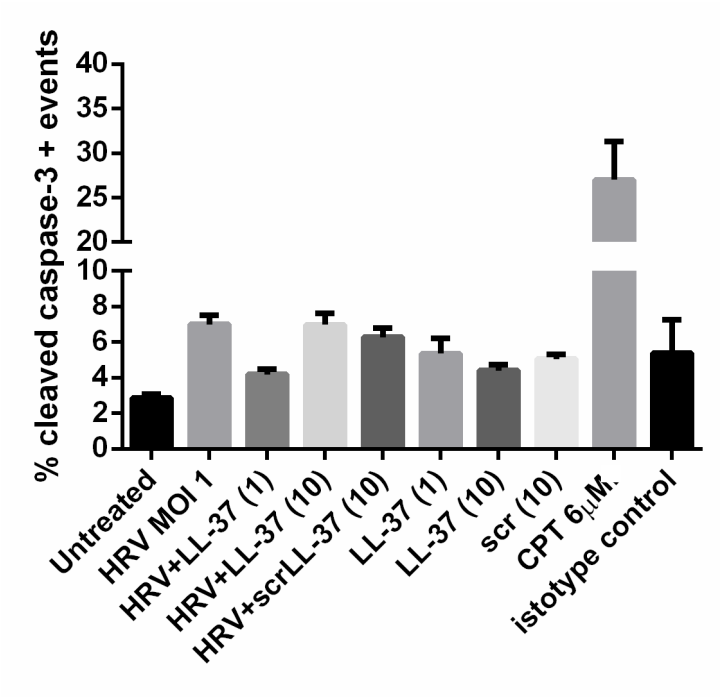
Figure 48. Rhinovirus activates caspase 3 in bronchial epithelial cells.

16HBE14^{o-} cells were left unstimulated or infected with purified HRV1B (MOI 1) for 2h, washed and re-immersed in media supplemented with 5% FBS in IMDM for 4, 16, 22 or 46h at 33^{o}C. As a positive control cells were treated with camptothecin (6µM) in 5% FBS in IMDM. Cleaved caspase-3 was detected using primary anti-cleaved caspase 3 (rabbit) antibody and secondary BV421 Goat Anti-Rabbit antibody. HRV1B VP3 protein was detected using primary pan-enterovirus monoclonal antibody (mouse) and secondary PE Goat anti-mouse antibody. Representative dot blots at each time point for uninfected, HRV-1B infected and CPT-treated 16HBE14^{o-} cells.

To determine if LL-37 exposure modulated caspase-3 activation in HRV-infected cells, 16HBE14^{o-} cells were infected with purified HRV (MOI 1) for 2h, washed and re-immersed in fresh media for 22h prior to treatment with low concentrations of LL-37 (1 and 10µg/ml) for 24h. Flow cytometry analysis indicated that rhinovirus infection alone increased cleaved caspase-3 in comparison to uninfected cells but this increase was not significant (Figure 49). LL-37 treatment of healthy cells also increased cleaved caspase-3 levels to a comparable extent as HRV only. Infected cells treated with 1µg/ml of LL-37 showed a reduction in cleaved-caspase-3 levels, in comparison to HRV alone, similar to levels observed in uninfected cells. The

scrambled LL-37 control did not have any effects on the levels of cleaved caspase-3 in any of the conditions tested (Figure 49). However, the isotype control used for detection of background cell staining did also show positive events for cleaved caspase-3 staining, which indicates that the observed staining for cleaved caspase-3 in the other samples may be partly due to background staining.

A.



B

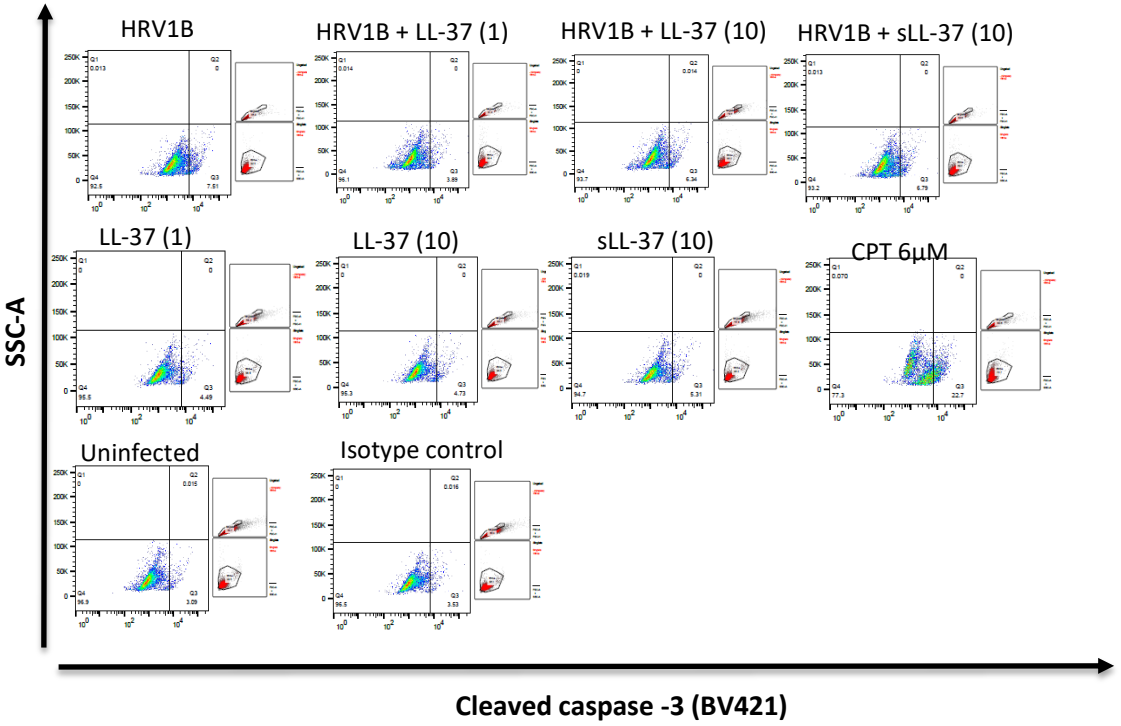


Figure 49. LL-37 does not alter caspase 3 activation in HRV-infected 16HBE cells.

16HBE14^{o} cells were left unstimulated or infected with purified HRV1B (MOI 1) for 2h, washed and left in contact with 5% FBS in IMDM for 22h. Cells were washed and LL-37 (1-10 µg/ml) was added to cells for 24h in 1%UltraGserum in IMDM. Cleaved caspase-3 was detected by flow cytometry. Cleaved caspase-3 was detected using primary anti-cleaved caspase 3 (rabbit) antibody and secondary BV421 Goat Anti-Rabbit. (A) Representative dot blots for each treatment. (B) Representative graph for % of positive events for cleaved caspase-3. Results are represented as the means +/- SEM from two independent experiments.

6.4. Discussion

The sole human cathelicidin, LL-37, is a pleiotropic peptide with the ability to modulate host immunity and inflammation, but the underlying mechanisms of action by which LL-37 contributes to host defense against infections is still unclear. Previously, LL-37 was shown to preferably promote the apoptosis of *Pseudomonas aeruginosa*-infected human bronchial epithelial cells (Barlow et al., 2010); and thus the modulation of apoptotic pathways in rhinovirus-infected airway epithelial cells could represent a novel peptide-mediated host cell mechanism to reduce viral infection. In this chapter, we investigated the potential for LL-37 to modulate cell death pathways in rhinovirus-infected airway epithelial cells in the context of varying peptide concentrations and exposure regimens using different experimental methods to assess apoptotic and necrotic cell death.

In this study we found that treating HRV-infected cells with physiological concentrations of LL-37 (10-30µg/ml) did not result in increased rates of apoptotic cell death, when cells were infected for 2h (MOI 1 and MOI 5) prior to LL-37 treatment for 22h. This was in contrast to previous studies using bacterial infection models (Barlow et al., 2010), although differences in experimental approach and treatment regimens should be noted. This indicates that, at this stage of viral infection and replication (24h post infection) alteration of apoptotic cell death may not represent a mechanism by which LL-37 reduces viral replication in epithelial cells. However, treatment of uninfected and HRV-infected 16HBE14^{o-} cells with physiological inflammatory concentrations of LL-37 (30µg/ml) did lead to a significant increase in necrosis (*$p \leq 0.05$) which was further confirmed by measurement of the cytosolic release of lactate dehydrogenase into cell supernatants.

In chapter 3 we observed a significant decrease in virus titer, measured by $TCID_{50}$ assay, in HRV-infected 16HBE14^{o-} cells treated with 30µg/ml LL-37 (Figure 21). Furthermore, we observed statistically significant increase in the release of the pro-inflammatory IL-8 chemokine in uninfected and HRV-infected cells treated with 30µg/ml LL-37. The prolonged exposure time utilised here (22h) and the relatively

high concentrations of LL-37, while still within physiological estimates, may therefore contribute to the cytopathic effects we observed in the 16HBE14^{o-} cells (Appendix 12). This indicates that LL-37 may have differential roles in the innate immune system depending on the concentration found at the site of infection.

In accordance with our findings, high concentrations of LL-37 (≥30µg/ml) have been shown to be cytotoxic to eukaryotic cells (Aarbiou et al., 2006; Barlow et al., 2006; Johansson et al., 1998; Thomas et al., 2017). In human primary nasal epithelial cells and in mouse macrophages, LL-37 was shown to cause cellular death through the pro-inflammatory necrotic and/or pyroptosis pathways (Thomas et al., 2017). Pyroptosis, also known as caspase-1-dependent cell death, has been described morphologically by the loss of plasma membrane integrity and the extracellular release of cytoplasmic content, followed by plasma membrane "re-seal" and swelling, DNA fragmentation and nuclear condensation (Labbé and Saleh, 2008). Caspase-1, also known as interleukin-1β converting enzyme, does not play a role in the classical apoptotic pathways and is required for the cleavage of pro-IL-1β, -IL-18 and, -IL-33 into their active cytokine forms, which have been shown to play an important role in inflammatory response to infection (Fantuzzi and Dinarello, 1999). Contrary to apoptotic cell death, the production of IL-1β and IL-18 during pyroptosis results in a highly inflammatory state (Rock and Kono, 2008). This further reveals the role of LL-37 in modulating alternative cell death pathways, such as pyroptosis. Future work would involve investigating the role of LL-37 in modulating pryroptosis in the context of viral infection.

By inducing necrotic cell death, LL-37 appears to damage both infected and non-infected cells inducing a pro-inflammatory response and impairing viral replication. However, it remains unclear if LL-37 induces cell death universally through a necrotic pathway or an apoptotic cell death pathway. Nevertheless, in *in vitro* experiments, apoptotic cells are not cleared by alveolar macrophages as they are *in vivo*. Therefore, cells will eventually lyse and gain a necrotic appearance, which is known as secondary necrosis (Arandjelovic and Ravichandran, 2015). Therefore, the increase in necrotic cell death observed in LL-37-treated 16HBE14^{o-} cells measured at 24h could be in fact secondary necrotic cell death.

Apoptosis and necrosis were evaluated by flow cytometry quantification of FITC-labelled annexin V and propidium iodide (PI) staining, and it is worth noting that this approach does not necessarily distinguish between cells that have undergone apoptosis and subsequently become necrotic from those that have died as a result of a necrotic cell death because cells can stain for both annexin V and PI. Therefore, different results may be obtained utilising an *in vivo* model of rhinovirus infection and this should be considered for future investigation in this area.

We further assessed apoptotic cell death by detecting cleaved-caspase 3 by flow cytometry. The cleavage of caspase-3 is generally considered a universal marker for cells undergoing apoptosis because caspase-3 activity is required for the morphological and biochemical changes that occur during apoptosis (Elmore, 2007; Poręba et al., 2013). Caspases, a family of cysteine proteases, are generally present in healthy cells as inactive zymogens, however when stimulated they undergo autolytic cleavage becoming active. Caspase-3 is cleaved in two sites, resulting in three fragments: the pro-domain, the large and small subunit. The first cleavage only partially activates caspase-3 and the second autolytic cleavage results in full activation (Martin et al., 1996). The cleaved fragments remain intact during apoptosis and can be detected using fragment-specific antibodies (Crowley and Waterhouse, 2016). The cleaved caspase-3 antibody used in our experiments detects endogenous levels of the large fragment (17/19kDa) of activated caspase-3, and does not recognise full length caspase-3 or other cleaved caspases. This antibody can then be used to specifically label cells in which caspase cleavage has occurred, therefore allowing for the quantification of apoptosis by flow cytometric detection of the cleaved caspase-3.

Interestingly, we found that physiologically relevant concentrations of LL-37 protected bronchial epithelial cells from HRV-induced apoptosis at later stages of rhinovirus infection. This was observed using two distinct exposure regimens: cells infected with HRV1B for 2h, washed and re-immersed in fresh maintenance media for 22 h prior to (1) treatment with LL-37 (10, 30 μg/ml) for 2h, and then washed and re-immersed in fresh media for 22h or (2) treatment with lower concentrations of LL-37 (1 and 10 μg/ml) for a period of 24h. Furthermore, we demonstrated that

HRV induced apoptosis via caspase-3 activation; however the protective effects of LL-37 were not associated with a decrease in caspase-3 activity, demonstrated by the quantification of apoptosis by flow cytometric detection of the cleaved caspase-3.

LL-37 has previously been shown to mediate contrasting effects on apoptotic cell death pathways (Alalwani et al., 2010; Barlow et al., 2010, 2006; Li et al., 2009). In agreement with our findings, LL-37 has been proposed to inhibit neutrophil apoptosis via activation of $P2X_7$ receptors and G-protein-coupled receptor FPRL1 with subsequent downstream activation of ERK 1/1 MAPK pathway (Nagaoka et al., 2006). In contrast to this study, Barlow et al. (2006) demonstrated that LL-37 acted as a potent inhibitor of spontaneous neutrophil apoptosis via P2X7 receptors and G-protein coupled receptors other than FPRL1 with subsequent downstream activation of PI3K pathway. Despite the contrasting results, this indicates that LL-37 signalling involves the combination of receptors types, which is in accordance with a previous study which argued against a highly structure-specific interaction between LL-37 and cell surface receptors (Braff et al., 2005). It was further demonstrated that LL-37 modulated the expression of the anti-apoptotic protein Mcl-1, and inhibited the cleavage of pro-apoptotic protein BID and pro-caspase 3, suggesting that LL-37 suppresses neutrophil apoptosis by acting on the intrinsic and extrinsic apoptotic pathways (Barlow et al., 2006). Furthermore, LL-37 was shown to protect keratinocytes from camptothecin-induced apoptosis by induction of COX-2, which catalyses the production of PGE_2 which subsequently induces the expression of inhibitor of apoptosis protein (IAP-2) genes (Chamorro et al., 2009). IAP-2 is known as a main regulator of apoptosis which acts through the direct inhibition of caspase-3 (Roy et al., 1997). In addition, LL-37 was shown to supress LPS-induced apoptosis of endothelial cells through LPS neutralization (Suzuki et al., 2011) as well as preventing RSV-induced cell death in Hep-2 cells when cells were simultaneously incubated with RSV and LL-37 or continuously exposed to LL-37 for up to 5 days correlating with a significant reduction in viral load (Currie et al., 2013).

While we have explored the ability of LL-37 to suppress HRV-induced apoptotic cell death in $16HBE14^{o-}$ cells, this did not translate to a corresponding reduction in viral load. Strikingly, we did not observe a reduction in detectable viral copies in cells

treated with LL-37 (1, 10 or 30µg/ml) compared to virus alone (see Appendix 8 and 9) using the two peptide exposure regimens (2h LL-37 treatment, wash and 22h in fresh media or 24h continuous LL-37 treatment) described previously. However, our findings should be considered in the context of other work which has shown that treating host cells with the pan-caspase inhibitor Z-VAD-FMK did not affect HRV14 replication in a HeLa cell model. Specifically, no drop in virus titer, determined in cell pellets, was observed in HRV14-infected HeLa cells treated with Z-VAD-FMK in comparison to virus alone. Nonetheless, the caspase inhibitor did reduce the amount of released virus, while unreleased virus appeared to accumulate inside of cells. This therefore indicates that apoptosis may be a critical mechanism to facilitate the release of intact rhinovirus from cells (Deszcz, 2005). We therefore suggest that, in similarity to what was shown for the major group strain HRV14, the minor group strain, HRV1B, might also modulate the induction of apoptosis to facilitate viral dissemination.

Several cells have been shown to undergo apoptosis in response to viral infections. There is evidence that some viruses induce apoptosis and utilise the cell remnants as vehicle for viral dissemination and as a measure to escape the immune system detection (Thomson, 2001). Influenza A virus has been shown to induce apoptosis in many cell types (Fesq et al., 1994; Stray and Air, 2001). Apoptosis was initially thought to be an anti-viral host defence mechanism, however later research showed that the overexpression of the anti-apoptotic protein Bcl-2 lead to a reduction in the viral load (Olsen et al., 1996). An *in vivo* study showed that IAV-induced apoptosis by modulating the expression of Annexin A1 (Arora et al., 2016). Annexin A1 (ANX1), an immune-modulatory protein, has been described as a pro-apoptotic protein, where the transfection of ANX1 in monocytic cells induced spontaneous apoptosis (Solito et al., 2001). ANX1 is shown to mediate caspase-3 activation and the translocation of the pro-apoptotic protein BAD to the mitochondria (Petrella et al., 2005; Solito et al., 2001). While our results demonstrated that HRV1B induced apoptosis at later stages of viral infection, leading to a time-dependent increase in the levels of cleaved caspase-3, we did not observe an increase in the levels of necrosis above those in uninfected cells. Apoptosis has been demonstrated in HeLa cells and 16HBE14^{o-} cells infected with

HRV1B, -9 and 14 (Deszcz, 2005; Taimen et al., 2004). This was predominantly triggered via the intrinsic pathway and involved translocation of cytochrome c into cytoplasm, DNA fragmentation and caspase-9 and -3 activation and cleavage of caspase substrates (Deszcz, 2005).

As previously described, LL-37 appears to inhibit caspase-3 activity via the activation of a number of different cellular receptors. Thus we proposed that LL-37 could also inhibit HRV-induced apoptosis by reducing caspase-3 activity, which in turn could result in the accumulation of viral particles inside the cells. This is consistent with our data which demonstrated no significant reduction in intracellular viral copies (Appendix 8 and 9), but a decrease in virus release following LL-37 exposure (Appendix 10). However, while our results indicated that rhinovirus induced caspase-3 activation in a time-dependent manner, we observed that treatment of HRV-infected 16HBE14^{o-} cells with LL-37 did not result in a significant reduction of caspase-3 activity. However, we would note that immuno-based detection of cleaved caspase-3 was challenging, reflected by isotype controls showing high background. Therefore, we believe that this data has to be viewed critically, and would propose that further studies are required to determine the exact pathway and mechanism by which LL-37 acts in this context.

In contrast to our own observations, several studies have convincingly shown the ability of LL-37 to induce apoptotic cell death in a variety of models. Higher concentrations of LL-37 (≥30 μg/ml) induced apoptosis in A549 and 16HBE14^{o-} cells in the absence of serum (Lau et al., 2006) and in primary bronchial epithelial cells at concentrations ≥10 μg/ml (Barlow et al., 2006). The mechanism by which LL-37 induced apoptotic cell death in these cells has not been fully elucidated, however, in human cervical epithelial cells the activation of $P2X_7$ receptors have been implicated in apoptosis induction (Q. Wang et al., 2004) which contrasts with the apoptosis-inhibitory role of $P2X_7$ receptors in neutrophils (Barlow et al., 2006; Nagaoka et al., 2006). While LL-37 preferentially promoted the apoptosis of *Pseudomonas aeruginosa*-infected 16HBE14^{o-} cells, this required specific pathogen-epithelial cell interaction with live bacteria, capable of airway epithelium invasion. Several other studies have reported LL-37 capability to induce apoptosis in the

context of the anti-tumorigenic activity of the peptide. A study by Mader et al. (2009) demonstrated that LL-37 induced apoptosis of Jurkat human T leukaemia cells in a caspase-independent manner. The pro-apoptotic mechanism of LL-37 resulted in the activation of Ca^{2+}/Calpain-dependent pathway which resulted in a calpain-dependent Bax translocation to mitochondria, causing the dissipation of mitochondrial membrane potential (ΔψM). The dissipation of ΔψM resulted in Apoptosis-Inducing Factor (AIF) release into the cytoplasm and subsequent migration to the nucleus, where AIF triggers DNA fragmentation and chromatin condensation. An interesting study looked at the potential anticancer effect of FK-16, a fragment of LL-37 corresponding to residues 17 to 32, and revealed that this fragment induced a distinctive pattern of cell death associated with the activation of caspase-independent apoptosis and autophagy. FK-16 induced apoptosis in colon cancer cells by promoting the nuclear translocation of AIF and Endonuclease G (EndoG) to the nucleus (Ren et al., 2013). EndoG is a key modulator protein of caspase-independent apoptosis via DNA fragmentation(Zhdanov et al., 2015). This suggests that LL-37 induces apoptotic cell death via different pathways in different cells.

In summary, our results indicate that LL-37 can protect airway epithelial cells from apoptosis induced by rhinovirus infection, which may represent a novel role for LL-37 in innate immune defence against viral infections. Apoptosis may play an important role by facilitating the release of newly synthesised viral particles and thus increasing the pathogenicity of rhinovirus infection by promoting the virus spread. Therefore, LL-37 represents a promising therapeutic target for the treatment of rhinovirus infections, by suppressing the apoptosis of infected cells thus inhibiting viral spread. However, the accumulation of viral particles inside cells may lead to an increase in the pro-inflammatory response that could be damaging to the airway environment. Nevertheless, in the natural physiological context, infected cells would be targeted by phagocytes (i.e. alveolar macrophages) which would ingest the infected epithelial cells, clearing these cells and avoiding tissue inflammation. Hence, we propose that the role of LL-37 in host innate immunity against viral infections represents a balancing act between the elimination of the virus and immune-mediated damage of the airway epithelium. The mechanism by

which LL-37 suppresses HRV-induced apoptosis remains to be established and we suggest that the use of air-liquid interface culture of differentiated bronchial epithelial cell monolayers in contact with immune cells (i.e. macrophages and dendritic cells) would be of value in this context. In addition, LL-37 has been shown to have the ability to modulate alternative cell death pathways, such as pyroptosis (Hu et al., 2016, 2014) as well as pro-survival pathways including autophagy (Yuk et al., 2009) and thus, these pathways should also be characterised in the context of viral infection.

CHAPTER 7

General Discussion and Future Considerations

Chapter 7. General Discussion and Future Considerations

This thesis has investigated the antiviral activity of the sole human cathelicidin LL-37 against human rhinovirus 1B and assessed the immunomodulatory properties of this peptide, including the modulation of inflammation and cell death, in the context of rhinovirus infection of airway epithelial cells

Rhinovirus replicates in the upper and lower respiratory tract, and with the development of more sensitive molecular techniques HRV was associated with severe respiratory tract infections, including bronchiolitis, pneumonia and exacerbations of COPD and asthma in younger children, immunocompromised individuals and elderly (Ghosh et al., 1999; Louie et al., 2009, 2005). In healthy individuals, rhinoviruses are the main etiological agent for the development of the common cold. The annual healthcare burden of human rhinovirus infections costs billions of dollars (Arden and Mackay, 2009). To date there are more than 140 serotypes identified which make the development of vaccines extremely challenging. Several antiviral drugs against rhinovirus have reached different phases of clinical trials; however, to date no anti-HRV drug has been approved by the EMEA or FDA. Consequently, there are no specific and effective therapies for rhinovirus infections. The development of alternative antiviral therapeutics is urgently required and in this thesis we provide evidence of the potential therapeutic role of the human cathelicidin LL-37 in protecting from and treating human rhinovirus infections.

Previous studies provided strong evidence for the antimicrobial, antiviral and immunomodulatory properties of LL-37 (Barlow et al., 2014; Kahlenberg and Kaplan, 2013; Putsep et al., 2002). Using different exposure regimens, peptide concentrations and different experimental techniques we demonstrated the potent antiviral activity of LL-37 against HRV1B. The concept exploited in this project is that exogenous LL-37, or derivatives could be potentially used as a prophylactic and/or as a therapeutic measure for rhinovirus infection and therefore we developed several different exposure regimens representing the use of exogenous LL-37 to prevent and protect cells from infection and to treat infected cells.

In this thesis, we demonstrate that LL-37 displays direct antiviral activity against rhinovirus 1B *in vitro,* and this effect was observed when the virus was exposed to the peptide prior to cell infection, and when cells were infected prior to the peptide treatment. In our study, the virus exposure to LL-37 prior to cell infection was more efficient than the LL-37 treatment of already infected cells at reducing viral load and chemokine expression in HRV-infected bronchial epithelial cells. These observations were consistent with previous studies, in which LL-37 reduced RSV infection of Hep-2 cells in similar experimental context, indicating that LL-37 may be effective partly by directly interacting with the virus, and in part through interaction with and uptake by host cells (Currie et al., 2013; Harcourt et al., 2016).

The interaction of LL-37 with airway epithelial cells was characterised by using DANSLY-labelled LL-37 and confocal microscopy. It was demonstrated that LL-37 was taken up into A549 and 16HBE14^{o-} cells revealing that the intracellular localization of the peptide was relatively diffuse although notably some peptide did localise around the perinuclear region of the cell. This was consistent with previous studies which demonstrated that LL-37 was actively taken up into A549 cells via a clathrin-mediated endocytosis and that trafficking into the perinuclear region was dependent on microtubules (Lau et al., 2005). This study further demonstrated that LL-37 itself is able to permeabilize the eukaryotic membrane without disturbing the integrity of the membrane. The porcine antimicrobial peptide, PR-39, in contrast to LL-37 is able to rapidly enter human microvascular endothelial cells without plasma membrane permeabilization (Chan and Gallo, 1998). Interestingly, a well-characterised antiviral and antimicrobial activity of the human LL-37 relates to its ability to target and disrupt the bacterial cell wall (Henzler Wildman et al., 2003; J. Turner et al., 1998; Zasloff, 2002)and the viral envelop (reviewed in Barlow et al.(2014)). In fact, it has been well documented that LL-37 can also be cytotoxic to certain eukaryotic cell lines (Oren et al., 1999). Nevertheless, we have shown that LL-37 is not cytotoxic to the respiratory epithelial cell lines (A549 and 16HBE14^{o-}) utilised in this study, at concentrations of ≤30µg/ml, unless cells were exposed to longer periods of time (around 24h) at inflammatory concentrations of LL-37 (i.e.30µg/ml). Importantly, LL-37 was shown to inhibit rhinovirus replication at the concentrations used without exhibiting cytotoxicity towards the airway epithelial

cells. A study by Sandgren et al., (2004) showed that LL-37 was not cytotoxic to eukaryotic cells in concentrations up to 10μM, whereas *E.coli* cells were killed at concentrations below 3 μM. The ability of the peptide to differentially target prokaryotic and eukaryotic cells is related to the peptides preferential binding to negatively charged outer leaflet of bacteria plasma membrane as compared to the predominantly zwitterionic outer leaflet (i.e. the phospholipids present in the outer leaflet have their head groups with a perfectly balanced positive and negative charge –essentially a neutral charge) as well as the presence of cholesterol in the eukaryotic plasma membrane (Zasloff, 2002). However, viral envelops have a very similar phospholipid profile to the host cellular lipid membrane (Aloia et al., 1993). In fact, viral envelopes lack the high negative charge that is the basis of the HDP interaction and disruption of bacterial cells (Chazal and Gerlier, 2003). Several studies of viral envelopes have identified a higher cholesterol-to-phospholipid ratio in comparison to host cell lipid membrane from which they are derived (Aloia et al., 1993; Cordero et al., 2014; Scheiffele et al., 1999). The selectivity of HDPs, therefore, relies on differences between the host cell and viral lipid membranes.

Host defense peptides have also revealed the ability to target non-enveloped virus; LL-37 was shown to inhibit adenovirus replication in A549 cells (Barlow et al., 2014), and HBD5 was shown to induce AdV aggregation (Gounder et al., 2012), which could impact viral infectivity by impeding cell binding. The mechanisms of action of host defense peptides are numerous and in addition to their positive charge, the structural diversity of theses peptides may account for the multiple mechanisms of action, including their role in modulating immune responses. Specifically regarding the antiviral mechanism of action of cathelicidins, several studies have provided evidence on their ability to inhibit viral replication and different mechanism of action have been proposed, including the ability of LL-37 to bind to the envelop E protein of dengue virus type 2 inhibiting entry into the cells (Alagarasu et al, 2018), the ability to inhibit the replication of HIV-1 in peripheral blood mononuclear cells by supressing HIV reverse transcriptase (Bergman et al., 2007), as well as, disrupting IAV membrane, preventing viral replication (Tripathi et al., 2013).

In addition to its direct antiviral activity, LL-37 has an active role in modulating the immune responses in the lung, displaying both pro- and anti-inflammatory properties, chemotactic activity, inducing the production of several chemokines and cytokines, and modulating dendritic cell maturation (Hiemstra et al., 2016; Mansour et al., 2014). In fact there is increasing evidence pointing towards LL-37 as a major player in local innate immunity, demonstrating that this may be its primary role *in vivo* at mucosal surfaces (Diamond et al., 2009; Scott et al., 2002). Cathelicidins have apparently paradoxical functions in regulating or modulating immune responses. In this study we assessed the cathelicidin immunomodulatory activity during rhinovirus infection of bronchial epithelial cells. Our data revealed that at early stages of rhinovirus infection, LL-37 reduced HRV-induced expression of IL-8, IL-6 and CCL5, but at later stages of infection, LL-37 enhanced HRV-induced expression of pro-inflammatory cytokines. Therefore, suggesting that at early stages of infection, LL-37 modulates inflammation primarily by direct anti-viral mechanisms, as indicated by the reduction in viral load and, at later stages of infection LL-37 may act as stimulatory agent in order to increase the sensitivity to rhinovirus infection. However a prolonged increase in pro-inflammatory cytokines can mediate the harmful inflammatory response which is often observed in diseases such as cystic fibrosis (Stecenko et al., 2001). During infection, the inflammatory response increases the local LL-37 concentration which could potentially lead to an even higher production of pro-inflammatory mediators (Pistolic et al., 2009; Tjabringa et al., 2003), which could disrupt the carefully regulated immune response and lead to excessive inflammation. The mechanism by which LL-37 modulates cell responses to infection is still unclear. These effects derive from the capacity to activate or transactivate host cell receptors. Several cellular receptors have been identified, such as FPRL1 (De Yang et al., 2000), EGFR (Tjabringa et al., 2003), $P2X_7$ (Elssner et al., 2004), and GAPDH (Mookherjee et al., 2009) and EGFR. The peptides ability to interact and utilise several different receptors may lead to different outcomes, and depending on the cell type and microenvironment this could be an anti- or a pro-inflammatory response. Inhibition studies, using specific toxins, for example pertussis toxin to inhibit Gi-coupled receptors, or CRISPR technology to knockdown the cell receptor genes activated by LL-37, could be applied to better understand the role of LL-37 in the regulation of the host cell

response to viral infections. The downstream signalling pathways activated by LL-37 should also be investigated and again knockdown of specific genes involved in these pathways could provide a more accurate idea of the mechanism behind LL-37 regulatory effects. Extensive studies, in different experimental settings, are required to fully understand the underlying mechanism by which LL-37 modulates the airway epithelial cell inflammation.

We further demonstrated that LL-37 was able to protect airway epithelial cells from apoptosis induced by rhinovirus infection, which could represent a novel role for LL-37 in innate immune defence against viral infections. Apoptosis may play an important role by facilitating the release of newly synthesised viral particles and thus increasing the pathogenicity of rhinovirus infection by promoting the virus spread. Therefore, LL-37 represents a promising therapeutic target for the treatment of rhinovirus infections, by suppressing the apoptosis of infected cells thus inhibiting viral spread. However, the accumulation of viral particles inside cells may lead to an increase in the pro-inflammatory response that could be damaging to the airway environment. Hence, we propose that the role of LL-37 in host innate immunity against viral infections represents a balancing act between the elimination of the virus and immune-mediated damage of the airway epithelium. The mechanism by which LL-37 suppresses HRV-induced apoptosis remains to be established and we suggest that the use of air-liquid interface culture of differentiated bronchial epithelial cell monolayers in contact with immune cells would be of value in this context. In addition, LL-37 has been shown to have the ability to modulate alternative cell death pathways, such as pyroptosis (Hu et al., 2016, 2014) as well as pro-survival pathways including autophagy (Yuk et al., 2009) and thus these pathways should also be characterised in the context of viral infection.

Chronic respiratory diseases are a growing global epidemic and a prominent cause of death and disability, and their prevalence is expected to increase significantly in the coming years (Mathers and Loncar, 2006). Rhinovirus infections are the most common cause of exacerbations in individuals with chronic respiratory diseases, such as asthma, cystic fibrosis and COPD. In these patients, during exacerbations,

bacterial colonization is commonly found and it is linked to severity and duration of these exacerbations (Arnason et al., 2014; Mallia et al., 2012). Co-infections with virus and bacteria are very common but are poorly understood phenomena in individuals with chronic lung diseases. Several studies have attempted to understand the mechanisms by which virus predispose the airways to secondary bacterial infections. Rhinovirus infection of the airways has been associated with a dysregulation of the innate host defense mechanisms, such as an impaired cationic host defense peptide expression in the lungs. Arnason et al., (2014) demonstrated that primary human bronchial epithelial cells infected with HRV16 and then subsequently bacteria (*H.influenzae/P.aeruginosa*) showed increased levels of β-defensins when compared to HRV and bacteria alone. A study by Mallia et al. (2012) showed that experimental rhinovirus infection was followed by secondary bacterial infections in subjects with moderate COPD but not in smokers and non-smokers with normal lung function. In addition, the sputum LL-37and α-defensins levels were increased significantly from baseline in the COPD group but this was not verified in either of the control groups; however only sputum LL-37 levels in subjects with COPD correlated with peak sputum viral load, inflammatory cells, neutrophils and bacterial load. Of note, the increased HDPs expression could be a secondary phenomenon of neutrophil activation and that other mechanisms, such as the activation of reactive oxygen species and the release of other pro-inflammatory mediators, may at least contribute to the exacerbations of chronic respiratory illnesses.

Patients frequently report cold-like symptoms before exacerbations (Hutchinson et al., 2007; Seemungal et al., 2001) and several *in vitro* studies have linked rhinovirus to increase susceptibility to bacterial infection. Primary human airway epithelia cells grown in ALI and bronchial epithelial cells (16HBE14^{o-}) infected with rhinovirus showed a significant increase in bound bacteria when compared to sham-infected cultures, as well as a decrease in transepithelial resistance and loss of zona occludins (ZO)-1 from tight junctions (Sajjan et al., 2008). Rhinovirus was also shown to induce the expression of fibronectin, platelet-activating factor receptor, and carcinoembryonic antigen-related cell adhesion molecule in nasal epithelial cells which significantly increased the adhesion of *S.aureus*, *S.pneumoniea* and

H.influenzae(Wang et al., 2009). Keeping this in mind, the development of effective anti-HRV therapies is highly required for this population group. The development of antiviral therapies as an early intervention at the onset of cold symptoms in subjects with chronic airway diseases could therefore raise the prospect that they not only decrease the severity and possibly prevent the virus-induced COPD exacerbations, and in addition they also have the potential to prevent secondary bacterial infections. This thesis provides relevant *in vitro* data on the direct antiviral activity of LL-37 against HRV1B, as well as, demonstrating the ability of LL-37 peptide to modulate innate immune responses to viral infection, by down-modulating pro-inflammatory mediators (e.g. IL-8, IL-6 and CCL5), and by protecting airway epithelial cells from virus-induced apoptosis. *In vivo* studies further demonstrated that influenza-infected mice treated with LL-37 had lower concentrations of pro-inflammatory cytokines detected in the BAL in comparison to infected mice that had not been treated with the human cathelicidin (Barlow et al., 2011). Patients with chronic lung diseases which are a risk population for rhinovirus infections require an effective and specific antiviral therapy, and the administration of exogenous cathelicidins, or their derivatives, could represent a promising novel therapy for this specific population group. However, it seems contradictory to administer a cathelicidin-based therapy, when studies have shown a dysregulation in the expression of these cationic host defense peptides, in particular a significant increase expression of LL-37 during viral and bacterial co-infection. Impaired HDP expression was only observed during co-infection of virus and bacteria, and not during viral infection only (Arnason et al., 2014; Mallia et al., 2012), and as previously mentioned, this could be an epiphenomenon of neutrophil activation and other mechanisms. However, it is important to fully understand the role of LL-37 in inflammation, because an increase in LL-37 concentration could potentially lead to an even higher cytokine/chemokine release, leading to excessive inflammation that could prolong the disease state. These observations further emphasise the importance of an early intervention at the onset of cold symptoms in subjects with chronic airway diseases.

An important factor to consider for the development of potential therapeutics based on administration of exogenous LL-37 is the proteolytic degradation of the

peptide. Peptides are susceptible to many host proteases, such as the digestive enzyme trypsin and chymotrypsin, which cleave proteins and peptides at basic and hydrophobic residues respectively, all of which are important for the structure and function of LL-37 (Kim et al., 2014). Several strategies have been proposed to block proteolytic degradation, such as incorporating non-natural D-isomers of amino acids, altering the stereochemistry of the peptide and making the peptides protease-resistance (Barlow et al., 2011; Molhoek et al., 2011). D-LL-37 and D-mCRAMP were shown to retain their potent antiviral activity against IAV *in vitro* and *in vivo* (Barlow et al., 2011). However, in the context of immunomodulation, little is known about the interaction of D-peptides with target host cells and whether the substitute of D-amino acids would compromise the receptor-ligand interactions (Mansour et al., 2014). Another strategy consists in incorporating unnatural amino acids into the peptide sequences providing an improved metabolic stability and preserving the positive charges, such as the replacement of one arginine residue by L-ornithine or L-homoarginine. The substitution for L-homoarginine was shown to increase the peptides stability in serum without affecting the anti-bacterial activity against *E.coli* (Berthold et al., 2013). A recent study demonstrated that LL-37 with Arg residues substituted by homoarginine, displayed full activity of native LL-37 despite peptidyl-arginine deiminases (PADs) treatment. PAD enzymes are responsible for the citrullination of cathelicidins/LL-37 which render the peptides inactive (Wong et al., 2018). Interesting future work would involve investigating the antiviral activity of homoarginine-LL-37/D-LL37 against rhinovirus infection, as well as, assessing the immunomodulatory properties of these peptides in the context of viral infections.

The differences in the ability of the peptides to modulate the pro-inflammatory response of bronchial epithelial cells to HRV infection were seen in the different exposure regimens. This indicates that the context of LL-37 exposure is a key determinant in the effect of the peptide and will likely determine the direction of cellular responses to viral infection. An important observation related to the immunomodulatory activity of LL-37, is that in healthy non-infected cells, none of the immunomodulatory effects were seen; LL-37 did not have any effects on cytokine and chemokine expression, or on cell death. This could suggest that some

aspects of immunoregulation mediated by the peptide are dependent upon infection, which should be considered when developing therapeutics relying up on exogenous peptide treatment, potentially allowing for preferential targeting of the viral infection.

The immunomodulatory activity of cathelicidins is very complex and mechanistic studies have proposed the involvement of several signalling pathways (i.e. NFkB, p38 and JNK, MAPK, and PI3K) which are activated depending on the intracellular uptake, endocytic mobilization and the interaction with several receptors (De Yang et al., 2000; Lau et al., 2005; Mookherjee et al., 2009; Nagaoka et al., 2006). Despite all the information on the immunomodulatory properties of cathelicidins, the role of LL-37 in protecting against viral infections is still far from being completely understood. This thesis provides a characterisation of the role of LL-37 against rhinovirus infection, investigating the antiviral activity, the inflammomodulatory properties and the modulation of cell death in a bronchial epithelial cell model of infection. In the context of these findings, and taking into account the limitations of our experimental approaches, we propose the use of a more physiological relevant model of infection, such as the development of air-liquid interface cell model using bronchial epithelial cells which could be co-cultured with alveolar macrophages and dendritic cells. An alternative to this would be through the use of a lung organoid model of infection, to better mimic the *in vivo* environment. In addition it would be relevant to evaluate the antiviral activity of LL-37 against a number of different serotypes of rhinovirus.

The data in this thesis indicates that LL-37 is able to inhibit rhinovirus 1B infection in bronchial epithelial cells. The possible mechanisms involved in the peptides-mediated antiviral activity are depicted in the figures 40 and 41. This thesis therefore provides strong evidence for the potential of LL-37 to inform the development of alternative antiviral therapies for rhinovirus infection.

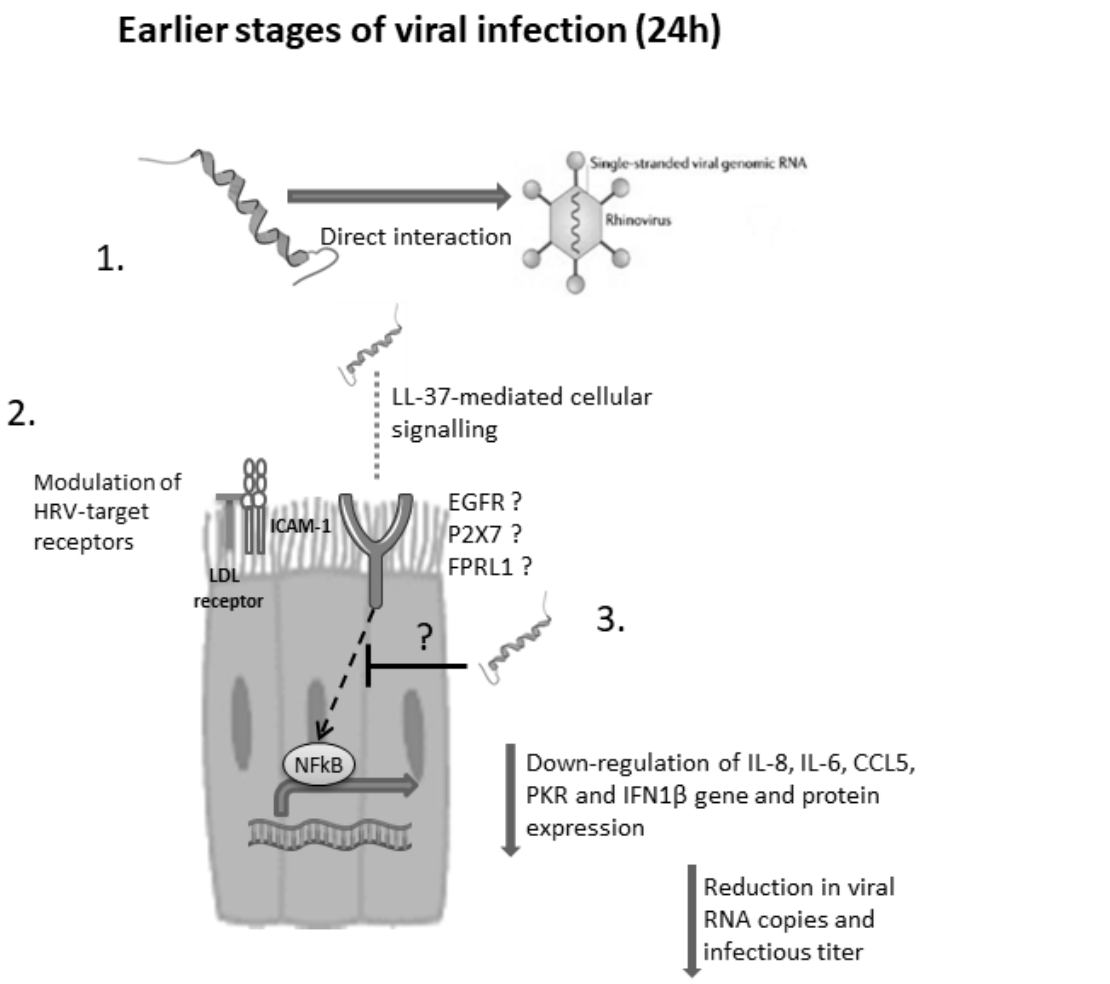


Figure 50. Proposed mechanisms of LL-37-mediated antiviral activity against human rhinovirus 1B at earlier stages of viral infection.

This figure highlights the possible mechanisms involved in LL-37-mediated antiviral activity against HRV1B during earlier stages of infection of 16HBE14^{o-} bronchial epithelial cells. LL-37 may be effective in part through direct interactions with HRV (1), and in part through interaction with and uptake by 16HBE14^{o-} cells. LL-37 may modulate the expression of HRV-targeted receptors (i.e ICAM-1 and LDLr) therefore limiting viral spread (2). In addition LL-37 may interact with airway epithelial cells through different cell receptors (i.e. EGFR, FPRL1, P2X$_7$) and may limit HRV-associated inflammation by inhibiting NF-kB activity (3).

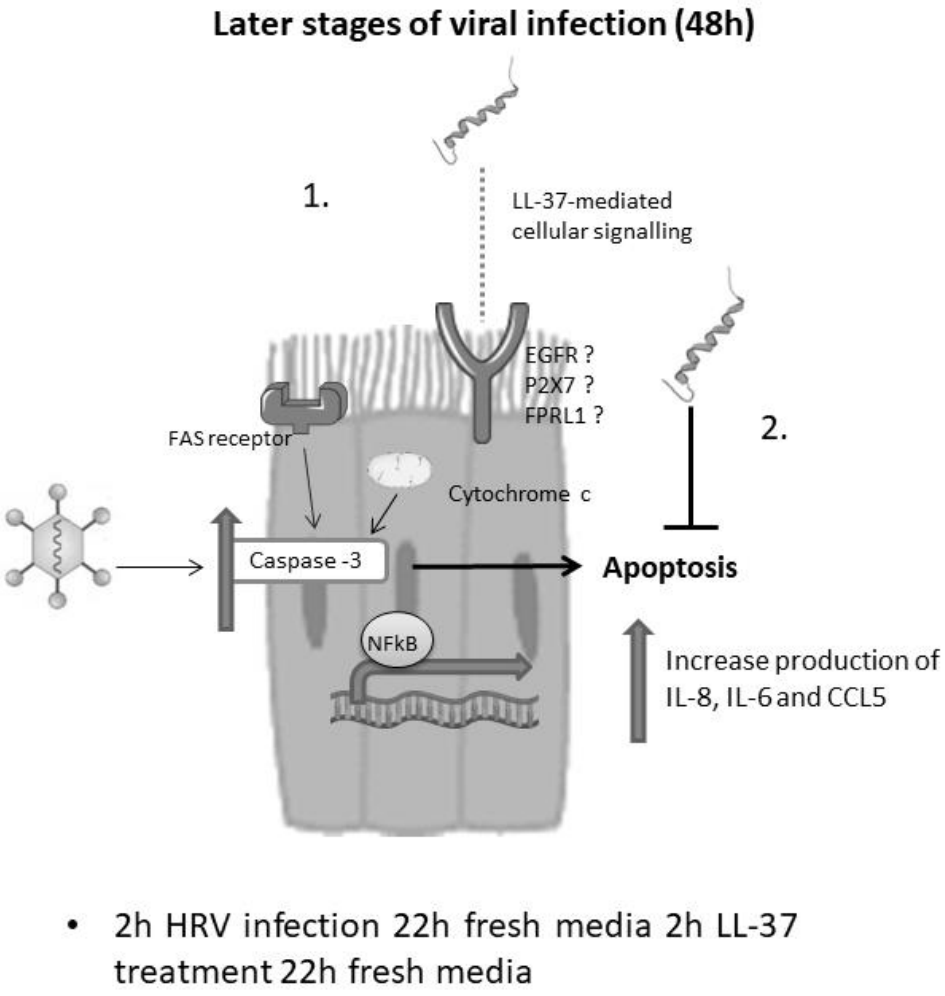


Figure 51. Proposed mechanisms of LL-37 mediated-antiviral activity against human rhinovirus 1B at later stages of viral infection.

This figure highlights the possible mechanisms involved in LL-37-mediated antiviral activity against HRV1B during later stages of infection of 16HBE14^{o-} bronchial epithelial cells. In later stages of infection, rhinovirus infection induces apoptotic cell death of bronchial epithelial cells which may facilitate the release of newly synthesised viral particles. LL-37 was shown to supresses the apoptosis of HRV-infected cells, which may represent a novel role for LL-37 in innate immune defence against viral infections (2). LL-37 increases the production of pro-inflammatory cytokines and chemokines in response to HRV infection possibly by mediating cellular signalling through the activation of host cell receptors (i.e. EGFR, FPRL1 and/or $P2X_7$) which could then lead to an increase in immune cell recruitment and likely aid in a more rapid and efficient infection clearance from airway epithelial cells (1).

References

Aarbiou, J., Tjabringa, G.S., Verhoosel, R.M., Ninaber, D.K., White, S.R., Peltenburg, L.T.C., Rabe, K.F., Hiemstra, P.S., 2006. Mechanisms of cell death induced by the neutrophil antimicrobial peptides alpha-defensins and LL-37. Inflamm. Res. Off. J. Eur. Histamine Res. Soc. Al 55, 119–127. https://doi.org/10.1007/s00011-005-0062-9

Abraham, G., Colonno, R.J., 1984. Many rhinovirus serotypes share the same cellular receptor. J. Virol. 51, 340–345.

Agerberth, B., Charo, J., Werr, J., Olsson, B., Idali, F., Lindbom, L., Kiessling, R., Jörnvall, H., Wigzell, H., Gudmundsson, G.H., 2000. The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. Blood 96, 3086–3093.

Alalwani, S.M., Sierigk, J., Herr, C., Pinkenburg, O., Gallo, R., Vogelmeier, C., Bals, R., 2010. The antimicrobial peptide LL-37 modulates the inflammatory and host defense response of human neutrophils: Innate immunity. Eur. J. Immunol. 40, 1118–1126. https://doi.org/10.1002/eji.200939275

Alexopoulou, L., Holt, A.C., Medzhitov, R., Flavell, R.A., 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413, 732–738. https://doi.org/10.1038/35099560

Aloia, R.C., Tian, H., Jensen, F.C., 1993. Lipid composition and fluidity of the human immunodeficiency virus envelope and host cell plasma membranes. Proc. Natl. Acad. Sci. U. S. A. 90, 5181–5185.

Alper, C.M., Doyle, W.J., Skoner, D.P., Buchman, C.A., Cohen, S., Gwaltney, J.M., 1998. Prechallenge antibodies moderate disease expression in adults experimentally exposed to rhinovirus strain hanks. Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 27, 119–128.

Amineva, S.P., Aminev, A.G., Gern, J.E., Palmenberg, A.C., 2011. Comparison of rhinovirus A infection in human primary epithelial and HeLa cells. J. Gen. Virol. 92, 2549–2557. https://doi.org/10.1099/vir.0.031302-0

Andries, K., Dewindt, B., Snoeks, J., Willebrords, R., van Eemeren, K., Stokbroekx, R., Janssen, P.A., 1992. In vitro activity of pirodavir (R 77975), a substituted phenoxy-pyridazinamine with broad-spectrum antipicornaviral activity. Antimicrob. Agents Chemother. 36, 100–107.

Ank, N., Paludan, S.R., 2009. Type III IFNs: new layers of complexity in innate antiviral immunity. BioFactors Oxf. Engl. 35, 82–87. https://doi.org/10.1002/biof.19

Arandjelovic, S., Ravichandran, K.S., 2015. Phagocytosis of apoptotic cells in homeostasis. Nat. Immunol. 16, 907–917. https://doi.org/10.1038/ni.3253

Arden, K.E., Mackay, I.M., 2009. Human rhinoviruses: coming in from the cold. Genome Med. 1, 44. https://doi.org/10.1186/gm44

Arnason, J., Jamieson, K., Kooi, C., Nikitenko, S., Shariff, S., Shelfoon, C., Proud, D., Leigh, R., 2014. Pre-existing human rhinovirus infection modulates host response to secondary bacterial infections. Allergy Asthma Clin. Immunol. Off. J. Can. Soc. Allergy Clin. Immunol. 10, A57. https://doi.org/10.1186/1710-1492-10-S1-A57

Arora, S., Lim, W., Bist, P., Perumalsamy, R., Lukman, H.M., Li, F., Welker, L.B., Yan, B., Sethi, G., Tambyah, P.A., Fairhurst, A.-M., Alonso, S., Lim, L.H.K., 2016. Influenza A virus enhances its propagation through the modulation of Annexin-A1 dependent endosomal trafficking and apoptosis. Cell Death Differ. 23, 1243–1256. https://doi.org/10.1038/cdd.2016.19

Arruda, E., Boyle, T.R., Winther, B., Pevear, D.C., Gwaltney, J.M., Hayden, F.G., 1995. Localization of human rhinovirus replication in the upper respiratory tract by in situ hybridization. J. Infect. Dis. 171, 1329–1333.

Arruda, E., Pitkäranta, A., Witek, T.J., Doyle, C.A., Hayden, F.G., 1997. Frequency and natural history of rhinovirus infections in adults during autumn. J. Clin. Microbiol. 35, 2864–2868.

Bals, R., Wang, X., Zasloff, M., Wilson, J.M., 1998. The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. Proc. Natl. Acad. Sci. U. S. A. 95, 9541–9546.

Bals, R., Weiner, D.J., Moscioni, A.D., Meegalla, R.L., Wilson, J.M., 1999. Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide. Infect. Immun. 67, 6084–6089.

Bandholtz, L., Ekman, G.J., Vilhelmsson, M., Buentke, E., Agerberth, B., Scheynius, A., Gudmundsson, G.H., 2006. Antimicrobial Peptide LL-37 Internalized by Immature Human Dendritic Cells Alters their Phenotype. Scand. J. Immunol. 63, 410–419. https://doi.org/10.1111/j.1365-3083.2006.001752.x

Barlow, P.G., Beaumont, P.E., Cosseau, C., Mackellar, A., Wilkinson, T.S., Hancock, R.E.W., Haslett, C., Govan, J.R.W., Simpson, A.J., Davidson, D.J., 2010. The Human Cathelicidin LL-37 Preferentially Promotes Apoptosis of Infected Airway Epithelium. Am. J. Respir. Cell Mol. Biol. 43, 692–702. https://doi.org/10.1165/rcmb.2009-0250OC

Barlow, P.G., Findlay, E.G., Currie, S.M., Davidson, D.J., 2014. Antiviral potential of cathelicidins. Future Microbiol. 9, 55–73. https://doi.org/10.2217/fmb.13.135

Barlow, P.G., Li, Y., Wilkinson, T.S., Bowdish, D.M.E., Lau, Y.E., Cosseau, C., Haslett, C., Simpson, A.J., Hancock, R.E.W., Davidson, D.J., 2006. The human cationic host defense peptide LL-37 mediates contrasting effects on apoptotic pathways in different primary cells of the innate immune system. J. Leukoc. Biol. 80, 509–520. https://doi.org/10.1189/jlb.1005560

Barlow, P.G., Svoboda, P., Mackellar, A., Nash, A.A., York, I.A., Pohl, J., Davidson, D.J., Donis, R.O., 2011. Antiviral Activity and Increased Host Defense against Influenza Infection Elicited by the Human Cathelicidin LL-37. PLoS ONE 6, e25333. https://doi.org/10.1371/journal.pone.0025333

Barral, P.M., Morrison, J.M., Drahos, J., Gupta, P., Sarkar, D., Fisher, P.B., Racaniello, V.R., 2007. MDA-5 Is Cleaved in Poliovirus-Infected Cells. J. Virol. 81, 3677–3684. https://doi.org/10.1128/JVI.01360-06

Basler, C.F., García-Sastre, A., 2002. Viruses and the type I interferon antiviral system: induction and evasion. Int. Rev. Immunol. 21, 305–337.

Beigelman, A., Bacharier, L.B., 2013. The role of early life viral bronchiolitis in the inception of asthma. Curr. Opin. Allergy Clin. Immunol. 13, 211–216. https://doi.org/10.1097/ACI.0b013e32835eb6ef

Bellamy, C.O., Malcomson, R.D., Harrison, D.J., Wyllie, A.H., 1995. Cell death in health and disease: the biology and regulation of apoptosis. Semin. Cancer Biol. 6, 3–16.

Belov, G.A., Romanova, L.I., Tolskaya, E.A., Kolesnikova, M.S., Lazebnik, Y.A., Agol, V.I., 2003. The Major Apoptotic Pathway Activated and Suppressed by Poliovirus. J. Virol. 77, 45–56. https://doi.org/10.1128/JVI.77.1.45-56.2003

Berthold, N., Czihal, P., Fritsche, S., Sauer, U., Schiffer, G., Knappe, D., Alber, G., Hoffmann, R., 2013. Novel Apidaecin 1b Analogs with Superior Serum Stabilities for Treatment of Infections by Gram-Negative Pathogens. Antimicrob. Agents Chemother. 57, 402–409. https://doi.org/10.1128/AAC.01923-12

Bierbaum, G., Sahl, H.G., 1985. Induction of autolysis of staphylococci by the basic peptide antibiotics Pep 5 and nisin and their influence on the activity of autolytic enzymes. Arch. Microbiol. 141, 249–254.

Blomqvist, S., Roivainen, M., Puhakka, T., Kleemola, M., Hovi, T., 2002. Virological and serological analysis of rhinovirus infections during the first two years of life in a cohort of children. J. Med. Virol. 66, 263–268.

Bochkov, Y.A., Watters, K., Ashraf, S., Griggs, T.F., Devries, M.K., Jackson, D.J., Palmenberg, A.C., Gern, J.E., 2015. Cadherin-related family member 3, a childhood asthma susceptibility gene product, mediates rhinovirus C binding and replication. Proc. Natl. Acad. Sci. U. S. A. 112, 5485–5490. https://doi.org/10.1073/pnas.1421178112

Bochkov, Y., Hanson, K., Keles, S., Brockman-Schneider, R., Jarjour, N., Gern, J., 2010. Rhinovirus-induced modulation of gene expression in bronchial epithelial cells from subjects with asthma. Mucosal Immunol. 3, 69–80. https://doi.org/10.1038/mi.2009.109

Bønnelykke, K., Sleiman, P., Nielsen, K., Kreiner-Møller, E., Mercader, J.M., Belgrave, D., den Dekker, H.T., Husby, A., Sevelsted, A., Faura-Tellez, G., Mortensen, L.J., Paternoster, L., Flaaten, R., Mølgaard, A., Smart, D.E., Thomsen, P.F., Rasmussen, M.A., Bonàs-Guarch, S., Holst, C., Nohr, E.A., Yadav, R., March, M.E., Blicher, T., Lackie, P.M., Jaddoe, V.W.V., Simpson, A., Holloway, J.W., Duijts, L., Custovic, A., Davies, D.E., Torrents, D., Gupta, R., Hollegaard, M.V., Hougaard, D.M., Hakonarson, H., Bisgaard, H., 2014. A genome-wide association study identifies CDHR3 as a susceptibility locus for early childhood asthma with severe exacerbations. Nat. Genet. 46, 51–55. https://doi.org/10.1038/ng.2830

Bossios, A., Psarras, S., Gourgiotis, D., Skevaki, C.L., Constantopoulos, A.G., Saxoni-Papageorgiou, P., Papadopoulos, N.G., 2005. Rhinovirus infection induces cytotoxicity and delays wound healing in bronchial epithelial cells. Respir. Res. 6, 114–114. https://doi.org/10.1186/1465-9921-6-114

Bowdish, D.M.E., Davidson, D.J., Hancock, R.E.W., 2006. Immunomodulatory properties of defensins and cathelicidins. Curr. Top. Microbiol. Immunol. 306, 27–66.

Bowdish, D.M.E., Davidson, D.J., Hancock, R.E.W., 2005a. A re-evaluation of the role of host defence peptides in mammalian immunity. Curr. Protein Pept. Sci. 6, 35–51.

Bowdish, D.M.E., Davidson, D.J., Lau, Y.E., Lee, K., Scott, M.G., Hancock, R.E.W., 2005b. Impact of LL-37 on anti-infective immunity. J. Leukoc. Biol. 77, 451–459. https://doi.org/10.1189/jlb.0704380

Braff, M.H., Hawkins, M.A., Nardo, A.D., Lopez-Garcia, B., Howell, M.D., Wong, C., Lin, K., Streib, J.E., Dorschner, R., Leung, D.Y.M., Gallo, R.L., 2005. Structure-Function Relationships among Human Cathelicidin Peptides: Dissociation of Antimicrobial Properties from Host Immunostimulatory Activities. J. Immunol. 174, 4271–4278. https://doi.org/10.4049/jimmunol.174.7.4271

Brown, K.L., Poon, G.F.T., Birkenhead, D., Pena, O.M., Falsafi, R., Dahlgren, C., Karlsson, A., Bylund, J., Hancock, R.E.W., Johnson, P., 2011. Host Defense Peptide LL-37 Selectively Reduces Proinflammatory Macrophage Responses. J. Immunol. 186, 5497–5505. https://doi.org/10.4049/jimmunol.1002508

Butler, W.T., Waldmann, T.A., Rossen, R.D., Douglas, R.G., Couch, R.B., 1970. Changes in IgA and IgG Concentrations in Nasal Secretions Prior to the Appearance of Antibody during Viral Respiratory Infection in Man. J. Immunol. 105, 584–591.

Carthy, C.M., Yanagawa, B., Luo, H., Granville, D.J., Yang, D., Cheung, P., Cheung, C., Esfandiarei, M., Rudin, C.M., Thompson, C.B., Hunt, D.W.C., McManus, B.M., 2003. Bcl-2 and Bcl-xL overexpression inhibits cytochrome c release, activation of multiple caspases, and virus release following coxsackievirus B3 infection. Virology 313, 147–157.

Chamorro, C.I., Weber, G., Grönberg, A., Pivarcsi, A., Ståhle, M., 2009. The Human Antimicrobial Peptide LL-37 Suppresses Apoptosis in Keratinocytes. J. Invest. Dermatol. 129, 937–944. https://doi.org/10.1038/jid.2008.321

Chan, Y.R., Gallo, R.L., 1998. PR-39, a Syndecan-inducing Antimicrobial Peptide, Binds and Affects p130Cas. J. Biol. Chem. 273, 28978–28985. https://doi.org/10.1074/jbc.273.44.28978

Chattoraj, S.S., Ganesan, S., Faris, A., Comstock, A., Lee, W.-M., Sajjan, U.S., 2011. Pseudomonas aeruginosa Suppresses Interferon Response to Rhinovirus Infection

in Cystic Fibrosis but Not in Normal Bronchial Epithelial Cells. Infect. Immun. 79, 4131–4145. https://doi.org/10.1128/IAI.05120-11
Chazal, N., Gerlier, D., 2003. Virus Entry, Assembly, Budding, and Membrane Rafts. Microbiol. Mol. Biol. Rev. 67, 226–237. https://doi.org/10.1128/MMBR.67.2.226-237.2003
Che, Z., Olson, N.H., Leippe, D., Lee, W.M., Mosser, A.G., Rueckert, R.R., Baker, T.S., Smith, T.J., 1998. Antibody-mediated neutralization of human rhinovirus 14 explored by means of cryoelectron microscopy and X-ray crystallography of virus-Fab complexes. J. Virol. 72, 4610–4622.
Chen, C.I.-U., Schaller-Bals, S., Paul, K.P., Wahn, U., Bals, R., 2004. β-defensins and LL-37 in bronchoalveolar lavage fluid of patients with cystic fibrosis. J. Cyst. Fibros. 3, 45–50. https://doi.org/10.1016/j.jcf.2003.12.008
Chen, X., Niyonsaba, F., Ushio, H., Nagaoka, I., Ikeda, S., Okumura, K., Ogawa, H., 2006. Human cathelicidin LL-37 increases vascular permeability in the skin via mast cell activation, and phosphorylates MAP kinases p38 and ERK in mast cells. J. Dermatol. Sci. 43, 63–66. https://doi.org/10.1016/j.jdermsci.2006.03.001
Chen, Y., Hamati, E., Lee, P.-K., Lee, W.-M., Wachi, S., Schnurr, D., Yagi, S., Dolganov, G., Boushey, H., Avila, P., Wu, R., 2006. Rhinovirus Induces Airway Epithelial Gene Expression through Double-Stranded RNA and IFN-Dependent Pathways. Am. J. Respir. Cell Mol. Biol. 34, 192–203. https://doi.org/10.1165/rcmb.2004-0417OC
Cho, Y., Challa, S., Moquin, D., Genga, R., Ray, T.D., Guildford, M., Chan, F.K.-M., 2009. Phosphorylation-Driven Assembly of RIP1-RIP3 Complex Regulates Programmed Necrosis and Virus-Induced Inflammation. Cell 137, 1112–1123. https://doi.org/10.1016/j.cell.2009.05.037
Chotjumlong, P., Bolscher, J.G., Nazmi, K., Reutrakul, V., Supanchart, C., Buranaphatthana, W., Krisanaprakornkit, S., 2013. Involvement of the P2X7 Purinergic Receptor and c-Jun N-Terminal and Extracellular Signal-Regulated Kinases in Cyclooxygenase-2 and Prostaglandin E2 Induction by LL-37. J. Innate Immun. 5, 72–83. https://doi.org/10.1159/000342928
Chromek, M., Slamová, Z., Bergman, P., Kovács, L., Podracká, L. ’udmila, Ehrén, I., Hökfelt, T., Gudmundsson, G.H., Gallo, R.L., Agerberth, B., Brauner, A., 2006. The antimicrobial peptide cathelicidin protects the urinary tract against invasive bacterial infection. Nat. Med. 12, 636–641. https://doi.org/10.1038/nm1407
Chun, Y.H., Park, J.Y., Lee, H., Kim, H.S., Won, S., Joe, H.J., Chung, W.J., Yoon, J., Kim, H.H., Kim, J.T., Lee, J.S., 2013. Rhinovirus-Infected Epithelial Cells Produce More IL-8 and RANTES Compared With Other Respiratory Viruses. Allergy Asthma Immunol. Res. 5, 216–223. https://doi.org/10.4168/aair.2013.5.4.216
Ciornei, C.D., Sigurdardottir, T., Schmidtchen, A., Bodelsson, M., 2005. Antimicrobial and Chemoattractant Activity, Lipopolysaccharide Neutralization, Cytotoxicity, and Inhibition by Serum of Analogs of Human Cathelicidin LL-37. Antimicrob. Agents Chemother. 49, 2845–2850. https://doi.org/10.1128/AAC.49.7.2845-2850.2005
Cirioni, O., Giacometti, A., Ghiselli, R., Bergnach, C., Orlando, F., Silvestri, C., Mocchegiani, F., Licci, A., Skerlavaj, B., Rocchi, M., Saba, V., Zanetti, M., Scalise, G., 2006. LL-37 protects rats against lethal sepsis caused by gram-negative bacteria. Antimicrob. Agents Chemother. 50, 1672–1679. https://doi.org/10.1128/AAC.50.5.1672-1679.2006
Cole, A.M., Hong, T., Boo, L.M., Nguyen, T., Zhao, C., Bristol, G., Zack, J.A., Waring, A.J., Yang, O.O., Lehrer, R.I., 2002. Retrocyclin: a primate peptide that protects cells from infection by T- and M-tropic strains of HIV-1. Proc. Natl. Acad. Sci. U. S. A. 99, 1813–1818. https://doi.org/10.1073/pnas.052706399
Conant, R.M., Hamparian, V.V., 1968. Rhinoviruses: basis for a numbering system. II. Serologic characterization of prototype strains. J. Immunol. Baltim. Md 1950 100, 114–119.

Cordero, J.G., Juárez, M.L., González-Y-Merchand, J.A., Barrón, L.C., Castañeda, B.G., 2014. Caveolin-1 in Lipid Rafts Interacts with Dengue Virus NS3 during Polyprotein Processing and Replication in HMEC-1 Cells. PLOS ONE 9, e90704. https://doi.org/10.1371/journal.pone.0090704

Couch, R.B., Cate, T.R., Douglas, R.G., Gerone, P.J., Knight, V., 1966. Effect of route of inoculation on experimental respiratory viral disease in volunteers and evidence for airborne transmission. Bacteriol. Rev. 30, 517–529.

Cozens, A.L., Yezzi, M.J., Kunzelmann, K., Ohrui, T., Chin, L., Eng, K., Finkbeiner, W.E., Widdicombe, J.H., Gruenert, D.C., 1994. CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. Am. J. Respir. Cell Mol. Biol. 10, 38–47. https://doi.org/10.1165/ajrcmb.10.1.7507342

Crowley, L.C., Marfell, B.J., Scott, A.P., Waterhouse, N.J., 2016. Quantitation of Apoptosis and Necrosis by Annexin V Binding, Propidium Iodide Uptake, and Flow Cytometry. Cold Spring Harb. Protoc. 2016, pdb.prot087288. https://doi.org/10.1101/pdb.prot087288

Crowley, L.C., Waterhouse, N.J., 2016. Detecting Cleaved Caspase-3 in Apoptotic Cells by Flow Cytometry. Cold Spring Harb. Protoc. 2016, pdb.prot087312. https://doi.org/10.1101/pdb.prot087312

Currie, S.M., Findlay, E.G., McHugh, B.J., Mackellar, A., Man, T., Macmillan, D., Wang, H., Fitch, P.M., Schwarze, J., Davidson, D.J., 2013. The Human Cathelicidin LL-37 Has Antiviral Activity against Respiratory Syncytial Virus. PLoS ONE 8, e73659. https://doi.org/10.1371/journal.pone.0073659

Currie, S.M., Gwyer Findlay, E., McFarlane, A.J., Fitch, P.M., Böttcher, B., Colegrave, N., Paras, A., Jozwik, A., Chiu, C., Schwarze, J., Davidson, D.J., 2016. Cathelicidins Have Direct Antiviral Activity against Respiratory Syncytial Virus In Vitro and Protective Function In Vivo in Mice and Humans. J. Immunol. 196, 2699–2710. https://doi.org/10.4049/jimmunol.1502478

Davidson, D.J., Currie, A.J., Reid, G.S.D., Bowdish, D.M.E., MacDonald, K.L., Ma, R.C., Hancock, R.E.W., Speert, D.P., 2004. The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. J. Immunol. Baltim. Md 1950 172, 1146–1156.

de Haro, C., Méndez, R., Santoyo, J., 1996. The eIF-2alpha kinases and the control of protein synthesis. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 10, 1378–1387.

De Smet, K., Contreras, R., 2005. Human antimicrobial peptides: defensins, cathelicidins and histatins. Biotechnol. Lett. 27, 1337–1347. https://doi.org/10.1007/s10529-005-0936-5

De Yang, Chen, Q., Schmidt, A.P., Anderson, G.M., Wang, J.M., Wooters, J., Oppenheim, J.J., Chertov, O., 2000. Ll-37, the Neutrophil Granule–And Epithelial Cell–Derived Cathelicidin, Utilizes Formyl Peptide Receptor–Like 1 (Fprl1) as a Receptor to Chemoattract Human Peripheral Blood Neutrophils, Monocytes, and T Cells. J. Exp. Med. 192, 1069–1074.

Denlinger, L.C., Sorkness, R.L., Lee, W.-M., Evans, M.D., Wolff, M.J., Mathur, S.K., Crisafi, G.M., Gaworski, K.L., Pappas, T.E., Vrtis, R.F., Kelly, E.A., Gern, J.E., Jarjour, N.N., 2011. Lower Airway Rhinovirus Burden and the Seasonal Risk of Asthma Exacerbation. Am. J. Respir. Crit. Care Med. 184, 1007–1014. https://doi.org/10.1164/rccm.201103-0585OC

Deszcz, L., 2005. Apoptotic events induced by human rhinovirus infection. J. Gen. Virol. 86, 1379–1389. https://doi.org/10.1099/vir.0.80754-0

Diamond, G., Beckloff, N., Weinberg, A., Kisich, K.O., 2009. The roles of antimicrobial peptides in innate host defense. Curr. Pharm. Des. 15, 2377–2392.

Dong, Y., Liu, Y., Jiang, W., Smith, T.J., Xu, Z., Rossmann, M.G., 2017. Antibody-induced uncoating of human rhinovirus B14. Proc. Natl. Acad. Sci. U. S. A. 114, 8017–8022. https://doi.org/10.1073/pnas.1707369114

Dotzauer, A., Kraemer, L., 2012. Innate and adaptive immune responses against picornaviruses and their counteractions: An overview. World J. Virol. 1, 91–107. https://doi.org/10.5501/wjv.v1.i3.91

Douglas, R.G., Cate, T.R., Gerone, P.J., Couch, R.B., 1966. Quantitative rhinovirus shedding patterns in volunteers. Am. Rev. Respir. Dis. 94, 159–167. https://doi.org/10.1164/arrd.1966.94.2.159

Duits, L.A., Nibbering, P.H., van Strijen, E., Vos, J.B., Mannesse-Lazeroms, S.P.G., Sterkenburg, V., A.j.a, M., Hiemstra, P.S., 2003. Rhinovirus increases human β-defensin-2 and -3 mRNA expression in cultured bronchial epithelial cells. FEMS Immunol. Med. Microbiol. 38, 59–64. https://doi.org/10.1016/S0928-8244(03)00106-8

Ehrhardt, C., Kneuer, C., Fiegel, J., Hanes, J., Schaefer, U., Kim, K.-J., Lehr, C.-M., 2002. Influence of apical fluid volume on the development of functional intercellular junctions in the human epithelial cell line 16HBE14o- : implications for the use of this cell line as an in vitro model for bronchial drug absorption studies. Cell Tissue Res. 308, 391–400. https://doi.org/10.1007/s00441-002-0548-5

Einarsson, O., Geba, G.P., Zhu, Z., Landry, M., Elias, J.A., 1996. Interleukin-11: stimulation in vivo and in vitro by respiratory viruses and induction of airways hyperresponsiveness. J. Clin. Invest. 97, 915–924. https://doi.org/10.1172/JCI118514

Elmore, S., 2007. Apoptosis: A Review of Programmed Cell Death. Toxicol. Pathol. 35, 495–516. https://doi.org/10.1080/01926230701320337

El-Sahly, H.M., Atmar, R.L., Glezen, W.P., Greenberg, S.B., 2000. Spectrum of clinical illness in hospitalized patients with “common cold” virus infections. Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 31, 96–100. https://doi.org/10.1086/313937

Elssner, A., Duncan, M., Gavrilin, M., Wewers, M.D., 2004. A Novel $P2X_7$ Receptor Activator, the Human Cathelicidin-Derived Peptide LL37, Induces IL-1β Processing and Release. J. Immunol. 172, 4987. https://doi.org/10.4049/jimmunol.172.8.4987

Erdag, G., Morgan, J.R., 2002. Interleukin-1alpha and interleukin-6 enhance the antibacterial properties of cultured composite keratinocyte grafts. Ann. Surg. 235, 113–124.

Fantuzzi, G., Dinarello, C.A., 1999. Interleukin-18 and interleukin-1 beta: two cytokine substrates for ICE (caspase-1). J. Clin. Immunol. 19, 1–11.

Fesq, H., Bacher, M., Nain, M., Gemsa, D., 1994. Programmed cell death (apoptosis) in human monocytes infected by influenza A virus. Immunobiology 190, 175–182. https://doi.org/10.1016/S0171-2985(11)80292-5

Filewod, N.C.J., Pistolic, J., Hancock, R.E.W., 2009. Low concentrations of LL-37 alter IL-8 production by keratinocytes and bronchial epithelial cells in response to proinflammatory stimuli. FEMS Immunol. Med. Microbiol. 56, 233–240. https://doi.org/10.1111/j.1574-695X.2009.00571.x

Fjell, C.D., Hiss, J.A., Hancock, R.E.W., Schneider, G., 2012. Designing antimicrobial peptides: form follows function. Nat. Rev. Drug Discov. https://doi.org/10.1038/nrd3653

Folkerts, G., Busse, W.W., Nijkamp, F.P., Sorkness, R., Gern, J.E., 1998. Virus-induced airway hyperresponsiveness and asthma. Am. J. Respir. Crit. Care Med. 157, 1708–1720. https://doi.org/10.1164/ajrccm.157.6.9707163

Folli, C., Descalzi, D., Scordamaglia, F., Riccio, A.M., Gamalero, C., Canonica, G.W., 2008. New insights into airway remodelling in asthma and its possible modulation. Curr. Opin. Allergy Clin. Immunol. 8, 367–375. https://doi.org/10.1097/ACI.0b013e32830a7086

French, C.A., 2009. Chapter 2 - Respiratory Tract, in: Cibas, E.S., Ducatman, B.S. (Eds.), Cytology (Third Edition). W.B. Saunders, Philadelphia, pp. 65–103.

Frohm, M., Agerberth, B., Ahangari, G., Stâhle-Bäckdahl, M., Lidén, S., Wigzell, H., Gudmundsson, G.H., 1997. The expression of the gene coding for the antibacterial

peptide LL-37 is induced in human keratinocytes during inflammatory disorders. J. Biol. Chem. 272, 15258–15263.
Fuchs, R., Blaas, D., 2010. Uncoating of human rhinoviruses. Rev. Med. Virol. 20, 281–297. https://doi.org/10.1002/rmv.654
Funderburg, N., Lederman, M.M., Feng, Z., Drage, M.G., Jadlowsky, J., Harding, C.V., Weinberg, A., Sieg, S.F., 2007. Human -defensin-3 activates professional antigen-presenting cells via Toll-like receptors 1 and 2. Proc. Natl. Acad. Sci. U. S. A. 104, 18631–18635. https://doi.org/10.1073/pnas.0702130104
Gale, M., Katze, M.G., 1998. Molecular Mechanisms of Interferon Resistance Mediated by Viral-Directed Inhibition of PKR, the Interferon-Induced Protein Kinase. Pharmacol. Ther. 78, 29–46. https://doi.org/10.1016/S0163-7258(97)00165-4
Gallo, R.L., Kim, K.J., Bernfield, M., Kozak, C.A., Zanetti, M., Merluzzi, L., Gennaro, R., 1997. Identification of CRAMP, a cathelin-related antimicrobial peptide expressed in the embryonic and adult mouse. J. Biol. Chem. 272, 13088–13093.
Ganz, T., Selsted, M.E., Szklarek, D., Harwig, S.S., Daher, K., Bainton, D.F., Lehrer, R.I., 1985. Defensins. Natural peptide antibiotics of human neutrophils. J. Clin. Invest. 76, 1427–1435.
García, M.A., Gil, J., Ventoso, I., Guerra, S., Domingo, E., Rivas, C., Esteban, M., 2006. Impact of Protein Kinase PKR in Cell Biology: from Antiviral to Antiproliferative Action. Microbiol. Mol. Biol. Rev. 70, 1032–1060. https://doi.org/10.1128/MMBR.00027-06
García-García, M.L., Calvo, C., Pozo, F., Villadangos, P.A., Pérez-Breña, P., Casas, I., 2012. Spectrum of respiratory viruses in children with community-acquired pneumonia. Pediatr. Infect. Dis. J. 31, 808–813. https://doi.org/10.1097/INF.0b013e3182568c67
Gavala, M., Bertics, P.J., Gern, J.E., 2011. Rhinoviruses, Allergic Inflammation, and Asthma. Immunol. Rev. 242, 69–90. https://doi.org/10.1111/j.1600-065X.2011.01031.x
Gee, P., Chua, P.K., Gevorkyan, J., Klumpp, K., Najera, I., Swinney, D.C., Deval, J., 2008. Essential role of the N-terminal domain in the regulation of RIG-I ATPase activity. J. Biol. Chem. 283, 9488–9496. https://doi.org/10.1074/jbc.M706777200
Gerber, H.P., Condorelli, F., Park, J., Ferrara, N., 1997. Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. J. Biol. Chem. 272, 23659–23667.
Gern, J.E., Galagan, D.M., Jarjour, N.N., Dick, E.C., Busse, W.W., 1997. Detection of rhinovirus RNA in lower airway cells during experimentally induced infection. Am. J. Respir. Crit. Care Med. 155, 1159–1161. https://doi.org/10.1164/ajrccm.155.3.9117003
Ghosh, S., Champlin, R., Couch, R., Englund, J., Raad, I., Malik, S., Luna, M., Whimbey, E., 1999. Rhinovirus Infections in Myelosuppressed Adult Blood and Marrow Transplant Recipients. Clin. Infect. Dis. 29, 528–532. https://doi.org/10.1086/598627
Ghosh, S., May, M.J., Kopp, E.B., 1998. NF-κB AND REL PROTEINS: Evolutionarily Conserved Mediators of Immune Responses. Annu. Rev. Immunol. 16, 225–260. https://doi.org/10.1146/annurev.immunol.16.1.225
Giacometti, A., Cirioni, O., Ghiselli, R., Bergnach, C., Orlando, F., D'Amato, G., Mocchegiani, F., Silvestri, C., Del Prete, M.S., Skerlavaj, B., Saba, V., Zanetti, M., Scalise, G., 2004. The antimicrobial peptide BMAP-28 reduces lethality in mouse models of staphylococcal sepsis. Crit. Care Med. 32, 2485–2490.
Giard, D.J., Aaronson, S.A., Todaro, G.J., Arnstein, P., Kersey, J.H., Dosik, H., Parks, W.P., 1973. In Vitro Cultivation of Human Tumors: Establishment of Cell Lines Derived From a Series of Solid Tumors2. JNCI J. Natl. Cancer Inst. 51, 1417–1423. https://doi.org/10.1093/jnci/51.5.1417
Girard, S., Couderc, T., Destombes, J., Thiesson, D., Delpeyroux, F., Blondel, B., 1999. Poliovirus Induces Apoptosis in the Mouse Central Nervous System. J. Virol. 73, 6066–6072.

Girnita, A., Zheng, H., Grönberg, A., Girnita, L., Ståhle, M., 2012. Identification of the cathelicidin peptide LL-37 as agonist for the type I insulin-like growth factor receptor. Oncogene 31, 352–365. https://doi.org/10.1038/onc.2011.239

Gombart, A.F., Borregaard, N., Koeffler, H.P., 2005. Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 19, 1067–1077. https://doi.org/10.1096/fj.04-3284com

Gordon, Y.J., Huang, L.C., Romanowski, E.G., Yates, K.A., Proske, R.J., McDermott, A.M., 2005. Human Cathelicidin (LL-37), a Multifunctional Peptide, is Expressed by Ocular Surface Epithelia and has Potent Antibacterial and Antiviral Activity. Curr. Eye Res. 30, 385–394. https://doi.org/10.1080/02713680590934111

Green, D.R., Ferguson, T., Zitvogel, L., Kroemer, G., 2009. IMMUNOGENIC AND TOLEROGENIC CELL DEATH. Nat. Rev. Immunol. 9, 353. https://doi.org/10.1038/nri2545

Grek, C.L., Newton, D.A., Qiu, Y., Wen, X., Spyropoulos, D.D., Baatz, J.E., 2009. CHARACTERIZATION OF ALVEOLAR EPITHELIAL CELLS CULTURED IN SEMIPERMEABLE HOLLOW FIBERS. Exp. Lung Res. 35, 155–174. https://doi.org/10.1080/01902140802495870

Greve, J.M., Davis, G., Meyer, A.M., Forte, C.P., Yost, S.C., Marlor, C.W., Kamarck, M.E., McClelland, A., 1989. The major human rhinovirus receptor is ICAM-1. Cell 56, 839–847.

Grünberg, K., Timmers, M.C., Smits, H.H., de Klerk, E.P., Dick, E.C., Spaan, W.J., Hiemstra, P.S., Sterk, P.J., 1997. Effect of experimental rhinovirus 16 colds on airway hyperresponsiveness to histamine and interleukin-8 in nasal lavage in asthmatic subjects in vivo. Clin. Exp. Allergy J. Br. Soc. Allergy Clin. Immunol. 27, 36–45.

Guillot, L., Le Goffic, R., Bloch, S., Escriou, N., Akira, S., Chignard, M., Si-Tahar, M., 2005. Involvement of Toll-like Receptor 3 in the Immune Response of Lung Epithelial Cells to Double-stranded RNA and Influenza A Virus. J. Biol. Chem. 280, 5571–5580. https://doi.org/10.1074/jbc.M410592200

Haines, L.R., Hancock, R.E.W., Pearson, T.W., 2003. Cationic antimicrobial peptide killing of African trypanosomes and Sodalis glossinidius, a bacterial symbiont of the insect vector of sleeping sickness. Vector Borne Zoonotic Dis. Larchmt. N 3, 175–186. https://doi.org/10.1089/153036603322662165

Hansdottir, S., Monick, M.M., Hinde, S.L., Lovan, N., Look, D.C., Hunninghake, G.W., 2008. Respiratory epithelial cells convert inactive vitamin D to its active form: potential effects on host defense. J. Immunol. Baltim. Md 1950 181, 7090–7099.

Harcourt, J.L., McDonald, M., Svoboda, P., Pohl, J., Tatti, K., Haynes, L.M., 2016. Human cathelicidin, LL-37, inhibits respiratory syncytial virus infection in polarized airway epithelial cells. BMC Res. Notes 9, 11. https://doi.org/10.1186/s13104-015-1836-y

Hayden, F.G., 2004. Rhinovirus and the lower respiratory tract. Rev. Med. Virol. 14, 17–31. https://doi.org/10.1002/rmv.406

Hayden, F.G., Coats, T., Kim, K., Hassman, H.A., Blatter, M.M., Zhang, B., Liu, S., 2002. Oral pleconaril treatment of picornavirus-associated viral respiratory illness in adults: efficacy and tolerability in phase II clinical trials. Antivir. Ther. 7, 53–65.

Hayden, F.G., Herrington, D.T., Coats, T.L., Kim, K., Cooper, E.C., Villano, S.A., Liu, S., Hudson, S., Pevear, D.C., Collett, M., McKinlay, M., Pleconaril Respiratory Infection Study Group, 2003a. Efficacy and safety of oral pleconaril for treatment of colds due to picornaviruses in adults: results of 2 double-blind, randomized, placebo-controlled trials. Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 36, 1523–1532. https://doi.org/10.1086/375069

Hayden, F.G., Turner, R.B., Gwaltney, J.M., Chi-Burris, K., Gersten, M., Hsyu, P., Patick, A.K., Smith, G.J., Zalman, L.S., 2003b. Phase II, randomized, double-blind, placebo-controlled studies of ruprintrivir nasal spray 2-percent suspension for prevention

and treatment of experimentally induced rhinovirus colds in healthy volunteers. Antimicrob. Agents Chemother. 47, 3907–3916.
Henzler Wildman, K.A., Lee, D.-K., Ramamoorthy, A., 2003. Mechanism of lipid bilayer disruption by the human antimicrobial peptide, LL-37. Biochemistry (Mosc.) 42, 6545–6558. https://doi.org/10.1021/bi0273563
Hicks, L.A., Shepard, C.W., Britz, P.H., Erdman, D.D., Fischer, M., Flannery, B.L., Peck, A.J., Lu, X., Thacker, W.L., Benson, R.F., Tondella, M.L., Moll, M.E., Whitney, C.G., Anderson, L.J., Feikin, D.R., 2006. Two outbreaks of severe respiratory disease in nursing homes associated with rhinovirus. J. Am. Geriatr. Soc. 54, 284–289. https://doi.org/10.1111/j.1532-5415.2005.00529.x
Hiemstra, P.S., Amatngalim, G.D., van der Does, A.M., Taube, C., 2016. Antimicrobial Peptides and Innate Lung Defenses: Role in Infectious and Noninfectious Lung Diseases and Therapeutic Applications. Chest 149, 545–551. https://doi.org/10.1378/chest.15-1353
Hiscott, J., 2007. Triggering the Innate Antiviral Response through IRF-3 Activation. J. Biol. Chem. 282, 15325–15329. https://doi.org/10.1074/jbc.R700002200
Hofer, F., Gruenberger, M., Kowalski, H., Machat, H., Huettinger, M., Kuechler, E., Blaas, D., 1994. Members of the low density lipoprotein receptor family mediate cell entry of a minor-group common cold virus. Proc. Natl. Acad. Sci. U. S. A. 91, 1839–1842.
Hu, Z., Murakami, T., Suzuki, K., Tamura, H., Kuwahara-Arai, K., Iba, T., Nagaoka, I., 2014. Antimicrobial cathelicidin peptide LL-37 inhibits the LPS/ATP-induced pyroptosis of macrophages by dual mechanism. PloS One 9, e85765. https://doi.org/10.1371/journal.pone.0085765
Hu, Z., Murakami, T., Suzuki, K., Tamura, H., Reich, J., Kuwahara-Arai, K., Iba, T., Nagaoka, I., 2016. Antimicrobial cathelicidin peptide LL-37 inhibits the pyroptosis of macrophages and improves the survival of polybacterial septic mice. Int. Immunol. 28, 245–253. https://doi.org/10.1093/intimm/dxv113
Huang, L.C., Reins, R.Y., Gallo, R.L., McDermott, A.M., 2007. Cathelicidin-deficient (Cnlp -/-) mice show increased susceptibility to Pseudomonas aeruginosa keratitis. Invest. Ophthalmol. Vis. Sci. 48, 4498–4508. https://doi.org/10.1167/iovs.07-0274
Hudy, M.H., Traves, S.L., Wiehler, S., Proud, D., 2010. Cigarette smoke modulates rhinovirus-induced airway epithelial cell chemokine production. Eur. Respir. J. 35, 1256–1263. https://doi.org/10.1183/09031936.00128809
Hultmark, D., Steiner, H., Rasmuson, T., Boman, H.G., 1980. Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of Hyalophora cecropia. Eur. J. Biochem. 106, 7–16.
Hung, I.F.N., Zhang, A.J., To, K.K.W., Chan, J.F.W., Zhu, S.H.S., Zhang, R., Chan, T.-C., Chan, K.-H., Yuen, K.-Y., 2017. Unexpectedly Higher Morbidity and Mortality of Hospitalized Elderly Patients Associated with Rhinovirus Compared with Influenza Virus Respiratory Tract Infection. Int. J. Mol. Sci. 18. https://doi.org/10.3390/ijms18020259
Hutchinson, A.F., Ghimire, A.K., Thompson, M.A., Black, J.F., Brand, C.A., Lowe, A.J., Smallwood, D.M., Vlahos, R., Bozinovski, S., Brown, G.V., Anderson, G.P., Irving, L.B., 2007. A community-based, time-matched, case-control study of respiratory viruses and exacerbations of COPD. Respir. Med. 101, 2472–2481. https://doi.org/10.1016/j.rmed.2007.07.015
Iimura, M., Gallo, R.L., Hase, K., Miyamoto, Y., Eckmann, L., Kagnoff, M.F., 2005. Cathelicidin Mediates Innate Intestinal Defense against Colonization with Epithelial Adherent Bacterial Pathogens. J. Immunol. 174, 4901–4907. https://doi.org/10.4049/jimmunol.174.8.4901
Islam, D., Bandholtz, L., Nilsson, J., Wigzell, H., Christensson, B., Agerberth, B., Gudmundsson, G.H., 2001. Downregulation of bactericidal peptides in enteric

infections: A novel immune escape mechanism with bacterial DNA as a potential regulator. Nat. Med. 7, 180–185. https://doi.org/10.1038/84627
Jacobs, S.E., Lamson, D.M., St. George, K., Walsh, T.J., 2013. Human Rhinoviruses. Clin. Microbiol. Rev. 26, 135–162. https://doi.org/10.1128/CMR.00077-12
Jagielo, P.J., Thorne, P.S., Watt, J.L., Frees, K.L., Quinn, T.J., Schwartz, D.A., 1996. Grain dust and endotoxin inhalation challenges produce similar inflammatory responses in normal subjects. Chest 110, 263–270.
J Huang, H., Ross, C., Blecha, F., 1997. Chemoattractant properties of PR-39, a neutrophil antibacterial peptide.
Jang, Y.J., 2006. Effect of clarithromycin on rhinovirus-16 infection in A549 cells. Eur. Respir. J. 27, 12–19. https://doi.org/10.1183/09031936.06.00008005
Jartti, T., Gern, J.E., 2011. Rhinovirus-associated wheeze during infancy and asthma development. Curr. Respir. Med. Rev. 7, 160–166.
Jennings, L.C., Dick, E.C., 1987. Transmission and control of rhinovirus colds. Eur. J. Epidemiol. 3, 327–335.
Johansson, J., Gudmundsson, G.H., Rottenberg, M.E., Berndt, K.D., Agerberth, B., 1998. Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. J. Biol. Chem. 273, 3718–3724.
Johnston, S.L., Papi, A., Bates, P.J., Mastronarde, J.G., Monick, M.M., Hunninghake, G.W., 1998. Low grade rhinovirus infection induces a prolonged release of IL-8 in pulmonary epithelium. J. Immunol. Baltim. Md 1950 160, 6172–6181.
Juvén, T., Mertsola, J., Waris, M., Leinonen, M., Meurman, O., Roivainen, M., Eskola, J., Saikku, P., Ruuskanen, O., 2000. Etiology of community-acquired pneumonia in 254 hospitalized children. Pediatr. Infect. Dis. J. 19, 293–298.
Kahlenberg, J.M., Carmona-Rivera, C., Smith, C.K., Kaplan, M.J., 2013. Neutrophil extracellular trap-associated protein activation of the NLRP3 inflammasome is enhanced in lupus macrophages. J. Immunol. Baltim. Md 1950 190, 1217–1226. https://doi.org/10.4049/jimmunol.1202388
Kahlenberg, J.M., Kaplan, M.J., 2013. Little Peptide, Big Effects: The Role of LL-37 in Inflammation and Autoimmune Disease. J. Immunol. 191, 4895–4901. https://doi.org/10.4049/jimmunol.1302005
Kang, D., Gopalkrishnan, R.V., Lin, L., Randolph, A., Valerie, K., Pestka, S., Fisher, P.B., 2004. Expression analysis and genomic characterization of human melanoma differentiation associated gene-5, *mda*-5: a novel type I interferon-responsive apoptosis-inducing gene. Oncogene 23, 1789–1800. https://doi.org/10.1038/sj.onc.1207300
Katze, M.G., He, Y., Gale Jr, M., 2002. Viruses and interferon: a fight for supremacy. Nat. Rev. Immunol. 2, 675–687. https://doi.org/10.1038/nri888
Keller, C., Keller, P., Marshal, S., Pedersen, B.K., 2003. IL-6 Gene Expression in Human Adipose Tissue in Response to Exercise – Effect of Carbohydrate Ingestion. J. Physiol. 550, 927–931. https://doi.org/10.1113/jphysiol.2003.044883
Kennedy, J.L., Turner, R.B., Braciale, T., Heymann, P.W., Borish, L., 2012. Pathogenesis of Rhinovirus Infection. Curr. Opin. Virol. 2, 287–293. https://doi.org/10.1016/j.coviro.2012.03.008
Kerr, J.F.R., Wyllie, A.H., Currie, A.R., 1972. Apoptosis: A Basic Biological Phenomenon with Wide-ranging Implications in Tissue Kinetics. Br. J. Cancer 26, 239–257.
Khaitov, M.R., Laza-Stanca, V., Edwards, M.R., Walton, R.P., Rohde, G., Contoli, M., Papi, A., Stanciu, L.A., Kotenko, S.V., Johnston, S.L., 2009. Respiratory virus induction of alpha-, beta- and lambda-interferons in bronchial epithelial cells and peripheral blood mononuclear cells. Allergy 64, 375–386. https://doi.org/10.1111/j.1398-9995.2008.01826.x

Killington, R.A., Stott, E.J., Lee, D., 1977. The effect of temperature on the synthesis of rhinovirus type 2 RNA. J. Gen. Virol. 36, 403–411. https://doi.org/10.1099/0022-1317-36-3-403
Kim, H., Jang, J.H., Kim, S.C., Cho, J.H., 2014. De novo generation of short antimicrobial peptides with enhanced stability and cell specificity. J. Antimicrob. Chemother. 69, 121–132. https://doi.org/10.1093/jac/dkt322
Kim, J., Sanders, S.P., Siekierski, E.S., Casolaro, V., Proud, D., 2000. Role of NF-kappa B in cytokine production induced from human airway epithelial cells by rhinovirus infection. J. Immunol. Baltim. Md 1950 165, 3384–3392.
Kim, J., Schleimer, R., 2009. Epithelial Cell Innate Responses to Rhinovirus Infection, in: Pawankar, R., Holgate, S.T., Rosenwasser, L.J. (Eds.), Allergy Frontiers: Classification and Pathomechanisms. Springer Japan, Tokyo, pp. 267–284.
Kin, N.W., Chen, Y., Stefanov, E.K., Gallo, R.L., Kearney, J.F., 2011. Cathelin-related antimicrobial peptide differentially regulates T- and B-cell function. Eur. J. Immunol. 41, 3006–3016. https://doi.org/10.1002/eji.201141606
Klotman, M.E., Chang, T.L., 2006. Defensins in innate antiviral immunity. Nat. Rev. Immunol. 6, 447–456. https://doi.org/10.1038/nri1860
Knott, J.A., Orr, D.C., Montgomery, D.S., Sullivan, C.A., Weston, A., 1989. The expression and purification of human rhinovirus protease 3C. Eur. J. Biochem. 182, 547–555.
Kögel, D., Prehn, J.H.M., 2013. Caspase-Independent Cell Death Mechanisms. Landes Bioscience.
Kościuczuk, E.M., Lisowski, P., Jarczak, J., Strzałkowska, N., Jóźwik, A., Horbańczuk, J., Krzyżewski, J., Zwierzchowski, L., Bagnicka, E., 2012. Cathelicidins: family of antimicrobial peptides. A review. Mol. Biol. Rep. 39, 10957–10970. https://doi.org/10.1007/s11033-012-1997-x
Kotla, S., Peng, T., Bumgarner, R.E., Gustin, K.E., 2008. Attenuation of the type I interferon response in cells infected with human rhinovirus. Virology 374, 399–410. https://doi.org/10.1016/j.virol.2008.01.022
Kovach, M.A., Ballinger, M.N., Newstead, M.W., Zeng, X., Bhan, U., Yu, F., Moore, B.B., Gallo, R.L., Standiford, T.J., 2012. Cathelicidin-related antimicrobial peptide is required for effective lung mucosal immunity in Gram-negative bacterial pneumonia. J. Immunol. Baltim. Md 1950 189, 304–311. https://doi.org/10.4049/jimmunol.1103196
Kumar, A., Haque, J., Lacoste, J., Hiscott, J., Williams, B.R., 1994. Double-stranded RNA-dependent protein kinase activates transcription factor NF-kappa B by phosphorylating I kappa B. Proc. Natl. Acad. Sci. U. S. A. 91, 6288–6292.
Kuo, R.-L., Kao, L.-T., Lin, S.-J., Wang, R.Y.-L., Shih, S.-R., 2013. MDA5 Plays a Crucial Role in Enterovirus 71 RNA-Mediated IRF3 Activation. PLOS ONE 8, e63431. https://doi.org/10.1371/journal.pone.0063431
Labbé, K., Saleh, M., 2008. Cell death in the host response to infection. Cell Death Differ. 15, 1339–1349. https://doi.org/10.1038/cdd.2008.91
Lai, Y., Adhikarakunnathu, S., Bhardwaj, K., Ranjith-Kumar, C.T., Wen, Y., Jordan, J.L., Wu, L.H., Dragnea, B., Mateo, L.S., Kao, C.C., 2011. LL37 and Cationic Peptides Enhance TLR3 Signaling by Viral Double-stranded RNAs. PLoS ONE 6, e26632. https://doi.org/10.1371/journal.pone.0026632
Lande, R., Gregorio, J., Facchinetti, V., Chatterjee, B., Wang, Y.-H., Homey, B., Cao, W., Wang, Y.-H., Su, B., Nestle, F.O., Zal, T., Mellman, I., Schröder, J.-M., Liu, Y.-J., Gilliet, M., 2007. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. Nature 449, 564–569. https://doi.org/10.1038/nature06116
Las Heras, J., Swanson, V.L., 1983. Sudden death of an infant with rhinovirus infection complicating bronchial asthma: case report. Pediatr. Pathol. 1, 319–323.
Lau, Y.E., Bowdish, D.M.E., Cosseau, C., Hancock, R.E.W., Davidson, D.J., 2006. Apoptosis of Airway Epithelial Cells: Human Serum Sensitive Induction by the Cathelicidin LL-37.

Am. J. Respir. Cell Mol. Biol. 34, 399–409. https://doi.org/10.1165/rcmb.2005-0170OC

Lau, Y.E., Rozek, A., Scott, M.G., Goosney, D.L., Davidson, D.J., Hancock, R.E.W., 2005. Interaction and Cellular Localization of the Human Host Defense Peptide LL-37 with Lung Epithelial Cells. Infect. Immun. 73, 583–591. https://doi.org/10.1128/IAI.73.1.583-591.2005

Lee, W.-M., Chen, Y., Wang, W., Mosser, A., 2015. Growth of Human Rhinovirus in H1-HeLa Cell Suspension Culture and Purification of Virions, in: Jans, D.A., Ghildyal, R. (Eds.), Rhinoviruses. Springer New York, New York, NY, pp. 49–61.

Leonova, L., Kokryakov, V.N., Aleshina, G., Hong, T., Nguyen, T., Zhao, C., Waring, A.J., Lehrer, R.I., 2001. Circular minidefensins and posttranslational generation of molecular diversity. J. Leukoc. Biol. 70, 461–464.

Li, H.-N., Barlow, P.G., Bylund, J., Mackellar, A., Björstad, Å., Conlon, J., Hiemstra, P.S., Haslett, C., Gray, M., Simpson, A.J., Rossi, A.G., Davidson, D.J., 2009. Secondary necrosis of apoptotic neutrophils induced by the human cathelicidin LL-37 is not proinflammatory to phagocytosing macrophages. J. Leukoc. Biol. 86, 891–902. https://doi.org/10.1189/jlb.0209050

Li, J., Post, M., Volk, R., Gao, Y., Li, M., Metais, C., Sato, K., Tsai, J., Aird, W., Rosenberg, R.D., Hampton, T.G., Li, J., Sellke, F., Carmeliet, P., Simons, M., 2000. PR39, a peptide regulator of angiogenesis. Nat. Med. 6, 49–55. https://doi.org/10.1038/71527

Linder, J.E., Kraft, D.C., Mohamed, Y., Lu, Z., Heil, L., Tollefson, S., Saville, B.R., Wright, P.F., Williams, J.V., Miller, E.K., 2013. Human rhinovirus C: Age, season, and lower respiratory illness over the past 3 decades. J. Allergy Clin. Immunol. 131, 69-77–6. https://doi.org/10.1016/j.jaci.2012.09.033

Liu, P., Jamaluddin, M., Li, K., Garofalo, R.P., Casola, A., Brasier, A.R., 2007. Retinoic acid-inducible gene I mediates early antiviral response and Toll-like receptor 3 expression in respiratory syncytial virus-infected airway epithelial cells. J. Virol. 81, 1401–1411. https://doi.org/10.1128/JVI.01740-06

Lopez-Souza, N., Dolganov, G., Dubin, R., Sachs, L.A., Sassina, L., Sporer, H., Yagi, S., Schnurr, D., Boushey, H.A., Widdicombe, J.H., 2004. Resistance of differentiated human airway epithelium to infection by rhinovirus. Am. J. Physiol.-Lung Cell. Mol. Physiol. 286, L373–L381. https://doi.org/10.1152/ajplung.00300.2003

Louie, J.K., Roy-Burman, A., Guardia-Labar, L., Boston, E.J., Kiang, D., Padilla, T., Yagi, S., Messenger, S., Petru, A.M., Glaser, C.A., Schnurr, D.P., 2009. Rhinovirus associated with severe lower respiratory tract infections in children. Pediatr. Infect. Dis. J. 28, 337–339. https://doi.org/10.1097/INF.0b013e31818ffc1b

Louie, J.K., Yagi, S., Nelson, F.A., Kiang, D., Glaser, C.A., Rosenberg, J., Cahill, C.K., Schnurr, D.P., 2005. Rhinovirus outbreak in a long term care facility for elderly persons associated with unusually high mortality. Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 41, 262–265. https://doi.org/10.1086/430915

Mader, J.S., Mookherjee, N., Hancock, R.E.W., Bleackley, R.C., 2009. The Human Host Defense Peptide LL-37 Induces Apoptosis in a Calpain- and Apoptosis-Inducing Factor–Dependent Manner Involving Bax Activity. Mol. Cancer Res. 7, 689–702. https://doi.org/10.1158/1541-7786.MCR-08-0274

Mak, R.K.Y., Tse, L.Y., Lam, W.Y., Wong, G.W.K., Chan, P.K.S., Leung, T.F., 2011. Clinical spectrum of human rhinovirus infections in hospitalized Hong Kong children. Pediatr. Infect. Dis. J. 30, 749–753. https://doi.org/10.1097/INF.0b013e31821b8c71

Malcolm, E., Arruda, E., Hayden, F.G., Kaiser, L., 2001. Clinical features of patients with acute respiratory illness and rhinovirus in their bronchoalveolar lavages. J. Clin. Virol. Off. Publ. Pan Am. Soc. Clin. Virol. 21, 9–16.

Mallia, P., Footitt, J., Sotero, R., Jepson, A., Contoli, M., Trujillo-Torralbo, M.-B., Kebadze, T., Aniscenko, J., Oleszkiewicz, G., Gray, K., Message, S.D., Ito, K., Barnes, P.J., Adcock, I.M., Papi, A., Stanciu, L.A., Elkin, S.L., Kon, O.M., Johnson, M., Johnston, S.L., 2012.

Rhinovirus Infection Induces Degradation of Antimicrobial Peptides and Secondary Bacterial Infection in Chronic Obstructive Pulmonary Disease. Am. J. Respir. Crit. Care Med. 186, 1117–1124. https://doi.org/10.1164/rccm.201205-0806OC

Mansour, S.C., Pena, O.M., Hancock, R.E.W., 2014. Host defense peptides: front-line immunomodulators. Trends Immunol. 35, 443–450. https://doi.org/10.1016/j.it.2014.07.004

Martin, S.J., Amarante-Mendes, G.P., Shi, L., Chuang, T.H., Casiano, C.A., O'Brien, G.A., Fitzgerald, P., Tan, E.M., Bokoch, G.M., Greenberg, A.H., Green, D.R., 1996. The cytotoxic cell protease granzyme B initiates apoptosis in a cell-free system by proteolytic processing and activation of the ICE/CED-3 family protease, CPP32, via a novel two-step mechanism. EMBO J. 15, 2407–2416.

Mathers, C.D., Loncar, D., 2006. Projections of global mortality and burden of disease from 2002 to 2030., Projections of Global Mortality and Burden of Disease from 2002 to 2030. PLoS Med. PLoS Med. 3, 3, e442–e442. https://doi.org/10.1371/journal.pmed.0030442, 10.1371/journal.pmed.0030442

Matsukura, S., Kokubu, F., Kurokawa, M., Kawaguchi, M., Ieki, K., Kuga, H., Odaka, M., Suzuki, S., Watanabe, S., Homma, T., Takeuchi, H., Nohtomi, K., Adachi, M., 2007. Role of RIG-I, MDA-5, and PKR on the Expression of Inflammatory Chemokines Induced by Synthetic dsRNA in Airway Epithelial Cells. Int. Arch. Allergy Immunol. 143, 80–83. https://doi.org/10.1159/000101411

Matsukura, S., Kokubu, F., Kurokawa, M., Kawaguchi, M., Ieki, K., Kuga, H., Odaka, M., Suzuki, S., Watanabe, S., Takeuchi, H., Kasama, T., Adachi, M., 2006. Synthetic double-stranded RNA induces multiple genes related to inflammation through Toll-like receptor 3 depending on NF-kappaB and/or IRF-3 in airway epithelial cells. Clin. Exp. Allergy J. Br. Soc. Allergy Clin. Immunol. 36, 1049–1062. https://doi.org/10.1111/j.1365-2222.2006.02530.x

Matsukura, S., Kokubu, F., Noda, H., Watanabe, H., Fukuchi, K., Gomi, K., Adachi, M., 1996. Expression of ICAM-1 on human bronchial epithelial cells after influenza virus infection. Allergol. Int. 45, 97–103. https://doi.org/10.2332/allergolint.45.97

Matsuzaki, K., 1999. Why and how are peptide-lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. Biochim. Biophys. Acta 1462, 1–10.

Matz, J., 2013. Vapendavir significantly improves upper respiratory symptoms of naturally acquired rhinovirus infection in asthmatic adults: Results of a phase 2 clinical trial. Eur. Respir. J. 42, 1493.

Message, S.D., Laza-Stanca, V., Mallia, P., Parker, H.L., Zhu, J., Kebadze, T., Contoli, M., Sanderson, G., Kon, O.M., Papi, A., Jeffery, P.K., Stanciu, L.A., Johnston, S.L., 2008. Rhinovirus-induced lower respiratory illness is increased in asthma and related to virus load and Th1/2 cytokine and IL-10 production. Proc. Natl. Acad. Sci. U. S. A. 105, 13562–13567. https://doi.org/10.1073/pnas.0804181105

Molhoek, E.M., van Dijk, A., Veldhuizen, E.J.A., Haagsman, H.P., Bikker, F.J., 2011. Improved proteolytic stability of chicken cathelicidin-2 derived peptides by d-amino acid substitutions and cyclization. Peptides 32, 875–880. https://doi.org/10.1016/j.peptides.2011.02.017

Monto, A.S., 2002. Epidemiology of viral respiratory infections. Am. J. Med. 112 Suppl 6A, 4S–12S.

Montreekachon, P., Chotjumlong, P., Bolscher, J.G.M., Nazmi, K., Reutrakul, V., Krisanaprakornkit, S., 2011. Involvement of P2X(7) purinergic receptor and MEK1/2 in interleukin-8 up-regulation by LL-37 in human gingival fibroblasts. J. Periodontal Res. 46, 327–337. https://doi.org/10.1111/j.1600-0765.2011.01346.x

Mookherjee, N., Brown, K.L., Bowdish, D.M.E., Doria, S., Falsafi, R., Hokamp, K., Roche, F.M., Mu, R., Doho, G.H., Pistolic, J., Powers, J.-P., Bryan, J., Brinkman, F.S.L., Hancock, R.E.W., 2006. Modulation of the TLR-mediated inflammatory response by

the endogenous human host defense peptide LL-37. J. Immunol. Baltim. Md 1950 176, 2455–2464.
Mookherjee, N., Hancock, R.E.W., 2007. Cationic host defence peptides: innate immune regulatory peptides as a novel approach for treating infections. Cell. Mol. Life Sci. CMLS 64, 922–933. https://doi.org/10.1007/s00018-007-6475-6
Mookherjee, N., Lippert, D.N.D., Hamill, P., Falsafi, R., Nijnik, A., Kindrachuk, J., Pistolic, J., Gardy, J., Miri, P., Naseer, M., Foster, L.J., Hancock, R.E.W., 2009. Intracellular Receptor for Human Host Defense Peptide LL-37 in Monocytes. J. Immunol. 183, 2688. https://doi.org/10.4049/jimmunol.0802586
Mosser, A.G., Brockman-Schneider, R., Amineva, S., Burchell, L., Sedgwick, J.B., Busse, W.W., Gern, J.E., 2002. Similar Frequency of Rhinovirus-Infectible Cells in Upper and Lower Airway Epithelium. J. Infect. Dis. 185, 734–743. https://doi.org/10.1086/339339
Murakami, M., Lopez-Garcia, B., Braff, M., Dorschner, R.A., Gallo, R.L., 2004. Postsecretory processing generates multiple cathelicidins for enhanced topical antimicrobial defense. J. Immunol. Baltim. Md 1950 172, 3070–3077.
Nagaoka, I., Tamura, H., Hirata, M., 2006. An antimicrobial cathelicidin peptide, human CAP18/LL-37, suppresses neutrophil apoptosis via the activation of formyl-peptide receptor-like 1 and P2X7. J. Immunol. Baltim. Md 1950 176, 3044–3052.
Nell, M.J., Tjabringa, G.S., Vonk, M.J., Hiemstra, P.S., Grote, J.J., 2004. Bacterial products increase expression of the human cathelicidin hCAP-18/LL-37 in cultured human sinus epithelial cells. FEMS Immunol. Med. Microbiol. 42, 225–231. https://doi.org/10.1016/j.femsim.2004.05.013
Newcomb, D.C., Sajjan, U.S., Nagarkar, D.R., Wang, Q., Nanua, S., Zhou, Y., McHenry, C.L., Hennrick, K.T., Tsai, W.C., Bentley, J.K., Lukacs, N.W., Johnston, S.L., Hershenson, M.B., 2008. Human Rhinovirus 1B Exposure Induces Phosphatidylinositol 3-Kinase–dependent Airway Inflammation in Mice. Am. J. Respir. Crit. Care Med. 177, 1111–1121. https://doi.org/10.1164/rccm.200708-1243OC
Nguyen, T.X., Cole, A.M., Lehrer, R.I., 2003. Evolution of primate theta-defensins: a serpentine path to a sweet tooth. Peptides 24, 1647–1654. https://doi.org/10.1016/j.peptides.2003.07.023
Nicholson, K.G., Kent, J., Hammersley, V., Cancio, E., 1996. Risk factors for lower respiratory complications of rhinovirus infections in elderly people living in the community: prospective cohort study. BMJ 313, 1119–1123.
Nicodemou, A., Petsch, M., Konecsni, T., Kremser, L., Kenndler, E., Casasnovas, J.M., Blaas, D., 2005. Rhinovirus-stabilizing activity of artificial VLDL-receptor variants defines a new mechanism for virus neutralization by soluble receptors. FEBS Lett. 579, 5507–5511. https://doi.org/10.1016/j.febslet.2005.09.013
Nijnik, A., Hancock, R., 2009. Host defence peptides: antimicrobial and immunomodulatory activity and potential applications for tackling antibiotic-resistant infections. Emerg. Health Threats J. 2. https://doi.org/10.3134/ehtj.09.001
Niyonsaba, F., Iwabuchi, K., Someya, A., Hirata, M., Matsuda, H., Ogawa, H., Nagaoka, I., 2002. A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis. Immunology 106, 20–26.
Niyonsaba, F., Someya, A., Hirata, M., Ogawa, H., Nagaoka, I., 2001. Evaluation of the effects of peptide antibiotics human beta-defensins-1/-2 and LL-37 on histamine release and prostaglandin D(2) production from mast cells. Eur. J. Immunol. 31, 1066–1075.
Nizet, V., Ohtake, T., Lauth, X., Trowbridge, J., Rudisill, J., Dorschner, R.A., Pestonjamasp, V., Piraino, J., Huttner, K., Gallo, R.L., 2001. Innate antimicrobial peptide protects the skin from invasive bacterial infection. Nature 414, 454–457. https://doi.org/10.1038/35106587

Noah, T.L., Becker, S., 1993. Respiratory syncytial virus-induced cytokine production by a human bronchial epithelial cell line. Am. J. Physiol.-Lung Cell. Mol. Physiol. 265, L472–L478. https://doi.org/10.1152/ajplung.1993.265.5.L472

Oberst, A., Dillon, C.P., Weinlich, R., McCormick, L.L., Fitzgerald, P., Pop, C., Hakem, R., Salvesen, G.S., Green, D.R., 2011. Catalytic activity of the caspase-8–FLIPL complex inhibits RIPK3-dependent necrosis. Nature 471, 363–367. https://doi.org/10.1038/nature09852

Olsen, C.W., Kehren, J.C., Dybdahl-Sissoko, N.R., Hinshaw, V.S., 1996. bcl-2 alters influenza virus yield, spread, and hemagglutinin glycosylation. J. Virol. 70, 663–666.

Ong, P.Y., Ohtake, T., Brandt, C., Strickland, I., Boguniewicz, M., Ganz, T., Gallo, R.L., Leung, D.Y.M., 2002. Endogenous Antimicrobial Peptides and Skin Infections in Atopic Dermatitis. N. Engl. J. Med. 347, 1151–1160. https://doi.org/10.1056/NEJMoa021481

Oren, Z., Lerman, J.C., Gudmundsson, G.H., Agerberth, B., Shai, Y., 1999. Structure and organization of the human antimicrobial peptide LL-37 in phospholipid membranes: relevance to the molecular basis for its non-cell-selective activity. Biochem. J. 341, 501–513.

Othumpangat, S., Noti, J.D., McMillen, C.M., Beezhold, D.H., 2016. ICAM-1 regulates the survival of influenza virus in lung epithelial cells during the early stages of infection. Virology 487, 85–94. https://doi.org/10.1016/j.virol.2015.10.005

Palmenberg, A.C., Rathe, J.A., Liggett, S.B., 2010. Analysis of the complete genome sequences of human rhinovirus. J. Allergy Clin. Immunol. 125, 1190-1199-1201. https://doi.org/10.1016/j.jaci.2010.04.010

Panyutich, A., Shi, J., Boutz, P.L., Zhao, C., Ganz, T., 1997. Porcine polymorphonuclear leukocytes generate extracellular microbicidal activity by elastase-mediated activation of secreted proprotegrins. Infect. Immun. 65, 978–985.

Papadopoulos, N.G., Bates, P.J., Bardin, P.G., Papi, A., Leir, S.H., Fraenkel, D.J., Meyer, J., Lackie, P.M., Sanderson, G., Holgate, S.T., Johnston, S.L., 2000. Rhinoviruses infect the lower airways. J. Infect. Dis. 181, 1875–1884. https://doi.org/10.1086/315513

Papadopoulos, N.G., Moustaki, M., Tsolia, M., Bossios, A., Astra, E., Prezerakou, A., Gourgiotis, D., Kafetzis, D., 2002. Association of rhinovirus infection with increased disease severity in acute bronchiolitis. Am. J. Respir. Crit. Care Med. 165, 1285–1289. https://doi.org/10.1164/rccm.200112-118BC

Papadopoulos, N.G., Papi, A., Meyer, J., Stanciu, L.A., Salvi, S., Holgate, S.T., Johnston, S.L., 2001. Rhinovirus infection up-regulates eotaxin and eotaxin-2 expression in bronchial epithelial cells. Clin. Exp. Allergy J. Br. Soc. Allergy Clin. Immunol. 31, 1060–1066.

Papadopoulos, N.G., Sanderson, G., Hunter, J., Johnston, S.L., 1999. Rhinoviruses replicate effectively at lower airway temperatures. J. Med. Virol. 58, 100–104.

Papi, A., Johnston, S.L., 1999. Rhinovirus infection induces expression of its own receptor intercellular adhesion molecule 1 (ICAM-1) via increased NF-kappaB-mediated transcription. J. Biol. Chem. 274, 9707–9720.

Parronchi, P., De Carli, M., Manetti, R., Simonelli, C., Sampognaro, S., Piccinni, M.P., Macchia, D., Maggi, E., Del Prete, G., Romagnani, S., 1992. IL-4 and IFN (alpha and gamma) exert opposite regulatory effects on the development of cytolytic potential by Th1 or Th2 human T cell clones. J. Immunol. Baltim. Md 1950 149, 2977–2983.

Perlman, D., Bodanszky, M., 1971. Biosynthesis of peptide antibiotics. Annu. Rev. Biochem. 40, 449–464. https://doi.org/10.1146/annurev.bi.40.070171.002313

Petrella, A., Festa, M., Ercolino, S.F., Zerilli, M., Stassi, G., Solito, E., Parente, L., 2005. Induction of annexin-1 during TRAIL-induced apoptosis in thyroid carcinoma cells. Cell Death Differ. 12, 1358–1360. https://doi.org/10.1038/sj.cdd.4401645

Pistolic, J., Cosseau, C., Li, Y., Yu, J. (Jessie), Filewod, N.C.J., Gellatly, S., Rehaume, L.M., Bowdish, D.M.E., Hancock, R.E.W., 2009. Host Defence Peptide LL-37 Induces IL-6

Expression in Human Bronchial Epithelial Cells by Activation of the NF-κB Signaling Pathway. J. Innate Immun. 1, 254–267. https://doi.org/10.1159/000171533
Pitkäranta, A., Virolainen, A., Jero, J., Arruda, E., Hayden, F.G., 1998. Detection of rhinovirus, respiratory syncytial virus, and coronavirus infections in acute otitis media by reverse transcriptase polymerase chain reaction. Pediatrics 102, 291–295.
Pitrez, P.M.C., Stein, R.T., Stuermer, L., Macedo, I.S., Schmitt, V.M., Jones, M.H., Arruda, E., 2005. [Rhinovirus and acute bronchiolitis in young infants]. J. Pediatr. (Rio J.) 81, 417–420. https://doi.org/10.2223/JPED.1394
Poręba, M., Stróżyk, A., Salvesen, G.S., Drąg, M., 2013. Caspase Substrates and Inhibitors. Cold Spring Harb. Perspect. Biol. 5. https://doi.org/10.1101/cshperspect.a008680
Proud, D., Gwaltney, J.M., Hendley, J.O., Dinarello, C.A., Gillis, S., Schleimer, R.P., 1994. Increased levels of interleukin-1 are detected in nasal secretions of volunteers during experimental rhinovirus colds. J. Infect. Dis. 169, 1007–1013.
Proud, D., Sanders, S.P., Wiehler, S., 2004. Human Rhinovirus Infection Induces Airway Epithelial Cell Production of Human β-Defensin 2 Both In Vitro and In Vivo. J. Immunol. 172, 4637–4645. https://doi.org/10.4049/jimmunol.172.7.4637
Proud, D., Turner, R.B., Winther, B., Wiehler, S., Tiesman, J.P., Reichling, T.D., Juhlin, K.D., Fulmer, A.W., Ho, B.Y., Walanski, A.A., Poore, C.L., Mizoguchi, H., Jump, L., Moore, M.L., Zukowski, C.K., Clymer, J.W., 2008. Gene expression profiles during in vivo human rhinovirus infection: insights into the host response. Am. J. Respir. Crit. Care Med. 178, 962–968. https://doi.org/10.1164/rccm.200805-670OC
Putsep, K., Carlsson, G., Boman, H.G., Andersson, M., 2002. Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. The Lancet 360, 1144(6).
Quiñones-Mateu, M.E., Lederman, M.M., Feng, Z., Chakraborty, B., Weber, J., Rangel, H.R., Marotta, M.L., Mirza, M., Jiang, B., Kiser, P., Medvik, K., Sieg, S.F., Weinberg, A., 2003. Human epithelial beta-defensins 2 and 3 inhibit HIV-1 replication. AIDS Lond. Engl. 17, F39-48. https://doi.org/10.1097/01.aids.0000096878.73209.4f
Rajan, D., Gaston, K.A., McCracken, C.E., Erdman, D.D., Anderson, L.J., 2013. Response to Rhinovirus Infection by Human Airway Epithelial Cells and Peripheral Blood Mononuclear Cells in an In Vitro Two-Chamber Tissue Culture System. PLOS ONE 8, e66600. https://doi.org/10.1371/journal.pone.0066600
REED, L.J., MUENCH, H., 1938. A SIMPLE METHOD OF ESTIMATING FIFTY PER CENT ENDPOINTS12. Am. J. Epidemiol. 27, 493–497. https://doi.org/10.1093/oxfordjournals.aje.a118408
Rehaume, L.M., Hancock, R.E.W., 2008. Neutrophil-derived defensins as modulators of innate immune function. Crit. Rev. Immunol. 28, 185–200.
Ren, S.X., Shen, J., Cheng, A.S.L., Lu, L., Chan, R.L.Y., Li, Z.J., Wang, X.J., Wong, C.C.M., Zhang, L., Ng, S.S.M., Chan, F.L., Chan, F.K.L., Yu, J., Sung, J.J.Y., Wu, W.K.K., Cho, C.H., 2013. FK-16 Derived from the Anticancer Peptide LL-37 Induces Caspase-Independent Apoptosis and Autophagic Cell Death in Colon Cancer Cells. PLoS ONE 8. https://doi.org/10.1371/journal.pone.0063641
Reza Etemadi, M., Ling, K.-H., Zainal Abidin, S., Chee, H.-Y., Sekawi, Z., 2017. Gene expression patterns induced at different stages of rhinovirus infection in human alveolar epithelial cells. PLOS ONE 12, e0176947. https://doi.org/10.1371/journal.pone.0176947
Rice, W.G., Ganz, T., Kinkade, J.M., Selsted, M.E., Lehrer, R.I., Parmley, R.T., 1987. Defensin-rich dense granules of human neutrophils. Blood 70, 757–765.
Rihkanen, H., Rönkkö, E., Nieminen, T., Komsi, K.-L., Räty, R., Saxen, H., Ziegler, T., Roivainen, M., Söderlund-Venermo, M., Beng, A.L., Anne, L., Hovi, T., Pitkäranta, A., 2008. Respiratory viruses in laryngeal croup of young children. J. Pediatr. 152, 661–665. https://doi.org/10.1016/j.jpeds.2007.10.043

Rock, K.L., Kono, H., 2008. The inflammatory response to cell death. Annu. Rev. Pathol. 3, 99–126. https://doi.org/10.1146/annurev.pathmechdis.3.121806.151456
Rodriguez, D.A., Weinlich, R., Brown, S., Guy, C., Fitzgerald, P., Dillon, C.P., Oberst, A., Quarato, G., Low, J., Cripps, J.G., Chen, T., Green, D.R., 2016. Characterization of RIPK3-mediated phosphorylation of the activation loop of MLKL during necroptosis. Cell Death Differ. 23, 76–88. https://doi.org/10.1038/cdd.2015.70
Roy, N., Deveraux, Q.L., Takahashi, R., Salvesen, G.S., Reed, J.C., 1997. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J. 16, 6914–6925. https://doi.org/10.1093/emboj/16.23.6914
Sajjan, U., Wang, Q., Zhao, Y., Gruenert, D.C., Hershenson, M.B., 2008. Rhinovirus Disrupts the Barrier Function of Polarized Airway Epithelial Cells. Am. J. Respir. Crit. Care Med. 178, 1271–1281. https://doi.org/10.1164/rccm.200801-136OC
Sajjan, U.S., 2006. H. influenzae potentiates airway epithelial cell responses to rhinovirus by increasing ICAM-1 and TLR3 expression. FASEB J. 20, 2121–2123. https://doi.org/10.1096/fj.06-5806fje
Sanders, S.P., Siekierski, E.S., Porter, J.D., Richards, S.M., Proud, D., 1998. Nitric Oxide Inhibits Rhinovirus-Induced Cytokine Production and Viral Replication in a Human Respiratory Epithelial Cell Line. J. Virol. 72, 934–942.
Sandgren, S., Wittrup, A., Cheng, F., Jönsson, M., Eklund, E., Busch, S., Belting, M., 2004. The human antimicrobial peptide LL-37 transfers extracellular DNA plasmid to the nuclear compartment of mammalian cells via lipid rafts and proteoglycan-dependent endocytosis. J. Biol. Chem. 279, 17951–17956. https://doi.org/10.1074/jbc.M311440200
Sarker, P., Ahmed, S., Tiash, S., Rekha, R.S., Stromberg, R., Andersson, J., Bergman, P., Gudmundsson, G.H., Agerberth, B., Raqib, R., 2011. Phenylbutyrate Counteracts Shigella Mediated Downregulation of Cathelicidin in Rabbit Lung and Intestinal Epithelia: A Potential Therapeutic Strategy. PLOS ONE 6, e20637. https://doi.org/10.1371/journal.pone.0020637
Savolainen, C., Blomqvist, S., Mulders, M.N., Hovi, T., 2002. Genetic clustering of all 102 human rhinovirus prototype strains: serotype 87 is close to human enterovirus 70. J. Gen. Virol. 83, 333–340. https://doi.org/10.1099/0022-1317-83-2-333
Schall, T.J., Bacon, K., Toy, K.J., Goeddel, D.V., 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. Nature 347, 669–671. https://doi.org/10.1038/347669a0
Schaller-Bals, S., Schulze, A., Bals, R., 2002. Increased Levels of Antimicrobial Peptides in Tracheal Aspirates of Newborn Infants during Infection. Am. J. Respir. Crit. Care Med. 165, 992–995. https://doi.org/10.1164/ajrccm.165.7.200110-020
Scheiffele, P., Rietveld, A., Wilk, T., Simons, K., 1999. Influenza Viruses Select Ordered Lipid Domains during Budding from the Plasma Membrane. J. Biol. Chem. 274, 2038–2044. https://doi.org/10.1074/jbc.274.4.2038
Schober, D., Kronenberger, P., Prchla, E., Blaas, D., Fuchs, R., 1998. Major and minor receptor group human rhinoviruses penetrate from endosomes by different mechanisms. J. Virol. 72, 1354–1364.
Schroth, M.K., Grimm, E., Frindt, P., Galagan, D.M., Konno, S.I., Love, R., Gern, J.E., 1999. Rhinovirus replication causes RANTES production in primary bronchial epithelial cells. Am. J. Respir. Cell Mol. Biol. 20, 1220–1228. https://doi.org/10.1165/ajrcmb.20.6.3261
Scott, M.G., Davidson, D.J., Gold, M.R., Bowdish, D., Hancock, R.E.W., 2002. The Human Antimicrobial Peptide LL-37 Is a Multifunctional Modulator of Innate Immune Responses. J. Immunol. 169, 3883–3891. https://doi.org/10.4049/jimmunol.169.7.3883
Seemungal, T., Harper-Owen, R., Bhowmik, A., Moric, I., Sanderson, G., Message, S., Maccallum, P., Meade, T.W., Jeffries, D.J., Johnston, S.L., Wedzicha, J.A., 2001.

Respiratory viruses, symptoms, and inflammatory markers in acute exacerbations and stable chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. 164, 1618–1623. https://doi.org/10.1164/ajrccm.164.9.2105011

Sethi, S.K., Bianco, A., Allen, J.T., Knight, R.A., Spiteri, M.A., 1997. Interferon-gamma (IFN-gamma) down-regulates the rhinovirus-induced expression of intercellular adhesion molecule-1 (ICAM-1) on human airway epithelial cells. Clin. Exp. Immunol. 110, 362–369.

Shai, Y., 1999. Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides. Biochim. Biophys. Acta 1462, 55–70.

Shiozaki, E.N., Chai, J., Shi, Y., 2002. Oligomerization and activation of caspase-9, induced by Apaf-1 CARD. Proc. Natl. Acad. Sci. U. S. A. 99, 4197–4202. https://doi.org/10.1073/pnas.072544399

Simmonds, P., McIntyre, C., Savolainen-Kopra, C., Tapparel, C., Mackay, I.M., Hovi, T., 2010. Proposals for the classification of human rhinovirus species C into genotypically assigned types. J. Gen. Virol. 91, 2409–2419. https://doi.org/10.1099/vir.0.023994-0

Sirén, J., Imaizumi, T., Sarkar, D., Pietilä, T., Noah, D.L., Lin, R., Hiscott, J., Krug, R.M., Fisher, P.B., Julkunen, I., Matikainen, S., 2006. Retinoic acid inducible gene-I and mda-5 are involved in influenza A virus-induced expression of antiviral cytokines. Microbes Infect. 8, 2013–2020. https://doi.org/10.1016/j.micinf.2006.02.028

Slater, L., Bartlett, N.W., Haas, J.J., Zhu, J., Message, S.D., Walton, R.P., Sykes, A., Dahdaleh, S., Clarke, D.L., Belvisi, M.G., Kon, O.M., Fujita, T., Jeffery, P.K., Johnston, S.L., Edwards, M.R., 2010. Co-ordinated Role of TLR3, RIG-I and MDA5 in the Innate Response to Rhinovirus in Bronchial Epithelium. PLOS Pathog. 6, e1001178. https://doi.org/10.1371/journal.ppat.1001178

Smith, T.J., Chase, E.S., Schmidt, T.J., Olson, N.H., Baker, T.S., 1996. Neutralizing antibody to human rhinovirus 14 penetrates the receptor-binding canyon. Nature 383, 350–354. https://doi.org/10.1038/383350a0

Snyers, L., Zwickl, H., Blaas, D., 2003. Human rhinovirus type 2 is internalized by clathrin-mediated endocytosis. J. Virol. 77, 5360–5369.

Soehnlein, O., Kai-Larsen, Y., Frithiof, R., Sorensen, O.E., Kenne, E., Scharffetter-Kochanek, K., Eriksson, E.E., Herwald, H., Agerberth, B., Lindbom, L., 2008. Neutrophil primary granule proteins HBP and HNP1-3 boost bacterial phagocytosis by human and murine macrophages. J. Clin. Invest. 118, 3491–3502. https://doi.org/10.1172/JCI35740

Solito, E., de Coupade, C., Canaider, S., Goulding, N.J., Perretti, M., 2001. Transfection of annexin 1 in monocytic cells produces a high degree of spontaneous and stimulated apoptosis associated with caspase-3 activation. Br. J. Pharmacol. 133, 217–228. https://doi.org/10.1038/sj.bjp.0704054

Sommergruber, W., Zorn, M., Blaas, D., Fessl, F., Volkmann, P., Maurer-Fogy, I., Pallai, P., Merluzzi, V., Matteo, M., Skern, T., Kuechler, E., 1989. Polypeptide 2A of human rhinovirus type 2: Identification as a protease and characterization by mutational analysis. Virology 169, 68–77. https://doi.org/10.1016/0042-6822(89)90042-1

Sørensen, O.E., Follin, P., Johnsen, A.H., Calafat, J., Tjabringa, G.S., Hiemstra, P.S., Borregaard, N., 2001. Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. Blood 97, 3951–3959.

Sørensen, O.E., Thapa, D.R., Rosenthal, A., Liu, L., Roberts, A.A., Ganz, T., 2005. Differential regulation of beta-defensin expression in human skin by microbial stimuli. J. Immunol. Baltim. Md 1950 174, 4870–4879.

Sousa, F.H., Casanova, V., Findlay, F., Stevens, C., Svoboda, P., Pohl, J., Proudfoot, L., Barlow, P.G., 2017. Cathelicidins display conserved direct antiviral activity towards rhinovirus. Peptides 95, 76–83. https://doi.org/10.1016/j.peptides.2017.07.013

Spurrell, J.C.L., Wiehler, S., Zaheer, R.S., Sanders, S.P., Proud, D., 2005. Human airway epithelial cells produce IP-10 (CXCL10) in vitro and in vivo upon rhinovirus infection. Am. J. Physiol. Lung Cell. Mol. Physiol. 289, L85-95. https://doi.org/10.1152/ajplung.00397.2004

Stecenko, A.A., King, G., Torii, K., Breyer, R.M., Dworski, R., Blackwell, T.S., Christman, J.W., Brigham, K.L., 2001. Dysregulated cytokine production in human cystic fibrosis bronchial epithelial cells. Inflammation 25, 145–155.

Steinmann, J., Halldórsson, S., Agerberth, B., Gudmundsson, G.H., 2009. Phenylbutyrate induces antimicrobial peptide expression. Antimicrob. Agents Chemother. 53, 5127–5133. https://doi.org/10.1128/AAC.00818-09

Steinstraesser, L., Kraneburg, U., Jacobsen, F., Al-Benna, S., 2011. Host defense peptides and their antimicrobial-immunomodulatory duality. Immunobiology 216, 322–333. https://doi.org/10.1016/j.imbio.2010.07.003

Stray, S.J., Air, G.M., 2001. Apoptosis by influenza viruses correlates with efficiency of viral mRNA synthesis. Virus Res. 77, 3–17.

Subauste, M.C., Jacoby, D.B., Richards, S.M., Proud, D., 1995. Infection of a human respiratory epithelial cell line with rhinovirus. Induction of cytokine release and modulation of susceptibility to infection by cytokine exposure. J. Clin. Invest. 96, 549–557. https://doi.org/10.1172/JCI118067

Suzuki, K., Murakami, T., Kuwahara-Arai, K., Tamura, H., Hiramatsu, K., Nagaoka, I., 2011. Human anti-microbial cathelicidin peptide LL-37 suppresses the LPS-induced apoptosis of endothelial cells. Int. Immunol. 23, 185–193. https://doi.org/10.1093/intimm/dxq471

Suzuki, T., Yamaya, M., Kamanaka, M., Jia, Y.X., Nakayama, K., Hosoda, M., Yamada, N., Nishimura, H., Sekizawa, K., Sasaki, H., 2001. Type 2 rhinovirus infection of cultured human tracheal epithelial cells: role of LDL receptor. Am. J. Physiol. - Lung Cell. Mol. Physiol. 280, L409.

Sykes, A., Macintyre, J., Edwards, M.R., Del Rosario, A., Haas, J., Gielen, V., Kon, O.M., McHale, M., Johnston, S.L., 2014. Rhinovirus-induced interferon production is not deficient in well controlled asthma. Thorax 69, 240–246. https://doi.org/10.1136/thoraxjnl-2012-202909

Taimen, P., Berghäll, H., Vainionpää, R., Kallajoki, M., 2004. NuMA and nuclear lamins are cleaved during viral infection—inhibition of caspase activity prevents cleavage and rescues HeLa cells from measles virus-induced but not from rhinovirus 1B-induced cell death. Virology 320, 85–98. https://doi.org/10.1016/j.virol.2003.11.026

Takahashi, T., Kulkarni, N.N., Lee, E.Y., Zhang, L.-J., Wong, G.C.L., Gallo, R.L., 2018. Cathelicidin promotes inflammation by enabling binding of self-RNA to cell surface scavenger receptors. Sci. Rep. 8, 4032. https://doi.org/10.1038/s41598-018-22409-3

Tarrats, N., Moles, A., Morales, A., García-Ruiz, C., Fernández-Checa, J.C., Marí, M., 2011. Critical role of tumor necrosis factor receptor 1, but not 2, in hepatic stellate cell proliferation, extracellular matrix remodeling, and liver fibrogenesis. Hepatology 54, 319–327. https://doi.org/10.1002/hep.24388

Terajima, M., Yamaya, M., Sekizawa, K., Okinaga, S., Suzuki, T., Yamada, N., Nakayama, K., Ohrui, T., Oshima, T., Numazaki, Y., Sasaki, H., 1997. Rhinovirus infection of primary cultures of human tracheal epithelium: role of ICAM-1 and IL-1β. Am. J. Physiol. - Lung Cell. Mol. Physiol. 273, L749.

Thibaut, H.J., De Palma, A.M., Neyts, J., 2012. Combating enterovirus replication: state-of-the-art on antiviral research. Biochem. Pharmacol. 83, 185–192. https://doi.org/10.1016/j.bcp.2011.08.016

Thomas, A.J., Pulsipher, A., Davis, B.M., Alt, J.A., 2017. LL-37 causes cell death of human nasal epithelial cells, which is inhibited with a synthetic glycosaminoglycan. PLOS ONE 12, e0183542. https://doi.org/10.1371/journal.pone.0183542

Thomson, B.J., 2001. Viruses and apoptosis. Int. J. Exp. Pathol. 82, 65–76. https://doi.org/10.1111/j.1365-2613.2001.iep0082-0065-x

Tjabringa, G.S., Aarbiou, J., Ninaber, D.K., Drijfhout, J.W., Sørensen, O.E., Borregaard, N., Rabe, K.F., Hiemstra, P.S., 2003. The antimicrobial peptide LL-37 activates innate immunity at the airway epithelial surface by transactivation of the epidermal growth factor receptor. J. Immunol. Baltim. Md 1950 171, 6690–6696.

Tjabringa, G.S., Ninaber, D.K., Drijfhout, J.W., Rabe, K.F., Hiemstra, P.S., 2006. Human Cathelicidin LL-37 Is a Chemoattractant for Eosinophils and Neutrophils That Acts via Formyl-Peptide Receptors. Int. Arch. Allergy Immunol. 140, 103–112. https://doi.org/10.1159/000092305

Tokumaru, S., Sayama, K., Shirakata, Y., Komatsuzawa, H., Ouhara, K., Hanakawa, Y., Yahata, Y., Dai, X., Tohyama, M., Nagai, H., Yang, L., Higashiyama, S., Yoshimura, A., Sugai, M., Hashimoto, K., 2005. Induction of keratinocyte migration via transactivation of the epidermal growth factor receptor by the antimicrobial peptide LL-37. J. Immunol. Baltim. Md 1950 175, 4662–4668.

Torres-Juarez, F., Cardenas-Vargas, A., Montoya-Rosales, A., González-Curiel, I., Garcia-Hernandez, M.H., Enciso-Moreno, J.A., Hancock, R.E.W., Rivas-Santiago, B., 2015. LL-37 Immunomodulatory Activity during Mycobacterium tuberculosis Infection in Macrophages. Infect. Immun. 83, 4495–4503. https://doi.org/10.1128/IAI.00936-15

Traub, S., Nikonova, A., Carruthers, A., Dunmore, R., Vousden, K.A., Gogsadze, L., Hao, W., Zhu, Q., Bernard, K., Zhu, J., Dymond, M., McLean, G.R., Walton, R.P., Glanville, N., Humbles, A., Khaitov, M., Wells, T., Kolbeck, R., Leishman, A.J., Sleeman, M.A., Bartlett, N.W., Johnston, S.L., 2013. An anti-human ICAM-1 antibody inhibits rhinovirus-induced exacerbations of lung inflammation. PLoS Pathog. 9, e1003520. https://doi.org/10.1371/journal.ppat.1003520

Triantafilou, K., Vakakis, E., Richer, E.A., Evans, G.L., Villiers, J.P., Triantafilou, M., 2011. Human rhinovirus recognition in non-immune cells is mediated by Toll-like receptors and MDA-5, which trigger a synergetic pro-inflammatory immune response. Virulence 2, 22–29. https://doi.org/10.4161/viru.2.1.13807

Troy, N.M., Bosco, A., 2016. Respiratory viral infections and host responses; insights from genomics. Respir. Res. 17. https://doi.org/10.1186/s12931-016-0474-9

Tsai, K.-W., Lai, H.-T., Tsai, T.-C., Wu, Y.-C., Yang, Y.-T., Chen, K.-Y., Chen, C.-M., Li, Y.-S.J., Chen, C.-N., 2009. Difference in the regulation of IL-8 expression induced by uropathogenic E. coli between two kinds of urinary tract epithelial cells. J. Biomed. Sci. 16, 91. https://doi.org/10.1186/1423-0127-16-91

Tsao, M.S., Zhu, H., Viallet, J., 1996. Autocrine growth loop of the epidermal growth factor receptor in normal and immortalized human bronchial epithelial cells. Exp. Cell Res. 223, 268–273. https://doi.org/10.1006/excr.1996.0081

Turner, J., Cho, Y., Dinh, N.-N., Waring, A.J., Lehrer, R.I., 1998. Activities of LL-37, a Cathelin-Associated Antimicrobial Peptide of Human Neutrophils. Antimicrob. Agents Chemother. 42, 2206–2214.

Turner, R.B., 1997. Epidemiology, pathogenesis, and treatment of the common cold. Ann. Allergy Asthma Immunol. Off. Publ. Am. Coll. Allergy Asthma Immunol. 78, 531-539-540. https://doi.org/10.1016/S1081-1206(10)63213-9

Turner, R.B., Wecker, M.T., Pohl, G., Witek, T.J., McNally, E., St George, R., Winther, B., Hayden, F.G., 1999. Efficacy of tremacamra, a soluble intercellular adhesion molecule 1, for experimental rhinovirus infection: a randomized clinical trial. JAMA 281, 1797–1804.

Turner, R.B., Weingand, K.W., Yeh, C.H., Leedy, D.W., 1998. Association between interleukin-8 concentration in nasal secretions and severity of symptoms of

experimental rhinovirus colds. Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 26, 840–846.
van Kempen, M., Bachert, C., Van Cauwenberge, P., 1999. An update on the pathophysiology of rhinovirus upper respiratory tract infections. Rhinology 37, 97–103.
Wan, H., Winton, H., Soeller, C., Stewart, G., Thompson, P., Gruenert, D., Cannell, M., Garrod, D., Robinson, C., 2000. Tight junction properties of the immortalized human bronchial epithelial cell lines Calu-3 and 16HBE14o-. Eur. Respir. J. 15, 1058.
Wang, J.H., Devalia, J.L., Xia, C., Sapsford, R.J., Davies, R.J., 1996. Expression of RANTES by human bronchial epithelial cells in vitro and in vivo and the effect of corticosteroids. Am. J. Respir. Cell Mol. Biol. 14, 27–35. https://doi.org/10.1165/ajrcmb.14.1.8534483
Wang, J.H., Kwon, H.J., Jang, Y.J., 2009. Rhinovirus enhances various bacterial adhesions to nasal epithelial cells simultaneously. The Laryngoscope 119, 1406–1411. https://doi.org/10.1002/lary.20498
Wang, Q., Wang, L., Feng, Y.-H., Li, X., Zeng, R., Gorodeski, G.I., 2004. P2X7 receptor-mediated apoptosis of human cervical epithelial cells. Am. J. Physiol. Cell Physiol. 287, C1349-1358. https://doi.org/10.1152/ajpcell.00256.2004
Wang, T., Liang, Z.-A., Sandford, A.J., Xiong, X.-Y., Yang, Y.-Y., Ji, Y.-L., He, J.-Q., 2012. Selection of Suitable Housekeeping Genes for Real-Time Quantitative PCR in CD4+ Lymphocytes from Asthmatics with or without Depression. PLoS ONE 7, e48367. https://doi.org/10.1371/journal.pone.0048367
Wang, T.-T., Nestel, F.P., Bourdeau, V., Nagai, Y., Wang, Q., Liao, J., Tavera-Mendoza, L., Lin, R., Hanrahan, J.W., Mader, S., White, J.H., Hanrahan, J.H., 2004. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. J. Immunol. Baltim. Md 1950 173, 2909–2912.
Wang, X., Lau, C., Wiehler, S., Pow, A., Mazzulli, T., Gutierrez, C., Proud, D., Chow, C.-W., 2006. Syk Is Downstream of Intercellular Adhesion Molecule-1 and Mediates Human Rhinovirus Activation of p38 MAPK in Airway Epithelial Cells. J. Immunol. 177, 6859. https://doi.org/10.4049/jimmunol.177.10.6859
Wark, P.A.B., Johnston, S.L., Bucchieri, F., Powell, R., Puddicombe, S., Laza-Stanca, V., Holgate, S.T., Davies, D.E., 2005. Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. J. Exp. Med. 201, 937–947. https://doi.org/10.1084/jem.20041901
Warnking, K., Klemm, C., Löffler, B., Niemann, S., van Krüchten, A., Peters, G., Ludwig, S., Ehrhardt, C., 2015. Super-infection with *S taphylococcus aureus* inhibits influenza virus-induced type I IFN signalling through impaired STAT1-STAT2 dimerization: Influenza virus- and *S. aureus* -mediated signalling. Cell. Microbiol. 17, 303–317. https://doi.org/10.1111/cmi.12375
Weber, G., Heilborn, J.D., Chamorro Jimenez, C.I., Hammarsjo, A., Törmä, H., Stahle, M., 2005. Vitamin D induces the antimicrobial protein hCAP18 in human skin. J. Invest. Dermatol. 124, 1080–1082. https://doi.org/10.1111/j.0022-202X.2005.23687.x
Westerhoff, H.V., Juretić, D., Hendler, R.W., Zasloff, M., 1989. Magainins and the disruption of membrane-linked free-energy transduction. Proc. Natl. Acad. Sci. U. S. A. 86, 6597–6601.
Wilson, S.S., Wiens, M.E., Smith, J.G., 2013. Antiviral Mechanisms of Human Defensins. J. Mol. Biol. 425. https://doi.org/10.1016/j.jmb.2013.09.038
Wimalasundera, S.S., Katz, D.R., Chain, B.M., 1997. Characterization of the T cell response to human rhinovirus in children: implications for understanding the immunopathology of the common cold. J. Infect. Dis. 176, 755–759.
Winther, B., Brofeldt, S., Christensen, B., Mygind, and N., 1984. Light and Scanning Electron Microscopy of Nasal Biopsy Material from Patients with Naturally Acquired

Common Colds. Acta Otolaryngol. (Stockh.) 97, 309–318. https://doi.org/10.3109/00016488409130994
Winther, B., Farr, B., Turner, R.B., Hendley, J.O., Gwaltney, J.M., Mygind, N., 1984. Histopathologic Examination and Enumeration of Polymorphonuclear Leukocytes in the Nasal Mucosa during Experimental Rhinovirus Colds. Acta Otolaryngol. (Stockh.) 98, 19–24. https://doi.org/10.3109/00016488409128537
Winther, B., McCue, K., Ashe, K., Rubino, J.R., Hendley, J.O., 2007. Environmental contamination with rhinovirus and transfer to fingers of healthy individuals by daily life activity. J. Med. Virol. 79, 1606–1610. https://doi.org/10.1002/jmv.20956
Wong, A., Bryzek, D., Dobosz, E., Scavenius, C., Svoboda, P., Rapala-Kozik, M., Lesner, A., Frydrych, I., Enghild, J., Mydel, P., Pohl, J., Thompson, P.R., Potempa, J., Koziel, J., 2018. A Novel Biological Role for Peptidyl-Arginine Deiminases: Citrullination of Cathelicidin LL-37 Controls the Immunostimulatory Potential of Cell-Free DNA. J. Immunol. Baltim. Md 1950. https://doi.org/10.4049/jimmunol.1701391
Wu, H., Zhang, G., Minton, J.E., Ross, C.R., Blecha, F., 2000. Regulation of cathelicidin gene expression: induction by lipopolysaccharide, interleukin-6, retinoic acid, and Salmonella enterica serovar typhimurium infection. Infect. Immun. 68, 5552–5558.
Xatzipsalti, M., Papadopoulos, N.G., 2007. Cellular and animals models for rhinovirus infection in asthma. Contrib. Microbiol. 14, 33–41. https://doi.org/10.1159/000107053
Xhindoli, D., Pacor, S., Benincasa, M., Scocchi, M., Gennaro, R., Tossi, A., 2016. The human cathelicidin LL-37 — A pore-forming antibacterial peptide and host-cell modulator. Biochim. Biophys. Acta BBA - Biomembr., Pore-Forming Toxins: Cellular Effects and Biotech Applications 1858, 546–566. https://doi.org/10.1016/j.bbamem.2015.11.003
Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., Akira, S., 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 301, 640–643. https://doi.org/10.1126/science.1087262
Yamasaki, K., Schauber, J., Coda, A., Lin, H., Dorschner, R.A., Schechter, N.M., Bonnart, C., Descargues, P., Hovnanian, A., Gallo, R.L., 2006. Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 20, 2068–2080. https://doi.org/10.1096/fj.06-6075com
Yang, D., Biragyn, A., Hoover, D.M., Lubkowski, J., Oppenheim, J.J., 2004. Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. Annu. Rev. Immunol. 22, 181–215. https://doi.org/10.1146/annurev.immunol.22.012703.104603
Yang, D., Chertov, O., Bykovskaia, S.N., Chen, Q., Buffo, M.J., Shogan, J., Anderson, M., Schröder, J.M., Wang, J.M., Howard, O.M., Oppenheim, J.J., 1999. Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. Science 286, 525–528.
Yang, L., Weiss, T.M., Lehrer, R.I., Huang, H.W., 2000. Crystallization of Antimicrobial Pores in Membranes: Magainin and Protegrin. Biophys. J. 79, 2002–2009. https://doi.org/10.1016/S0006-3495(00)76448-4
Yasin, B., Harwig, S.S., Lehrer, R.I., Wagar, E.A., 1996. Susceptibility of Chlamydia trachomatis to protegrins and defensins. Infect. Immun. 64, 709–713.
Yeung, A.T.Y., Gellatly, S.L., Hancock, R.E.W., 2011. Multifunctional cationic host defence peptides and their clinical applications. Cell. Mol. Life Sci. 68, 2161. https://doi.org/10.1007/s00018-011-0710-x
Yim, S., Dhawan, P., Ragunath, C., Christakos, S., Diamond, G., 2007. Induction of cathelicidin in normal and CF bronchial epithelial cells by 1,25-dihydroxyvitamin D3. J. Cyst. Fibros. Off. J. Eur. Cyst. Fibros. Soc. 6, 403–410. https://doi.org/10.1016/j.jcf.2007.03.003

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., Fujita, T., 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat. Immunol. 5, 730–737. https://doi.org/10.1038/ni1087

Yu, J., Mookherjee, N., Wee, K., Bowdish, D.M.E., Pistolic, J., Li, Y., Rehaume, L., Hancock, R.E.W., 2007. Host defense peptide LL-37, in synergy with inflammatory mediator IL-1beta, augments immune responses by multiple pathways. J. Immunol. Baltim. Md 1950 179, 7684–7691.

Yu, K., Park, K., Kang, S.-W., Shin, S.Y., Hahm, K.-S., Kim, Y., 2002. Solution structure of a cathelicidin-derived antimicrobial peptide, CRAMP as determined by NMR spectroscopy. J. Pept. Res. Off. J. Am. Pept. Soc. 60, 1–9.

Yuk, J.-M., Shin, D.-M., Lee, H.-M., Yang, C.-S., Jin, H.S., Kim, K.-K., Lee, Z.-W., Lee, S.-H., Kim, J.-M., Jo, E.-K., 2009. Vitamin D3 Induces Autophagy in Human Monocytes/Macrophages via Cathelicidin. Cell Host Microbe 6, 231–243. https://doi.org/10.1016/j.chom.2009.08.004

Zanetti, M., 2005. The role of cathelicidins in the innate host defenses of mammals. Curr. Issues Mol. Biol. 7, 179–196.

Zasloff, M., 2002. Antimicrobial peptides of multicellular organisms. Nature 415, 389–395. https://doi.org/10.1038/415389a

Zasloff, M., 1987. Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc. Natl. Acad. Sci. U. S. A. 84, 5449–5453.

Zhdanov, D.D., Fahmi, T., Wang, X., Apostolov, E.O., Sokolov, N.N., Javadov, S., Basnakian, A.G., 2015. Regulation of Apoptotic Endonucleases by EndoG. DNA Cell Biol. 34, 316–326. https://doi.org/10.1089/dna.2014.2772

Zhu, Z., Tang, W., Gwaltney, J.M., Wu, Y., Elias, J.A., 1997. Rhinovirus stimulation of interleukin-8 in vivo and in vitro: role of NF-kappaB. Am. J. Physiol. 273, L814-824.

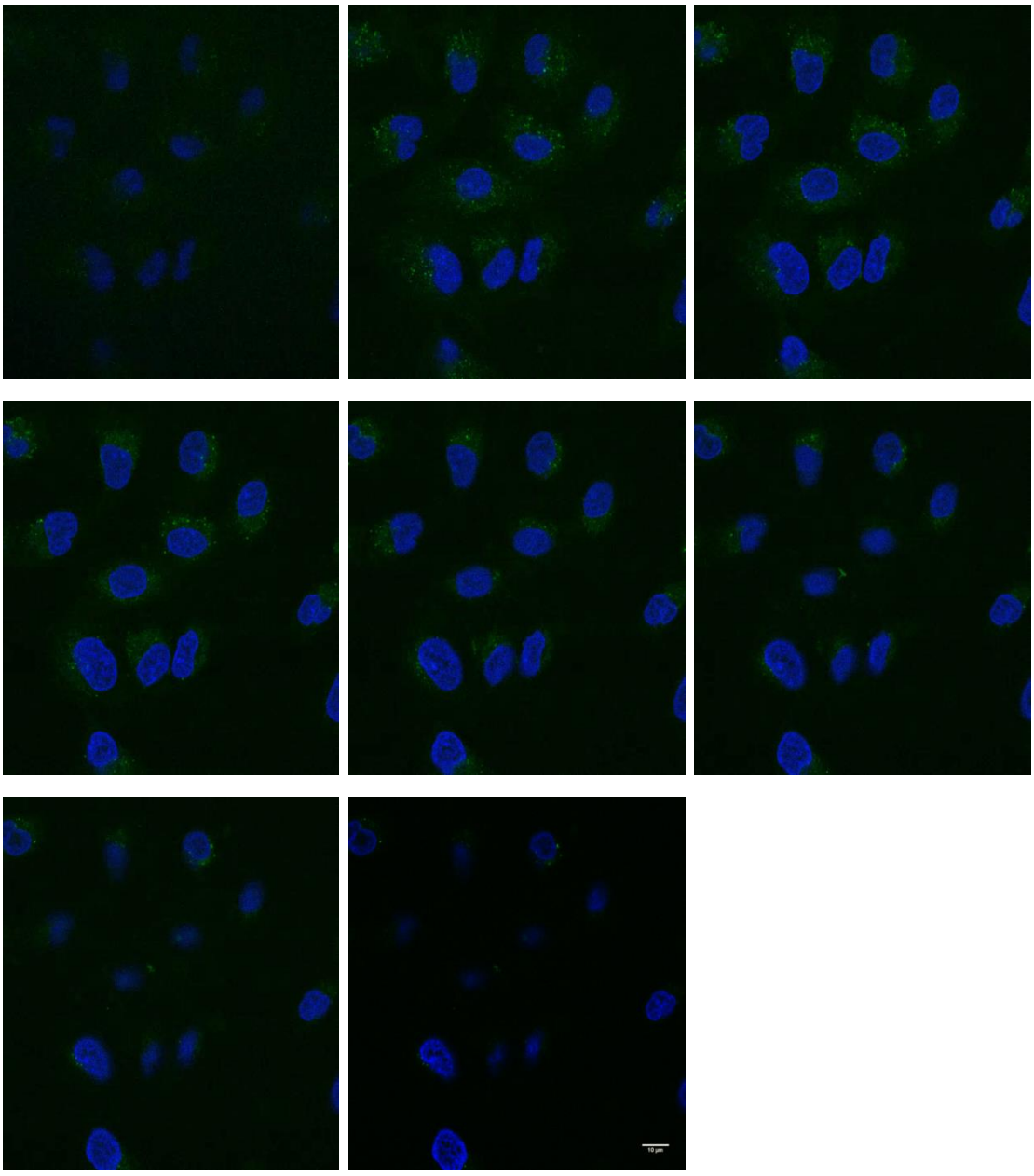
Zhu, Z., Tang, W., Ray, A., Wu, Y., Einarsson, O., Landry, M.L., Gwaltney, J., Elias, J.A., 1996. Rhinovirus stimulation of interleukin-6 in vivo and in vitro. Evidence for nuclear factor kappa B-dependent transcriptional activation. J. Clin. Invest. 97, 421–430. https://doi.org/10.1172/JCI118431

Zlotnik, A., Morales, J., Hedrick, J.A., 1999. Recent advances in chemokines and chemokine receptors. Crit. Rev. Immunol. 19, 1–47.

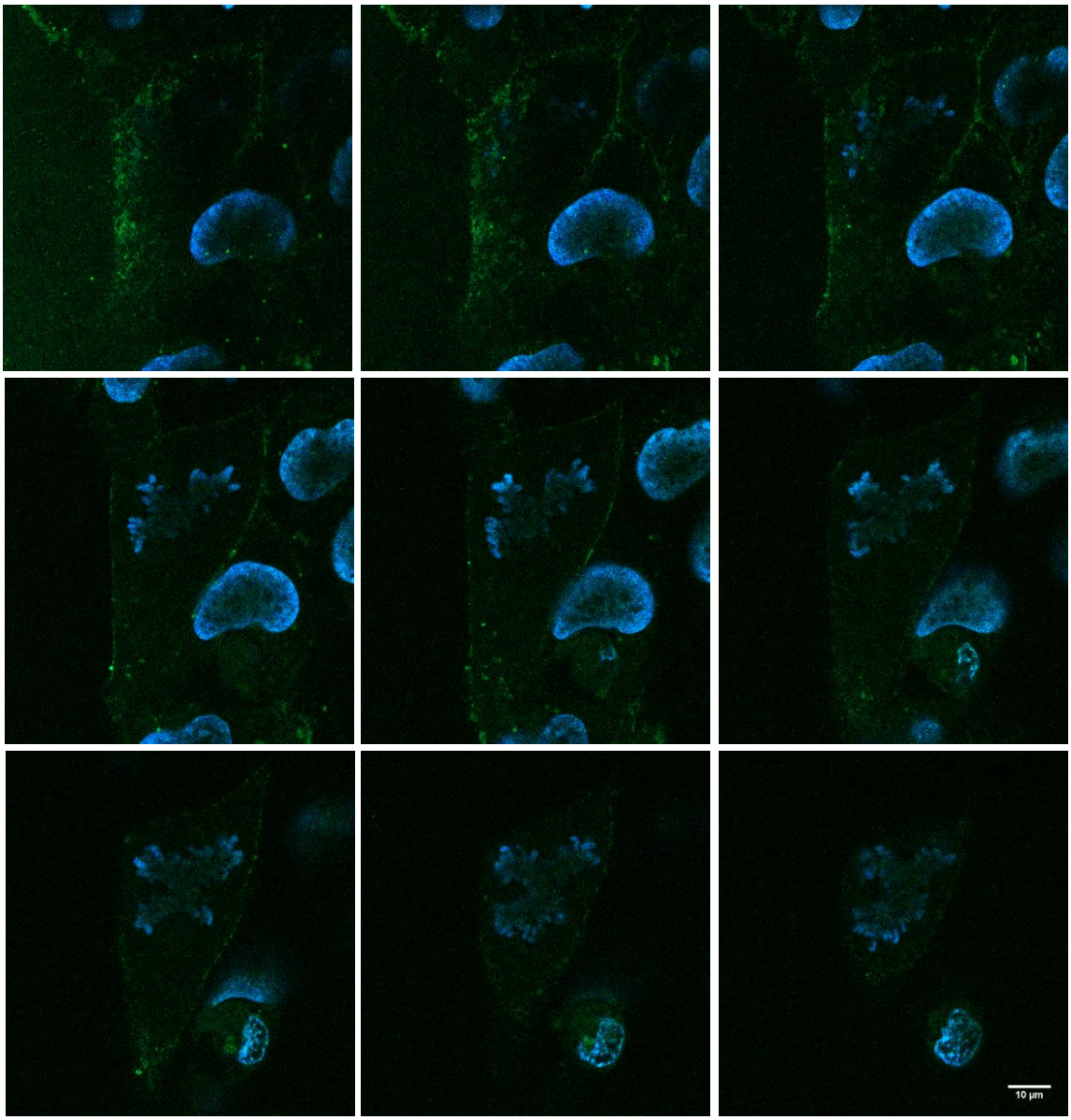
Zughaier, S.M., Svoboda, P., Pohl, J., Stephens, D.S., Shafer, W.M., 2010. The Human Host Defense Peptide LL-37 Interacts with Neisseria meningitidis Capsular Polysaccharides and Inhibits Inflammatory Mediators Release. PLOS ONE 5, e13627. https://doi.org/10.1371/journal.pone.0013627

APPENDIX

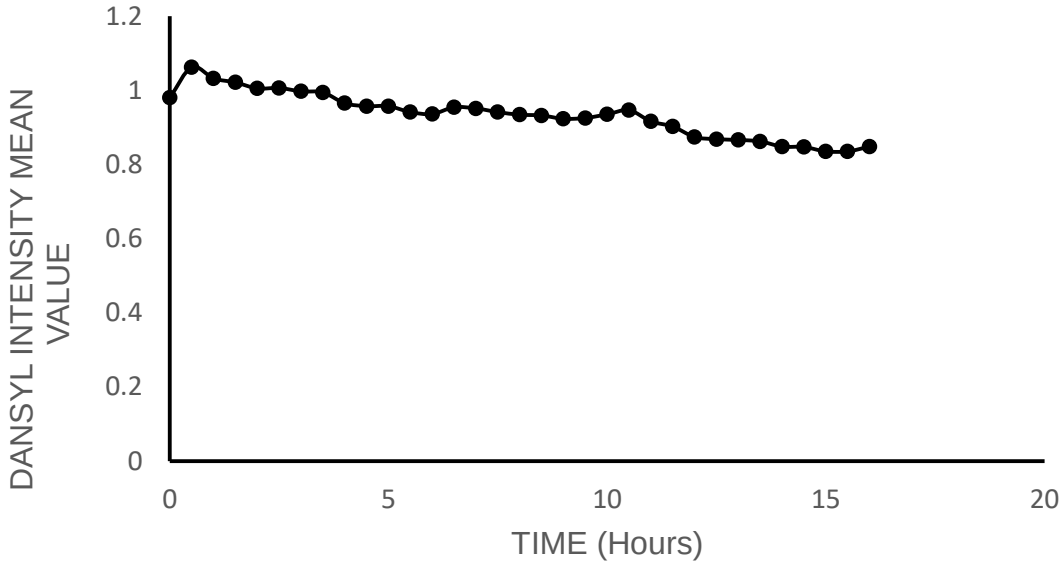
1. Intracellular localization of DANSYL-labelled LL-37 in A549 cells (Z-stack analysis)



2. Intracellular localization of DANSYL-labelled LL-37 in 16HBE14^{o-} cells (Z-stack analysis)

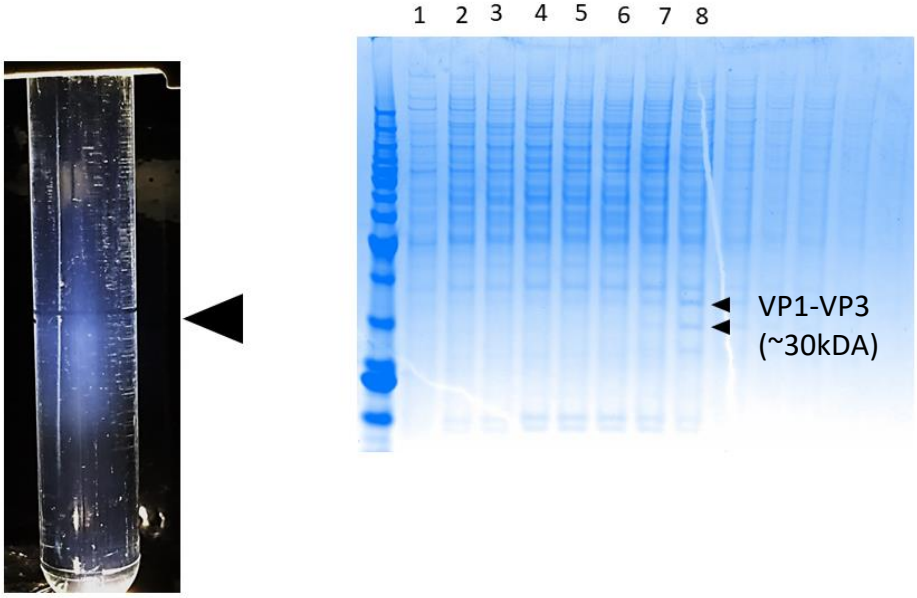


3. **Fluorescence intensity of DANSYL-LL-37 through time.**

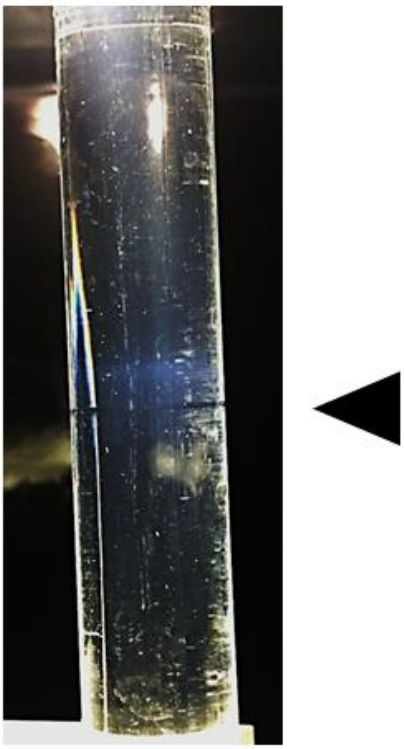


A549 cells were treated with DANSYL-labelled LL-37 (10µg/ml) for 30 minutes, washed with saline and left in contact with 5% FBS DMEM for further 16h. Live-cell imaging was set up using confocal microscopy immediately after the addition of fresh media (5% FBS DMEM) and images were taken every 2 minutes for 16 hours. The graph represents the DANSYL intensity mean value of images taken every 0.5 h up to 16h.

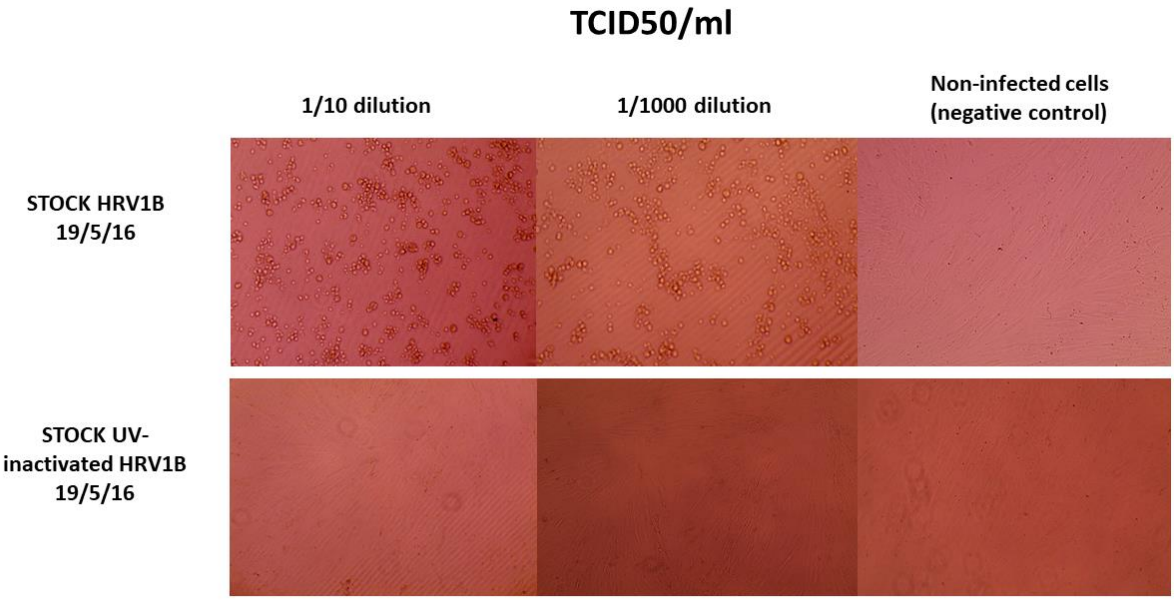
4. **SDS-PAGE performed with virus samples obtained after the first sucrose gradient purification**



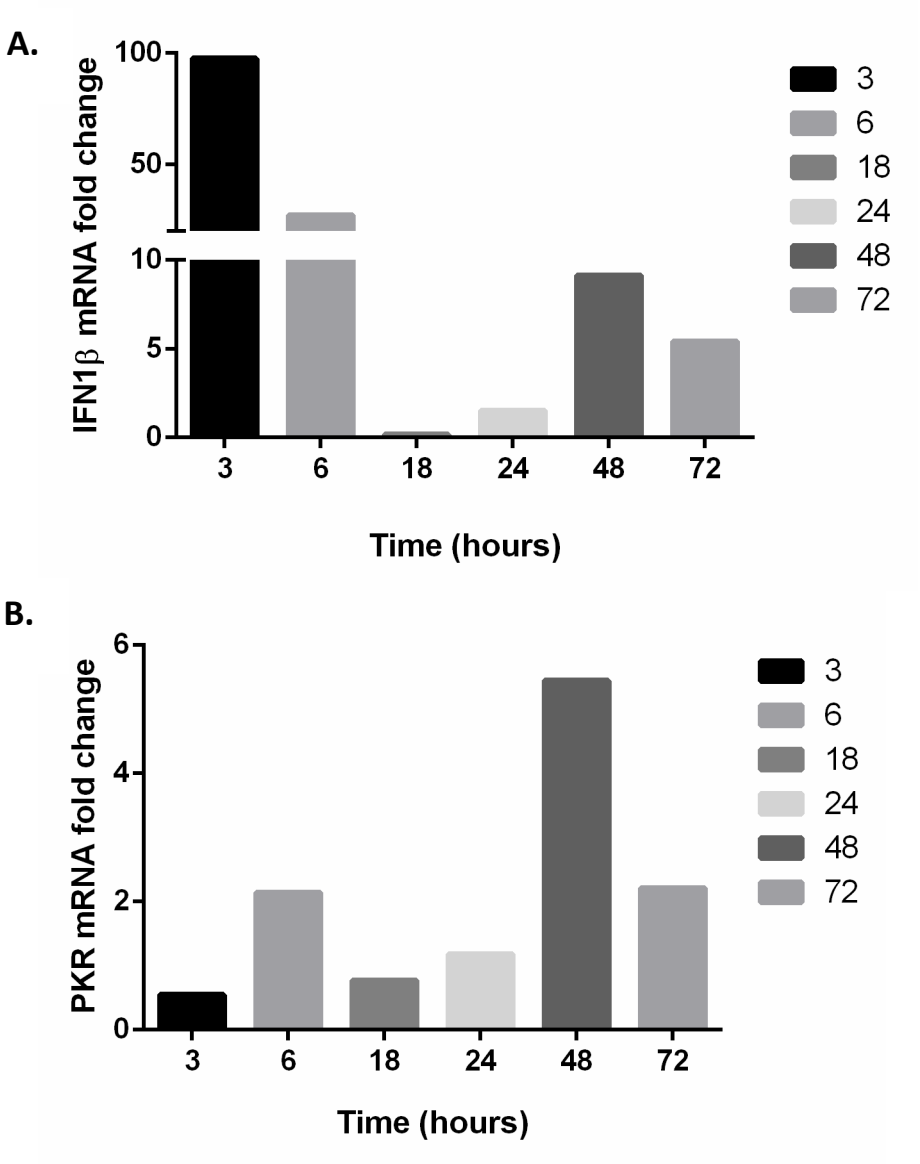
5. Second sucrose gradient purification



6. UV inactivation of HRV1B

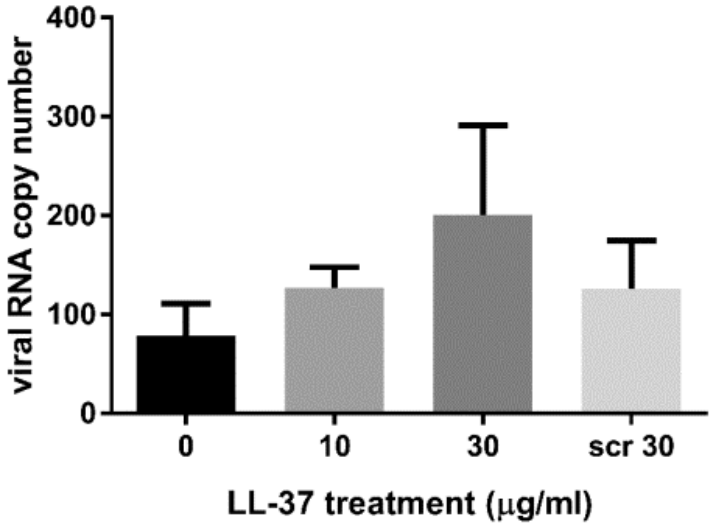


7. Time-course analysis of IFN1β (A) and PKR (B)gene expression in 16HBE14^{o-} treated with poly I:C (10µg/ml)



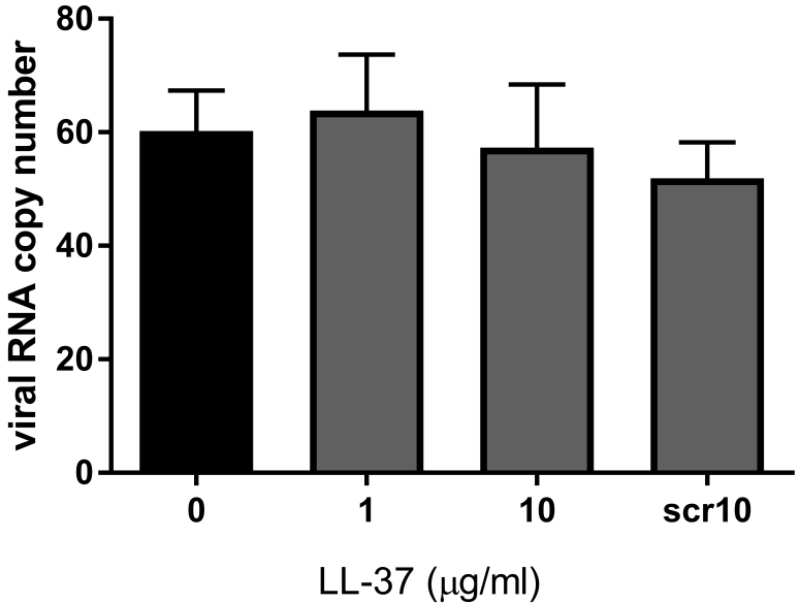
Bronchial 16HBE cells were exposed to poly I:C (10µg/ml) for 2h washed and re-immersed in IMDM supplemented with 5%FBS for 1, 4, 16,22,46 or 70h at 33^{o}C. Total RNA was extracted from 16HBE cell lysates and qPCR was performed to determine the relative expression of INF1β (A), and PKR (B). The GAPDH and β-actin genes were used as reference genes. Results represent one independent experiment.

8. Viral RNA copies extracted from 16HBE14^{o-} cell lysates



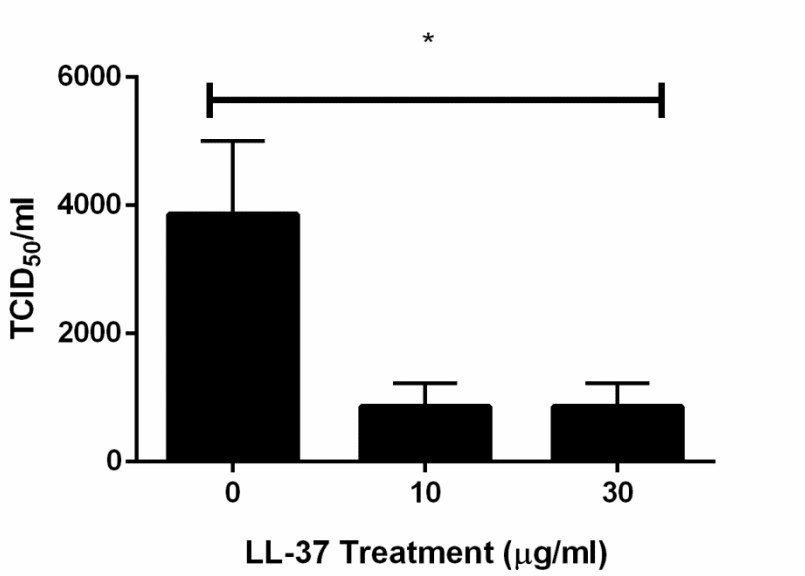
Bronchial epithelial cells (16HBE14^{o-}) were infected with HRV1B (MOI 1) or left unstimulated in serum-free media for 2H, washed and re-immersed in fresh maintenance media for further 22h. Cells were treated with LL-37 for 2h, washed and re-immersed in fresh maintenance media for further 22h at 33^{o}C. After 48h, cells were lysed and viral RNA copies were determined by qPCR. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-test to compare virus only to LL-37 treated cells.

9. Viral RNA copies extracted from 16HBE14^{o-} cell lysates



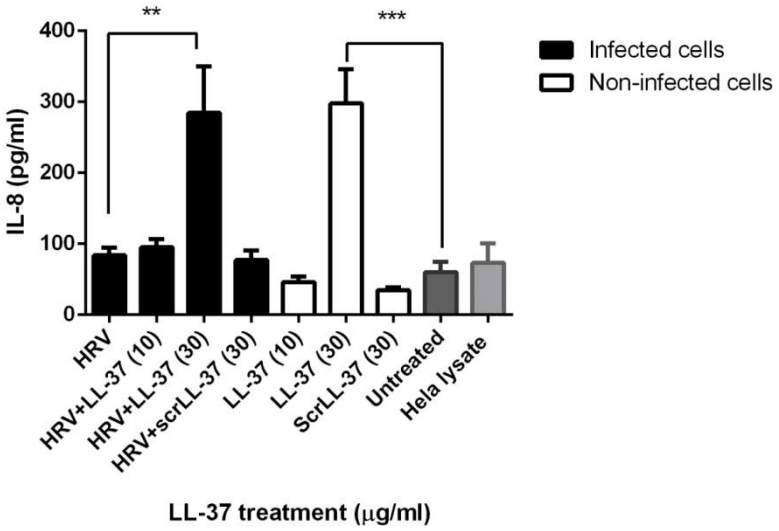
Bronchial epithelial cells (16HBE14^{o-}) cells were left untreated or infected with purified HRV1B (MOI 1) for 2h in serum-free media, washed and re-immersed in media supplemented with 5% FBS for 22h at 33^{o}C. Cells were washed and treated with LL-37 (1 and 10μg/ml) for 24h in media supplemented with 1% Ultroser G. After 48h, cells were lysed and viral RNA copies were determined by qPCR. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-test to compare virus only to LL-37 treated cells.

10. Virus infectious titer determined by $TCID_{50}$ assay in 16HBE14^{o-} cells



Bronchial epithelial cells (16HBE14^{o-}) were infected with HRV1B (MOI 1) or left unstimulated in serum-free media for 2H, washed and re-immersed in fresh maintenance media for further 22h. Cells were treated with LL-37 for 2h, washed and re-immersed in fresh maintenance media for further 22h at 33^{o}C. After 48h, cell supernatants were used to determine the infectious titer by $TCID_{50}$ assay. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-test to compare virus only to LL-37 treated cells (* $p \leq 0.05$).

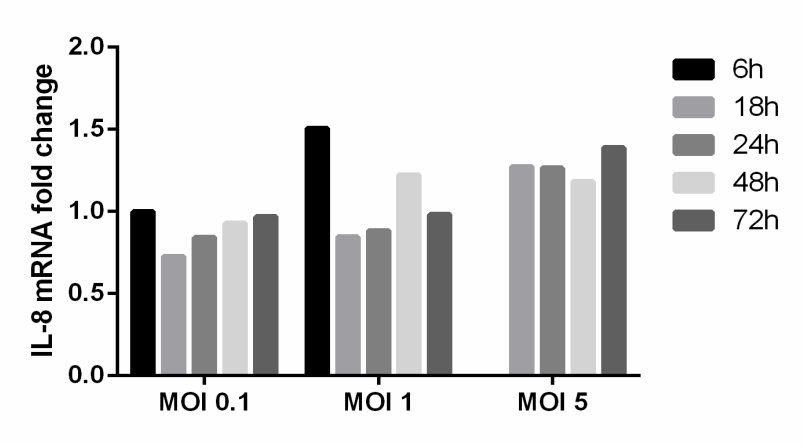
11. Inflammatory concentrations of LL-37 significantly enhance IL-8-release from rhinovirus-infected 16HBE14^{o-} cells.



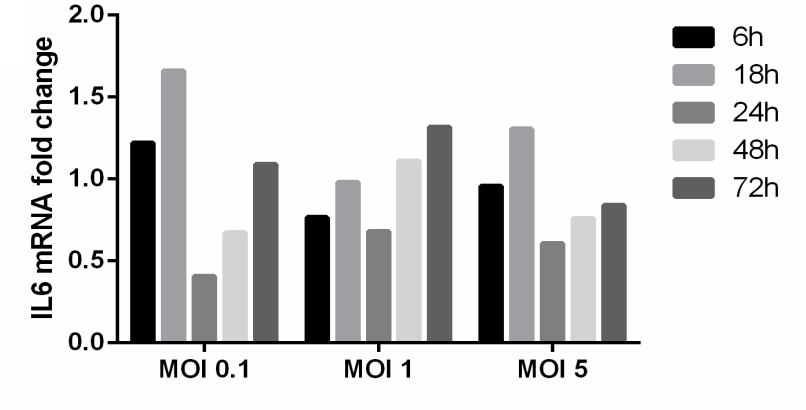
16HBE14^{o-} cells were left unstimulated (control) or were exposed to HRV (MOI 5) for 2 hours at 33^{o}C in serum-free media, washed with saline before incubating with LL-37 at concentrations indicated for 22 hours at 33^{o}C. IL-8 protein levels released into cell supernatants were assessed by ELISA. Results are represented as the means +/- SEM from four independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons post-test (**p<0.01; ***p<0.001).

12. Time-course analysis of IL-8 (A), IL-6 (B), CCL5 (C) and IFN1β (C) gene expression in A549 cells treated with HeLa Lysates

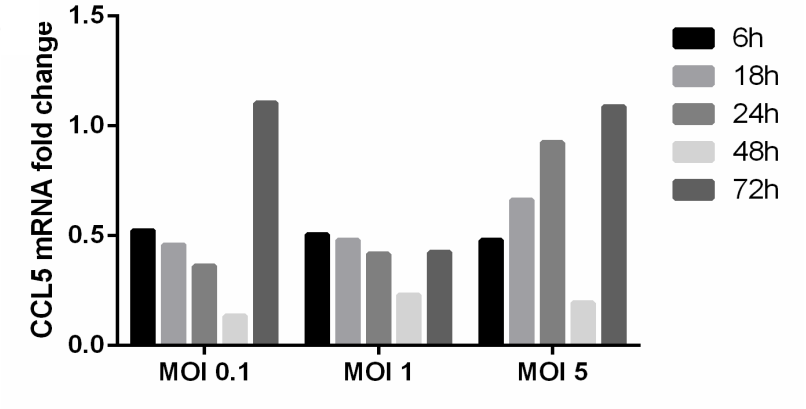
A.



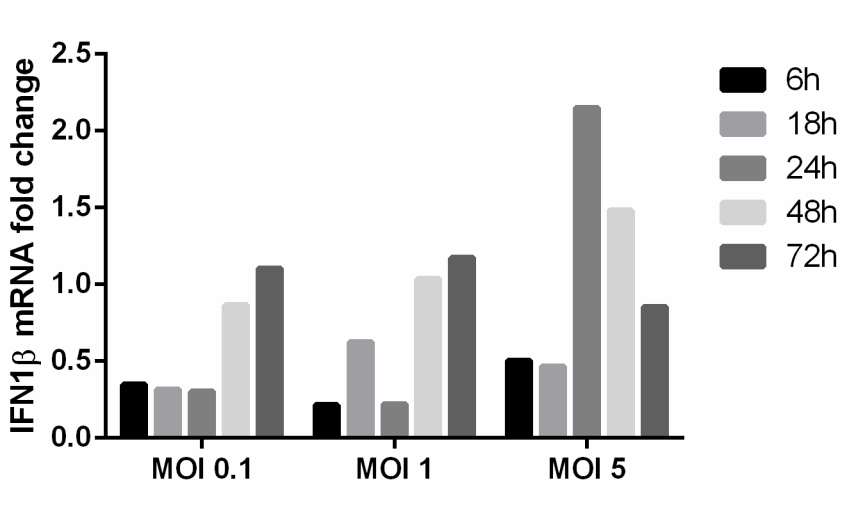
B.



C.



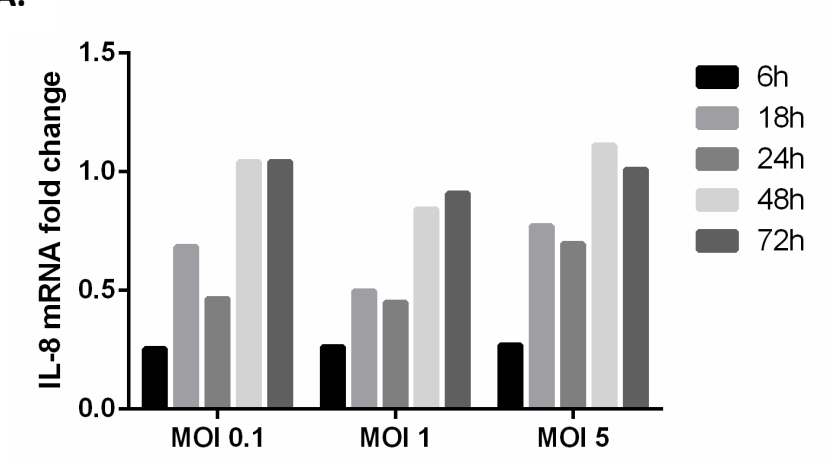
D.



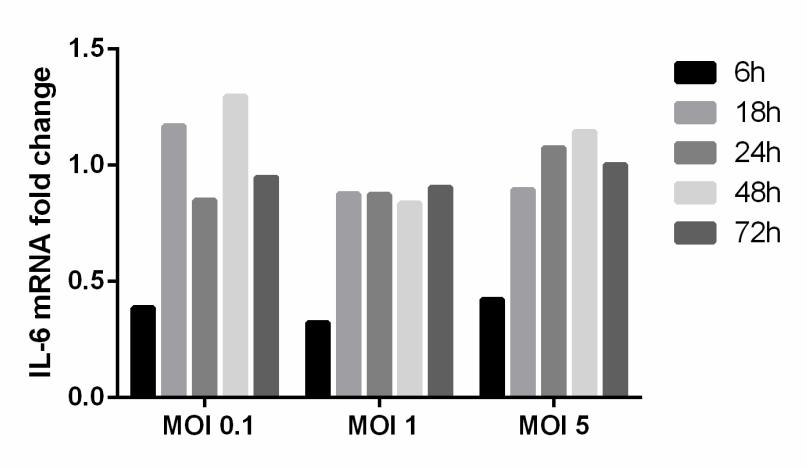
A549 cells were exposed to HeLa lysates for 2h, washed and re-immersed in fresh maintenance media for 4h, 16h, 22h, 46h or 72h prior to determination of gene expression by qPCR.

13. Time-course analysis of IL-8 (A), IL-6 (B), CCL5 (C) and IFN1β (C) gene expression in 16HBE14^{o-} cells treated with HeLa Lysates

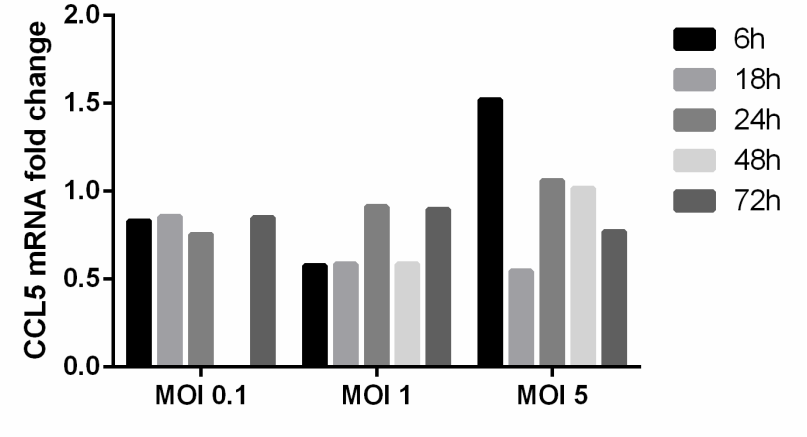
A.



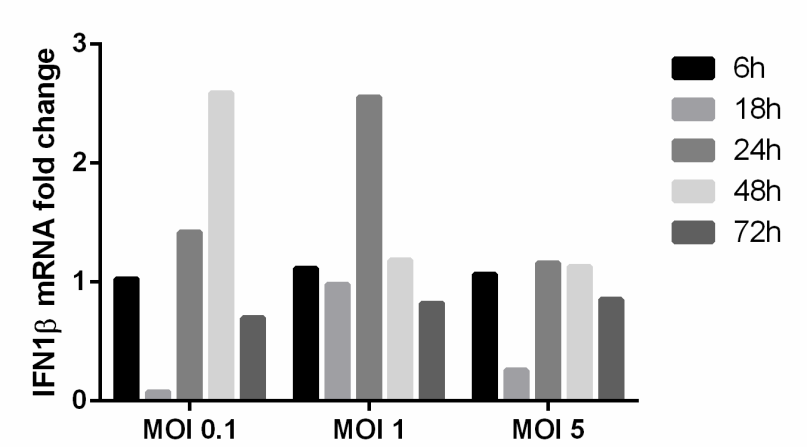
B.



C.



D.



16HBE14^{o-} cells were exposed to HeLa lysates for 2h, washed and re-immersed in fresh maintenance media for 4h, 16h, 22h, 46h or 72h prior to determination of gene expression by qPCR.