IL-36 and IL -37 cytokines, mediators or potential modulators of airway infection and inflammation?

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By

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Abstract

Respiratory tract infections caused by viruses or bacteria are among the most common human diseases worldwide. More specifically, respiratory syncytial virus (RSV) is a major cause of acute lower respiratory infection (ALRI), and this frequently results in hospitalisation in young children, particularly among those under the age of 5, and the elderly. Infection with RSV is also correlated with various airway respiratory diseases, such as bronchiolitis and asthma. In vivo testing suggests that levels of IL-36 alpha, IL-36 gamma, and IL-37 protein can be very high in nasopharyngeal aspirate (NPA) during RSV and rhinovirus (RV) infections. This work thus hypothesises that, during RSV and RV infections, the epithelial cells in the airways may express IL-36α, IL-36γ, and their respective receptors in a manner that plays a crucial pro-inflammatory role in RSV and RV infections. In addition, this work hypothesises the possibility of the further expression of IL-36Rα and IL-37 cytokines by airway epithelial cells (AECs) in response to RSV and RV infections. The main aim of this work, as described in this thesis, is thus to investigate whether RSV infection can trigger AECs to express IL-36 and IL-37 cytokines and their receptors.

IL-36α, IL-36γ, and IL-37 proteins were measured in NPAs obtained from PCR positive RSV or RV infected infants under the age of 5. The results were classified by disease severity, patient age, and oxygen requirements during hospital admission based on both RSV and RV patient samples. Human adult nasal airway epithelial cells (HNAECs) and A549 and BEAS-2B cultured AEC cells were used to characterise the expression of IL-36α, IL-36γ, and IL-36Rα cytokines and the relevant IL-36 receptors (IL-1RL2 and IL-1Rap) following RSV infection. The expression of IL-37 cytokine and its receptors (IL-18Ra and IL-1R8) was also examined in HNAECs and A549 and BEAS-2B. This work then extended this investigation to determine whether other pro-inflammatory cytokines, including IL-1β, TNF-α, IL-17, IL-22, and IFNγ, might influence the expression of IL-36γ in A549 cells during RSV infections, or whether there is any combined effect of these cytokines on the expression of IL-36γ. LPS was also examined with respect to the expression of IL-36γ cytokines in infected or non-infected cells.

The levels of IL-36α protein were significantly higher than those of IL-36γ protein in NPAs from RSV and RV infected infants, though IL-36α, IL-36γ, and IL-37 were all seen in significantly higher proportions in NPAs from RV infected infants than in those from infants with RSV. RSV infection, based on HNAECs and cultured bronchial epithelial cell lines (A549 and BEAS-2B), induces

increased expression of IL-36α, IL-36γ, and IL-36Rα mRNA. In both HNAECs and cultured cell lines (A594 and BEAS-2B), IL-36γ was seen to be the most upregulated IL-36 cytokine. It is also worth nothing that the IL-36 protein requires ultracentrifugation to enable its detection within the supernatant of infected HNAECs and cultured cell lines (A594 and BEAS-2B), while xo-stimulation with ATP is required for optimal secretion of IL-36γ from RSV infected cells. There was, nevertheless, a significant expression of IL-37 in both HNAECS and cultured cell lines (A594 and BEAS-2B).

With regard to IL-37 receptors, the results showed significantly increased IL-18R1 mRNA expression in both HNAECs and culture cell lines A549 and BEAS2B. In addition, there was a significant increase in the expression of IL-1R8 mRNA in infected A549 and BEAS-2B cells; however, this was not found in HNAECs. The stimulation of A549 cells with either IL-1β or TNF-α played a significant role in the expression of IL-36γ mRNA, though the results showed no effects from these cytokines with respect to the expression of IL-36γ mRNA in RSV infected cells. The combination of IL-1β and TNF-α with other cytokines such as IL-17, IL-22, IFNγ, IFNβ and IL-4 also did not affect the expression of IL-36γ cytokine by A549 cells.

Overall, the expression of IL-36 cytokines, and particularly the IL-36γ cytokine, by AECs was found to significantly increase as a response to RSV infection. IL-37 cytokines and their receptors were also expressed by airway epithelial cells in response to RSV infection, with the exception of IL-1R8 in HNAECs. These findings support the idea of a regulatory role for cytokines in terms of limiting the immune response induced by RSV infection. IL-1β and TNF-α cytokines may thus increase the expression of IL-36γ cytokines by A549 cells, although they appear to have no effect when combined with other cytokines.

Declaration

I declare that the work presented in this thesis was carried out by myself unless stated in the Department of Women and Children's Health, Institute of Child Health, Institute in the Park's Wolfson Laboratory, University of Liverpool.

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Dad are you hearing me? I did it. It was hard after your leaving. Thank you, Dad, for your support, love, for all the nice memories in my mind and my heart. Dad, my husband is amazing, brave, and supportive, he helped me for doing this. Thank you my love for everything you did for me. For my kids, one day you will read this, I did all of this for you, thank you for being nice all the time, for the everyday hugs, We did it.

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A549 Adenocarcinomic human alveolar basal epithelial cell line

aBA allergic bronchial asthma

ACD Allergic contact dermatitis

AEC Airway epithelial cell

AJC apical junctional complex

APRIL proliferation-inducing ligand

AR Allergic Rhinitis

ARI Acute respiratory illness

ARDS Acute respiratory distress syndrome

ASL airway surface liquids

ATP Adenosine triphosphate

B cell B lymphocyte

BAFF B cell activating factor

BAL Bronchoalveolar lavage

BEAS-2B Human bronchial epithelium cell line

BEGM Bronchial epithelium growth medium

BSA Bovine Serum Albumin

BMDCs Bone marrow Dendritic Cells

BoV Boca virus

Cat Cathepsin

CCL Chemokine (C-C motif) ligand

CD Cluster of differentiation

cDCs conventional DCs

cDNA Complimentary deoxyribonucleic acid

CHB Chronic hepatitis B

CHD congenital heart disease

CIA Collagen-induced arthritis

CLRs C-type lectin receptors

COPD Chronic obstructive pulmonary disease

Covid-19 Corona virus

CRS Chronic Rhinosinusitis (CRS

CXC C-X-C motif chemokine

CX3CR1 CXC chemokine receptor 1

CXCL8 (IL-8) Chemokine (C-X-C motif) ligand 8

CXCL9 (MIG) Chemokine (C-X-C motif) ligand 9

CXCL10 (IP-10) Chemokine (C-X-C motif) ligand 10

CXCL11 (I-TAC) Chemokine (C-X-C motif) ligand 11

DAPI 4',6-diamidino-2-phenylindole

DC Dendritic cell

DMEM Dulbecco's Modified Eagle Medium

dNTP Deoxyribonucleotide triphosphate

ds Double stranded

DSS Dextran sodium sulfate

ECP eosinophil cationic protein

EDN eosinophil-derived neurotoxin

EDTA Ethylene diamine tetra acetate

ELISA Enzyme-linked immunosorbent assay

ERK extracellular signal regulating kinase IKK

FBS Fetal bovine serum

FI-RSV formalin-inactivated RSV

HBEC Human bronchial airway epithelial cells

HBV Hepatitis B virus

HCAECs Human coronary artery endothelial cells

HCV Hepatitis C virus

HEp-2 Human epithelial type 2

HLA Human leukocyte antigen

HNAEC Human nasal airway epithelial cells

HMPV human metapneumo virus

IBD Inflammatory bowel disease

ICAM-1 Intercellular adhesion molecule-1

IFN Interferon Ig Immunoglobulin

IKK IκB kinase

IRF Interferon regulatory factors

KCl Potassium chloride L

Kp Klebsiella pneumoniae

LILRB1 leukocyte immunoglobulin-like receptor B1

LL Lepromatous leprosy

LPS Lipopolysaccharide

LRTIs Lower respiratory tract

MAVS Mitochondrial antiviral-signaling protein

MCP Monocyte chemoattractant protein

MFI Mean fluorescence intensity

MIP Macrophage Inflammatory Proteins

MAPK mitogen-activated protein kinase

MBP major basic protein

mDCs myeloid DCs

MIF macrophage inhibitory factor

MIP-1 α macrophage inflammatory protein-1α

MHC Major Histocompatibility Complex

MP Microparticle

MOI Multiplicity of infection

mRNA Messenger RNA

MyD88 Myeloid differentiation primary response 88

NaCl Sodium chloride

NAL nasal lavage

ng Nanogram

NF-kB Nuclear factor kappa-light-chain-enhancer of activated B cells

NK Natural killer

NPA Nasopharyngeal aspirate

NLRs NOD-like receptors

NOD2 Nucleotide-binding oligomerization domain-containing protein 2

OD Optical Density

PAMP Pathogen-associated molecular patterns

PAH pulmonary arterial hypertension

PARs protease-activated receptors

PBMC Peripheral blood mononuclear cells

PBS Phosphate buffered saline

PCR Polymerase chain reaction

pDCs plasmacytoid DCs

PFU Plaque-forming units

pg Picogram

PMs Pulmonary macrophages

PRR Pattern Recognition Receptors

qPCR Quantitative polymerase chain reaction

RA Rheumatoid arthritis

RANTES Chemokine (C-C motif) ligand 5 (CCL5)

RIG-I Retinoic acid inducible gene

RLRs RIG-I-like receptors

rpm Revolutions per minute

RPMI Roswell Park Memorial Institute medium

RSV Respiratory syncytial virus

RT-PCR Real time polymerase chain reaction

RV Rhinovirus

SEM Standard error of the mean

SH Small hydrophobic protein

SEMFs sub epithelial myofibroblasts

SLE Systemic lupus erythematosus

SNPs Single-nucleotide polymorphisms

Sp Streptococcus pneumoniae

TB Tuberculosis diseases

TCM T central memory cell

TEM T effector cell

TGF Transforming growth factor

Th1 T cell helper 1

Th2 T cell helper 2

TLR Toll like receptor

TNF Tumor necrosis factor

TRAM (TRIF)–related adaptor molecule

TRIF TIR-domain-containing adapter-inducing interferon-β

TSLP Thymic stromal lymphopoietin

µg Microgram

µl Microliter

URTIs Upper respiratory tract infections

WD-AECs well-differentiated human airway epithelial cells

WHO World Health Organization

Chapter 1. Introduction

Globally, respiratory diseases are associated with significant morbidity and mortality in children less than five years of age worldwide including the viruses and bacteria infections. Recently, a novel coronavirus (SARS-CoV-2), caused an outbreak of acute respiratory infections that resulted in global social and economic disruption. In January 2020, the World Health Organization declared the outbreak, known as COVID-19, to be a Public Health Emergency of International Concern, upgrading this designation to a pandemic in March 2020. As of 5 February 2021, more than 105 million cases were confirmed, with more than 2.29 million deaths attributed to COVID-19.

Respiratory syncytial virus (RSV) is one of the major causes of acute respiratory infection (ARI), and it thus frequently results in hospitalisation, as well as occasionally death, in both the elderly and young children aged 5 and under (Nair *et al*., 2010; Shi *et al*., 2017). RSV causes acute bronchiolitis in various upper and lower respiratory tract infections. The risk of severe bronchiolitis and its related complications is also higher among preterm infants with severe congenital heart disease (CHD), and immunological disorders. Bronchiolitis is an infection of the lower respiratory tract most commonly found among new-borns (American Academy of Paediatrics, 2006; Cincinnati Children's Hospital Medical Centre, 2006), and the annual incidence of bronchiolitis in new-borns is 10% (Callen *et al.,* 2009), leading to an admission rate of between 2% and 5%; this has (Callen *et al.,* 2009; Scottish Intercollegiate Guidelines Network., 2006), however, been subject to substantial increases in recent years. Rhinovirus (RV) and respiratory syncytial virus (RSV) are also the predominant viruses linked to the development of chronic airway inflammatory diseases (Jartti and Gern, 2017).

Innate immune responses in the lung serve as a first line of defence against infection by respiratory viruses. Respiratory viral infections induce a variety of inflammatory cytokines, chemokines, and immunoregulatory cytokines that interact with host proteins to affect viral replication, immune regulation and induce programmed death (Short *et al.,* 2014). The importance of the innate immune response in respiratory viral disease was to shape the adaptive immune response through the production of immune mediators. Early cytokine and chemokine expression induce trafficking of immune cells such as macrophages, eosinophils, neutrophils and

NK cells to the lungs, leading to the activation of B and T cells. The importance of innate immunity is critical in infants, in whom the immune system is still developing and often lack immunologic memory. Impaired innate immune responses may lead to slow and inadequate viral clearance, enhanced pathology and greater disease severity during the acute disease, with possible long-term consequences. Further, impaired innate immune responses lead to inadequate adaptive immune responses, poor immunological memory and recurrent infections.

The work described in this thesis focuses on how the innate immunity of the airway epithelium responds to RSV infection by expressing the pro-inflammatory cytokine IL-36 and the immunoregulatory cytokine IL-37. These cytokines were measured in respiratory secretions from infants with RSV and RV disease. Expression of these cytokines and their receptors is investigated in virus-infected respiratory epithelial cell line cultures and primary airway epithelial cell cultures. Finally, the influence of other pro-inflammatory cytokines and immune stimulators on airway epithelial cell IL-36 expression is investigated.

1.1. Human respiratory syncytial virus

1.1.1 Epidemiology

RSV is a common global cause of respiratory infections in infants and children (Hall *et al.,* 2013). Such infection is characterised by wheezing, coughing, and fever. There is a strong association between a history of RSV infections and increased prevalence of asthma in later childhood (Sigurs *et al.,* 2005). In addition, RSV can cause severe upper respiratory tract infections, especially in infants, but RSV commonly presents in young children as bronchiolitis, a lower respiratory tract illness with small airway obstruction, and can rarely progress to pneumonia, respiratory failure, apnea, and death (Jain *at al*., 2022).

Worldwide, RSV is the second largest cause of death in children under one year of age. In 2017, the World Health Organization (WHO) estimated that RSV causes around 33 million severe respiratory infections, more than 3 million hospitalisations, and nearly 60,000 deaths in children under 5 years of age, annually and around half of these hospitalisations and deaths are among children under 6 months of age (Shi *et al.,* 2017).

Bronchiolitis is responsible for around 1 in 6 of all UK paediatric admissions in the winter months, and it is estimated that over 30,000 babies and children aged under 5 are hospitalised every year in the UK because of RSV infections (Public Health, England). Around one in twenty, (6%), of these hospitalisations require admission to intensive care. Most of these hospital admissions occur in otherwise healthy babies. However, babies with congenital heart or lung disease or delivered prematurely, are at high risk of developing more severe RSV disease. RSV thus causes the deaths of around 30 babies a year in the UK. This virus also carries a significant economic burden. In the UK. This virus cost of healthcare for children younger than 5 years of age due to RSV infection has been estimated at £50 to 57 million annually, with around £37 million of this attributed to children requiring hospitalization (Barr *et al.,* 2019). These hospitalisation costs are nearly equally divided between infants less than 6 months old and those aged 6 months to 5 years old (Cromer *et al.,*2017). However, annual costs related to GP consultations alone are estimated to be in the range of £16 to 19 million (Cromer *et al.,* 2017).

In the UK, RSV seasons normally occur during the winter months, from October to March (Public health England, 2013). Before the COVID pandemic, RSV infections would tend to peak between late November and the middle of January (White *et al.,* 2005). Figure 1.1.1 shows the seasonal associations of RSV in England and Wales, based on national laboratory surveillance data. In contrast, RSV spread in tropical areas tends to occur during the hot, wet days of the summer season (Al-Toum *et al.,* 2006).

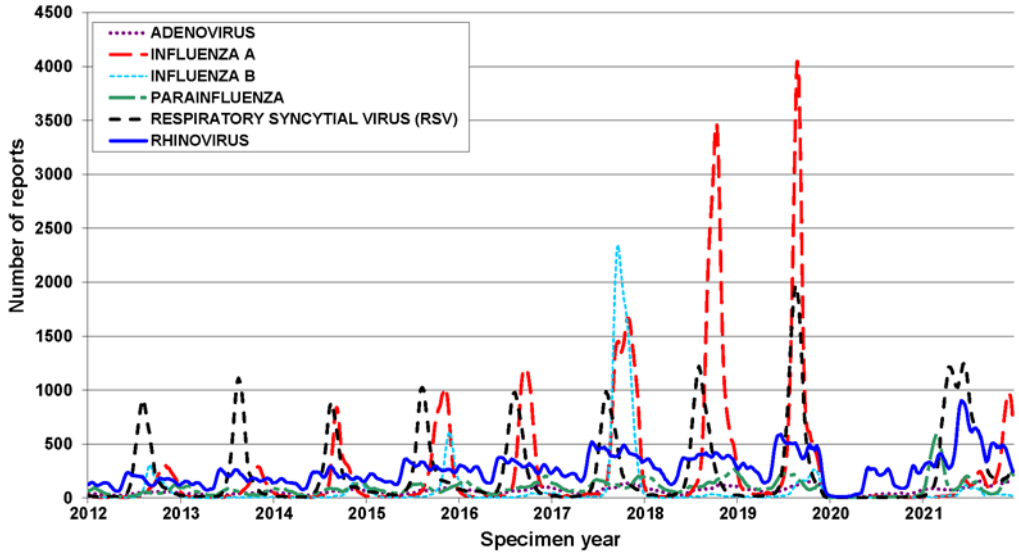

Figure 1.1.1. Weekly distribution of major viruses in England and Wales. Data is collected from Public Health England and NHS laboratories surveillance from 2012 to 2022 and shows a 3-week moving average. Lines represent adenovirus (purple), influenza A (red), influenza B (aqua), parainfluenza (green), RSV (black) and RV (blue). (Reproduced from Public Health England, 2022).

As alluded to above, the epidemiology of respiratory viral infections underwent significant changes following the emergence of COVID-19, with the number of RSV cases decreasing significantly. This has been attributed to the decrease in travel, and the use of additional precautionary hygiene and infection control measures (Wagatsuma *et al.,* 2021). Zheng *et al.* stimulated using mathematical models annual epidemics of RSV before and after the COVID-19

pandemic in New York and California to explore associations between factors such as duration of maternal-derived immunity, mitigation strategies and the importation of external infections with respect to the dynamics of RSV epidemics. They found that among children aged below 1 year, the incidence of RSV hospitalizations was around 0.7 % annually in the 2021 and 2022 RSV seasons, which represented a increase around 0.35% annually in a typical RSV season (Zheng *et al.,* 2021).

Groves *et al.* conducted a population-based study in Canada to examine the effects of the COVID-19 pandemic on rates of seasonal respiratory infections. As with Zheng *et al.,* they found a dramatic decrease in cases of seasonal respiratory viruses, including RSV and RV, in 2020/2021. Their found that the levels of positive cases of RSV decreased to 0.0169 times pre-pandemic levels, while levels of enterovirus/rhinoviruses decreased to 0.531 times pre-pandemic levels. They attributed this decrease to multiple factors, including the implementation of multiple public health precautions such as social distancing and travel restrictions. The findings of the studies are thus consistent with the evidence in the literature regarding the roles of handwashing, wearing facemasks in public, border closures, and lockdowns with respect to reducing the impact of epidemic respiratory viruses (Groves *et al.,* 2021). These findings were also confirmed by the work of Varela *et al.,* who performed a prospective study in Brazil of RSV and influenza infections rates during the COVID-19 pandemic. They reported the complete absence of influenza and RSV in the study cohort, despite Brazil having well-documented influenza and RSV seasons every other year (Varela *et al.,* 2021). They concluded that measures to reduce SARS-CoV-2 transmission significantly impacted the spread of these other respiratory pathogens, and that these findings could contribute significantly to existing knowledge about the dynamics of virus spread, as well as potentially guiding therapeutic choices for a range of viruses.

Another nationwide registry-based study using data from the Finnish Infectious Disease Register was conducted to examine the epidemiology of RV among children during the COVID-19 pandemic. This study showed that the largest initial positive impact from public health restrictions was seen among children aged 0 to 4 years in weeks 14 - 22 of 2020. However, later on in 2020, incidence rates remained near baseline across all age groups. They concluded that although strict restrictions played a temporary role in decreasing infection rates, the looser restrictions adopted later during the pandemic barely changed the prevalence of RV among children (Kuitunen *et al.,* 2021).

1.1.2. Classification and structure

RSV was first isolated in 1956 from cultures taken from a laboratory chimpanzee (Blount *et al.,* 1956). One year after its initial isolation, RSV was confirmed in humans, and it was then shown to be the leading viral cause of severe respiratory illness in children worldwide (Collins *et al.,* 2008). RSV is a single-stranded RNA virus of negative genome polarity (15.2 kb). It is a member of the Orthopneumovirus genus belonging to the Pneumoviridae family (Amarasinghe *et al.,* 2017). Based on RSV's antigenic and genetic characteristics, it can be divided into two antigenic subtypes: RSV A and RSV B (Anderson *et al.* 1985; Oliveira *et al.* 2008). Both antigenic subtypes A and B can then be divided into genotypes based on their G gene variations (Johnson *et al.,* 1987).

RSV is an enveloped virus with a genome located in a helical nucleocapsid (McNamara *et al.,* 2002). The genome encodes 10 mRNA that can be translated into 11 proteins: two are non-structural proteins, while the remaining nine are structural proteins. The non-structural proteins direct viral replication within the infected host cell (Chatterjee *et al.,* 2017). There are three transmembrane surface glycoproteins: the fusion protein (F) the attachment protein (G), and a hydrophobic protein (SH). The composition of G protein is variable across the RSV A and B subtypes, whereas the F protein is conserved between the two strains (Welliver, 2003; Hacking and Hull, 2002). The G glycoprotein facilitates RSV attachment to the infected cell (Teng *et al.,* 2001), and previous studies have also demonstrated that the G protein interacts with the cell surface proteins of airway epithelial cells (AECs) such as CX3CR1 to promote cell binding (Tripp *et al.,* 2001; Jeong *et al.,* 2015).

RSV F protein is responsible for cell entry, based on it mediating fusion between the virus and the host plasma membrane of the cell (Gonzalez-Reyes *et al.,* 2001). The virus penetrates the plasma membrane of the host cell when the F protein binds to the ICM-1 of the cell (Behera *et al.,* 2001; Krzyzaniak *et al.,* 2013). However, the role of the SH protein in RSV replication and pathogenesis remains unclear, though previous studies have shown that the inflammatory cytokine TNF-α may be inhibited by SH protein, hereby enhancing viral replication *in vivo* (Li *et al.,* 2011). Overall, the functions of G and F proteins include regulating the initial stages of RSV infection: the target of the G protein is the ciliated cells of the airways, while the F protein directs viral penetration to the host plasma membrane (McNamara *et al.,* 2002).

One of RSV structural proteins essential in assembling RSV to produce infectious viral particles is the matrix protein (M). The M protein accumulates in the inner layer of the lipid bilayer and envelops the nucleocapsid. This nucleocapsid has a helix shape and is surrounded by four nucleocapsid proteins, identified as N, P, M2-1, and L (Lay *et al.* 2013). The N and P proteins are essential for transcriptional activity (Hacking & Hull 2002), while the L protein initiates viral transcription on infection, after which replication proceeds (Fearns & Collins 1999; Oshansky *et al.,* 2009). The M2-1 is a transcription elongation factor, whereas M2-2 regulates viral transcription (Hacking & Hull 2002). Notably, the non-structural proteins NS1 and NS2 alter host response to infection by interrupting interferon signalling via anti alpha and anti-beta activity (Spann, Tran & Collins 2005).

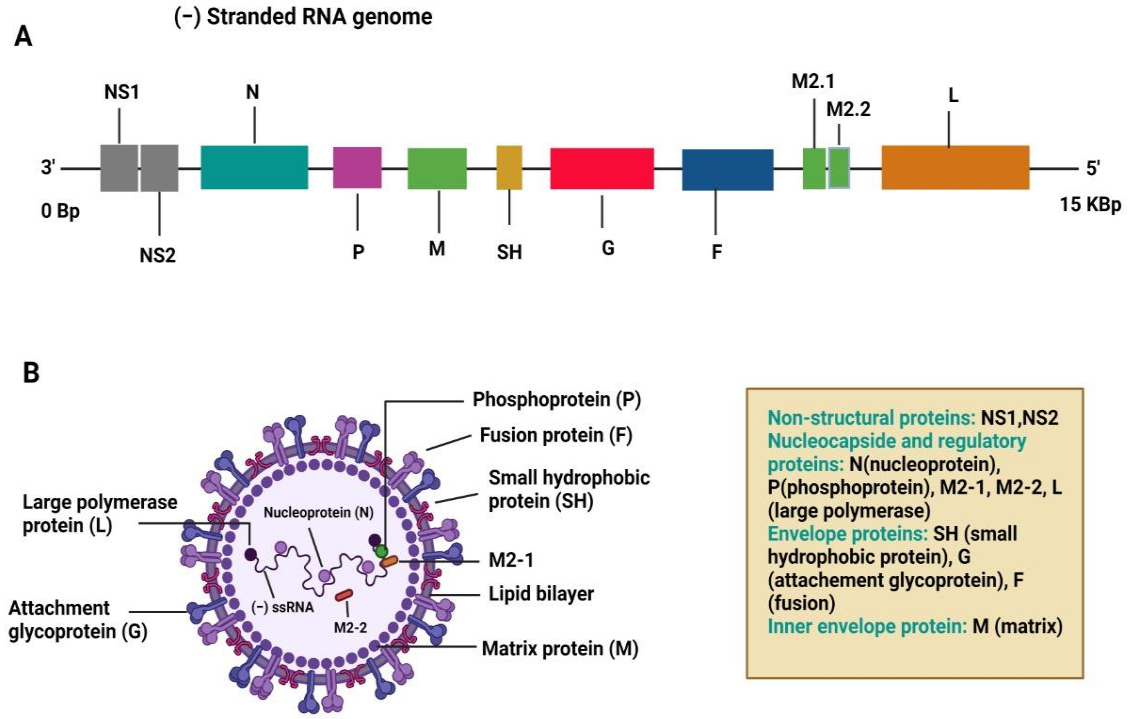

Figure 1.1.2. Structure and genome organization of RSV. (A) The genome of RSV is non-segmented RNA, negative sense, 15.2 kb, Nucleocapsid is surrounded by to structure proteins, including (N), (P), (M2-1), and (L), non-structural protein-1; and NS2, non-structural protein-2. (B) Cartoon diagram of RSV structure, shown the helical nucleocapsid which surrounded by M, matrix protein and an envelope containing host cell membrane and viral glycoproteins: G, attachment glycoprotein; F, fusion glycoprotein; and SH, small hydrophobic (Adapted from Nam and Ison., 2019).

1.1.3. Transmission and replication of RSV

RSV infection occurs either after inhalation of aerosolised particles or by hand to nose transmission after contact with contaminated surfaces. In the respiratory tract, RSV replicates over an incubation period of 3 to 5 days in epithelial cells in the upper respiratory tract before spreading to the lower respiratory tract (Bagga *et al.,* 2013; Gonzàlez *et al.,* 2018). Figure 1.1.3. shows the RSV replication cycle in the respiratory tract epithelial cells. The resulting high viral load in the lower respiratory tract can lead to the development of bronchiolitis, characterised by mucus production and oedema of the airway walls, as well as infiltration of the airways by various inflammatory cells, including neutrophils and lymphocytes (Collins & Graham 2008; Hall *et al.,* 2001; McNamara & Smyth 2002).

RSV is not cytopathic; however, the replication of the virus in apical ciliated airway epithelial cells can cause airway damage (Zhang *et al.,* 2002), although this is generally superficial. Such damage can, however, increase the host's susceptibility to bacterial infections (Damasio *et al.,* 2015), encourage apoptosis of ciliated epithelial cells, and increase goblet cell numbers, potentially leading to prolonged mucus overproduction (Villenave *et al.,* 2012; Jumat *et al.,* 2015). Moreover, RSV has been shown to infect non-ciliated bronchial, bronchiolar, and alveolar epithelial cells (Persson *et al.,* 2014), as well as non-ciliated epithelium cells, intraepithelial DCs, and alveolar macrophages in some cases (Johnson *et al.,* 2006).

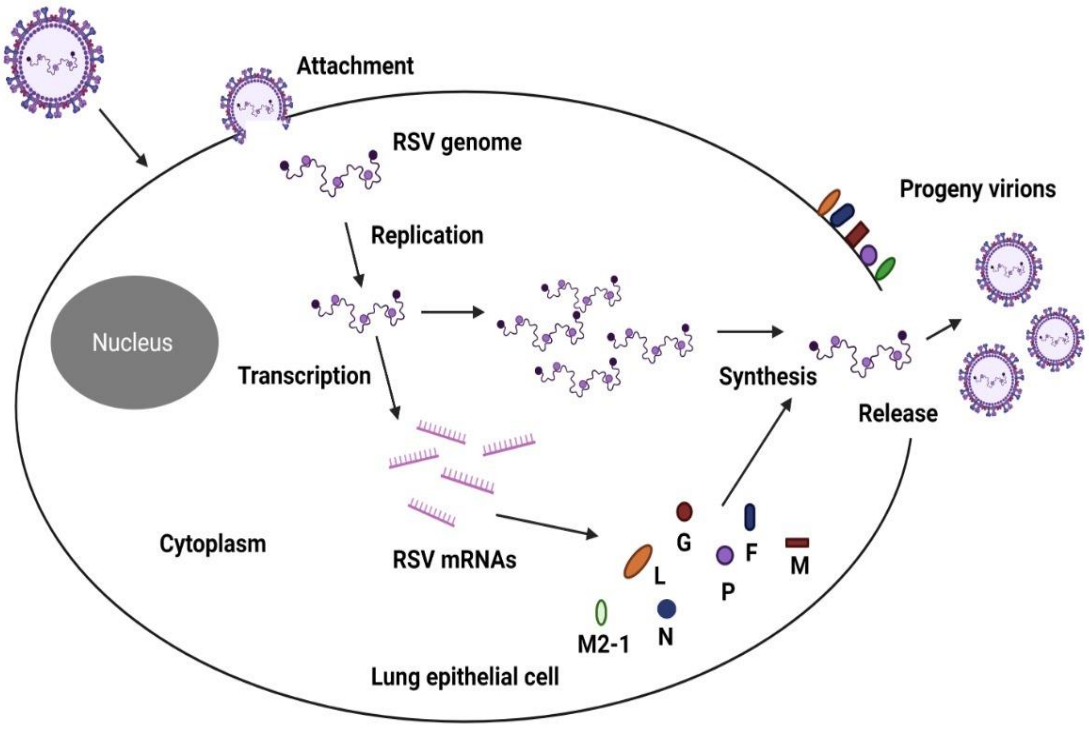

Figure 1.1.3. The RSV replication cycle. The virus enters to the host cells through G protein, the fusion (F) protein mediates entry of the nucleocapsid into the cytoplasm, releasing the encapsidated genome RNA (blue) which serves as a template for the RSV polymerase complex. The genome is transcribed into mRNAs, which are translated into viral proteins, and genome RNAs. The resulting encapsidated genomes are assembled with other viral proteins and RNA to produce progeny virions from the plasma membrane (Adapted from Fearns & Deval, 2016).

1.1.4 Recognition of RSV by airway epithelium

Airway epithelial cells (AECs) are the first target of RSV, as these provide a mechanical barrier as part of the innate immune response that also regulates the adaptive immune response. The airway epithelium is a distinct complex system that includes ciliated epithelial cells, secretory cells, and migrated immune cells (Knight and Holgate, 2003). RSV induces infected cells to produce a variety of chemokines and cytokines with related inflammatory and immune-regulatory functions after it is recognised by three main groups of pattern recognition receptors (PRRs), which are Toll like receptors (TLRS), RIG-I-like receptors (RLRS), and NOD-like receptors (NLRs) (Kumar *et al.,* 2009; Loo *et al.,* 2008). These receptors can recognise many pathogen-associated molecular patterns (PAMPs), and they thus play a crucial role in initiating innate immune responses. In response to RSV, several cell types, including epithelial cells, dendritic cells, macrophages, monocytes, and granulocytes, express receptors on the cell's surface and endosomal compartments (Amanatidou *et al.,* 2009; Murawski *et al.,* 2009). The binding of PRRs with PAMPs leads to numerous signalling cascades, although the most critical involve the activation of MyD88-dependent and TRIF-dependent/MyD88-independent pathways. The MyD88-dependent pathway leads to the induction of Nuclear Factor (NF)-κB-dependent, pro-inflammatory genes, while the MyD88-independent pathway leads to the induction of interferon regulatory factor (IRF-dependent), which regulates the production of type I (IFNα and IFNβ) and III interferons (IFNλ); this in turn results in the development of inflammation in response to infection (Fig. 1.1.4) (Baum *et al.,* 2010; Tian *et al.,* 2002).

One of the primary receptors for PRRs, which play a crucial role in initiating immune signalling, is the TLR (Leulier *et al.,* 2008). Ten different TLRs have been identified in humans, with TLR 1, 2, 4, 5, 6, and 10 expressed on the cell surface, and TLR 3, 7, 8, and 9 (Kawai *et al.,* 2010; He *et al.,* 2013) located on the endosomal compartments. TLR 3, 4, 7, 8 and 9 have all emerged as important in the regulation of innate immunity in response to viral infection (Kawai and Akira 2006; Takeda and Akira, 2005), and TLR3 has also been shown to recognize RSV dsRNA. All TLRs except for TLR 3 stimulate the MyD88-dependent signalling pathway (Chen, 2005; Sato *et al.,* 2005), while TLR 3 uses only the MyD88 independent pathway (Funami *et al.,* 2004; Meylan *et al.,* 2004). TLR 4, however, activates both MyD88 and TRIF dependent signalling, using all four adapter molecules (Yamamoto *et al.,* 2003).

Various cells, including epithelial cells, macrophages, and dendritic cells express TLRs in the lungs. TLR 1-7 and TLR 9 are expressed by the tracheal, bronchial, and alveolar epithelial cells (AECs) (Mayer *et al.,* 2007; Ioannidis *et al.,* 2013). Alveolar macrophages have been shown to express TLR 1, 2, 4, 6, 7 and 8, but not TLR 3, 5 and 9 in human and mice (Maris *et al.,* 2006; Suzuki *et al.,* 2005), however, whereas lung fibroblasts have been shown to express TLR 2, 3, 4 and 9 (Brant *et al.,* 2008; Sugiura *et al.,* 2009).

In the A549 cell line, knockdown of TLR3 decreases the immune response-initiating gene expression of IFNβ, CXCL10, and CCL5 (Liu *et al.,* 2007). RSV NS2 protein has also been shown to inhibit the TLR3 pathway in A549s, leading to reduced interferon responses (Ling *et al.,* 2009). The expression of TLR 4 is also increased in A549 cell lines and primary human airway epithelial cells during RSV infections (Monick *et al.,* 2003). On a host cell surface, the fusion protein (F protein) of RSV induces translocation of TLR4 to the endosome together with TRAM. In endosomes, TLR3 is expressed in response to double-stranded RNA (dsRNA), which is formed as a product of the replication of most RNA viruses, including RSV. Both TLR3 and TLR4 can also activate TRIF-dependent signalling, which activates nuclear factor κB (NF-κB) and interferon regulatory factor 3 (IRF-3), leading to the induction of proinflammatory cytokines such as IL-6, CXCL8 (Bueno *et al.,* 2008; Tripp. 2004) and type I IFNs. Signalling of MyD88-dependent and independent pathways by TLR4 then results in the activation of IRF-3, NF-κB, AP-1, extracellular signal regulating kinase (ERK), and IKK, and RSV is recognised by all of these receptors, which induce appropriate antiviral innate immune response in the infected cells (Hirokazu *et al.,* 2013).

Both RIG-I and NOD2 are cytoplasmic receptors, which play a significant role in detecting RSV inside epithelial cells, macrophages, and dendritic cells (DCs). Several studies have shown that RIG-I is upregulated during RSV infections, and there is a significant positive correlation between RSV viral load and RIG-I mRNA levels (Scagnolari *et al.* 2009). When RIG-I detects RSV infection, it can stimulate NF-KB and IRF3 pathways by complexing with the MAVS adaptor in the mitochondrial membrane, leading to production of IFNβ, IP-10, and CCL5 by airway epithelial cells. Furthermore, a study by Sabbah demonstrated that NOD2 expression and activated IRF3 production was increased during RSV infection as the activated NOD2 interacted with MAVS and translocated to the mitochondria to produce IFNβ (Sabbah *et al.,* 2009).

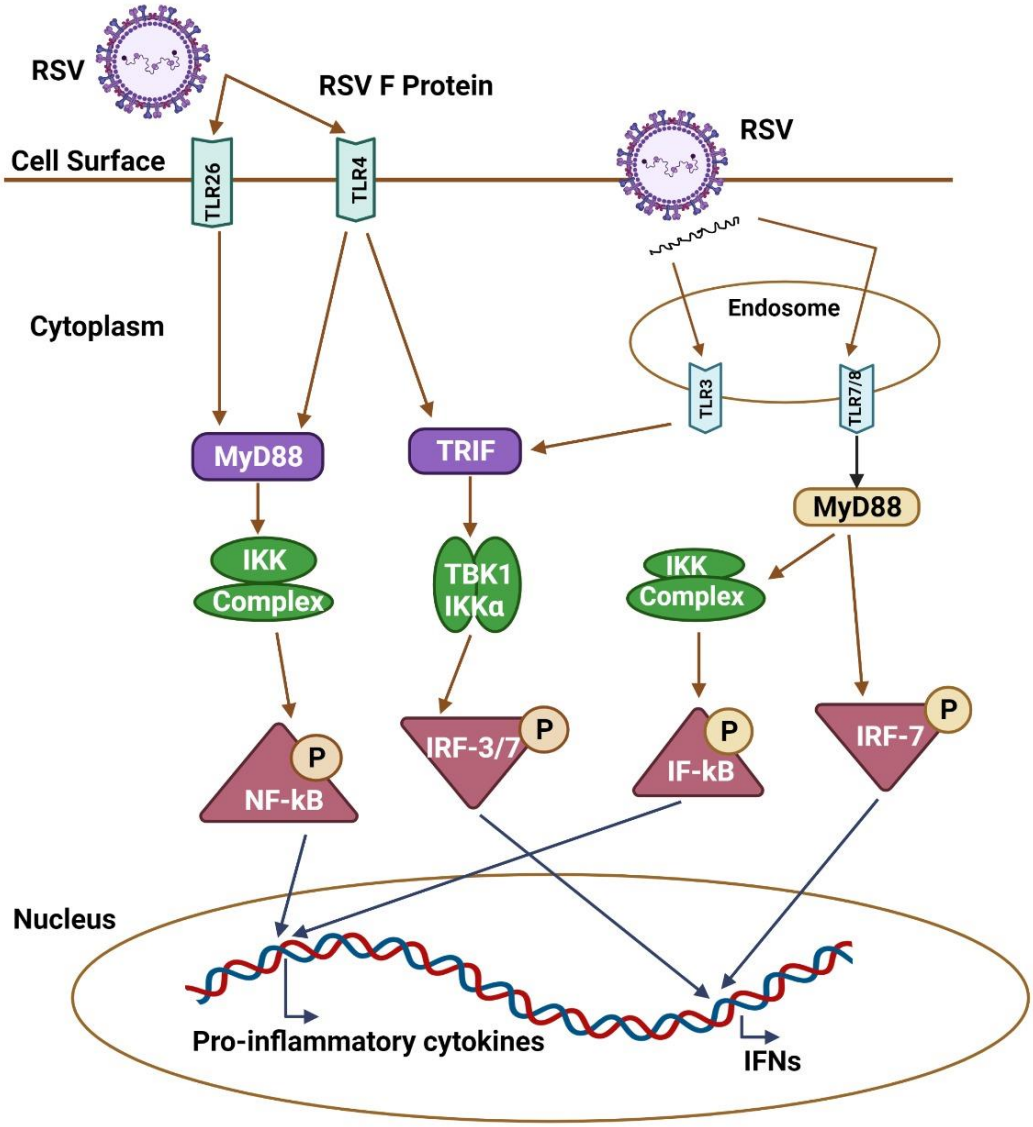

Figure 1.1.4 RSV-induced toll-like receptor (TLR) signalling pathway. RSV proteins and RNA are recognised by extracellular and intracellular TLRs which signal through MyD88 or TRIF adaptor molecule pathways to induce transcription factors NF-κB and IRFs 3 and 7. This leads to production of interferons (IFNs) and pro-inflammatory cytokines (Adapted from Kolli *et al.,* 2013).

1.1.5 Pathophysiology of RSV

When RSV reaches the human respiratory tract, innate immune cells recognise viral antigen and induce an inflammatory response through interactions between pathogen and host receptors. The Th2 and/or Th17 immune responses trigger overproduction of mucus in the airways and the lungs' inflammatory immune response. As mentioned above, AECs recognize RSV components such as the F protein and virus-related nucleic acids by means of Toll-like receptors (in the form TLR3 or 4), retinoic-acid inducible gene I (RIG-I) and nucleotide-binding oligomerization domain containing 2 (NOD-2) receptors. Various signalling pathways then activate transcription factors, such as IRF-3 and NF-κB, which in turn promote the transcription of several anti-viral genes and soluble molecules. AECs produce pro-inflammatory agents in the form of type-I and type-III interferons (IFN), which bind to IFN receptors and activate signal transducers and activators of transcription 1 (STAT-1) and transcription 2 (STAT-2). STATs 1 and 2 bind to IFN-regulatory factors to promote the transcription of interferon-stimulated genes (ISGs). At the same time, pro-inflammatory cytokines such as IL-6, tumour necrosis factor-alpha (TNF-α), and chemokines such as CXCL8, CCL3, CCL2, and CCL5 are induced and then secreted into the extracellular space. Importantly, some of these molecules (CCL2 and CCL5) promote the recruitment of leukocytes monocytes, neutrophils, dendritic cells, macrophages, natural killer cells, and CD4+ T cells to the site of infection. Effective clearance of RSV requires a balanced Th1 and Th2 adaptive immune response, which promotes IFNγ production by cytotoxic CD8+ T cells. Type 1 IFN is a weak host response, allowing viral replication to remain effective in infected cells and a pro-inflammatory Th2-response to be generated, which may account for why RSV infection does not produce a memory response that guarantees complete immunity to later viral exposure. This permits frequent re-infections, recurrent wheezing, increased susceptibility to developing asthma, and hyperreactivity (Vázquez *et al.,* 2019). The antibodies induced after RSV infection are short-lived, and it is also hypothesised that viral immunomodulatory mechanisms impair anti-RSV humoral memory responses (Ascough *et al.*, 2018).

Following RSV infection, CD8 T cells start initiating viral clearance, as well as helping protect against secondary RSV infection by producing cytokines. It is hypothesised that IFNγ and TNF produced by CD8 T cells could also play a role in viral clearance and the induction of immunopathology following RSV infection; however, the exact mechanisms governing CD8 T cell-

mediated viral control following RSV infection are unknown. It is, however, known that CD8 T cells can induce immunopathology following RSV infection (Schmidt & Varga, 2020).

In vivo animal models of innate immune stimulation have been helpful in the characterisation of immune responses following RSV infection. Studies using models in mice, ferrets, and chimpanzees have provided valuable insight into the pathogenesis of viral respiratory tract infections and immunological mechanisms. In most animal models, RSV induces a typical antiviral adaptive immune response, with resolution of primary infection resulting in high titres of virus-specific antibodies and large numbers of antigen-specific T cells. This response helps weaken the immune response to future infections, as reinfection limits the transient virus replication, causing more minor illness (Openshaw *et al.,* 2017). In mice and cotton rats, both CD4+ and CD8+ T cells are essential in terms of eliminating the virus from the respiratory tract. They also play a part in generating immunopathology during RSV infection (Chiu & Openshaw, 2015).

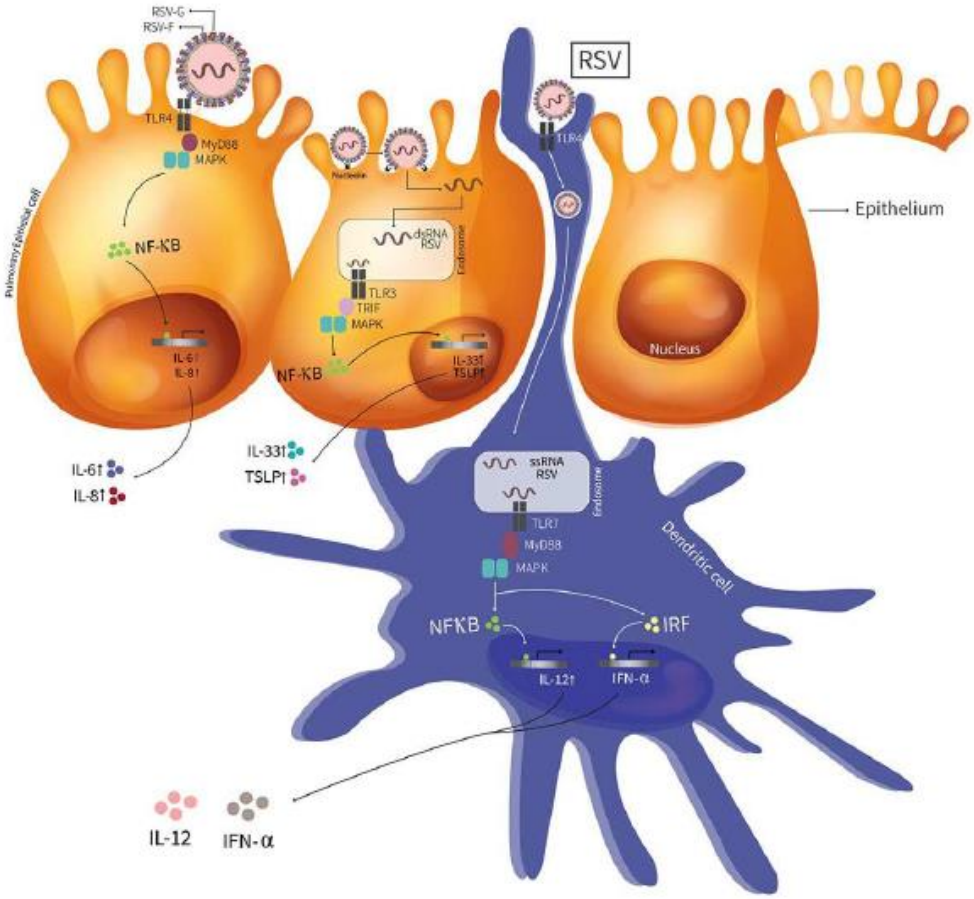

Figure 1.1.5 Pathogenesis of RSV and molecules with a biomarker potential induced in the airways during RSV infection. RSV attaches to AECs and this binding is mediated by the interaction between the RSV F or G protein. When RSV F protein binds to TLR4, this triggers a cascade of signaling, where the protein myeloid differentiation primary response (MyD88) is activated. The activation of MyD88 leads to activation of mitogen-activated protein kinase (MAPK), and the NF-kB transcription factor. Activated NF-kB translocates to the nucleus and promotes the production of Th1 cytokines. TLR3 triggers a cascade of signaling by the TIR-domain-containing adapter-inducing interferon-b (TRIF), MAPKs and NF-kB transcription factor. This signaling pathway promotes the IL-33 and TSLP production. RSV also can infect Dendritic Cells (DCs) and the virus mediates its entry by TLR4 receptor, present on the surface of the DC. DCs are then infected and the genetic material of the virus enters the cell. dsRNA binds TLR7 receptor, present in the endosome produced by the fusion, which one TLR3 triggers a cascade of signaling by the MyD88 protein, MAPKs and NF-kB transcription factor or interferon-regulatory factor (IRF). Those signaling pathways promote the IL-12 and IFNα production, respectively. (Vázquez *et al.,* 2019).

1.1.6 RSV in Omics-based studies

The term "omics" can be defined as the use of biotechnology to examine biological functions at a deeper level. Omics-based approaches and technologies have been used in clinical research for the last two decades in an increasing manner that may be attributed to advances in technology that have enabled better laboratory protocols, data storage, and analysis (D'Adamo *et al.,* 2020).

Omics technologies now include metabolomics (technologies that quantify endogenous as well as exogenous chemicals produced by biological systems); proteomics (those that address protein levels and their chemical modification); transcriptomics (those that measure transcript expression through methods such as RNA-Sequencing (RNA-Seg)); epigenomics (those that measure modifications of the DNA through chromatin immunoprecipitation sequencing (ChIP-Scq) and epigenome-wide Association Studies (EWAS); genomics (those that study variations in DNA sequences, including genome-wide Association Studies (GWAS), whole-genome sequencing (WGS), and whole-Exone Sequencing (WES)); metaomics, which can be divided into metatranscriptomics, metagenomics, and metaproteomics; and, finally, combined omics approaches that combine two omics technologies to address a particular research question, such as combining metabolomic approaches with genomics analysis (Palazzotto & Tilmann, 2018).

Since DNA microarrays were first used in the 1990s and, spurred on by advancements in next-generation sequencing technologies, biomedical researchers have collected large quantities of data to create large omics datasets that capture a variety of biological measurements. Such data expansion is expected to continue to increase exponentially, providing opportunities to develop unbiased characterisation of biological systems. The advancement of omics has thus enabled development of a deeper understanding of diseases and the generation of various new diagnostic and therapeutic methods to address a plethora of medical conditions. Recently, omics technologies have helped advance knowledge for multiple respiratory diseases, including idiopathic pulmonary fibrosis (IPF), acute respiratory distress syndrome (ARDS), asthma, pulmonary arterial hypertension (PAH), and chronic obstructive pulmonary disease (COPD) (Kan *et al.,* 2017).

An examples of the clinical applications of omics technology is seen in the work of Raita *et al.*, who integrated clinical data, information on viruses, data on the nasopharyngeal microbiome and metabolome, and transcriptome data from a multicentric prospective cohort study conducted on 221 cases of paediatric RSV bronchiolitis. This research helped characterise four distinct biologically meaningful RSV endotypes as well as demonstrating their longitudinal effect on the risk of chronic morbidities. They found that infants with RSV endotype B had a higher prevalence of parental asthma, coinfection with RV, high IFNα and IFNγ response, S. pneumoniae/M. catarrhalis codominance, and IgE sensitization, as well as a greater risk for developing asthma later in life, compared to infants with endotype A or the a classical RSV endotype. This data could aid researchers in further investigating the development of endotype-specific strategies for preventing asthma and treating bronchiolitis (Raita *et al.,* 2021), while advances in proteomic and transcriptomic technologies have increased knowledge of host responses to RSV. Similarly, Dapat and Oshitani conducted a metanalysis on microarray and proteomics datasets from high-throughput experiments that identified hundreds of host factors affected during RSV replication (Dapat & Oshitani 2016). Integrating proteome and transcriptome datasets allowed them to instigate a global approach, and they thus constructed a virus-host interaction network to identify affected host factors during RSV infection to explore potential drug targets.

1.1.7 Prevention

There are currently no vaccines licensed for use in pregnancy or infancy. During the 1960s, a formalin-inactivated RSV (FI-RSV) vaccine was evaluated in children. Unfortunately, vaccinated individuals exhibited increased disease severity upon subsequent natural RSV infection compared to the control group (Kim *et al.,* 1969). The vaccine's tendency to enhance disease is though to result from the failure of the vaccine to elicit either potent neutralizing antibodies or memory CD8+ T cells, alongside the induction of a strong inflammatory CD4 T cell response (Delgado *et al.,* 2009).

Currently, development of a vaccine against RSV depends on impairing the life cycle of the virus at entry, replication, and/or transcription. Such methods directly weaken the viral infective cycle without compromising the host immune response, a feature essential for treating new-borns, who have inefficient immune responses. Based on this, a drug that can directly inhibit virus spreading by targeting either RSV function or viral entry events by blocking the interaction between host cell receptors and viral proteins, or RSV function is the aim (Andries *et al.,* 2003; Sun *et al.,* 2013).

The only treatment option otherwise available for RSV is a humanised monoclonal antibody to the RSV F surface protein, known as palivizumab (Village *et al.,* 1998), which was first used in 1998. Palivizumab binds with RSV F protein on the host cell's surface to prevent viral entry into host cells. It has strong neutralisation and inhibitory effects against RSV subgroups A and B, and the results of two randomized controlled clinical trials showed that the efficacy rate of palivizumab is 39 to 78%. As no approved vaccine for RSV has emerged in over 50 years of research, such passive immunisation with the monoclonal antibody palivizumab is the only regulatory-approved option for preventing the serious LRTI that may be caused by RSV in paediatric patients, and it is thus given to those at high risk of RSV. Wegzyn *et al.* performed a systematic review that assessed results from seven RCTs, four open-label non-comparative trials, and eight prospective observational studies or registries, thus gathering data for over 42,000 high-risk infants/children from across 34 countries. They found that palivizumab showed consistent efficacy in terms of reducing hospitalisations related to RSV, especially among high-risk populations. The safety profile was different from that of the placebo in blinded randomised controlled clinical trials, with palivizumab prophylaxis proven to have a good safety profile in the identified studies. Studies have shown that palivizumab is a safe and well-tolerated prophylactic

option that can reduce the risk of severe RSV infection and the need for hospitalisation in high-risk infants. A consensus has emerged, however, that palivizumab prophylaxis should be used only in well-defined high-risk populations, rather than arbitrarily, to ensure cost-effective use (Wegzyn *et al.,* 2014). Palivizumab does not seem to interfere with routine immunisations, but there is no data regarding its reproductive toxicity, mutagenesis, carcinogenesis, and reproductive toxicity (Resch, 2017).

Palivizumab treatment has been shown to reduce the overall risk of hospitalisations due to RSV infection by about 50% in infants born at term and by 78% for infants born prematurely. The disadvantage of treatment with palivizumab, however, is that is leads to passive immunisation, which exposes the individuals involved to the risk of later RSV infection, which may be problematic for infants born prematurely and those with other underlying diseases (Paes *et al.,* 2012). Monthly injections of palivizumab are also required to provide and maintain protection throughout the relevant season.

Nirsevimab is a monoclonal antibody developed as a passive immunisation drug against RSV to prevent LRTI in infants experiencing an initial RSV infection (Domachowske *et al.,* 2018). Nirsevimab may be the most advanced prophylactic option currently under development (Path *et al.,* 2019), and the available data supports its efficacy and safety in preventing medically-attended LRTI and hospitalisations in healthy preterm infants, based on as a single intramuscular dose. Furthermore, nirsevimab has been proven to have a longer serum half-life and a more significant neutralising activity than palivizumab, with one injection of nirsevimab proven to protect an individual for a full typical RSV season (Griffin *et al.,* 2020). Currently, nirsevimab is being studied for use in both healthy full-term and late-preterm infants due to its unique effects and sustained response from only a single dose (Griffin *et al.,* 2020).

1.2 Human Rhinovirus

1.2.1 Epidemiology

Human rhinoviruses (RVs) were first discovered in the 1950s during attempts to identify the aetiology of the common cold. They generally cause upper respiratory tract infections, but they may also infect the lower respiratory tract. RV infection can lead to various further complications, such as otitis media, sinusitis, chronic bronchitis, and asthma exacerbation. Classified as a respiratory virus, RV is responsible for approximately 80% of asthma exacerbations in children and around 50% of those in adults (Johnston *et al.,* 1995). RV is also the most frequent pathogen cause the common cold (Mäkelä *et al,* 1998). Worldwide, rhinovirus infection occurs among all age groups during all seasons, suggesting that infections occur all year round; however, before COVID, two peaks of infection were typically reported in temperate regions, the first being between April and May and the second between September and October (Monto *et al.,* 2002; Winther *et al.,* 2006).

1.2.2 Classification and Structure

Rhinoviruses (RV) are small, positive-stranded RNA viruses that belongs to the Picornaviridae family. The diameter of these viruses is between 24 to 30 nm, and they contain a simple viral capsid with a single strand of positive sense RNA (Pitkäranta and Hayden, 1997). The capsid contains four proteins, VP1, VP2, VP3, and VP4, which are arranged in 60 repeating protomeric icosahedral units (Figure 1.2.1). On the viral surface, VP1, VP2, and VP3 are responsible for the antigenic diversity of the virus, while the other capsid protein, VP4, is located inside the virus, anchoring the RNA core to the viral capsid (Rotbart, 1997). There are 169 HRV genotypes distributed across three species: these are the HRV-A (80), HRV-B (32), and HRV-C (57) genotypes (Simmonds *et al.,* 2020). Several studies in children suggest that, in comparison to HRV-A and B, HRV-C may be associated with a higher frequency of lower respiratory tract disease (Piralla *et al,* 2009). Intercellular Adhesion Molecule 1 (ICAM-1) is the main receptor for more than 90% of RV serotypes seeking to enter the host cell and initiate infection. The remaining RV serotypes enter cells via the low density lipoprotein receptor (Rotbart, 1997).

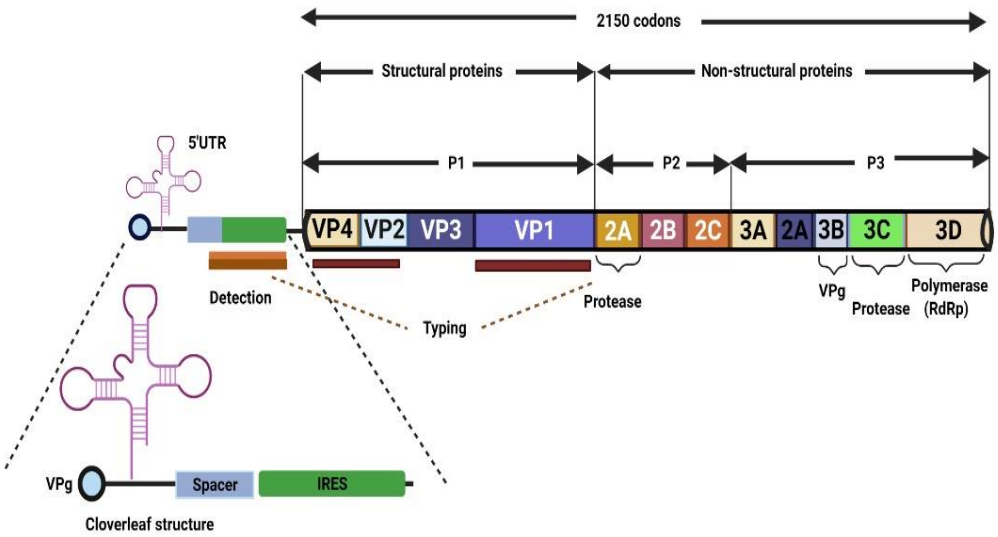

Figure 1.2.2 Schematic organization of rhinovirus genome. The RV single-stranded positive-sense RNA genome is about 7.1-7.2 kb long and consist of 50 and 30-UTRs. There are around 2150 codons divided into the P1 (structural proteins codon), P2 and P3 (non-structural proteins codon) regions. The coding region is surrounded by the 50UTR and 30UTR. The 50UTR is essential for translation and replication of viral RNA. It's also has the IRES sequence and a cloverleaf structure. 30UTR organize the structure of a conserved stem-loop preceding a poly (A) tail. A VPg protein is covalently bound to the 50-end of the genome. Genomic regions used for RV detection and typing are indicated by orange and red lines, respectively. IRES, internal ribosome entry site; UTRs, untranslated regions; VPg, viral protein genome-linked. (Adapted from Niespodziana *et al.,* 2022).

1.2.3 Transmission and replication of RV

Rhinoviral infections are spread from person to person by means of contaminated nasal secretions; these may enter a new host's respiratory tract as part of an aerosol, through direct contact, or through indirect contact from a contaminated object. When the virus enters the nasal cavity, HRVs are transported to the nasopharynx by ciliated epithelial cells. Through attachment to its receptor ICAM–1, the virus can enter host nasal epithelial cells causing a host immune response which results in cold symptoms (Igarashi *et al.,* 1993). Such symptoms can occur within 16 h of inoculation, and they generally peak between 24 and 48 hours post inoculation. A sore throat is usually the first sign of infection, with other symptoms including rhinorrhoea and nasal congestion, facial or sinus pressure, headaches, and coughs, and low-grade fevers may also occur (Arruda *et al*, 1997).

The RV replication cycle consists of four main steps: the first step is the binding of the virus to its receptor ICAM-1 and its internalisation into the host cell by means of endocytosis. The second step is called uncoating, and this is where the viral RNA is released into the cytoplasm of the host cells thought pore-like structures. The third step occurs when the viral RNA translates into a polyprotein and replicates in the cytoplasm. Finally, viral protein assembly and release of the new infectious particles completes the cycle (Figure 1.2.3).

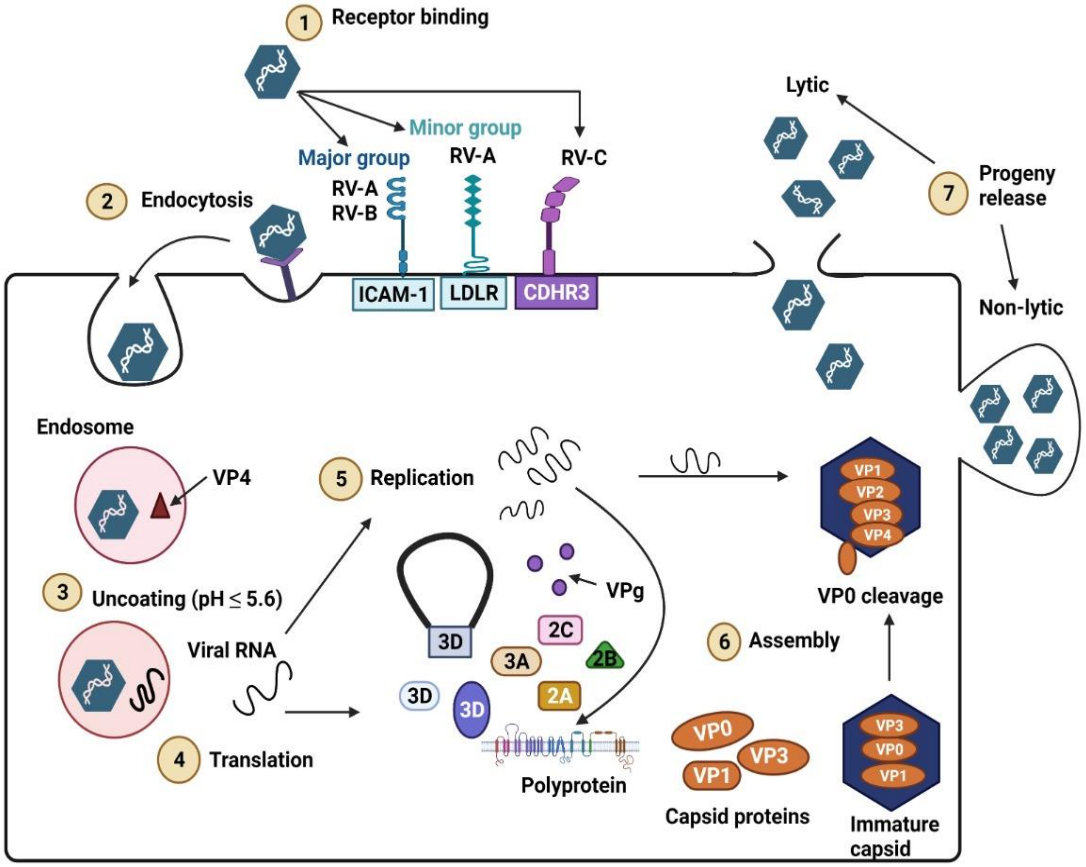

Figure 1.2.3 Schematic representation of rhinovirus infectious cycle. Rhinovirus enters the cell via endocytosis. Following uncoating at pH<5.6, the viral RNA genome translates to a polyprotein, which convert to mature viral proteins. A template viral RNA allow the viral polymerase to produce a new viral genomes. Structural proteins VP1 and VP3 and the VP0 precursor assemble into empty capsids. Mature viral particles (complete with the viral RNA) are formed upon the final cleavage of VP0 into VP2 and VP4. Finally, the new infectious virus are released by cell lysis or non-lytic exocytosis (Adapted from Niespodziana *et al.,* 2019).

1.3 Host immune response to RSV

1.3.1 Innate immune response to RSV

Both innate and the adaptive immune responses contribute to the control of viral infections. The innate immune response is the host's first line of defence against various infectious pathogens and other stimuli that may cause cell damage. It thus has a crucial role in determining the nature of the subsequent protective adaptive immune response. As part of the innate immune response, various elements contribute to the control of viral infection, including the actions of various cell types (epithelial cells, and immune cells such as dendritic cells, macrophages, monocytes, and granulocytes), work by multiple pattern recognition receptors (PRRs) and numerous cytokines and chemokines.

1.3.1.1 Cytokines and chemokines arising from airway epithelial cells

Airway epithelial cells play a critical role in creating a healthy lung environment by acting as a barrier to maintain tight and adherent junctions within the apical junctional complex (AJC). These are responsible for the paracellular transport of environmental viral and microbial antigens from the lumen of the airways to immune cells in the underlying tissue. In addition, their cilia and the mucus and airway surface liquids (ASL) they produce allow the mucociliary escalator to function effectively allowing the clearance of pathogens and other inhaled foreign material from the respiratory tract (Bustamante *et al.,* 2017). Upon infection, airway epithelial cells act as innate immune sensors through their PRRs, which include (TLRs), RLRs, C-type lectin receptors (CLRs), and protease-activated receptors (PARs). TLRs have been shown to contribute to antiviral immune responses in both *in vivo* and *in vitro* models; and apical surface expression of TLR3, TLR4, and TLR7 in human tracheal sections and well-differentiated human airway epithelial cells (WD-AECs) cultures has also been reported. RSV infection increases the expression of TLR4 in monolayer AECs cultures (Monick *et al*., 2003), and it is also known to interact with TLR4 on airway epithelial cells (Kurt-Jones *et al.,* 2000). Interactions between RSV and TLR4 were recently implicated in the synthesis of type III interferons by the airway epithelium (Rallabhandi *et al.,* 2012). Similarly, RSV infection upregulates the expression of TLR3 in monolayer AECs cultures (Groskreutz *et al.,* 2016), and the interactions between TLR3 and RSV have thus been implicated in the secretion of the cytokines CCL5 and CXCL10 (Rudd *et al.,* 2005).

AECs are the source of over 20 pro-inflammatory cytokines, chemokines, and growth factors during RSV infections (Zhang *et al.,* 2001), Interactions between RSV and host airway epithelial cell PRRs have been suggested to induce the release of these cytokines/chemokines (Oshansky *et al.,* 2010). Table 1.3.1 shows the various AEC-derived chemokines that arise during RSV infection. During RSV infection, two types of cytokines and chemokines are produced: primary, which are directly induced by virus infection, and secondary, which are induced by other inflammatory mediators. The autocrine action of the secreted TNF-α, IL-1α, and IL-1β following RSV infection results in the secretion of IL-6 and CXCL8 (Jiang *et al.,* 1998), while the secretion of TNF-α from RSV infected airway epithelial cells has been shown to increase secretion of CCL5, as shown in Figure 1.3.1A (Das *et al.,* 2005).

Airway epithelial cells also play an important role in the recruitment of immune cells from the peripheral circulation. CXCL8, CCL5, and CXCL10 have been demonstrated to be chemo-attractants for innate immune cells, such as neutrophils, eosinophils, and natural killer cells (NKs) (Openshaw *et al.,* 2005). As shown in Figure 1.3.1B, epithelial-derived CCL11 promotes eosinophil recruitment (Mellow *et al.,* 2004), while CCL2, CCL3, and CCL4 secreted from RSV infected airway epithelial cells are important for the efficient recruitment of monocytes as well as natural killer cells (Tregoning *et al.,* 2010).

Another class of primary cytokines are Interferons (IFNs), which can be classified as type I, including IFNα and IFNβ; type II, including IFNγ, or type III. Each class binds to specific receptors. Recent data from mouse models suggests that alveolar macrophages (AM) might be a major source of type I IFNs following RSV infection (Goritzka *et al.,* 2015). However, Schijf *et al.* demonstrated the significant secretion of IFNβ from A549 cell lines *in vitro* (Schijf *et al.,* 2013). Other studies investigating the secretion of INFβ have shown this to be cell line-specific, as well as dependent on high levels of infection (Hillyer *et al.,* 2018). RSV infection induces high expression levels of IFNγ in the lungs, a response that is associated with more severe disease in children (Selvaggi *et al.,* 2014). Such IFN induction by RSV involves the recognition of RSV by TLRs, which activates both innate and adaptive immunity (Caballero *et al.,* 2015). However, two RSV non-structural proteins, NS1 and NS2, as well as G protein, are known to inhibit type I IFNα production.

Chemokine/ (known as)	Receptor/s	Target cells
CCL2 /(MCP-1)	CCR2	Basophils, monocytes
CCL3 /(MIP-1α)	CCR1/5	Lymphocytes, monocytes, macrophages, immature DC
CCL4 /(MIP-1β)	CCR5/8	Monocytes, macrophages
CCL5 /(RANTES)	CCR1/3/4/5	Eosinophils, monocytes, T lymphocytes, DC
CCL17 /(TARC)	CCR4/8	Th2 lymphocytes, NK cells
CCL20 (MIP-3α)	CCR6	T lymphocytes, naïve B cells, DC
CXCL2 (MIP-2)	CXCR2	Monocytes, neutrophils, basophils
CXCL8 (IL-8)	CXCR1/2	Neutrophils, eosinophils, T lymphocytes
CXCL9 (MIG)	CXCR3	Activated Th1 lymphocytes, NK cells
CXCL10 (IP-10)	CXCR3	Activated Th1 lymphocytes, NK cells
CXCL11 (I-TAC)	CXCR3	Activated Th1 lymphocytes, NK cells
CX3CL1 (Fractalkine)	CXCR1/2	Monocytes, lymphocytes

Table 1.3.1. AEC-derived chemokines during RSV infection. (Modified from Zhang *et al.* 2001).

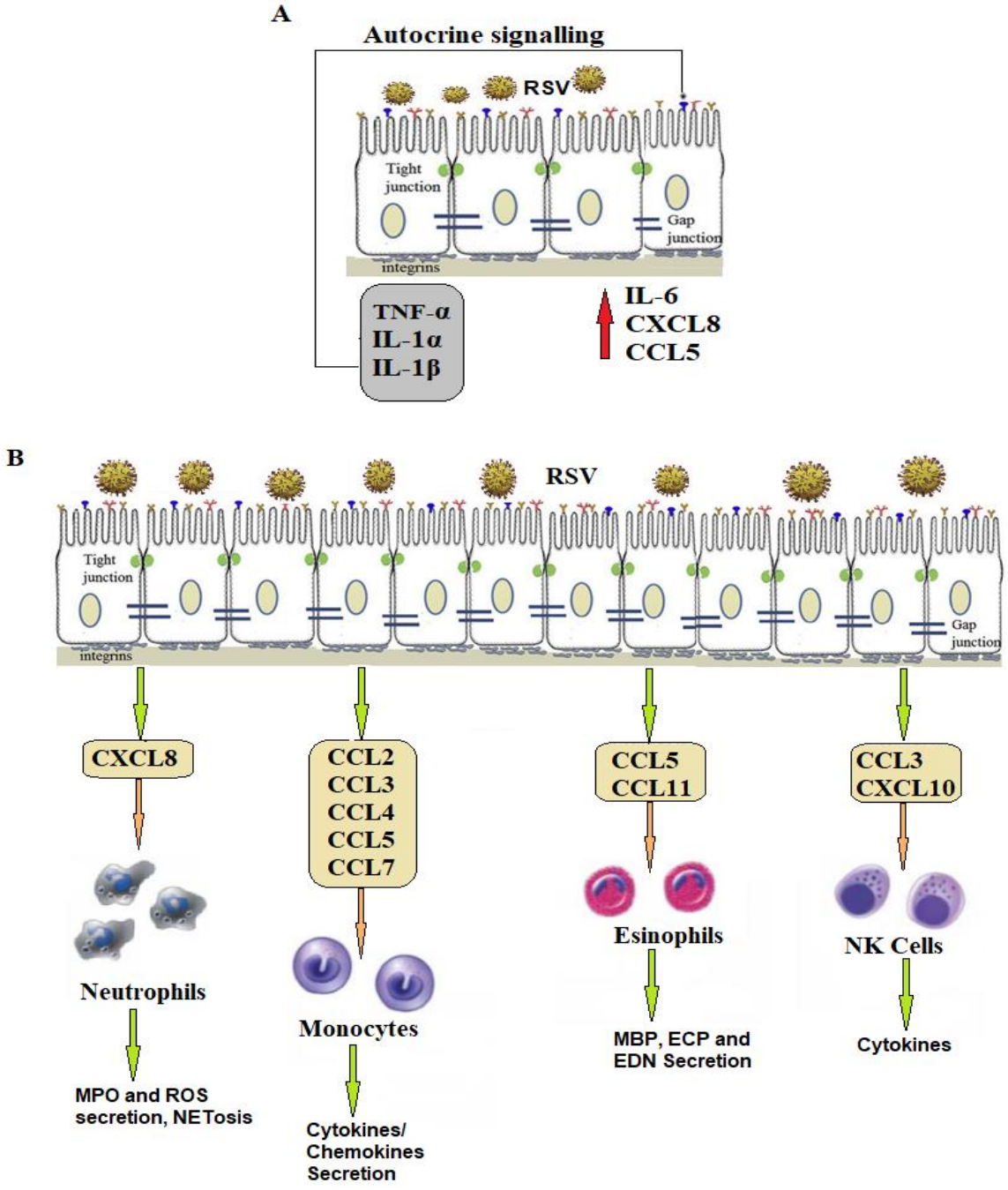

Figure 1.3.1 Autocrine signalling of TNF-α, IL-1α, and IL-1β amplifies the secretion of the pro-inflammatory cytokine IL-6 and the chemokines CXCL8 and CCL5 following RSV infection (A); Primary airway epithelial cells and airway epithelial cell lines secrete chemokines. The chemokines facilitate the recruitment of innate immune cells from the periphery to the lung where they implement antiviral actions through various cell-specific mechanisms **(B).** Major Basic Protein (MBP), Eosinophil Cationic Protein (ECP), Eosinophil-derived neurotoxin (EDN), Myeloperoxidase (MPO), reactive oxygen species (ROS) (Adapted from Glaser *et al.,* 2019).

1.3.1.2 Immune cells during RSV infection

Neutrophils are the most common cell type found in airway lumens during RSV infection in infants (Kerrin *et al.,* 2017). The exact function of neutrophils in RSV infection is not yet clear, but it has been hypothesised that they contribute to both viral clearance and tissue damage (Openshaw *et al.,* 2017). Based on bronchoalveolar lavage (BAL) of infants with severe RSV bronchiolitis, neutrophils accounted for about 76 to 93% of the innate immune cells present (Openshaw *et al.,* 2005). Epithelial-derived CXCL8 is induced by neutrophils in response to RSV infection (Figure 1.3.1), with the activation of neutrophils depending on the presence of cytokines and chemokines; this results in degranulation, oxidative bursts, inflammatory mediator release, and NETosis (Bataki *et al.,* 2005). The presence of Myeloperoxidase (MPO) has previously been reported on the DNA fibres of NETs formed in response to RSV exposure (Geerdink *et al.,* 2015). Thus, alongside MPO, neutrophil elastase has been implicated in contributing to RSV pathology and inducing tissue damage. Neutrophils also contribute to immunopathology by producing the pro-inflammatory cytokine TNF-α, which suggests that neutrophilia plays a major role in enhancing respiratory disease (Geerdink *et al.,* 2015).

Alveolar macrophages (AMs) are present in the lumen of the alveolar lung space (Joshi *et al.,* 2018). RSV infected AMs induce TNF-α through the RIPK1/3/MLKL expression pathways (Santos *et al.,* 2020), and the replication of RSV thus leads to increases in the expression of migration of macrophage inhibitory factor (MIF), leading to modifications in the production of AMs cytokines (De Souza *et al.,* 2019). Interestingly, this pathway is also associated with the levels of TNF-α and IL-10, which play a critical role in balancing protection and pathology. AMs express IL-33, a driver of Th2-associated cytokine production, through a mitogen-activated protein kinase (MAPK)-dependent pathway leading to activation of the nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) (Qi *et al.,* 2017). AMs also express CD169+, which is responsible for the capture of pathogens. Mouse macrophage studies show that macrophages play a role in recruiting and activating different immune cell populations, while other studies have shown that the depletion of alveolar macrophages from mouse models reduces the innate immune response to RSV infection and leads to increased viral load and airway obstruction (Pribul *et al.,* 2008; Kolli *et al.,* 2008). During RSV infection, alveolar macrophages induce the production of various cytokines, such as IFNα, IFNβ, TNF-α, IL-6, CCL3, and CXCL10 (Panuska *et al.,* 1995; Makris *et al.,* 2016).

Eosinophils are recruited to the lungs by epithelium-derived chemokines, such as CCL3, CCL5, and CCL11 (Jung *et al.,* 2013) (Figure 1.3.1B). These also recruit other immune cells to the inflammation site during RSV pathogenesis. Eosinophils contribute to viral clearance by producing cytokines and other inflammatory mediators, including eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN), which has been found in the BAL and nasal lavage (NAL) fluids of infants with severe RSV (Dimova-Yaneva *et al.,* 2004). *In vitro*, increased production of superoxide from eosinophils has been seen after exposure to RSV, indicating their activity to be antiviral in response to direct contact with the virus (Kimpen *et al.,* 1992). Furthermore, a recent study identified major basic protein (MBP) as another eosinophil antiviral protein, with its function being to promote the cell death of RSV-infected epithelial cells (Ishioka *et al.,* 2011). Saito *et al.* presented the observation that human epithelial cells induce the expression of the eosinophil chemo-attractant RANTES in response to RSV infection (Saito *et al.,* 1997), though the role of eosinophils during RSV infection is not fully understood. Nevertheless, previous studies suggest that eosinophils can produce cytokines and other inflammatory mediators, including nitric oxide, which may aid viral clearance (Phipps *et al.,* 2007); they also have a prominent role in allergic asthma (Ishioka *et al.,* 2011).

Monocytes have been implicated as facilitator cells during RSV infection, supporting the proliferation, activation, and survival of other immune cells. They are also thought to be the precursors of differentiated macrophages and dendritic cells in inflamed tissues (Bohmwald *et al.,* 2017). The incubation of peripheral blood monocytes with a conditioned medium from RSV-infected airway epithelial cells induced the expression of the chemokine receptors CCR1, CCR2 and CCR5 on the monocytes' surface which indicates the ability of epithelial cell to recruit monocytes to the inflammation site (Morrison *et al.,* 2007). The absence of monocytes delays viral clearance in mice, though it has also been seen to reduce the spread of the virus to adjacent, uninfected cells (Soukup *et al.,* 2003). The secretion of cytokines and chemokines from monocytes such as IL-1β, IL-1RA, IL-6, CXCL8, IL-10, TNF-α, G-CSF, IFN-γ, CXCL10, CCL2, CCL3, CCL4, and CCL5 has been idintified as a major function of these cells during RSV infection (Bohmwald *et al.,* 2017).

NK cells are innate immune cells that play a critical role against respiratory viruses by facilitating the elimination of virus infected cells. Cytokines and chemokines have been shown to enhance NK cell recruitment and activity, including IFN-α/β and macrophage inflammatory protein-1α

(MIP-1 α)/CCL3 (Anfossi *et al.,* 2006). NK cells are rapid and efficient producers of cytokines, such as IFNγ, that are an important part of early antiviral responses, as well as acting in the antigen-independent activation of antigen-presenting cells. They also act as regulatory cells and interact with other immune cells, such as dendritic cells and T cells, which support a key a role in the modulation of adaptive immunity (Bhat *et al.*, 2020). Single-nucleotide polymorphisms (SNPs) that increased leukocyte immunoglobulin-like receptor B1 (LILRB1+) NK cells (Noyola *et al.,* 2015) are more prevalent in infants with severe RSV disease. The numbers of natural killer (NK) cells in peripheral blood is reduced in a manner that correlates with increased disease severity, indicating that these are recruited to the infection site (Larrañaga *et al.,* 2009). Mice infected with recombinant RSV expressing IL-18 to enhance NK cell activation also had reduced lung viral loads (Harker *et al.,* 2010).

DCs play a crucial role both in the innate immune responses and the initiation of adaptive immune responses in lung viral infections by inducing the proliferation and activation of T cells (Grayson and Holtzman., 2007; Cormier *et al.,* 2014). DCs are enhanced and then migrate into the epithelium through CCL20 induction by viral infected airway epithelial cells (Wareing *et al.,* 2004). There are two major types of pulmonary DCs: conventional DCs (cDCs), formerly called myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). The cDCs express specific chemokine receptors, such as CCR6, the ligand for the chemokine CCL20 (Power *et al.,* 1997). They lie under the airway epithelial layer, in a position that allows them to sense and take up foreign antigens. Upon antigen recognition, they undergo migration to the lymph nodes and maturation via the expression of the chemokine receptor CCR7 (Saeki *et al.,* 1999). In the lymph nodes, cDCs mature into antigen-presenting cells and express molecules such as CD80, CD86, and major histocompatibility complex (MHC) class I and II molecules (Lukens *et al.,* 2009). In addition, airway epithelial cells contribute towards the differentiation and maturation of local cDCs via the production of various cytokines, including IL-15, thymic stromal lymphopoietin (TSLP), and type I IFNs (Van *et al.,* 2010).

In contrast, pDCs are not efficient antigen presenting cells; however, they have a strong antiviral action driven by their production of large amounts of IFNα. The migration of pDCs into lymph nodes is a modulated adaptive immune response that promotes Th1 response through the secretion of type I IFNs and IL-12 (Smit *et al.,* 2008). Upon viral infection, pDCs are thus recruited rapidly from the bone marrow, and within a few days, they form the most of the DCs in the lungs

(Wang *et al.,* 2006). Both cDCs and pDCs express different type of TLRs on their surfaces: human cDCs express typically TLR2 and TLR4 in response to viral proteins such as the RSV fusion protein, whereas pDCs primarily express TLR7 and TLR9 in response to single-stranded RNA and viral DNA, such as influenza viruses (Finberg *et al*., 2007). Previous studies have shown that the number of DCs in nasal washes increases in children with RSV infections, with an associated decrease in the number of these cells in the peripheral blood (Gill *et al.,* 2005). In mice, depletion of DCs leads to enhanced lung inflammation and mucus production during RSV infection, and thus to decreased expression of IFNα protein (Smit *et al.,* 2006).

There are several evidence for the functional role of IL-36 in infectious diseases. IL-36 cytokines are involved in regulation of early immune response by induce type I and II IFN leading to produce inflammatory cytokines and immune cells recruitment in the skin (Swindell *et al*., 2018; Foster *et al.,* 2014). In human keratinocytes, IL-36 cytokines trigger various inflammatory cytokines such as IL-17, IL-20, IL-22, IL-23, IFNγ and TNF-α leading to the recruitment of macrophages, T cells and neutrophils (Foster *et al.,* 2014). It was report that SARS-CoV-2 infection can induce endothelia cells to secrete IL36γ leading to leukocytes infiltration (Xue *et al.*, 2021). In severe COVID-19 patient, it has found that IL-36 may drive pulmonary infiltration of myeloid cells including neutrophils and macrophages/monocytes recruitment and activation (Dietrich *et al.,* 2016; Wang *et al.,* 2018). This finding suggest the critical role of IL-36 cytokines in innate and adaptive immune response and could be a biomarker to the infectious diseases.

1.3.2 Host Adaptive Immune Response to RSV

In contrast to the primary innate immune response, the adaptive immune response could be considered a second line of defence, which emerges in a more specific manner that makes it high effective against pathogens. It thus takes a longer time to complete viral clearance by preventing viral replication or growing a new generation of virions and developing immunological memory cells (Newton *et al.,* 2016), though it is then characterised by immunological memory, which refers to the ability to quickly and robustly respond to previously encountered pathogens in an antigen-specific manner (Bergeron and Tripp., 2021). There are two types of adaptive immunity: cell-mediated immunity and humoral immunity.

1.3.2.1 Cell-mediated immunity

The induction of an adaptive immune response can begin with the activation of DCs cells that as mentioned above, link innate and adaptive immune responses. DCs induce and regulate the adaptive immune response by presenting antigens that interact with T cells (Wieczorek *et al.,* 2017). T cells are divided into two main groups, CD4+ and CD8+. CD4+ T cells can recognise viral antigens via MHC class II molecules in antigen presenting cells and, after such recognition, CD4+ T cells differentiate into Th (T helper)1, Th2, Th9, Th17, Th22, Treg (regulatory), and Tfh (follicular helper) cells (Golubovskaya and Wu., 2016). The production of IFN-γ characterises Th1 cells, while Th2 cells produce various cytokines, including IL-4, IL-5, and IL-13, and drive differentiation and antibody production by B cells. Treg cells are characterised by the expression of transcription factor Foxp3 and the production of IL-10 and TGF-β cytokines (Rosendahl Huber *et al.,* 2014; Zajac & Harrington 2008), while Th17 cells produce IL-17, IL-21, and IL-22 cytokines and play a role in gut and mucosal defences (Ouyang *et al.,* 2008).

CD8+ cells includes naive, TCM (central memory), TEM (effector) and TEMRA (effector memory) cells (Geginat *et al.,* 2003). After viral recognition, CD8+ T cells generate a cytotoxic response and induce apoptosis of the infected cell. Granzymes and perforin are the cytotoxic proteins produced by CD8+ T cells during infection (Janeway 2001), and CD8 T cells also produce cytokines such as IFN-γ, TNF-α, and TNF-β, which aid in viral clearance.

RSV has been shown to impair DC function, with these cells migrating to lymph nodes and activating immature, inefficient T cells. RSV infection thus triggers an inefficient T cells response with respect to virus clearance, promoting a Th2 response and decreasing the effectiveness of infected cell removal due to the downregulation of essential proteins such as granzyme B and perforin (Gomez *et al.,* 2014). After RSV infection, T cells fail to upregulate the production of granzyme B, perforin and IFNs, as well as downregulating TCR surface expression (Olson and Varga., 2008; Varga *et al.,* 2001). RSV also induces a strong Th2 response in the host and promotes the recruitment of pro-inflammatory cells, such as eosinophils, neutrophils, and monocytes, based on the product identification of IL-4, IL-5, and IL-13. This combination of immune cells and cytokines dampens CD8+ T cell immunity and prevents virus clearance (Vallbracht and Unsold., 2006; Varga *et al.,* 2001). Some studies have reported that children with defective T-cell responses show higher severity of disease in RSV with high viral loads. These

observations indicate that T cells participate in the resolution of such infection (Fishaut *et al.,* 1980; Hall *et al.,* 1986). RSV has also been reported to directly infect CD4+ T cells, which results in reduced expression of the Th1 associated cytokines IL-2 and IFN-γ (Raiden *et al.,* 2017). Overall, the response of impaired CD4+ T cells in humans appears to result in prolonged RSV illness and the potential for subsequent repeated infections.

Whether a Th2 immune response plays a role in disease pathogenesis during RSV infection in infancy is unclear (Lambert *et al.,* 2014; Connors *et al.,* 1994). Some studies have reported a correlation between levels of Th2 cytokines in bronchoalveolar lavage and RSV disease severity (Legg *et al.,* 2003; Kristjansson *et al.,* 2005); however, other studies have failed to detect this (Collins *et al.,* 2008; Christiaansen *et al.,* 2014). Th17 cells have been found in tracheal aspirate and in infants' peripheral blood of during severe RSV infections (Faber *et al.,* 2012; Stoppelenburg *et al.,* 2014). Moreover, experiments in mice have revealed that cytokines associated with the Th17 profile, such as IL-6, IL-23, and IL-17, can be detected in the airways post RSV infection (Mukherjee *et al.,* 2011; Nagata *et al.,* 2014). Th17-derived cytokines appear to promote three significant negative responses: infiltration of the lungs by neutrophils, stimulation of IL-13 synthesis and mucus overproduction, and inhibition of cytotoxicity mediated by CD8+ T cells (Bystrom *et al.,* 2013; Lambert *et al.,* 2014).

1.3.2.2 Humoral immunity

B cells contribute to virus clearance through the production of virus-specific antibodies. These antibodies prevent the spread of infectious virions from infected cells to neighbouring cells by neutralising the relevant F or G protein binding sites, which can lead to increased phagocytosis of the virus by macrophages and neutrophils. Additionally, antibodies can bind to viral proteins, particularly G and F, when expressed on the surface of infected cells in a manner that induces a complement cascade and antibody-mediated cell-mediated cytotoxicity; these processes then eventually eliminate infected cells (Newton *et al.,* 2016). It has also been observed that a virus specific serum, featuring IgM, IgG (IgG2a), and RSV-specific IgA, has been seen in the BAL fluid of mice infected with RSV (Borchers *et al.,* 2013; Kamphuis *et al.,* 2012; Singleton *et al.,* 2003), and previous studies have confirmed that in mice with deficient B cells, no change in viral clearance was observed during RSV infection, while viral replication was upregulated (Graham *et al.,* 1991a).

During RSV infection, B-cell-stimulating factors, a proliferation-inducing ligand (APRIL) and B-cell-activating factor (BAFF), are both present and colocalised with infected epithelial cells. APRIL and BAFF receptors are also expressed on perialveolar plasma cells. BAFF mRNA and protein levels are similarly increased in bronchial epithelial cells of infants with severe bronchiolitis (McNamara *et al.,* 2012; Fonceca *et al.,* 2011), while RSV IgA, IgG, and IgM are present in the lungs of infants with RSV LRTI, together with higher quantities of BAFF and APRIL and lower levels of T-cell-dependent cytokines (IL-2, IL-4, and IL-10) (Reed *et al.,* 2009; Jensen *et al.,* 1997). The concentration of APRIL is positively associated with RSV IgA and IgM levels and inversely linked to hypoxia. Thus, the pulmonary antibody response to RSV seems to be driven predominantly by T-cell independent antibody production via B-cell-stimulating factors (APRIL and BAFF), most likely derived from infected pulmonary epithelial cells.

Infants aged less than 3 months old are more susceptible to more severe RSV disease due to their lack of mature B cells (Novak *et al.,* 2009). Other studies have demonstrated that children aged between 4 and 8 months old may show an 8- to 10-fold lower rate of neutralising antibodies than children aged between 9 to 21 months old after RSV infections (Murphy *et al.,* 1986). These differences may be explained by the lack of maturity of the immune system among very young children (Adkins *et al.,* 2004; Levy, 2007), which naturally reduces the response of antibodies induced by RSV (Kasel *et al.,* 1987; Murphy *et al.,* 1986).

In addition, a significant decrease in both levels of IgA and IgG antibodies titres has been seen after acute RSV infection in elderly adults (Walsh & Falsey 2004). In around 75% of adults, a rapid decline in serum neutralizing anti-RSV F and anti-RSV G IgG titres was seen as compared to that observed in uninfected controls (Falsey *et al.,* 2006). Such decreases may account for the increased likelihood of re-infection with RSV (Falsey & Walsh 1998).

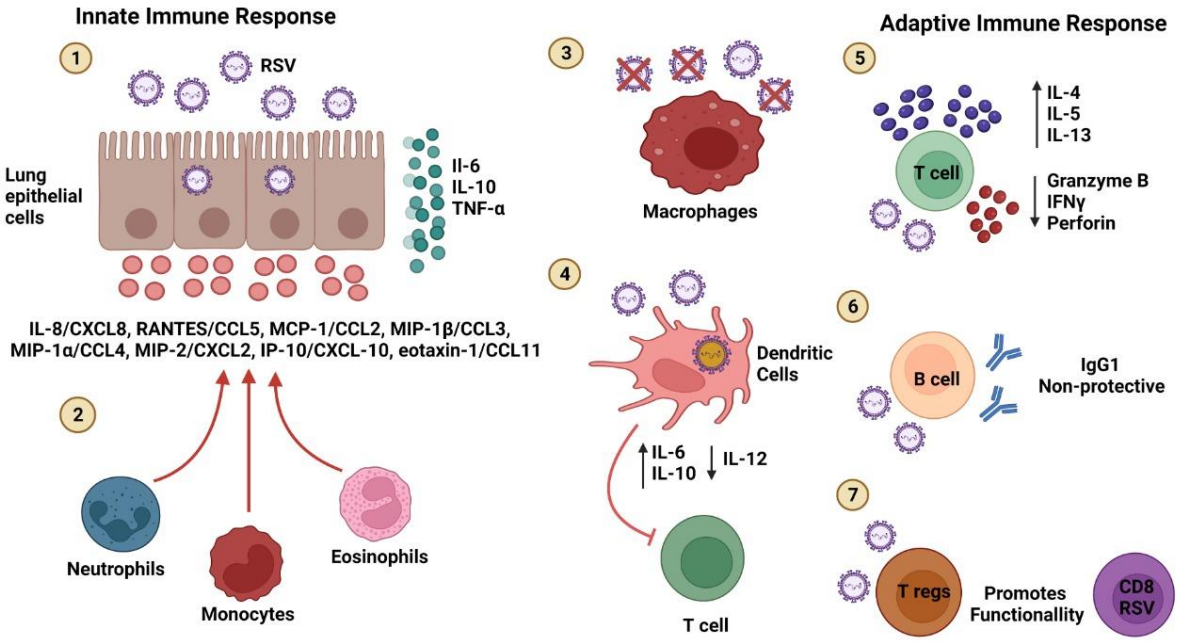

Figure. 1.3.1.2 Innate and Adaptive response against Respiratory Syncytial Virus. (1) RSV infects the airways, epithelial cells which secrete inflammatory cytokines and chemokines. (2) Cytokines and Chemokines start to attack and activate neutrophils, monocytes and eosinophils, generating the recruitments of these cells into the airways. (3) Alveolar macrophages play an important role decreasing the viral loads in the lungs. (4) In the border between innate and adaptive response are DCs, that are infected by the virus and are able to migrate to the lymph nodes, where impair the T cell activation. (5) RSV infection triggers an inefficient T cells response for virus clearance, promoting a Th2 response and decreasing the effectiveness to kill infected cells, due to down-regulation of important proteins such as Granzyme B and Perforin. (6) B cells, after RSV infection, generate non-protective antibodies, which normally are IgG1 that promote a Th2 response and non-neutralizating antibodies. (7) Tregs play an important role promoting the functionality of RSV-specific CD8' T cells (Adapted from Gomez *et al.,* 2014).

1.3.2.3 Imbalance in Th1/Th2 responses during RSV infection.

Imbalances in the Th1/Th2 cytokine immune response have been related to pathogenesis of RSV and to increased severity of infection. Th1 responses are characterised by production of IFNγ, IL-1, IL-2, IL-12, IL-18, and TNFα, and the full Th1 response is important in the generation of the cell-mediated immunity required for the control of intracellular pathogens and expression of CXCR3 and CCR5. The Th2 response is characterised by IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 production, and this is involved in the generation of antibody and eosinophil responses and the expression of CCR2, CCR3 and CCR4 (Bermejo *et al*., 2007; Mella *et al.,* 2013).

As mentioned above, previous studies have demonstrated that severe RSV infections in early life are associated with a persistent increase in the production of type 2 cytokines. The expression of Th1 cytokine IFNγ was lower in nasal lavage fluid taken from infants with severe RSV bronchiolitis (Legg *et al.*, 2003), and an association was made between this and the observed IL-4/IFNγ ratio, based on a lower IFNγ concentration and higher IL-4 concentration (Garofalo *et al.,* 2001; Hassan *et al*., 2008, Caballero *et al.,* 2015). In addition, the levels of circulating $CXCR3^+$ T-cell (Th1) were significantly reduced during acute RSV infections, while $CCR4^+$T cells (Th2) were not (Roe *et al.,* 2010). Excessive Th2 or deficient Th1 response may thus be associated with more severe RSV (Legg *et al.,* 2003).

Legg *et al*. (2003) examined immune response against RSV infection *in vivo*. They detected RSV in 28 children out of 88 with an upper respiratory infection, of whom nine later developed bronchiolitis (Legg *et al.,* 2003). The IFN-γ, IL-4, IL-10, and IL-12 mRNA extracted from NPA were then measured, showing that the IL-4/IFN-γ ratio for infants with acute bronchiolitis was upregulated in NPA on days 1 to 2 days and 5 to 7 days of the illness as compared to that in children with simple upper respiratory tract infections. In addition, the ratio of IL-10/IL-12 was higher on days 1 to 2. It was thus concluded that an excessive type 2 and/or deficient type 1 immune response plays a role in RSV pathogenesis.

Recently, studies have proven the role of two other T-cell subsets determining during RSV infection, namely the interleukin IL-17 producing T helper 17 cells (Th17) and regulatory T-cells (Treg) (Kimura and Kishimoto*.,* 2010). The balance between Th17 and Treg cells has been suggested in the setting of several autoimmune diseases including rheumatoid arthritis, lupus,

and inflammatory bowel disease (Heylen *et al.,* 2014). In infants, a high levels of IL-17 in tracheal aspirates and serum during RSV bronchiolitis which suggest association with disease severity (Mukherjee *et al.,* 2011). IL-17 induce the expression of pro-inflammatory cytokines and chemokines from AECs (Bystrom *et al.,* 2013). Mukherjee *et al.,* (2011) study found that IL-17 induce mucus production and inhibition of viral clearance and enhanced a Th2 cytokine profile. During RSV infection, Treg cells have also an important role in viral clearance by regulating recruitment of CD8+ T cells to the lungs, avoiding an excessive the response of CD4+ and CD8+ T cells to RSV infection and thus an excessive inflammatory response, limiting inefficient Th2 immune responses and controlling the innate immune response by neutrophils and NK cells (Openshaw *et al.,* 2013). Production of IL-10 by Treg cells also contributes to maintaining an adequate immune response during RSV infection (Weiss *et al.,* 2011).

1.4 IL-1 family

The interleukin-1 (IL-1) family of cytokines and their receptors play a central role in the modulation of innate immunity and inflammation (Dinarello, 2009; Garlanda *et al,* 2013). The IL-1 family is closely linked to several innate inflammatory and immune responses that trigger the production of IL-1 family cytokines. The IL-1 family itself consists of 11 members; seven of these have pro-inflammatory activity (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β and IL-36γ), while the remaining four have antagonistic (IL-1Rα, IL-36Rα, IL-38) and anti-inflammatory (IL-37) properties.

The IL-1 receptor family consists of 10 members: IL-1R1; IL1R2; IL-1R accessory protein (IL-1RAcP); IL-18Rα; IL-18Rβ; ST2 (IL-33R); IL-36R (previously IL-1Rrp2); single Ig IL-1R-related molecule (SIGIRR or TIR8); the three Ig domain, containing IL-1R related-2 (TIGIRR-2 or IL-1RAPLI); and TIGIRR-1 (IL-1RAPL2) (Garlanda *et al.* 2013). Most of the genes encoding IL-1Rs map onto chromosome 2 in humans, except for SIGGIR, which maps onto a gene located on chromosome 11, and those encoding TIGGIR-1 and TIGIRR-2, which lie on the X chromosome in humans (Palomo *et al.,* 2015).

All IL-1 family members bind to the same conserved receptors, which consist of both extracellular immunoglobulin domains and intracellular Toll/ IL-1 (TIR) domains. All IL-1Rs have three extracellular immunoglobulin (Ig) domains with the exception of SIGIRR, which contains one extracellular immunoglobulin (Ig). Further, with the exception of IL-1R2, which lacks a TIR domain, all the intracellular domains of the IL-1Rs share some homology with the Toll-like receptors (TLR), thus being known as Toll-like / IL-1R (TIR) domains.

All the signalling pathways of pro-inflammatory agonists of the IL-1 family lead to activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the subsequent gene transcription of proteins involved in the regulation of both local and systemic inflammatory reactions (Palomo *et al.,* 2015) (Figure 1.4). The antagonist members of the IL-1 family inhibit this signalling by binding the main receptors, preventing co-receptor recruitment and thus ultimately inhibiting the immune response.

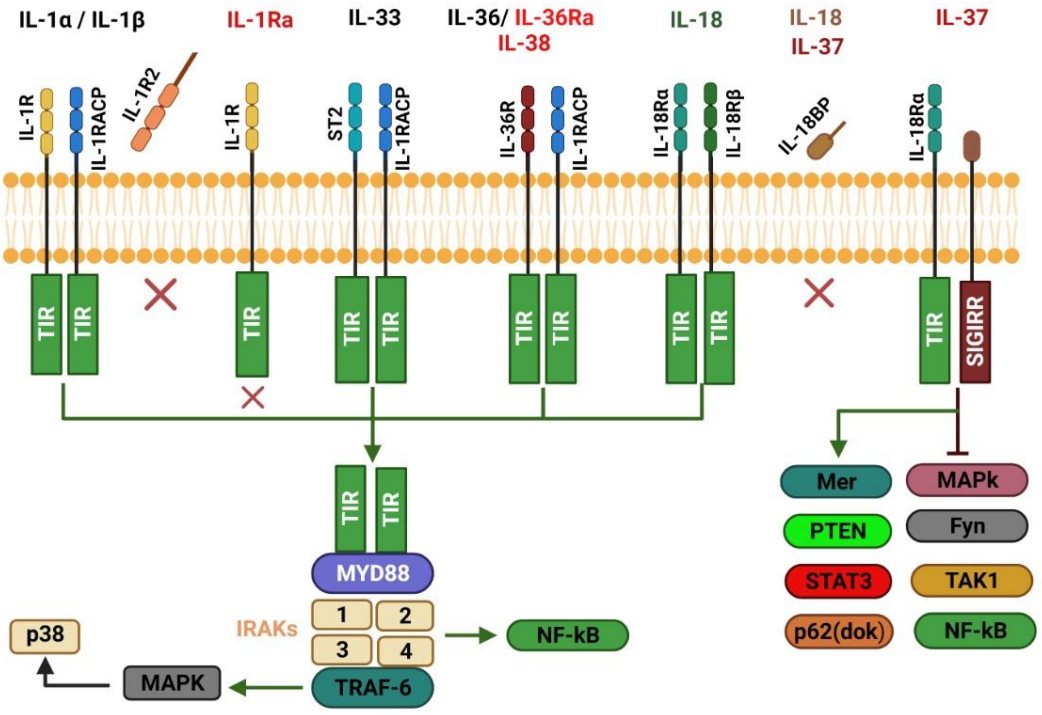

Figure. 1.4 IL-1 family cytokines and their known receptors. The IL-1 cytokine family consists of seven agonists (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β and IL-36γ), three antagonists (IL-1Ra, IL-36Ra and IL-38) and one anti-inflammatory cytokine (IL-37). IL-1 family receptors consist of extracellular receptors (TLR) and intracellular receptors (TIR) (Adapted from Moorlag *et al,* 2018).

All IL-1 family members with the exception of IL-1Rα are translated as precursors; they thus lack a signal peptide for secretion via the Golgi apparatus. These precursors are found in the cytosol, and they exit the cell following its death by necrosis rather than apoptosis. IL-1-receptor antagonist (IL-1Rα) is instead translated by means of a signal peptide and secreted, although an intracellular form also exists (Arend, 2002). The precursors of all IL-1 family members except IL-1α are thus inactive at first; neutrophil proteases must be processed for them to gain full activation, and eventually, the infection is contained as the invading pathogen is eliminated (Afonina *et al.,* 2015). Most members of the IL-1 family promote inflammation and enhance specific acquired immune responses, though some members provide a brake on inflammation (Table 1.4).

Cytokine	Alternative Name	Receptor	Co-receptor	Inhibitory Ligands and Receptors	Main Function
IL-1α	IL-1F1	IL-1R1	IL-1RAcP	IL-1RA, IL-1R2	pro-inflammatory
IL-1β	IL-1F2	IL-1R1	IL-1RAcP	IL-1RA, IL-1R2	pro-inflammatory
IL-18	IL-1F4	IL-18Rα	IL-18Rβ	IL-18BP	pro-inflammatory
IL-33	IL-1F11	ST2 (IL-1RL1)	IL-1RAcP	sST2	pro-inflammatory
IL-36α IL-36β IL-36γ	IL-1F6 IL-1F8 IL-1F9	IL-36R (IL-1Rrp2)	IL-1RAcP	IL-36RA	pro-inflammatory
IL-37	IL-1F7	IL-18Rα	SIGIRR (TIR8, IL-1R8)	unknown	anti-inflammatory
IL-38	IL-1F10	IL-36R (IL-1Rrp2)	–	unknown	anti-inflammatory
IL-1RA	IL-1F3	IL-1R1	–	NA	IL-1R antagonist
IL-36RA	IL-1F5	IL-36R	–	NA	IL-36R antagonist

Abbreviation is as follows: NA, not applicable.

Table 1.4 Overview of IL-1 Family Members, Their (Co)-receptors, Inhibitory Ligands plus Receptors, and Main Functions. IL-1 receptor family consist of 10 members, namely, IL-1R1, IL1R2, IL-1R accessory protein (IL-1RAcP), IL-18Rα, IL-18Rβ, ST2 (IL-33R), IL-36R (previously IL-1Rrp2), single Ig IL-1R-related molecule (SIGIRR or TIR8), three Ig domain containing IL-1R related-2 (TIGIRR-2 or IL-1RAPLI) and TIGIRR-1 (IL-1RAPL2) (Afonina *et al.,* 2015).

1.4.1 IL-36 cytokines

The IL-36 subfamily of IL-1 related cytokines includes three pro-inflammatory agonists, IL-36α (length 158 AA, mass 17,684 Da), IL-36β (length 164 AA, mass 18,522 Da), and IL-36γ (length 169 AA, mass 18,721 Da), previously known as IL-1F6, IL-1F8, and IL-1F9, and one antagonist, the IL-36 receptor antagonist (IL-36Rα) (length 155 AA, mass 16,962 Da), formerly known as IL-1F5. IL-36 cytokines are expressed widely in T cells, keratinocytes, and skin, lung, and gut cells, while IL-36 agonists bind to the same heterodimeric receptor complex, which consists of IL-36R (also known as IL-1RL2) and a co- receptor subunit (IL-1RAcP) (Dinarello *et al.,* 2010). All IL-36 agonists bind to the same heterodimeric receptor to generate intracellular signalling cascades that lead to the activation of NF-kB and MAPKs to induce inflammatory responses (Bassoy *et al.,* 2018). IL-36Ra binds to IL-36R to prevent recruitment of IL-1RAcP, however, and therefore does not initiate a signalling response (Towne *et al.,* 2011).

The IL-36 cytokines and their receptors are located on human chromosome 2 in the same locus as the genes for the most of other IL-1 family cytokines and their receptors (Sims *et al.,* 2002; Taylor *et al.,* 2002). IL-36 cytokines are inactive as full-length proteins, and they thus require post-translational processing to elicit pro-inflammatory activity. Towne reported that IL-36 agonists and IL-36Rα require N-terminal processing at specific peptide locations to become active, suggesting that such processing may produce up to 1,000-fold increases in activity compared to full-length forms (Figure 1.4.1) (Towne *et al.,* 2011). Other studies have shown that incubation of the three IL-36 cytokines with neutrophil proteases allows them to be processed and converted to the active form (Clancy *et al.*, 2017).

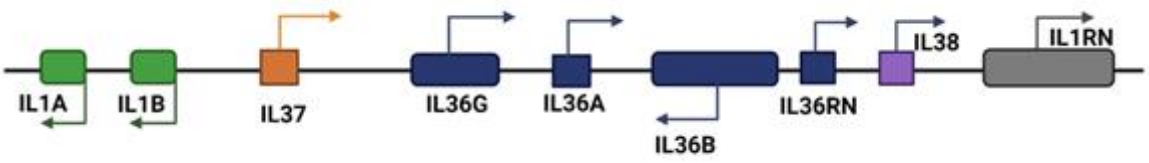

Figure 1.4.1 Schematic illustration of the human IL-1 family gene cluster. Chromosomal locations and organization of the genes encoding the human IL-1 family members. Arrows indicate the direction of transcription (Adapted from Jensen *et al.,* 2017).

1.4.1.1 Expression of IL-36

IL-36 cytokines are expressed at low levels in many tissue types, including skin, colon, tonsil, lung, gut, and brain. IL-36 can also be expressed by immune cells, including monocytes, macrophages, and dendritic and T cells (Sim and Smith, 2010; Gresnigt *et al.,* 2013). IL-36 cytokines can be induced in keratinocytes and other epithelial cell types upon the stimulation with various agents, which include cytokines, bacteria, viruses, and other pathologic conditions (Gabay *et al.,* 2016; Gresnigt *et al.,* 2013; Afonina *et al.,* 2015). Tracheal and bronchial epithelial cells express IL-36α and IL-36γ, which are induced most clearly in response to stimuli that incorporate inflammatory cytokines such as IL-1, TNF, IL-17, TLR ligands, bacteria, and smoke (Chustz *et al.,* 2011; Vos *et al.,* 2005). IL-36 may plays an important role in protecting against viral and bacterial pulmonary infection: in mice, IL-36α has been observed to exert strong pro-inflammatory effects in the lung (Ramadas *et al.,* 2012), while in human lung fibroblasts, IL-36 induces the chemokine IL-8 and the Th17 chemokine CCL20 (Chustz *et al.,* 2011).

1.4.1.2 Processing and secretion of IL-36 cytokines

The secretion mechanism of IL-36 family members as part of the pathogenesis of inflammatory diseases is still poorly understood. IL-36 cytokines are synthesised without a signal peptide; IL-36 cytokines are thus not secreted from cells through an endoplasmic reticulum-Golgi pathway. IL-36 cytokines are synthesised as precursor inactive proteins and require translational processing to deliver their active form. However, there are no caspase cleavage sites in the IL-36 amino acid sequences, suggesting that caspases play no part in IL-36 cytokine processing (Afonina *et al.,* 2011).

Previous studies have demonstrated that neutrophil proteases do however have significant roles in IL-36 N- terminal processing and activation, facilitating these functions by amplifying inflammatory responses (Ikeda *et al.,* 2013). The incubation of IL-36α cytokine with either Cathepsin G or elastase lead to processing and activation by cleavage at lysine 3 and alanine 4, respectively (Conor *et al.,* 2016). In comparison, IL-36γ is activated by elastase or proteinase3 by cleavage at the valine 15 (Val15). In human skin, Cathepsin S can process and activate IL- 36γ between the Glut17 and Ser18 cleavage sites (Ainscough *et al.,* 2017), while IL-36Rα is cleaved into an active form by neutrophil derived protease elastase (Macleod *et al.,* 2016).

Several studies have demonstrated that extracellular adenosine triphosphate (ATP) is required for the secretion of intracellularly produced IL- 36γ (Carrier *et al.,* 2011). Lian *et al.* (2012) demonstrating that secretion of IL-36γ by keratinocytes in response to bacterial flagellin was dependent on co-stimulation with ATP (Lian *et al.,* 2012). Similarly, Johnston *et al.* (2011) observed that keratinocytes stimulated with IL-1α or TNF-α require ATP for the secretion of IL-36γ. Co-stimulation with LPS and ATP is also necessary for IL-36α secretion from bone marrow–derived macrophages (Martin *et al.,* 2009), and a significant increase in IL-36γ secretion occurs in response to the combination of LPS plus ATP in the presence of Klebsiella pneumoniae (Kp) in pulmonary macrophages (Kovach *et al.,* 2016). These finding suggest that IL-36γ is most likely secreted in a nonconventional, Golgi-independent, manner. It has also been shown that IL-36γ protein can be released in microparticles from macrophages upon bacterial stimulation in conjunction with ATP (Aoyagi *et al.,* 2016). Kovach *et al* study, have also reported that IL-36γ protein is secreted within microparticles from activated pulmonary macrophages in BAL after infected with Streptococcus pneumoniae. They also found that, there is a significant increase in extracellular IL-36γ protein after sonicate the conditioned medium (Kovach *et al.,* 2016). Sonication of conditioned medium is a protocol of cell lysis that using the sound wave to disrupt the cellular membrane and release the component of microparticles which Kovach study suggest that IL-36 produced and packed in microparticles more details of this protocol in (section 2.5.3.1). These findings suggest that IL-36γ protein is contained within membrane-bound structures after infection.

1.4.1.3 The role of IL-36 cytokines in inflammatory diseases

In lungs, Influenza virus infection can induce the expression of IL-36 cytokines from AECs (Aoyagi *et al.,* 2017), which activated NF-κB signaling and increased inflammatory cytokines IL-6 and IL-8 (Chustz *et al.,* 2011). However, IL-36γ was upregulated in the lungs and played a protective role in severe H1N1 and H3N2 influenza infection via modulating macrophage polarization and activity (Wein *et al.,* 2018). Expression of IL-36 cytokines has been noted that IL-36γ expression is strongly induced in bronchial epithelium exposed to inflammatory stimuli (Kovach *et al.,* 2016). Several studies believe that SARS-CoV-2 infection may induce IL-36 secretion from endothelia cells, leading to leukocytes infiltration and skin symptoms in COVID-19 patients (Dietrich *et al.,* 2016). In addition, pulmonary infiltration of myeloid cells such as neutrophils and macrophages/monocytes have been found in COVID-19 patients with severe symptoms (Sanchez-Cerrillo *et al.,* 2020; Merad *et al.,* 2020). This finding suggest that IL-36 may drive these myeloid cell recruitment and activation, resulting in pulmonary inflammation (Wang *et al.,* 2018) which suggest that IL-36 might be a potential biomarker of severity COVID-19 disease. While IL-36γ mRNA, but not IL-36α or IL-36β mRNA, is induced in the lungs during Streptococcus pneumoniae, Klebsiella pneumoniae, P. Aeruginosa infection, or stimulation with LPS and flagellin in mice (Aoyagi *et al.,* 2016; Kovach *et al.,* 2016; Kovach *et al.,* 2017).

With respect to the skin, several lines of evidence indicate that IL-36 is involved in the pathogenesis of psoriasis. IL-36α and IL-36γ mRNA are upregulated in skin psoriatic lesions, and there is a positive correlation between expression of IL-36α and IL- 36γ and the expression of IL-17, IL- 23, TNF- α, and IFNγ cytokines (Blumberg *et al.,* 2007; Carrier *et al.,* 2011). Besides being implicated in psoriasis, IL-36 has been studied with respect to the pathogenesis of allergic contact dermatitis (ACD), with the expression of all IL-36 agonists except IL- 36Rα induced in ACD, as shown by the immunohistochemistry of the epidermal layers (Mattii *et al.,* 2013).

A recent study also demonstrated that in colonic epithelial cells from patients with inflammatory bowel disease (IBD) (Nishida *et al.,* 2016), while in human colon carcinoma cell lines (HT-29 and Widr), IL-36α and IL- 36γ enhance the production of chemokines CXCL1, CXCL2, CXCL3 (Nishida *et al.,* 2017). When combined with either IL- 17A or TNF-α, IL-36α and IL-36γ induce the production of IL-6 and CXC chemokines in human colonic subepithelial myofibroblasts (SEMFs) (Kanda *et al.,* 2015). The expression of IL-36α and IL-36γ mRNA is upregulated in the colons of

mice treated with dextran sodium sulphate (DSS), with levels correlating with those of IL-1β and IL-17A (Dunn *et al.,* 2001).

1.4.1.4 Regulation of expression of the IL-36 subfamily by other factors and cytokines

The expression of IL-36α mRNA is induced in adipose tissue-associated macrophages and murine dendritic cells (DC) upon stimulation with lipopolysaccharide (LPS) (Van *et al.,* 2010; Vigne *et al.,* 2011). LPS also induces IL-36γ mRNA in pulmonary macrophages after 4 h in a manner that persists to around 18 h after stimulation (Kovach *et al.,* 2016).

Cytokines that have been shown to stimulate IL-36 agonist expression include IL-1β and TNF-α. The combination of IL-1β, TNF-α, and IFNγ can also stimulate the expression of IL-36 cytokines in monocyte dendritic cells (MDC) (Bachmann *et al.,* 2012), while the induction of IL-36α and IL-36γ is upregulated when keratinocytes are cultured with TNF-α, IL-17 or IL-22 (Carrier *et al.,* 2011; Johnston *et al.,* 2011).

In human lungs, TNF-α, IL-1β, and IL-17 stimulate the expression of IL-36 in bronchial epithelial cells and IL-36α also increases the expression of its specific receptor, IL-36R (Chustz *et al.,* 2011; Parsanejad *et al.,* 2008). In general, the expression of IL-36 in human pulmonary disease has not been investigated thoroughly, however, and there are only a few studies estimating the expression of IL-36 in the lungs. IL-36γ mRNA expression was found to be increased in biopsies of recurrent respiratory papillomas, with expression levels correlated with disease severity (Devoti *et al.,* 2008). Kovach *et al.* found that TNF-α and IL-1β also induced IL-36γ mRNA in pulmonary macrophages (Kovach *et al.,* 2016).

IL-1β has emerged as a potent inducer for IL-36γ mRNA and protein in colonic myofibroblasts, and this induction has been observed as early as 3 hours post-stimulation (Takahashi *et al.,* 2015). TNF-α has similarly been reported to induce IL-36γ in human bronchial epithelial cells (Chustz *et al.,* 2011). Whereas colonic myofibroblast exposure to TNF-α had a minimal effect on IL-36 expression. Separate exposure to IL-1β and TNF-α encouraged the expression of IL-36γ, and simultaneous stimulation with IL-1β and TNF-α induced a synergistic increase in IL-36γ mRNA expression (Takahashi *et al.,* 2015). Keratinocyte stimulation with IL-17, either alone or in combination with poly (I:C), results in a synergistic increase in both IL-36γ mRNA and protein (Liu *et al.,* 2019).

In primary human keratinocytes, Th17 cell cytokines, including IL-22, IL-17A, TNF-α, and IL-17F, have been observed to stimulate IL-36s expression both separately and in combination. The addition of IL-22 along with IL-17A cytokines induced all IL-36 cytokines and had a synergistic effect in the regulation of IL-36 cytokines. This expression was higher in terms of IL-36γ protein as compared with expression of other IL-36 agonists. In contrast, no effect was observed in terms of the expression of IL-36 protein stimulated with IL-17F alone or in combination with IL-22. TNF-α stimulation has also been shown to induce expression of IL-36 cytokines in keratinocyte, with such induction increasing when the culture medium is treated with IL-22 (Carrier *et al.,* 2011).

The effect of Th1 cytokines on the expression of IL-36 agonists indicates that IFNγ alone stimulates an approximate a 10-fold increase in IL36β mRNA while having minimal effect on IL36α and IL-36γ mRNA (Lowes *et al.,* 2007). However, no effect was observed when keratinocytes were treated with IL-12, either alone or alongside IFNγ. These observations indicate that Th17 cytokines predominantly regulate the expression of IL-36 in keratinocytes, alongside contributions by Th1 cytokines. The Wang *et al.* (2017) study on psoriatic skin reported increased expression of IL-36α, IL-36γ, and IL-36Rα mRNA in lesional psoriatic skin as compared with non-lesional psoriatic skin or with controls. They also found an increase in the expression of IL-36γ mRNA and protein, supporting the synergistic effects of TNF-α and IL-17A (Wang *et al.,* 2017).

1.4.2 Interleukin-37 isoforms (IL-37)

Interleukin-37 (IL-37) is a member of the IL-1 family with five isoforms, designated IL-37(a-e). The IL-37 gene is located on chromosome 2, with a length of 3.617 kb (Boraschi *et al.,* 2011). IL-37 has a molecular weight of approximately 17-25 kDa (Tet`e *et al.,* 2012). It is a vital immune-regulator that inhibits inflammatory responses by reducing levels of pro-inflammatory cytokines (Nold *et al.,* 2010; Boraschi *et al.,* 2011; Al-Anazi *et al.,* 2019). IL-37 binds to the IL-18Rα chain before recruiting TIR-8/IL-1R8/SIGIRR to execute anti- inflammatory effects and suppress both innate and acquired immune responses (Kumar *et al.,* 2002; Boraschi and Tagliabue, 2013; Dinarello and Bufler, 2013; Lunding *et al.,* 2015; Nold-Petry *et al.,* 2015).

IL-37 isoforms are produced by several cell types, including peripheral blood mononuclear cells (PBMCs), plasma cells, synovial cells, tonsillar B cells, epithelial cells, macrophages, and DCs (Moretti *et al.,* 2014). However, IL-37 is not expressed by blood monocytes within healthy subjects, being instead induced by IL-1β and TLR agonists (Boraschi *et al.,* 2011). Several studies have revealed that levels of IL-37 are promptly increased on inflammation, potentially to prevent excessive tissue damage resulting from host immune responses to microbial infections (Bulau *et al.,* 2014). In addition, IL-37 has been shown to play a crucial role in the pathogenesis of several infectious diseases. However, the mechanisms underlying the anti-inflammatory effect of IL-37 during infection are poorly understood.

The human IL-37 gene is located between the IL-1β and IL36γ genes (Figure 1.4.2). The IL-37 gene is absent in mice, however and mice thus do not express IL-37 (Boraschi *et al.,* 2011). The IL-37 gene consists of six exons and can encode five isoforms: IL-37a (21.55 kDa), IL-37b (24.13 kDa), IL-37c (19.61 kDa), IL-37d (21.95 kDa), and IL-37e (17.46 kDa). Exons 1 to 3 encode the N terminal domain while exons 4 to 6 encode the C-terminal domain of these IL-37 isoforms. Exons 4 to 6 have 12 putative β-strands, forming a β-trefoil protein structure characteristic of the IL-1 family (IL-1 like domain) that can bind to IL-1Rs for signalling.

IL-37β is the longest among the IL-37 isoforms encoded by exons 1, 2, 4, 5, and 6, and a caspase-1 cleavage site has been detected in its N-terminal domain. This cleavage site exists in all IL-37 (b-e) isoforms except IL-37a. In contrast, IL-37α is encoded by exons 3, 4, 5, 6, and it is the only isoform that uses exon 3 to encode protein (Taylor *et al.,* 2002). IL-37a lacks the caspase-1 cleavage site; however, it has an elastase cleavage site at the N-terminus upstream of the β-

trefoil structure (Dinarello *et al.,* 2016). All three isoforms can be processed by caspase-1 (Figure 1.4.2).

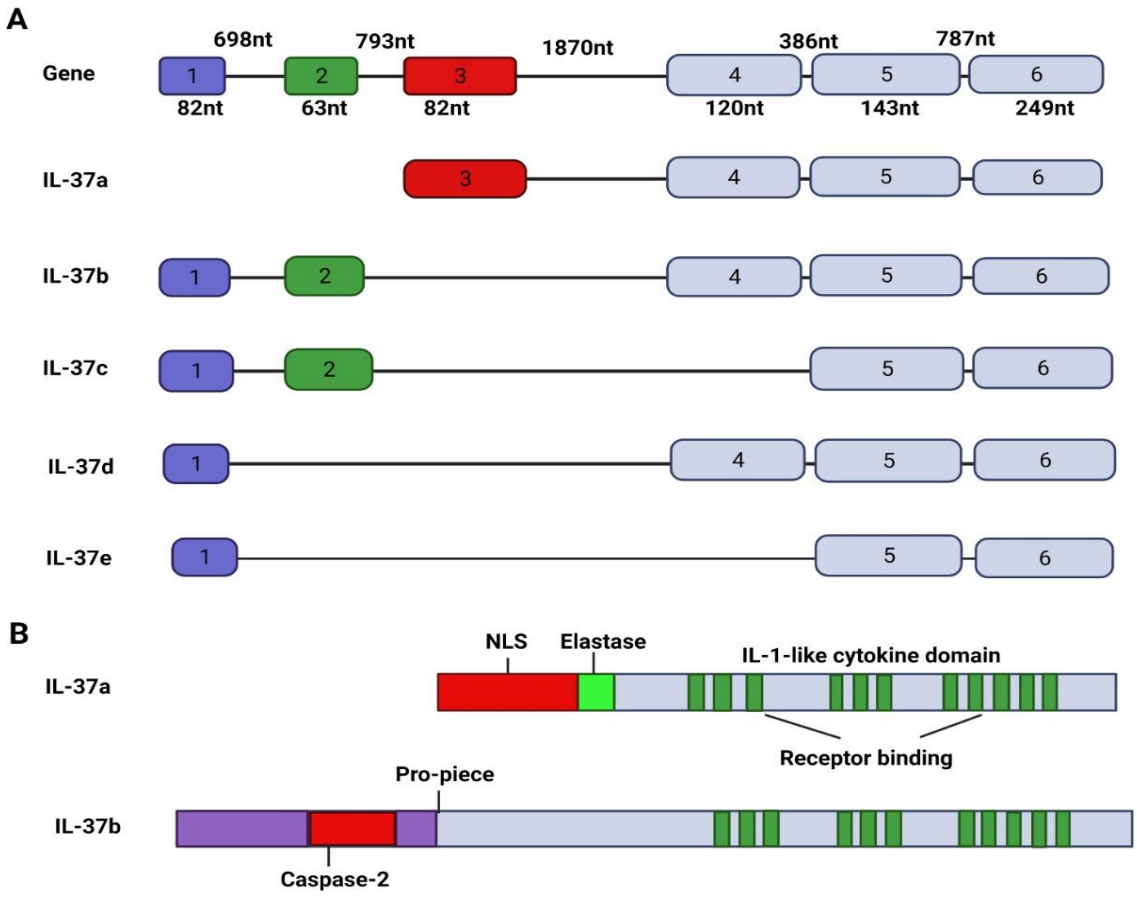

Figure 1.4.2. Structure of the IL-37 gene. IL-37 gene is composed of 6 exons (1-6) as shown. A) Illustrates IL-37 gene and related 5 isoforms; B) illustrates the protein domains of IL-37a and IL-37b isoforms, IL-37a possesses NLS and elastase putative site in exon 3, while exons 4-6 encode the receptor binding sequence. IL-37b has exon 1, which contains a caspase-1 cleavage site, while IL-37b shares exons 4-6 with IL-37a and express the receptor-binding site. Size of the exons indicated as numbers down each exon (Adapted from Hameed, 2018).

1.4.2.1 IL-37 receptors

As a cytokine, IL-37 sends signals via a receptor complex, which consists of IL-18R and IL-1R8. The extracellular IL-37 first binds to IL-18Rα and then recruits the IL-1R8 receptor to form this receptor complex for intracellular signal transduction (Nold-Petry *et al.,* 2015). IL37, IL-1R8, and IL-18Rα on the cell surface then trigger an anti-inflammatory response (Schmidt *et al.,* 2009). The IL-1R8 receptor consists of only one extracellular Ig-like domain with a long "tail" that is immersed in the cytoplasm (Vorobyeva *et al.,* 2019). IL-37 has shown anti-inflammatory properties in both extracellular and intracellular conditions. After intracellular synthesis, a portion of the precursor protein is processed by caspase-1, and this then negatively regulates pro-inflammatory genes through the Smad3 pathway. Another portion of the IL-37 precursor protein is secreted into the extracellular space, where it is processed further to induce an anti-inflammatory effect by competitively inhibiting the pro-inflammatory IL-18 and activating the anti-inflammatory signalling pathway via the IL-1R8 and IL-18Rα receptors (Figure. 1.4.2.1).

Nold *et al.* found that IL37 can exert an anti-inflammatory effect in combination with IL-18Rα, while the anti-inflammatory effect of IL-37 disappeared in SIGIRR knockout mice (IL-37tg SIGIRR-KO mice). This suggests that IL-1R8 and IL-18Rα are required for IL-37 to trigger anti-inflammatory effects both *in vivo* and *in vitro* (Nold *et al.,* 2013).

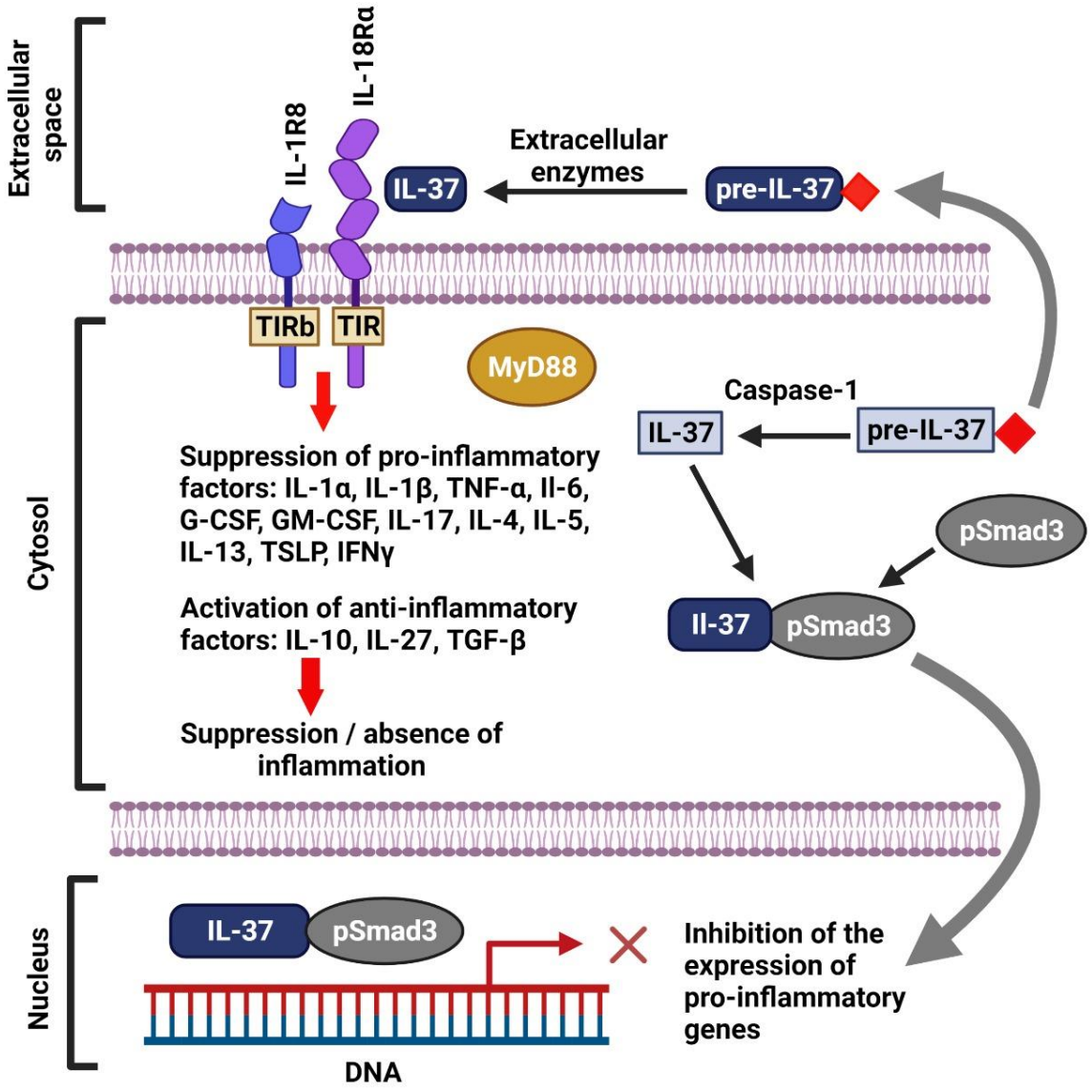

Figure. 1.4.2.1. Mechanisms of the anti-inflammatory effects of IL-37. IL-37 is synthesized as a precursor (pre-IL-37) which is capable of secreting into the extracellular space, where it is processed to a mature form through an unidentified mechanism. Mature IL-37 binds to the chains IL-18Rα and IL-1R8 (instead of IL-18Rβ); at the same time, the IL-1R8 chain carries the mutant TIRb domain (instead of functional TIR), which does not allow realization of the MyD88-mediated inflammatory effect. The precursor of IL-37 is also capable of being processed intracellularly into mature form by Caspase-1. In the cytosol, IL-37 binds to the phosphorylated form of the Smad3 factor (pSmad3). Apparently, The IL-37 / Smad3 complex is able to translocate into the nucleus and inhibit the transcription of pro-inflammatory genes (Adapted from Shilovaskiy *et al.,* 2019).

1.4.2.2 Expression and processing of IL-37

Several cells and tissues express IL-37 isoforms, including natural killer (NK) cells, stimulated B cells, monocytes, skin keratinocytes, epithelial cells, lymph node, thymus, lung, colon, uterus, and bone marrow (Boraschi *et al.,* 2011; Busfield *et al.,* 2000; Kumar *et al.,* 2000). IL-37β was first discovered in the bone marrow as synthesized by neutrophils, and it has since been found mainly in blood cells, skin keratinocytes, and the respiratory and gastrointestinal tracts (Li *et al.,* 2014). Several studies have demonstrated that IL-37 is expressed at low levels in human cells and increased by various inflammatory stimuli, including TLR agonists, IL-18, interferon IFNγ, IL-1β, TGFβ1 and TNF (Nold *et al.,* 2010; He *et al.,* 2015; McNamee *et al.,* 2011). Li *et al.* proved that IL-37 increases after stimulation with TLR agonists in monocytes (Li *et al.,* 2014), while Nold *et al.* clarified that IL-37β protein expression increases dose-dependently when the macrophage RAW cell line that stably expresses human IL-37β is stimulated with LPS (Nold *et al.,* 2010).

Cytokines of the IL-1 family are synthesised as precursor molecules containing a pro-peptide domain, and it has been established that caspase-1 is the main enzyme necessary for processing precursor molecules into mature cytokine forms to allow subsequent secretion (Deeks, 2017). IL-37β is thus synthesised as a precursor protein and processed into a mature form after cell stimulation (Meric-Bernstam *et al.,* 2019). The caspase-1 cleavage site is located in the sequence encoded by exon 1 in isoforms b, c, d, e and by exon 3 in isoform IL-37a (Shilovskiy *et al.,* 2019).

1.4.2.3 The role of IL-37 in inflammatory disease

Several strands of evidence have confirmed that IL-37 is an anti-inflammatory cytokine. The work of Ye *et al.* showed that IL-37 decreased IL-17 expression and the proliferation of Th17 cells in PBMCs and CD4+ T cells in both rheumatoid arthritis and collagen-induced arthritis patients, while IL-37 suppresses Th17 cell proliferation but has no effect on Th17 cell differentiation (Ye *et al.,* 2015). IL-37 suppresses both NF-κB and ICAM-1 expression on TLR2 activation in human coronary artery endothelial cells (Xie *et al.,* 2016). However. Li *et al.* similarly found that epithelial cell-derived IL-37 inhibits T cell and DCs activation in the inflammatory mucosa in cases of inflammatory bowel disease (Li *et al.,* 2014).

IL-37 translocates into the nucleus, decreasing cytokine production and affecting both innate and adaptive immune responses (Sharma *et al.,* 2008; Li *et al.,* 2015). Li *et al* reported an extracellular function for the IL-37 precursor that suppresses LPS-induced IL-6 production in human differentiated macrophages (Li *et al.,* 2015). However, the specific mechanism of IL-37-mediated suppression in adaptive immunity remains unclear.

Several studies have shown that IL-37 may affect human immunodeficiency virus-1 (HIV-1) infection by increasing IL-10 and TGF-β levels, which induces IL-37 production and eventually inhibits IFNγ mediated immune responses to HIV-1 infection (Wiercinska-Drapalo *et al.,* 2004; Garba *et al.,* 2002). However, Hojen *et al.* reported a positive correlation between IL-37 mRNA expression and HIV-1 infection reservoirs in the PBMC of chronically infected individuals. This correlation leads to an increase in CD14, the inflammatory marker of monocytes (Hojen *et al.,* 2015). IL-37 has also been reported as acting as a targeted therapy to decrease the mortality rate of viral myocarditis. Myocarditis is an inflammatory disease that cause heart tissues damage that is triggered by many viral infections (Zhao *et al.,* 2018). These viruses upregulate TLR4 on macrophages, enhancing pro-inflammatory cytokine production (Fairweather *et al.,* 2005) and inhibiting Treg cell functions (Frisancho-Kiss *et al.,* 2007). IL-37 decreases the functions of Th17 cells and enhances the immunomodulatory role of Treg cells in the spleen, increasing IL-10 levels in the hearts of myocarditis patients. Changes in the levels of IL-37 are also associated with increased liver damage in patients with Hepatitis B virus (HBV) and hepatitis C virus (HCV) (Jung *et al.,* 2016). De Sousa *et al.* observed that the expression of IL-37 levels was increased in keratinocytes and lymphocytes among patients with the tuberculoid form of leprosy as

compared to among those with the lepromatous (LL) form (De Sousa *et al.,* 2018). They thus suggested that IL-37 could alter the activities of such cells in the two forms of leprosy (Shuai *et al.,* 2015). Moretti *et al.* (2014) also found that IL-37 can significantly supress the activation of Th2/Th17 cells in mice with allergic bronchopulmonary aspergillosis, while a clinical study on chronic hepatitis B confirmed that serum levels of IL-37 in chronic hepatitis B patients with high viral loads were significantly higher than those in the control population (Li *et al.,* 2013).

Previous studies have also found that IL-37 is excessively expressed in the synovial tissue of patients with active rheumatoid arthritis (Nold *et al.,* 2010). While Song *et al.* (2013) demonstrated that serum levels of IL37 were significantly higher in patients with systemic lupus erythromatosis than in healthy controls. In addition, it has been found that IL-37 mRNA levels in the PBMC and serum IL-37 of SLE patients are significantly higher than in healthy controls (Ye *et al.,* 2014).

1.4.2.4 The role of IL-37 in lung disease

There are only a limited number of studies examining the role of IL-37 in respiratory infection. It has been found that a significant elevated plasma IL-37 in patients COVID-19, suppressing inflammatory responses and restraining the occurrence of cytokine storms (Li *et al.,* 2021). Qi *et al* (2019) showed that IL-37 treatment attenuates influenza virus (H1N1) induced lung tissue damage *in vivo* in BALB/c mice (Qi *et al.,* 2019). IL-37 also protects the lungs from H1N1 infection by regulating the levels of inflammatory cytokines, particularly macrophage cytokine production, and there is a growing body of evidence that suggests that IL-37 inhibits the production of proinflammatory cytokines. It has been reported that serum levels of IL-37 and mRNA expression in PBMCs are significantly higher in patients with active Mycobacterium tuberculosis (Mtb) than in healthy controls (Huang *et al.,* 2015; Allam *et al.,* 2016), while serum IL-37 levels in patients with active TB are positively correlated with the levels of IL-10 and TGF-β and negatively correlated with IL-6, IL-12, and IFNγ (Huang *et al.,* 2015). Schauer *et al.* (2017) reported that RAW macrophages transfected with human IL-37 showed a 70% reduction in pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β) production and approximately a two-fold decrease in killing capacity in response to pneumococcal infection (Schauer *et al.,* 2017). However, the regulatory role of IL-37 during RSV infection and the expression of IL-37 mRNA and protein in infected

human nasal cells, particularly with respect to the expression of IL-37 protein in patients with RSV or RV, remains unknown.

1.5 Aims of thesis

The immune response of AECs against RSV infection characterised by, the production of antiviral factors and pro-inflammatory cytokines has not been fully characterised. The aim of the work described in this thesis was to investigate the ability of RSV infected AECs to support immune responses through production of the pro-inflammatory cytokines IL-36s.

The main aim of this study was to examine whether pro-inflammatory IL-36α and IL-36γ cytokines, play a role in the immune response in infants NPAs against RSV and RV infections. This study also aim to investigate the ability of RSV to induce the expression of pro-inflammatory IL-36α and IL-36γ cytokines, and their receptors in HNAECs and cell lines (A549 and BEAS-2B) cells. To investigate whether IL-36α and IL-36γ cytokines are expressed *in vivo* during RSV and RV infection, the study measured the expression of these cytokines in (NPAs) from infants (see chapter 3, section 3.3.1). Also, the expression of IL-36α and IL-36γ or IL-36Rα mRNA and proteins, and their receptors, were measured in culture HNAECs and cell line (A549 and BEAS-2B) cells. These experiments are described in Chapter 3.

The second aim was to determine whether anti-inflammatory cytokine IL-37 could offer a therapeutic target against RSV and RV infection in infant NPAs. This study also aim to investigate the expression of IL-37 cytokines, and their receptors in HNAECs and cell lines (A549 and BEAS-2B) cells during RSV infection. These experiments are described in Chapter 4. Another objective of this study was to investigate whether IL-37 cytokine is expressed *in vivo* in infants NPAs with RSV or RV infection (see chapter 4, section 4.3.1). Determining whether RSV infection of culture HNAECs and cell line (A549 and BEAS-2B) cells results in the release of IL-37 and its receptors (IL-1R8 and IL-18R1) was achieved by measuring mRNA and protein expression, as well as examining the localisation of intracellular IL-37 protein in cultured BEAS-2B cells during RSV infection (see chapter 4, sections 4.3.2 and 4.3.3).

The third aim of this study was to investigate whether other inflammatory cytokines, such as IL-1β, TNF-α, IL-17, IL-22, IFNγ, IFNβ, IL-4 and LPS, could act as amplifier factors with respect to the

expression of IL-36γ cytokine in RSV-infected airway epithelial cells. To determine the influence of pro-inflammatory cytokines, including IL-1β, IL-17, IL-22, IFNγ, IFNβ and IL-4, on the expression of IL-36γ by A549 cells; this included examining the optimum timing and concentration for the introduction of each cytokine. In addition, a further objective of the study was to determine the best combinations of these cytokines with respect to expression of IL-36γ; to achieve this, mRNA and protein from infected and non-infected A549 cells was combined of IL-1β or TNF-α cytokines with IL-17, IL-22, IFNγ, IFNβ and IL-4 cytokines. LPS was also examined in relation to the expression of IL-36γ cytokines in both infected and non-infected cells (see Chapter 5, sections 5.3.2 and 5.3.3).

Chapter 2. Materials and methods

Overview of methodology

Human nasal airways epithelial cells (HNAECs) were isolated and grown; the cultures were taken from healthy adult donors and developed in 96 well tissue culture plates. A549 and BEAS-2B cells were grown in 96-well tissue culture plates until they were 80% confluent. HNAECs were infected with RSV A2 at MOIs of 0.1, 1 and 2.5 which were allowed to grow for 48 hours. Palivizumab is a humanized monoclonal antibody that targets the F glycoprotein on the surface of the RSV virus (Teusink-Cross *et al.,* 2016), thereby preventing viral replication, in this study palivizumab was used with as an anti-RSV control at an MOI of 2.5. In addition, cultured A549 and BEAS-2B colonies were then infected with RSV A2 at MOIs of 0.1, 1, and 2.5 for 48 hours, with non-infected cells used as a control. RNA was extracted from the cells using Qiagen and reverse transcribed. The resulting mRNA expression was the determined using TaqMan primer-probe assays, with L32, IL-36α, IL36γ, IL- 36Rα, IL-37 isoforms, IL-36Rs (IL1RL2 and IL1RAP) and IL-37Rs (IL-1R8 and IL-8R1) normalised to the housekeeping gene L32 (Figure 2.1).

The culture supernatant and pellet were both collected and IL-37 protein levels measured using ELISA. For IL-36 Proteins measurement, HNAECs, A549, and BEAS-2B cells were grown and infected with RSV at MOIs 0.1, 1, and 2.5 at 48 hours. Then, 5mM of ATP were added to the cultured cells for 30 minutes. Then, cell culture supernatants were collected and centrifuged at 1,500 g for 30 min at 4 °C. The supernatant was then collected and stored at -80°C until required. All supernatants were then thawed and ultracentrifuged at 25,000 g for 30 min at 10 °C. Supernatants were sonicated three times for 10 seconds each time to release the IL-36 proteins from their microparticles. Culture pellets were resuspended with PBS at 0.5 mM EDTA and IL-36 proteins were measured using ELISA (Figure 2.1).

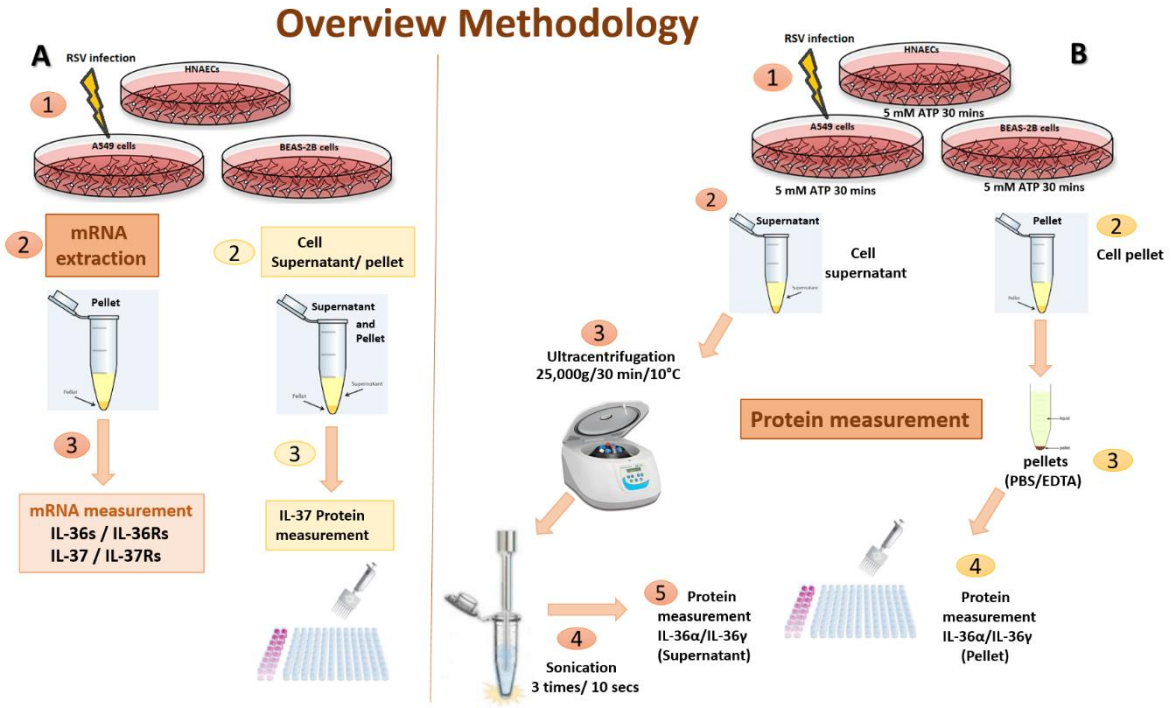

Figure 2.1 Overview of methodology showing mRNA extraction and protein measurement in HNAECs, A549, and BEAS-2B cells. A) mRNA measurement 1) HNAECs, A549, and BEAS-2B cells were grown and infected with RSV at MOIs 0.1, 1, and 2.5 at 48 hours. **2/3)** mRNA samples were extracted from the cultured cells pellet and IL-36s, IL-37, IL-36Rs, and IL-37Rs mRNA expression measured. **2/3)** The culture supernatant and pellet were both collected and IL-37 protein levels measured using ELISA. **B) Protein measurement 1)** HNAECs, A549, and BEAS-2B cells were grown and infected with RSV at MOIs 0.1, 1, and 2.5 at 48 hours. Then, 5mM of ATP were added to the cultured cells for 30 minutes. **2)** Cell culture supernatants were collected and centrifuged at 1,500 g for 30 min at 4 °C. The supernatant was then collected and stored at -80°C until required **3)** All supernatants were then thawed and ultracentrifuged at 25,000 g for 30 min at 10 °C. **4)** Supernatants were sonicated three times for 10 seconds each time to release the IL-36 proteins from their microparticles. **2)** Culture pellets were resuspended with PBS at 0.5 mM EDTA. **4/5)** IL-36 proteins were measured using ELISA.

2.1. Processing and Culturing of Human Nasal Airway Epithelial Cells (HNAECs)

2.1.1. Preparation of medium

Human nasal airway epithelial cells (HNAECs) were grown in Airway Epithelial Cell Growth Medium (AECGM) (Promo Cell, Cat# C-21060, Germany) in combination with mix supplement (Promo Cell, Cat# C-39165, Germany) and amphotericin B solution (Sigma, Cat# A2942, UK) (Table 2.1) (Davies., 2019).

2.1.2. Culturing of HNAECs

This study was approved by Liverpool central research ethics committee, and given study number 17/NW/0044, IRAS number 212223. HNAECs were obtained from healthy consenting adult volunteers with the help of Professor Paul McNamara and then cultured and grown *in vitro*. The volunteers' nasal passages were swabbed with a ConMed® Cytology Brush (Ring Handle 3.00mm x 120.00cm) (ConMed; 149R), and, after each collection phase, the brush was vigorously pressed against the sides of a centrifuge tube containing 7ml RPMI media (collection medium) (Thermo Fisher Scientific, Cat# 61870-010) to release the nasal cells. Cells were centrifuged at 1,500 rpm for 5 minutes, and the supernatant was then discarded. The pellet was resuspended and 500µl trypsin/EDTA solution was added (Lonza, Cat# CC5012). The cells were then incubated for 5 minutes at 37 °C to allow for digestion of epithelial sheets. After that, 500 µl of trypsin neutralising solution (TNS) (Lonza, Cat# CC-5002) was added to neutralise the trypsin, along with 2 ml of AECGM (Promo cells, C21060). Cells were centrifuged at 1,500 rpm for a further 5 minutes to pellet the cells, then the medium was discarded and the cells resuspended in 1 ml of AECGM. A further 2 ml of AECGM was added, and 1 ml of cell suspension added to each well of a 12-well plate pre-coated with 10 µg/ml collagen (working concentration of 50ug/ml). Typically, cells were incubated for 7 days until they were 100% confluent before subculturing occurred (Table 2.1, Figure 2.1) (Fonceca *et al.,* 2012).

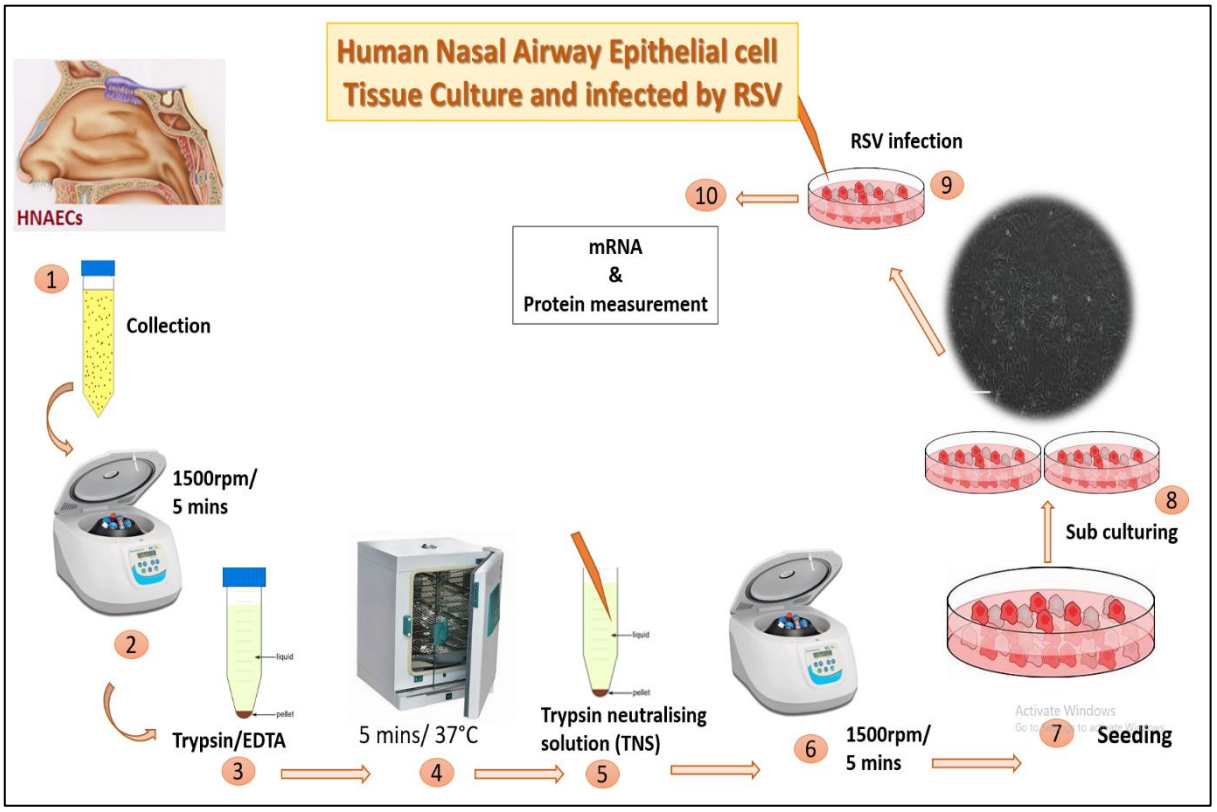

Figure 2.1.2. General methodology and culturing of human airway epithelial cells (HNAECs). **1)** HNAECs were obtained from consenting healthy adult volunteers. Nasal passages were abraded using the brush, while t release the nasal cells, the brush was then vigorously pressed against the sides of a centrifuge tube containing the collection medium. **2)** Cells were centrifuged at 1,500rpm for 5 minutes, and the supernatant was then discarded. **3)** The pellet was resuspended with 500µl trypsin/EDTA solution. **4)** The cells were incubated for 5 minutes at 37 °C. **5)** 500 µl of trypsin neutralising solution was added (TNS). **6)** Cells were centrifuged at 1,500 rpm for 5 minutes to pellet the cells, the medium was discarded, and cells were resuspended in 1 ml AECGM. **7)** Cell suspension was added to each well of a 12-well plate. **8)** After 7 days, when the cells achieved 90% confluence, the cells were subcultured then left to incubate for another 4 to 5 days before the step was repeated. **9)** When the cells reached 100% confluence, they were infected with RSV at specific MOIs and at specific time. **10)** The cells were then ready for the extraction of the mRNA and protein measurement.

Table 2.1. Media and media supplements used with HNAECs.

Name	Composition	Application	Supplier/Cat no.
Airway epithelial cell growth medium (AECGM)	Refer to manufacturer's stated composition	Human nasal airway epithelial cells culture	Promo Cell/ C-21060
Supplement Mix / Airway epithelial cell growth medium	Refer to manufacturer's stated composition	Human nasal airway epithelial cells culture	Promo Cell/ C-39165
RPMI 1640 Medium, Gluta MAX™ Supplement	Refer to manufacturer's stated composition	Human nasal airway epithelial cells	Thermo Fisher Scientific/ 61870-010
Ultroser-G	Refer to manufacturer's stated composition	Human nasal airway epithelial cells	Pall Life Sciences/15850-017
Collagen I Rat Protein, Tail	Working concentration of 50 ug/ml	Human nasal airway epithelial cells (10 ug/ml) seeding	Thermo Fisher Scientific/ A1048301
0.25% trypsin/EDTA solution	Refer to manufacturer's stated composition	Human nasal airway epithelial cells sub-culture	Lonza/ CC-5012
Trypsin neutralising solution (TNS)	Refer to manufacturer's stated composition	Human nasal airway epithelial cells sub-culture	Lonza/ CC-5002
Complete AECGM (cAECGM) medium	2% Ultroser-G	Human nasal airway epithelial cells culture	As stated above
Amphotericin B solution	Refer to manufacturer's stated composition	Human nasal Airway epithelial cells culture	Sigma/ A2942

2.1.3. Subculturing of HNAECs

After removing the media, the cells were washed with PBS, and 200 µl/well of 0.25% trypsin/EDTA was added for 3 to 5 minutes at 37 °C. Trypsinization is the process of cell dissociation using trypsin enzyme which breaks down proteins, to dissociate adherent cells from the flask in which they are being cultured. When added to a cell culture, trypsin breaks down the proteins that enable the cells to adhere to the flask and the cells will be in suspension. Plates were visualised under the microscope to identify when 90% dissociation was achieved, when 200 µl/well of trypsin neutralising solution (TNS) added to each well, The cell suspension was then transferred into a 10 ml sterile tube, and 1 ml AECGM added before being centrifuged at 1,500 rpm for 5 minutes to pellet the cells. The supernatant was discarded, and the cells were resuspended in 1 ml of media for counting. Cells were seeded at $1x10^4$ across 96-well plates and $1x10^5$ across 12-well plates. After subculturing, the cells were incubated for 4 to 5 days, until 100% confluence was reached, at which point they were subcultured again. The maximum repetition of cell culturation is usually only two, with three passes for experimentation. Subculturing was thus stopped at passage three (Fonceca *et al.,* 2012) (Figure 2.1.3).

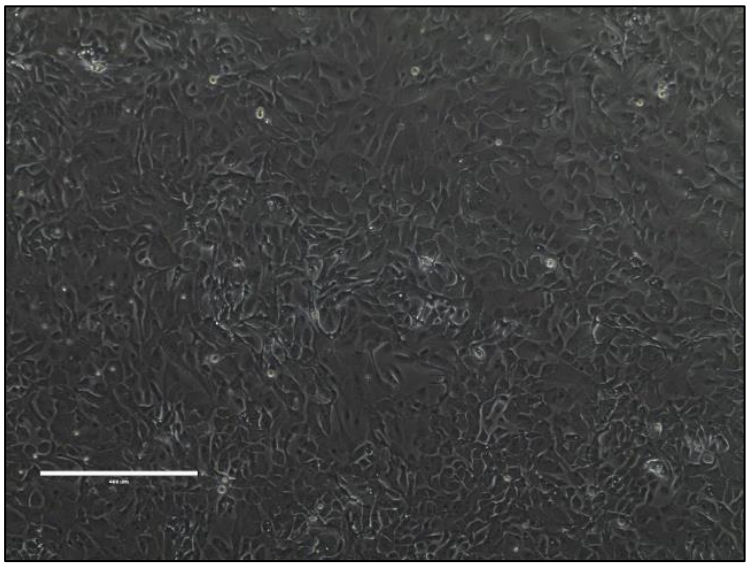

Figure 2.1.3 Culturing human nasal airway epithelial cells to form a confluent monolayer. Image representative of donor nasal AECs taken from the first passage at day 7 after seeding. Image taken using a light microscope. Scale bar represents 400 µm.

2.2. Culture of A549 and BEAS-2B cells

2.2.1. Preparation of medium

Airway epithelial A549 and BEAS-2B cells were grown in Dulbecco's Eagle Epithelial Media (DMEM) (Sigma, Cat# D5796, UK) supplemented with 10% Fetal Bovine Serum (FBS) (Thermo scientific, Cat# Fisher-16000, UK) and Gentamicin (Sigma, Cat# G1397, UK) (Canton *et al.,* 2013; Ghio *et al.,* 2013). The growth of human bronchial epithelial cells at an air-liquid interface alters their response to particle exposure and particle and fibre toxicology (https://doi.org/10.1186/1743-8977-10-25) (Fonceca *et al.,* 2012) (Table 2.2.A).

2.2.2. Culture of A549 and BEAS-2B cells

A549 is a human epithelial cell line derived from lung carcinoma tissue, whereas BEAS-2B cells are derived from human bronchial epithelium transformed by a hybrid adenovirus 12-SV40. A549 and BEAS-2B cells were pipetted into sterile universal tubes, which contained 15 ml of warm cDMEM medium (10% FBS, 1% Gentamicin) for A549 cells and 5 ml of cDMEM for BEAS-2B cells. Cells were cultured in T75 for A549 cells and T25 for BEAS-2B cells in tissue culture flasks (Nunc, Thermo Fisher Scientific) at 37 °C with 5% CO2. After 24 hours of culturing, the culture media was changed to remove any dead cells and the remaining cells were examined under the light microscope to ensure that they were attached. BEAS-2B cells' flasks and plates were coated with 3 µg/ml collagen (working concentration of 50 ug/ml) (Thermo scientific, Cat# Fisher A1048301) diluted in 20 mM acetic acid. Both the plates and flasks were incubated for one hour at room temperature, then washed thrice with sterile phosphate buffered saline (PBS) (Sigma, Cat# P3813, UK) before the addition of the cells (Fonceca *et al.,* 2012) (Table 2.2.A and B).

2.2.3. Subculturing of A549 and BEAS-2B cells

Cells were subcultured twice weekly as soon as 80 to 90% confluence was achieved in the flasks (Corning Costar); the media was changed every 2 to 3 days. A549 cells were seeded at 10,000 cells/cm^2, and BEAS-2B cells were seeded at 5,000 cells/cm^2. Culture media were removed, and cells washed with 5 to 10 ml of sterile PBS. To release the cells from the flask surface, 1 to 3 ml of 0.25% trypsin (Sigma, Cat# 59427C) was added into each set of cells, which were then placed directly into the incubator at 37 °C, with 5% CO2 for 3 to 5 minutes. The cells were examined

under the microscope to identify when 90% of cells had detached from the flask surfaces. A dose of 6 to 7 ml of cDMEM was then added to neutralise the trypsin, and the full 7 to 10 ml of liquid transferred into a 10 ml sterile tube to be centrifuged at 2,200 rpm for 5 minutes to pellet the cells. After the supernatant was discarded, the pellet was resuspended in 1 ml of complete media. The total number of cells was then counted using a Bright-Line™ haemocytometer (Reichert, USA). The maximum number of cells subcultures was 15 passages before a new cell vial was used. Depending on how many cells were needed, cells were diluted with the complete DMEM medium and pipetted into 12 well plates (2.5 ×105 cells/ml), T25 flasks (1× 106 cells/ml), or T75 flasks (2.5 ×106 cells /ml) for 3 days. Subsequently, cells were placed in an incubator at 37 °C, with 5% CO2, and the media was changed every 2 days. Cells were thus grown until confluence was again reached (Table 2.2.A and B).

2.2.4. Freezing of A549 and BEAS-2B cells

Cells were only selected for long-term storage up to passage 4. After centrifugation, the culture medium was removed, and the cells were washed twice with sterile PBS. Cells were dissociated with trypsin, placed in 10 ml of media and transferred into sterile centrifuge tube and centrifuged at 2200 rpm for 5 minutes, the supernatant was discarded, and the pellet cells were resuspended in 1 ml of cDMEM containing 10% DMSO (Table 2.2). The density of the cells was set at 1.5x106 cells/ml and 1 ml of this was added to each cryovial (Fisher, UK). Cryovials were placed in a CoolCell® freezing container (BioCision) and incubated at -80 °C for 24 hours before being transferred to 180 °C freezer for long-term storage (Table 2.2.A and B).

2.2.5. Stimulation of A549 cells with cytokines and other factors

A549 cells were seeded at 1.5 × 104 cells/ml in cDMEM medium in uncoated 12-well plates. Cells at 70% confluence were treated with cytokines with different concentrations (0.1, 1, 5, 10, 50, 100, and 200ng/ml) that included IL-β (PEPROTECH, Cat# 200-01B), TNF-α (PEPROTECH, Cat# 300-01A), IFNγ (PEPROTECH, Cat# 300-02), IL-17A (PEPROTECH, Cat# 200-17), IL-22 (PEPROTECH, Cat# 22-22), and LPS (SIGMA, Cat# L2630) for 0, 3, 6, 12, 24, and 48 hours. The cells were also treated with 5 mM Adenosine 5′-triphosphate disodium salt solution (ATP) (Sigma, Cat# A6559, UK) for 30 minutes before cell collection. Supernatants were then collected and analysed for the expression of IL-36 cytokines. RNA was also isolated and examined for the expression of IL-36 mRNA cytokines (Table 2.2.B and C).

Table 2.2.A. Media and Media Supplements for A549 and BEAS-2B cells.

Name	Composition	Application	Supplier/Cat no.
Dulbecco's modifiedEagle's medium (DMEM)	Refer to manufacturer's stated composition	A549, BEAS-2B and Hep2 culture	Sigma/ D5796
Collagen I RatProtein, Tail	Working concentration of 50ug/ml	BEAS-2B (3ug/ml) and human nasal airway epithelial cells (10ug/ml) seeding	Thermo Fisher Scientific/ A1048301
Foetal Bovine Serum(FBS)	Heat inactivated (60 ˚C for 30 minutes in water bath)	A549, BEAS-2B and Hep2 cells culture	Thermo Fisher Scientific/ Fisher-16000
Gentamicin solution	Refer to manufacturer's stated composition	A549, BEAS-2B and Hep2 cells culture	Sigma/ G1397
Trypsin 10x solution	2.5% trypsin diluted in PBS to 0.025%	A549, BEAS-2B and Hep2 culture	Sigma/ 59427C
Complete DMEM (cDMEM)	10% FBS, 1% gentamicin in DMEM	A549, BEAS-2B and Hep2 culture	As stated above
Serum-free DMEM	1% gentamicin in DMEM	A549, BEAS-2B and Hep2 infection culture	As stated above

Table 2.2.B. Reagent, Buffer, and Kits used for A549 and BEAS-2B cells.

Name	Composition	Application	Supplier/Cat no.
phosphate buffered saline (PBS)	0.014M KH2PO4, 0.008M Na2HPO4-7H20, 0.0026M KCl, 0.137M NaCl	Cell Culture	Sigma/ P3813
Dimethyl sulfoxide (DMSO)	Dimethyl sulfoxide (DMSO) (C_2H_6OS)	Cell Culture	Sigma/ 2650
Adenosine 5'-triphosphate disodium salt solution (ATP)	20 mM Adenosine 5′-triphosphate disodium salt solution	A549, BEAS-2B and human airway epithelial cell culture	Sigma/ A6559
Lipopolysaccharide (LPS)	100 ng/ml of LPS in culture medium	A549 cell stimulation	Sigma/ L2630

Table 2.2.C. Human Recombinant proteins.

Name	Working concentration	Supplier/ Cat no.
IL-1β	100 ng/ml	Peprotech/ 200-01B
TNF-α	100 ng/ml	Peprotech/ 300-01A
IFN-γ	100 ng/ml	Peprotech/ 300-02
IL-17A	100 ng/ml	Peprotech/ 200-17
IL-22	100 ng/ml	Peprotech/ 22-22

2.3. RSV preparation

2.3.1. RSV propagation

Hep2 cells were used for propagation of RSV A2. These were seeded in uncoated T75 flasks at a concentration of 30,000 cells/cm^2 in a total volume of 15ml of complete DMEM medium plus 10% FBS and 1% Gentamicin. Cells were grown at 37 °C with 5% CO_2 until 60 to 70%, confluent. The media was then removed, and the cells washed twice with 15ml PBS. RSV A2 stock was added in 5 ml of serum-free DMEM (Sigma, Cat# D5796, UK) and this was placed onto the Hep-2 cells. To ensure infection over the whole flask, each flask was rocked gently for two hours at 37 °C with 5% CO_2 before 11 ml of complete cDMEM plus 10% FBS was added. Each flask was then left overnight in the incubator. Cells were examined daily until a 50% cytopathic effect was visible, typically at 48 hours, at which point 9 ml of the media was removed and discarded. The monolayer was then harvested using a cell scraper and the cells were collected in a 10ml sterile tube. Cell were lysed using10 passes on the ice through a 2 ml syringe and a 25-gauge needle. The lysate was then centrifuged at 1,500 rpm in a pre-cooled centrifuge at 4 °C for 5 minutes. The supernatant was aliquoted into pre-labelled cryovials and placed in liquid nitrogen. These cryovials were then quickly transferred to -80 °C storage (Table 2.1.A and B) (McNamara *et al*., 2005).

2.3.2. Determination of RSV titre by plaque assay

RSV viral titres were measured by titration on monolayers of the cell line A549. A549 cells were seeded into 96-well plates at 10,000 cells/cm^2 and incubated at 37 °C with 5% CO2 for 24 hours. Cells were washed with PBS before the serial dilution of RSV in serum-free DMEM, then added in triplicate wells (50 µl/well) for two hours, after which 150 µl of complete media was added and cells were incubated for 24 hours. Cells were washed with PBS fixed with methanol containing 2% hydrogen peroxide (Sigma, Cat#H1009, UK), and then stained with goat anti-RSV biotinylated antibody (1/200 dilution) (Bio Rad; Cat#7950-0104) before being labelled using an Extravidin® peroxidise colour development substrate for 30 minutes at room temperature. After washing with PBS, 3-Amino-9-ethylcarbazole substrate was added for 20 minutes until plaques were formed. The plaques were then diluted and examined under a light microscope to achieve a count of 100 to 200 /well (Figure 2.3). Plaque forming units (PFU) were then calculated as the number of plaques/dilution factor x volume of added fluid (µl) (Table 2.1.B and Table 2.3) (Alturaiki., 2014).

Table 2.3. Reagent, Buffer, and Kits of RSV preparation.

Name	Composition	Application	Supplier/Cat no.
Extravidin-Peroxidase	Mixture of peroxidase and 2-Methyl-4-isothiazolin-3-one	RSV plaque assay	Sigma/ E2886
3-Amino-9-ethyl carbazole substrate	AEC 9-Ethylcarbazol-3-amine ($C_{14}H_{14}N_2$)	RSV plaque assay	Sigma/ 03005
hydrogen peroxide	Hydrogen peroxide solution, 30% in H_2O, containing a stabiliser	RSV plaque assay	Sigma/ H1009

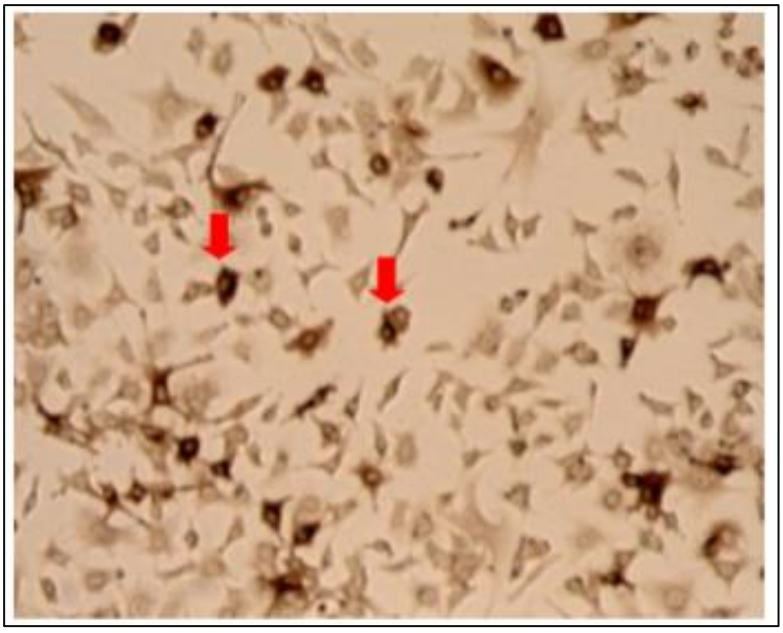

Figure 2.3. RSV plaque assay. RSV positive A549 cells are shown as dark brown spots. Those indicated by arrows were formed after staining with an anti-RSV biotinylated goat anti-RSV and labelled using an Extravidin peroxidise colour development substrate. Image taken using the EVOS XL cell imaging system (Alturaiki., 2014).

2.4. Polymerase Chain Reaction (PCR)

2.4.1. RNA extraction and isolation

Following the manufacturer's instructions, total RNA was extracted from the cells using an RNeasy Mini kit (QIAGEN, Cat# 74106). This product combines the selective binding properties of a silica-based membrane with the rapidity of microspin technology, with a specialised high-salt buffer system allowing up to 100 µg of RNA over 200 bases long to bind to the RNeasy silica membrane. Briefly, 350 µl of lysis buffer RLT was added to each well of a 12–well plate along with about 2.5×10^5 cells. The cells were mixed well and transferred into autoclaved Eppendorf tubes before 350 µl of 70% sterile ethanol was added to the homogenised lysate in each tube. The tube contents were mixed, and the tubes placed into the RNeasy Mini QIAcube kit columns with the aim of providing appropriate binding conditions. Tubes were centrifuged at full speed for 15 seconds, after which the collection tubes were discarded, and 500 µl of wash buffer RWI added and centrifuged at full speed for 15 seconds. Subsequently, the cells were washed twice with 500 µl of RPE, once for 15 seconds and once for two minutes. The collection tubes were again removed and replaced with new tubes, and the RNeasy Mini columns centrifuged at full speed for two minutes. Finally, 50 µl of DNAse/RNAse free water was added into each tube for one minute at room temperature. The total RNA was then collected by centrifuging the column into sterile Eppendorf tubes and measured by NaNoDrop to determine the concentration of RNA (Biotech International) (McNamara *et al.,* 2012) (Table2.4.A).

2.4.2. Reverse transcription (RT)

Reserve transcription combined with the reverse polymerase chain reaction (RT-PCR) has proven to be a powerful method for quantifying gene expression (Livak and Schmittgen, 2001). A cDNA single-strand was thus synthesised with a high capacity cDNA Reverse Transcription Kit (A&B applied biosystem, Cat#4368814). A 10 µl extract of each RNA sample, containing around 1 µg of total RNA, was combined with 10 µl of freshly prepared reverse transcription master mix in a pyrogen- free sterile tube containing RNase and DNase (Thermo Fisher Scientific; 3451). The master mix consisted of 2 µl 10X RT buffer, 2 µl 10X RT Buffer, 2 µl 10X RT Random Primers, 1 µl 25X dNTP Mix, 1 µl MultiScribe® Reverse Transcriptase, and 4 µl nuclease-free water to each 10 µl RNA. To complete cDNA synthesis, samples were incubated in a TC-512 thermal cycler

(Techne, USA) for 1 hour at 37 °C. The cDNA samples were then diluted 1:10 with the addition of 180 µl nuclease-free water. Samples were then either stored at -20 °C or carried forward to qPCR analysis (Section 2.4.3) (McNamara *et al.,* 2005) (Table2.4.A).

2.4.3. Quantitative Polymerase Chain Reaction (qPCR)

TaqMan real-time RT-PCR was performed using an Applied Biosystems 7500 Sequence Detection System. TaqMan® probes (Table 2.4 B) were added into TaqMan® gene expression Precision PLUS MasterMix with ROX buffer (Applied Biosystems, Cat#4369016) (Table2.4.A). Samples of cDNA (10µl) were then added into each well, and 1 µl of TaqMan® primer/probes mixed with 10 µl of Precision PLUS MasterMix to give a final volume of 21 µl (Table 2.4.A and B). Each sample was prepared in duplicate, and each plate was sealed with a cover and centrifuged for 10 seconds.

The control housekeeping gene for each cDNA sample was identified as the ribosomal L32 gene. Housekeeping genes are typically constitutive genes expressed relatively constantly in all cells under normal and pathophysiological conditions, and L32 is a key example of a housekeeping gene widely used for experimental purpose as a reference gene to analyse changes in the levels of target genes. In a 96-well PCR plate (Starlabs, UK), each sample was run in duplicate on a 7300 Real-Time PCR System (Applied Biosystems, UK) using Sequence Detection Software V1.4 (Applied Biosystems, UK). The PCR amplification required three steps: 1. Denaturing: dsDNA was separated to yield single stranded DNA at 95 °C.

2. Annealing: primers were encouraged to bind the single target DNA at 40 to 65 °C.

3. Extending: DNA polymerase extended the primers at 72 °C.

Amplification plots for each gene allowed the threshold cycle (Ct) (the point between the amplification curve and the threshold line) to be detected by means of a fluorescent signal

Real-Time PCR focuses on the exponential phase, which provides the most precise and accurate data for quantitation. During the exponential phase, the real-time PCR instrument calculates values CT– the PCR cycle at which the sample reaches the threshold. The CT value is used in relative quantitation (Table 2.4.A and 2.4.B).

2.4.4. TaqMan RT-PCR chemistry

TaqMan real -time PCR uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR cycles. The availability of these fluorogenic probes enabled the development of a real-time method for detecting only specific amplification products. TaqMan probes are dual labeled, hydrolysis probes that increase the specificity of real-time PCR assays. TaqMan probes contain: a reporter dye (for example, FAM™ dye) linked to the 5′ end of the probe, a nonfluorescent quencher (NFQ) at the 3′ end of the probe and MGB moiety attached to the NFQ TaqMan MGB probes also contain a minor groove binder (MGB) at the 3´ end of the probe (Afonina *et al.*, 1997; Kutyavin *et al.,* 1997) (Figure 2.4.4).

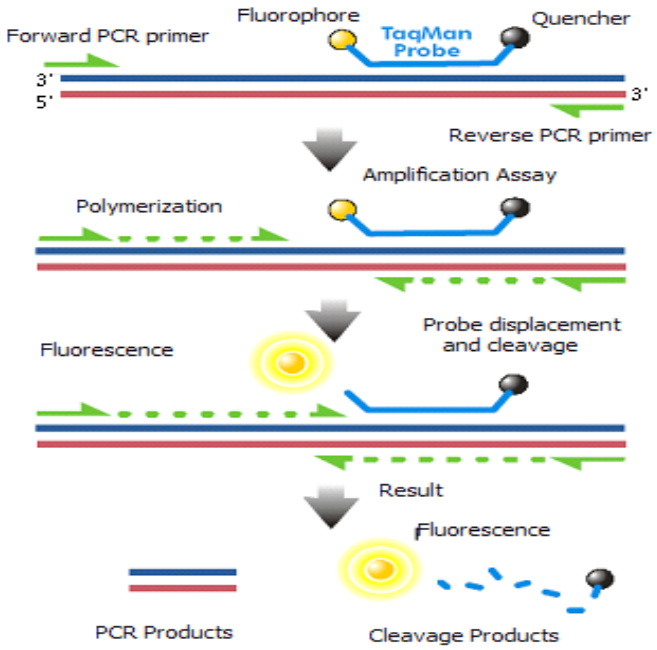

Figure 2.4.4 TaqMan probe based assay chemistry. 1) An oligonucleotide probe is constructed with a fluorescent reporter dye bound to the 5′ end and a quencher on the 3′ end. While the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer through space. **2)** If the target sequence is present, the probe anneals between primer sites and is cleaved by the 5′ nuclease activity of the taq DNA polymerase during extension. This cleavage of the probe: Separates the reporter dye from the quencher, increasing the reporter dye signal. Removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. **3)** Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed (Kutyavin *et al.,* 1997).

2.4.5. Analysing RT-PCR Data

This study use the relative quantitation of gene expression method to quantify differences in the expression level of a specific target (gene) between different samples. The data output is expressed as a fold-change of expression levels that use the comparative CT (ΔCT) method (relative quantitation) Relative quantitation is a technique used to analyze changes in gene expression in a given sample relative to a reference sample (such as L32) (Livak and Schmittgen., 2001). Ideally, real-Time PCR focuses on the exponential phase, which provides the most precise and accurate data for quantitation. During the exponential phase, the real-time PCR instrument calculates values CT– the PCR cycle at which the sample reaches the threshold. The CT value is used in relative quantitation (Figure 2.4) (Table 2.4.A and 2.4.B).

The 2(-Delta CT) method is a convenient way to analyse the relative changes in gene expression seen in real-time quantitative PCR experiments (Livak and Schmittgen, 2001), and this was selected in this case. Analysis of each gene was done using the comparative CT method (2- ΔCT) using the equations below, with housekeeping gene L32 used as the internal reference gene (McNamara *et al.,* 2013). The comparative CT method makes several assumptions, including that the efficiency of the PCR is close to 1 and the PCR efficiency of the target gene is similar to that of the internal control gene. The PCR efficiency of the target and internal control genes are thus included in the equation to ensure that differences in efficiency between the target and internal control are accounted for in the calculation. The comparative CT method thus promotes both ease of use and the ability to present data in the form of “fold change” in expression. In this study, a 60-cycles programme was run but 30 cycles were used to detect the target genes (McNamara *et al.,* 2000). Amplification plots for each gene allowed the threshold cycle (Ct) (the point between the amplification curve and the threshold line) to be detected by means of a fluorescent signal.

1) Duplicates' Ct values were averaged for both the housekeeping gene and gene of interest

2) Ct values were corrected to the housekeeping gene, L32, to give a relative Ct value. This was then used to calculate the delta (ΔCt) = Ct (target gene) – Ct (housekeeping gene)

3) The fold changes in target gene expression relative to the internal housekeeping gene (L32) were calculated using (2^ - ΔCT) = 2^ - (ΔCT)

4) Relative gene expression values were then used to create a graphical presentation and expressed as percentages of L32 housekeeping gene expression by multiplying the value given for relative gene expression by 100.

Table 2.4.A. Reagent, Buffer, and PCR Kits.

Name	Composition	Application	Supplier/Cat no.
High-capacity cDNART kit	10x RT Buffer, 10x RT Random Primers,25X dNTP Mix (100 mM), MultiScribe Reverse Transcriptase (50U/ul)	Reverse transcription	Applied Biosystems/ 4368814
TaqMan™ Universal PCR Master Mix	Refer to manufacturer's stated composition	qPCR	Life technologies/ 4369016
PrecisionPLUS MasterMix with ROX	Refer to manufacturer's stated composition	qPCR	Primer Design/ PPLUS-R-XXML
RNeasy Mini kit	Refer to manufacturer's stated composition	RNA extraction from A549 and BEAS-2B cells and human nasalepithelial cells	Qiagen/ 74106
Rnase-Free Dnaseset (50)	Refer to manufacturer's stated composition	RNA extraction from A549 and BEAS-2B cells and human nasalepithelial cells	Qiagen/ 79254

Table 2.4.B. Taqman® primer/probe assays for PCR.

Primer/ Probe	Gene	Assay ID	Amplicon length
L32	MRPL32	Hs00388301_m1	81
Interleukin-8	CXCL8	Hs00174103_m1	101
Interleukin-36α	IL-36α	Hs00205367_m1	88
Interleukin-36γ	IL-36γ	Hs00219742_m1	64
Interleukin-36RN	IL-36RN	Hs01104220_m1	58
Interleukin-37	IL-37C	Hs01020567_m1	123
Interleukin-37	IL-37	Hs00205363_m1	79
Interleukin-37	IL-37β	Hs00367199_m1	134
Interleukin-37	IL-37	Hs00367201_m1	60
Interleukin-1RL2	IL-1RL2	Hs00909276_m1	90
Interleukin-1RAPL1	IL-1RAPL1	Hs00990788_m1	60
Interleukin-18R1	IL-18R1	Hs00175381_m1	63
Interleukin-1RAP	IL-1RAP	Hs00895050_m1	116

2.5. Enzyme Linked Immunosorbent Assay (ELISA)

When testing took place in 2017, no commercial IL-36γ ELISA was available. Attempts were thus made by the researcher to create an IL-36γ cytokine ELISA based on the results of the Kovach study; however, no IL-36γ cytokines appeared in the samples (Kovach *et al*., 2016). A sandwich approach with the relevant antibody was thus used (Cat# AF2320). Figure 2.5 shows the concentration in each sample plate, ranging from high to low concentrations, with the left-hand side of the plate reserved for standard peptides. This was used to select a concentration pair that offered a decent standard curve from high to low background levels (Table 2.5.B) (Figure 2.5).

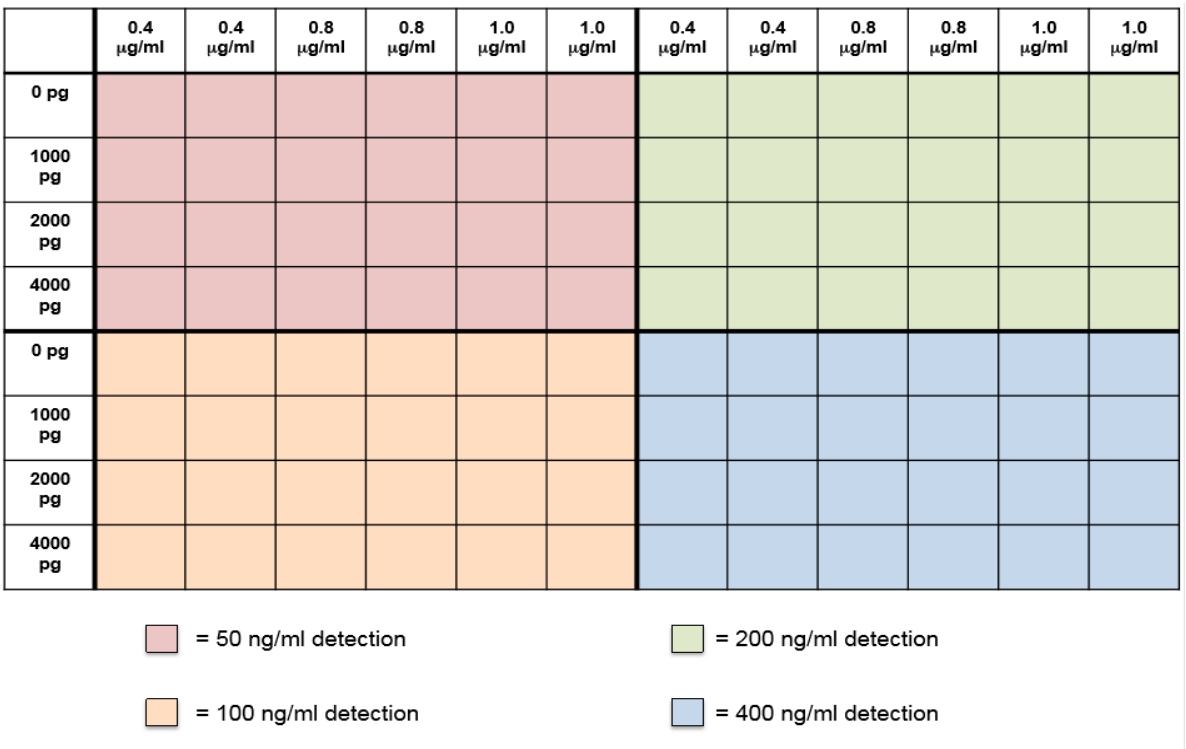

Figure 2.5 Experimental ELISA plate showing the concentrations for IL-36γ protein. 1) The plate was coated with a capture antibody (in 0.4, 0.8, and 1 µg/ml concentrations); **2)** the sample was added, and any antigen present thus bound to the capture antibody, with protein concentrations of 0, 1,000, ,2000, and 4,000 pg/ml; **3)** the detecting antibody was added, at 50, 100, 200 and 400 ng/ml, again binding to the antigen; **4)** an enzyme-linked secondary antibody was added to bind to the detecting antibody; **5)** a substrate was added, and this was converted by enzymatic action to detectable form.

All ELISA assays were examined according to the manufacturer's instructions. Table 2.4 illustrates the details of the capture, and the standard and detection antibodies were all aliquoted and stored at -70 °C (Table 2.5.A and B). All assays were supplied by R&D systems and were carried out on MaxiSorp® flat bottom 96-well plates (Nunc, Thermo Fisher Scientific). No protein should be detected in the blank graphs, as these should be at a level below the detection limit (negative control samples). The absorbance for each sample was read at 540 nm using an ELx800 (BioTek) absorbance microplate reader, while Optical Density (OD) values were collected using KC junior software (version 1.4.1.8) (Davies., 2019) (BioTek).

2.5.1. Plate Preparation

The capture antibody was diluted to the working concentration in PBS without a carrier protein. Using a 96-well plate, 100 µl of diluted capture antibodies were added in each well, and plates were sealed and incubated overnight at room temperature. The next day, the plate was washed three times with wash buffer (0.05% Tween-20 in 400 µl PBS) in an auto washer (ELx50 Microplate Strip Washer-Bio-Tek). After the final wash, any remaining wash buffer was removed by aspirating and by inverting the plate and blotting it with clean paper towels. After that, 300 µl of reagent diluent (1% BSA (Sigma, Cat#A7030) in PBS) was added in each well to block the plate. All plates were incubated for one hour at room temperature and then washed three times with wash buffer (Table 2.5. A and C).

2.5.2. Preparation of standard

A seven-point standard curve was prepared using two-fold serial dilutions of reagent diluent. A high standard was required, with 800 pg/ml recommended for human IL-36α, 1,200 pg/ml recommended for human IL-36γ, and 2,000 pg/ml recommended for human IL-37. A dose of 100 µl of each standard was added in duplicate wells and incubated at room temperature for two hours before being washed three times with wash buffer. The standard curve was then calculated using the KC Junior program to generate a four-parameter logistic (4-PL) curve-fit (Table 2.5.A and C).

2.5.3. Sample preparation

Human IL-36α, IL-36γ, and IL-37 were measured in the culture supernatants and pellets of the A549, BEAS-2B, and HNAECs. The supernatants were collected and centrifuged at 1,500 g for 30 min at 4 °C to remove all cell debris and apoptotic bodies. The supernatant was then collected and stored at -80°C until further use, for a maximum period of 2 weeks. Supernatants were thawed as required and ultracentrifuged at 25,000 g for 30 min at 10 °C for testing. The pellets were resuspended in a 1:1 ratio of RIPA buffer (Sigma Cat#R0278) to PBS, with the addition of 0.5 mM EDTA. Supernatants were sonicated three times, for 10 seconds a time (section 2.5.3.1) and 100 µl of each sample was added in duplicated wells (Table 2.2) (Kovach *et al*., 2016). The plate was then sealed and incubated for two hours at room temperature before being washed three times. Any remaining samples were removed from the wells before washing and the addition of the detection antibodies (Table 2.5.A and C).

2.5.3.1. Sonication Samples

Sonication is defined as a process in which sound waves are used for the lysis of cells, disrupting them, and agitating the particles in the solutions to facilitate dissolution of a solid into a liquid. A sonicator is made up of three major components: a generator, a transducer, and a probe. The generator is to transform input electrical power into an electrical signal that drives the transducer (Figure 2.5.3.1). The transducer is then used to convert the electrical signal into vibration, which is used in the probe tip based on amplification into a longitudinal vibration that creates a cavity in the sample. The ultrasound energy caused by the creation of cavitation generates disruption of the sample and helps break down any large particles into smaller ones (Figure 2.5.3.1).

The principle of the sonication process is to harness ultrasonic sound waves. During the process, thousands of microscopic vacuum bubbles are produced in the solution due to the applied pressure. These bubbles then collapse into the solution during the process of cavitation, and the collapsing of bubbles that takes place in the cavitation field leads to the generation of enormous energy and the production of waves. This results in the disruption of the molecular interactions between the water molecules, and this reduction in molecular interactions causes the particles to begin to separate, allowing a mixing process to take place (https://byjus.com/physics/sonication/).

Sonication is physical method to disrupt the cells and vesicles by using sound waves. There are two sonication methods: direct and indirect, with the former being the most common. This study also used the direct method, which involves inserting the probe directly into the sample (culture medium) so that energy is transmitted from the probe to the sample directly. This is a high-intensity process, and the processing of the sample thus takes place quickly. The determination of the volume of the liquid required for processing is done by studying the diameter of the probe's tip.

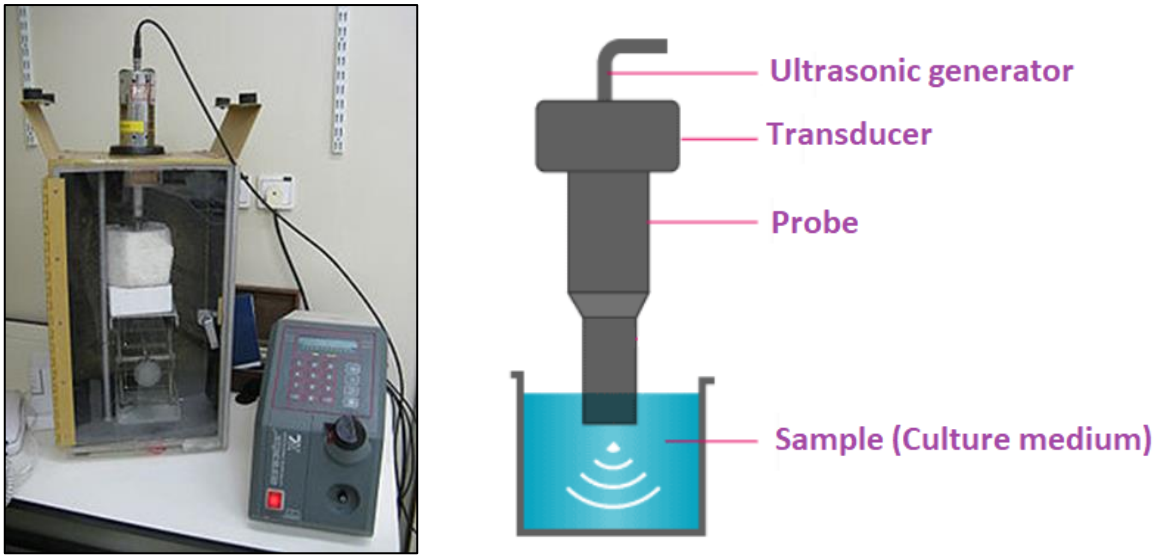

Figure 2.5.3.1. Sonicator instrument showing generator, transducer, and probe. The generator is used to transform the electrical power input into an electrical signal that drives the transducer. The transducer converts the electrical signal into vibration, which is used in the probe tip after amplification into longitudinal vibration, which causes a cavity in the sample. Ultrasound energy is created by this cavitation, which causes the disruption of the sample and begins break down particles into smaller ones (https://byjus.com/physics/sonication/; Li *et al.,* 2021).

2.5.4. Preparation of nasopharyngeal aspirates (NPA) for ELISA

NPAs were vortexed for 10 seconds at 1,500 rpm and centrifuged for 5 minutes at 2,000 rpm to pellet the cell debris and mucus before being diluted 1:1 with a PBS plus 1% BSA buffer.

2.5.5. Assay Procedure

A total volume of 100 µl/sample, or the applicable standard, of reagent diluent was added to the plate per well, after the plate was coated with capture antibody and blocked with BSA. The plate was then covered with an adhesive strip and incubated for 2 hours at room temperature before being washed three times with a wash buffer (0.05 % Tween 20 in PBS). After that, 100 µl of the detection antibody, diluted in reagent diluent (1% BSA in PBS), was added per well, and the plate was incubated for a further 2 hours at room temperature. The plate was then again washed three times with the wash buffer before 100 µl of Streptavidin-HRP, diluted in reagent diluent at 1:50 for IL-36α and IL-36γ and 1:200 for IL-37, was added to each well and incubated for 20 minutes at room temperature without light. The plates were then washed three times with the wash buffer again before 100 µl of substrate solution (TMB Substrate solution, Thermo scientific, Cat# N301 Solution) was added to each well and incubated for 20 minutes at room temperature without light.

The development of the colour of each well was monitored at that time, and 50 µl of Stop Solution (H_2SO_4) was added to each well so that the colour changed from blue to yellow. The optical density (OD) of each well was determined using a microplate reader (BioTek ELx800 Absorbance Microplate Readers) set to 450 nm (Davies., 2019) (Table 2.5. A and C).

Table 2.5.A.Reagent, Buffer, and ELISA Kits.

Name	Composition	Application	Supplier/Cat no.
Bovine Serum Albumin (BSA)	Refer to manufacturer's composition	ELISA	Sigma/ A7030
Substrate solution (TMB)	Refer to manufacturer's composition	ELISA	Thermo scientific/N301
RIPA	Octylphenol polyethoxyethanol (mixture)	ELISA	Sigma/ R0278

Table 2.5.B Commercially IL-36γ Antibody

Name	Composition	Application	Supplier/Cat no.
IL-36γ	Refer to manufacturer's composition	ELISA	R&D systems/ AF2320

Table 2.5.C. Commercially available ELISA kits.

ELISA (human)	Standard curve range (pg/ml)	Supernatant dilutionfactor	Cell pellet dilution factor	Nasopharyngeal aspirate dilution factor	Supplier/ Cat no.
IL-36α	12.5-800	1	2	vary	R&D systems/ DY1078-05
IL-36γ	18.75-1200	1	2	vary	R&D systems/ DY2320-05
IL-37	31.3-2000	1	2	vary	R&D systems/ DY1975

2.6. Immunofluorescence staining

Confocal microscopy was conducted on a Leica DM2500 microscope at 40x magnification using LAS X Core (version 3.3.0).

2.6.1. BEAS-2B cells Intracellular staining

BEAS-2B cells were grown on sterile 13 mm glass coverslips in 12-well plates that were infected at 50% confluency. RSV infected BEAS-2B cells were incubated for 24 hours, then the medium was removed and cells washed with PBS before 4% paraformaldehyde was added (Table 2.2) for 10 minutes at room temperature. This was followed by the addition of ice-cold methanol, which was fixed for 2 minutes at room temperature. The slips were then washed three times with PBS. Coverslips were permeabilised in PBS containing 0.3% Triton X-100 for 5 min and blocked for 1 hour at room temperature in PBT buffer (PBS with 1% BSA, 0.1% Triton X-100, 0.05% sodium azide) (Table 2.2). They were then incubated with IL-37 primary antibodies (R&D systems, Cat#AF1975) and Isotype control antibody (R&D systems, Cat#AB-108-C) (Table 2.5) overnight at 4 °C in PBT before being washed three times for 5 min with PBT and then incubated for two hours at room temperature with the secondary antibodies (Invitrogen, Cat#A11055), appropriately diluted in PBT. After further washing, the slides were counter stained with DAPI (Sigma, cat# D9542) for 10 minutes at room temperature before being washed again and allowed to dry before being mounted in VECTASHIELD Antifade Medium (Vector laboratories, Cat#H-1000) (Table 2.6.A and B) (Davies., 2019).

Table 2.6.A. Reagent, Buffer, and Immunofluorescence Kits.

Name	Composition	Application	Supplier/Cat no.
Bovine Serum Albumin (BSA)	Refer to manufacturer's composition	ELISA	Sigma/ A7030
Substrate solution (TMB)	Refer to manufacturer'scomposition	ELISA	Thermo scientific/ N301
RIPA	Refer to manufacturer's composition	ELISA	Sigma/ R0278

Table 2.6.B. Antibodies used in Immunofluorescence.

Name	Composition	Application	Supplier/Cat no.
IL-37	Refer to manufacturer's composition	Immunofluorescence	R&D systems/ DY1975

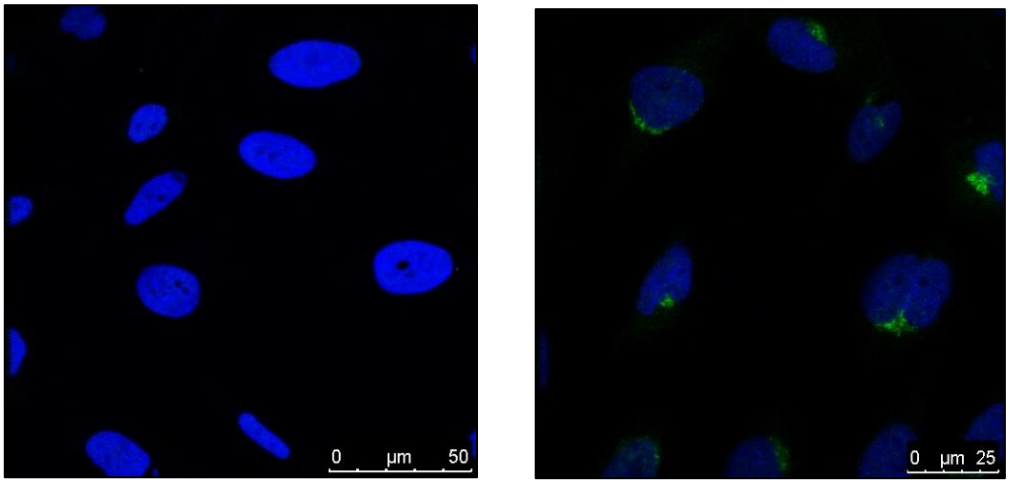

Figure 2.6. BEAS-2B cells expressing IL-37 protein. BEAS-2B cells were stained for IL-37 to determine whether the cells were of airway epithelial origin. Images represent **A)** isotype control and **B)** IL-37 protein (green). The IL-37 proteins shown in green are located around the nuclei, which are stained in blue (DAPI).

2.7. Nasopharyngeal aspirates

This study was approved by Liverpool central research ethics committee study number 17/NW/0044 IRAS number 212223. Access was thus provided to residual NPA samples previously collected as part of routine virological assessment of children attending Alder Hey Children's Hospital. The NPAs used had been collected between 1 November 2016 and 6 April 2017 during the RSV winter season; these were obtained with the kind help of the Alder Hey Children's Hospital Microbiology Department.

PCR analysis of respiratory virus present in the samples had previously been carried out within the microbiology laboratory in Alder Hey Children's Hospital.

Samples which met the selection criteria were thus selected for analysis:

- From infants aged between 21 days and 4 years old.

- From cases in general paediatrics services only.

  - Infants who were already in different departments at Alder Hey for potentially unrelated issues were excluded.

  - Only samples from children admitted to hospital for respiratory illness were included.

- NPA taken within 2 days of admission.

- Positive for RSV or RV only.

- No comorbidities.

Eighty RV samples were collected, with eight severe cases included (10%): 51 samples were thus collected from mild cases, and 19 were moderate. Two were unclassified.

Ninety-five RSV samples were collected, of which 12 were severe (12.6%): 30 samples were collected from mild cases, and from 39 moderate cases. Fourteen were unclassified.

Severity was classified based on the following conditions

- Mild: no oxygen required at all during admission

- Moderate: some oxygen needed

- Severe: admission to the paediatric critical care unit

2.8. Statistical Analysis

Paired, non-parametric Friedmans testing followed by Dunn's multiple comparison test were used to calculate statistical significance. AEC culture experiments comparing three or more groups were also required. Chapter 5 discusses the multi- comparison of groups using one-way and two-way ANOVA statistical tests, which was followed by Sidak's multiple comparison test and the Friedmans statistic test/Dunn's multiple comparison test combination.

For the NPA protein analysis discussed in chapter 3 and chapter 4, the results did not follow a normal distribution; thus, non-parametric tests were used. Analysis between two groups was done using the Wilcoxon signed rank test, while comparisons between three or more groups were done using the Kruskal-Wallis test. Spearman's rank correlation coefficient analysis was also used to derive correlations between NPA cytokine levels.

The data were collected and entered into an Excel sheet, and statistical analysis was completed using GraphPad Prism 8. Several p value levels were used to indicate significance. (* for $p<0.05$, ** for $p<0.001$ and *** for $p<0.0001$).

2.9. Ethical Considerations

Consent was obtained from all the participants, and the study overall was approved by the Liverpool central research ethics committee, study number 17/NW/0044, IRAS 212223.

Chapter 3. Induction of IL-36 cytokines and their receptors by RSV infections in nasopharyngeal aspirate, human nasal epithelial cells, and cultured airway epithelial cell lines

3.1 Introduction

The primary target of respiratory viruses are the airway epithelial cells, and the immune response of airway epithelial cells against viruses is characterised by the production of cytokines and chemokines, which act to limit the spread of such viruses. The work described in this chapter thus focused on investigating the expression of IL-36α and IL-36γ in NPAs in patients with RSV and RV diseases, as well as on the ability of cultured airway epithelial cells, whether HNAECs or cell lines (A549 and BEAS-2B), to express IL-36α, IL-36γ, and IL-36Rα in response to RSV infection. To support this, the work also examined the expression of receptors IL-1RL2 and IL-1RAcp, alongside investigating whether they play a role in initiating the associated inflammatory response. In various models of asthma, IL-36 cytokines are produced by lung epithelial cells in response to inflammatory stimuli (Bochkov *et al.*, 2010; Chustz *et al.*, 2011); however, to date, no studies have investigated the role of IL-36 cytokines as a mediator or potential modulator of the airway epithelial cells in cases of RSV infection.

Further, the full role of IL-36 protein in RSV and RV infection and its relationship to disease severity has not previously been established. To determine whether IL-36 protein may also play a role in RSV and RV infections. Particularly with respect to any relationship to disease severity, the current work measured the expression of IL-36α and IL-36γ proteins in nasopharyngeal aspirate (NPAs) from infants under the age of 4 years who had been naturally infected with RSV and RV. The relationships between cytokine expression and group and disease severity and oxygen requirements for these patients were also examined, and a comparison between cytokine expression in RSV and RV infection made. Furthermore, this study also focused on the ability of human nasal airway epithelial cells (HNAECs) and bronchial epithelial cell lines (A549 and SEAS-2B) to express IL-36 cytokine mRNA and proteins in response to RSV infections.

IL-36 cytokines may play a crucial role in supporting host immunity against respiratory pathogens by enhancing the innate adaptive immune response (Gabay *et al.,* 2016). Moreover, the mechanism of IL-36 cytokines secretion and activation in the lung during infection has previously been understudied. In this chapter, the relevant work on how IL-36 cytokines are activated and then secreted by cultured epithelial cells during RSV infection is thus discussed in detail.

Hypothesis: IL-36 alpha and IL-36 gamma, and their receptors, may highly expressed by airway epithelial cells in response to RSV infection while, in contrast, the inhibitory cytokine IL-36 receptor antagonist, which may also be expressed, regulates or inhibits the immune response.

3.2 Objectives

1) To investigate whether IL-36α and IL-36γ cytokines are present *in vivo* during RSV and RV infections by measuring their expression in nasopharyngeal aspirate (NPAs) samples from infected infants using the necessary specific ELISA (section 3.3.1).
2) To determine whether the expression of IL-36α and IL-36γ proteins correlates with patient age or disease severity, with the latter defined by the need for oxygen administration (section 3.3.1).
3) To determine whether *in vitro* RSV infection of human nasal airway epithelial cells results in the release of IL-36α, IL-36γ, or IL-36Rα by measuring mRNA and protein expression (section 3.3.2).
4) To determine whether RSV infection of cultured bronchial epithelial cell lines (A549 and BEAS- 2B) results in the release of IL36α, IL-36γ, or IL-36Rα, based on measuring mRNA and protein expression (section 3.3.3).
5) To demonstrate whether the receptors for IL-36 are expressed in human nasal airway epithelial cells and cultured bronchial epithelial cell lines (A549 and BEAS-2B), which might indicate that these cells could respond to cytokines after RSV infection (sections 3.3.2.6, 3.3.2.7, 3.3.3.6, and 3.3.3.7).
6) To explore whether RSV induced expression of intracellular or extracellular IL-36α and IL-36γ proteins can be triggered by co-stimulation with ATP in infected HNAECs or A549 and BEAS-2B cell lines (sections 3.3.2.8, 3.3.2.9, 3.3.3.8, and 3.3.3.9).

3.3 Results

3.3.1 Detection of IL-36α and IL-36γ protein in nasopharyngeal aspirate samples from children with RSV and RV infection

3.3.1.1. The Relation between Age and Expression of IL-36α and IL-36γ Protein in RSV and RV patient Samples

3.3.1.2. The Relation between Severity and Expression of IL-36α and IL-36γ Protein in RSV and RV patient Samples

3.3.1.3. The relation between a patient need oxygen and supplementation expression of IL36α and IL-36γ protein in RSV and RV patient Samples

3.3.1.4 The Relation between Expression of IL-36α and IL-36γ Protein in RSV and RV patient Samples

3.3.2 Expression of IL-36 cytokines and their receptors in human nasal epithelial cells during RSV infection

3.3.2.1. RSV replication in human nasal airways epithelial cells (HNAECs)

3.3.2.2. IL-8 mRNA expression in human nasal epithelial cells in response to RSV infection

3.3.2.3. Human nasal epithelial cell expression of IL-36 αmRNA following RSV infection: the effect of viral concentration

3.3.2.4. Human nasal epithelial cell expression of IL-36 γmRNA following RSV infection: the effect of viral concentration

3.3.2.5. Human nasal epithelial cell expression of IL-36RαmRNA following RSV infection: the effect of viral concentration

3.3.2.6. Human nasal epithelial cell expression of IL-36 Receptors (IL-1RL2 and IL-1RAP) mRNA following RSV infection: the effect of viral concentration

3.3.2.7. IL-36α protein expression by human nasal airway epithelial cells after RSV infection

3.3.2.9. IL-36γ HNAEC protein expression following RSV infection

3.3.3 Expression of IL-36 cytokines and their receptors by the bronchial epithelial cell lines, A549 and BEAS-2B, following RSV infection

3.3.3.1. RSV replication in the bronchial epithelial cell lines A549 and BEAS-2B

3.3.3.2. IL-8 mRNA expression in bronchial epithelial cells (A549 and BEAS-2B) cells in response to RSV infection

3.3.3.3. Increased IL36α mRNA expression by the bronchial epithelial cell lines (A549 and BEAS-2B) cells after RSV infection

3.3.3.4. Increased IL36γ mRNA expression by the bronchial epithelial cell lines (A549 and BEAS-2B) cells after RSV infection

3.3.3.5. Increased IL36Rα mRNA expression by the bronchial epithelial cell lines (A549 and BEAS-2B) cells after RSV infection

3.3.3.6. Expression of IL1RL2 mRNA by human RSV infection in bronchial epithelial cells (A549 and BEAS-2B) cells

3.3.3.7. Expression of IL1RAP mRNA by human RSV infection in airways epithelial cells (A549 and BEAS-2B) cells

3.3.3.8. Expression of IL-36α protein by human RSV infection in bronchial epithelial cells (A549 and BEAS-2B) cells

3.3.3.9. Expression of IL-36γ protein by human RSV infection in bronchial epithelial cells (A549 and BEAS-2B) cells

3.3.1 Detection of IL-36α and IL-36γ proteins in nasopharyngeal aspirate samples from children with RSV and RV infection

The first aim of the current work was to measure expression of IL-36α and IL-36γ in nasopharyngeal aspirate samples (NPAs) from RSV and RV infected children. NPAs samples were collected as routine sample for viral infection diagnosis and the remaining sample material subsequently made available for use in this study.

A total of 175 NPAs from RSV (n=95) and RV (n=80) infected infants were examined. In each sample section, the IL-36α and IL-36γ protein concentrations were measured using a dedicated ELISA (enzyme-linked immunosorbent assay), as discussed in section 2.5. Section 2.7 further details the selection criteria for the NPAs used. The level of protein expression was subsequently compared across:

1) Patients by age group, with groups of less than one-month-old (<1M), one to three months old (1-3M), 3 months to nine months old (3-9M), and more than 9 months.

2)Patient disease severity, ranging from mild (no oxygen required), though moderate (oxygen required), to severe (oxygen and a stay in a paediatric critical care unit).

3) Whether patients required any oxygen.

No protein values followed a normal distribution. In the RSV NPAs, the mean of IL-36α protein levels in RSV patient samples was 2,278 pg/ml. In contrast, the mean value for the IL-36γ protein level in RSV patient samples was 454 pg/ml (Figure 3.3.1 A). In RV NPAs, the mean of IL-36α levels in RV patient samples was 7,954 pg/ml, while the mean value of IL-36γ protein levels in RV patient samples was 552 pg/ml (Figure 3.3.1 B). Figure 3.3.1 B also shows a significant difference between the expression of IL-36α and the expression of IL-36γ proteins in RV NPAs, based on the Wilcoxon matched-pairs signed-rank test (* $p< 0.05$).

Figure 3.3.1 C shows a significant difference in the expression of IL-36α protein between RSV virus patient and RV virus patient sample groups ($p<0.001$). Expression of IL-36α protein was higher in RV virus patients' samples than in RSV virus patients' samples (Mann-Whitney/ U-test,

p<0.001) (Figure 3.3.1 C). As shown in Figure 3.3.1 D, however, no significant difference was observed in the expression of IL-36γ protein between RSV virus patients and RV virus patients.

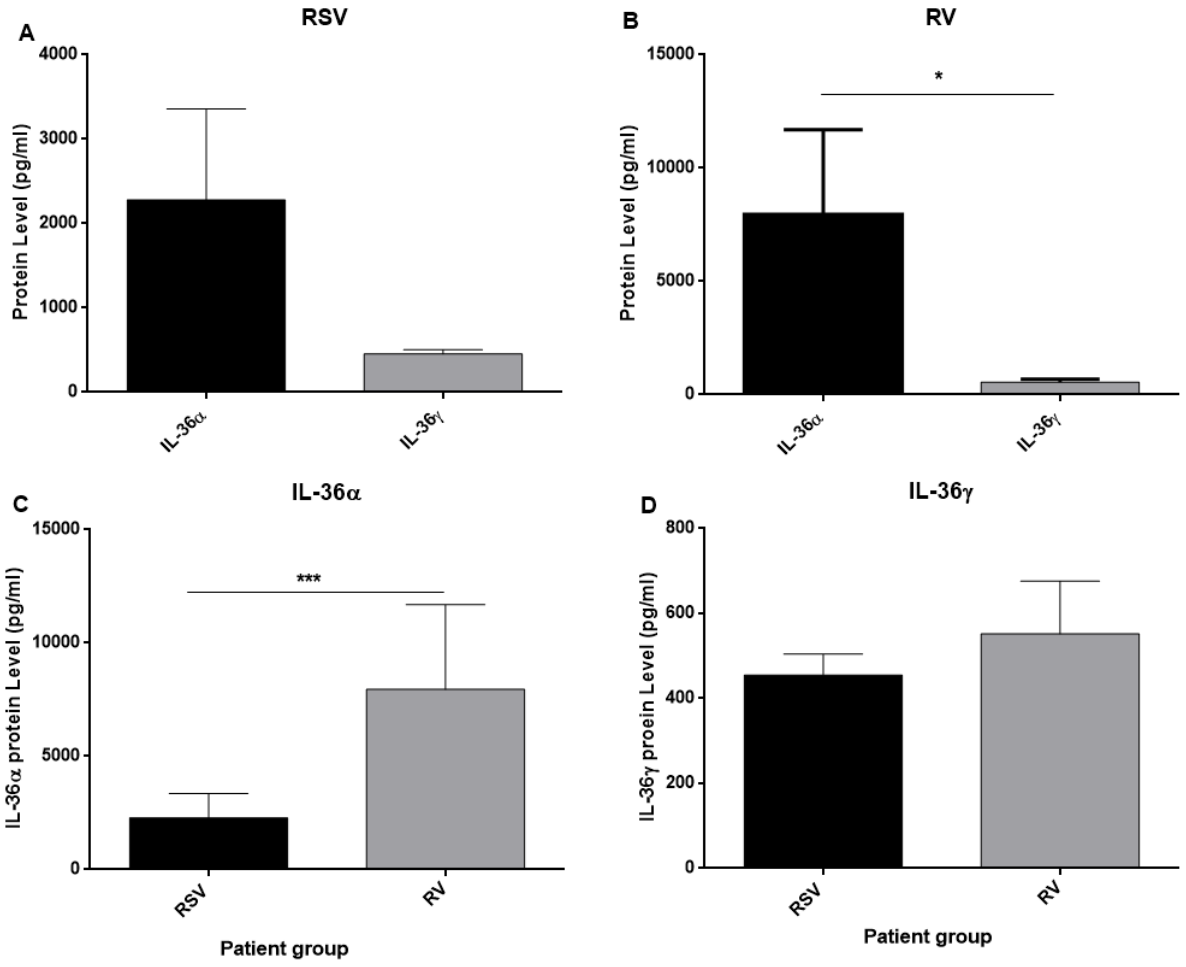

Figure 3.3.1 IL-36α and IL-36γ protein expression in the NPAs of RSV and RV patients. A) IL-36α and IL-36γ in RSV patients (n=92), **B)** IL-36α and IL-36γ in RV patients (n= 73). Data expressed as the mean ± SEM (Wilcoxon matched-pairs signed-rank test, * p< 0.05) **C)** IL-36α level in RSV and RV patients, **D)** IL-36γ level in RSV and RV NPAs. Data expressed as the mean ± SEM (Mann-Whitney/ U-test, ***p<0.001).

3.3.1.1 The relationship between age and Expression of IL-36α and IL-36γ Proteins in RSV and RV Patient Samples

To confirm if the expression of IL-36 α/γ proteins could be change during the age in children with RSV and RV diseases and if the immune response to the viral infection could be change in different age, IL-36 α/γ proteins were examined. As shown in Figure 3.3.1.1 A, there was no significant difference in levels of IL36α between age groups across RSV patient samples (p=0.301, n=91). The mean for patients aged less than 1 month was 6,498 pg/ml (n= 20), while for patients between 1 to 3 months, it was 1,664 pg/ml (n= 37); similarly mean=768 pg/ml (n= 18) for patients aged from 3 months to 9 months and 923.4 pg/ml (n= 16) in patients aged over 9 months. In addition, no significant differences in IL36α protein expression were observed between age groups in RV patient samples (p= 0.09, n= 73). The mean for patients less than 1 month in age was 30,883 pg/ml (n= 10), while mean= 672 pg/ml (n= 20) for patients between 1 to 3 months, 4,451 pg/ml (n= 25) for patients aged from 3 months to 9 months, and 2,595 pg/ml (n= 18) for patients aged over 9 months (Figure 3.3.1.1 B).

For the IL-36γ protein, there were again no significant differences in level observed between age groups in RSV patient samples (p= 0.9, n= 92). The mean from patients less than 1 month in age was 449 pg/ml (n= 20), while mean= 488 pg/ml (n= 38) for patients aged between 1 to 3 months, 455 pg/ml (n= 18) for patients aged from 3 months to 9 months, and 406 pg /ml (n= 16) for patients over 9 months old (Figure 3.3.1.1 C). There were also no significant differences in IL-36γ protein expression between age groups in RV patient samples (p= 0.6, n=73). The mean for patients less than 1 month old was 599 pg/ml (n= 10), while mean= 526 pg/ml (n= 20) for patients between 1 and 3 months, 335 pg/ml (n= 25) for patients aged from 3 months to 9 months, and 608 pg/ml (n= 18) for patients aged more than 9 months old (Figure 3.3.1.1 D). These results indicate that no associations exist between age group and IL-36α and IL-36γ protein expression in NPAs, based on the Kruskal-Wallis test and Dunn's multiple comparison test.

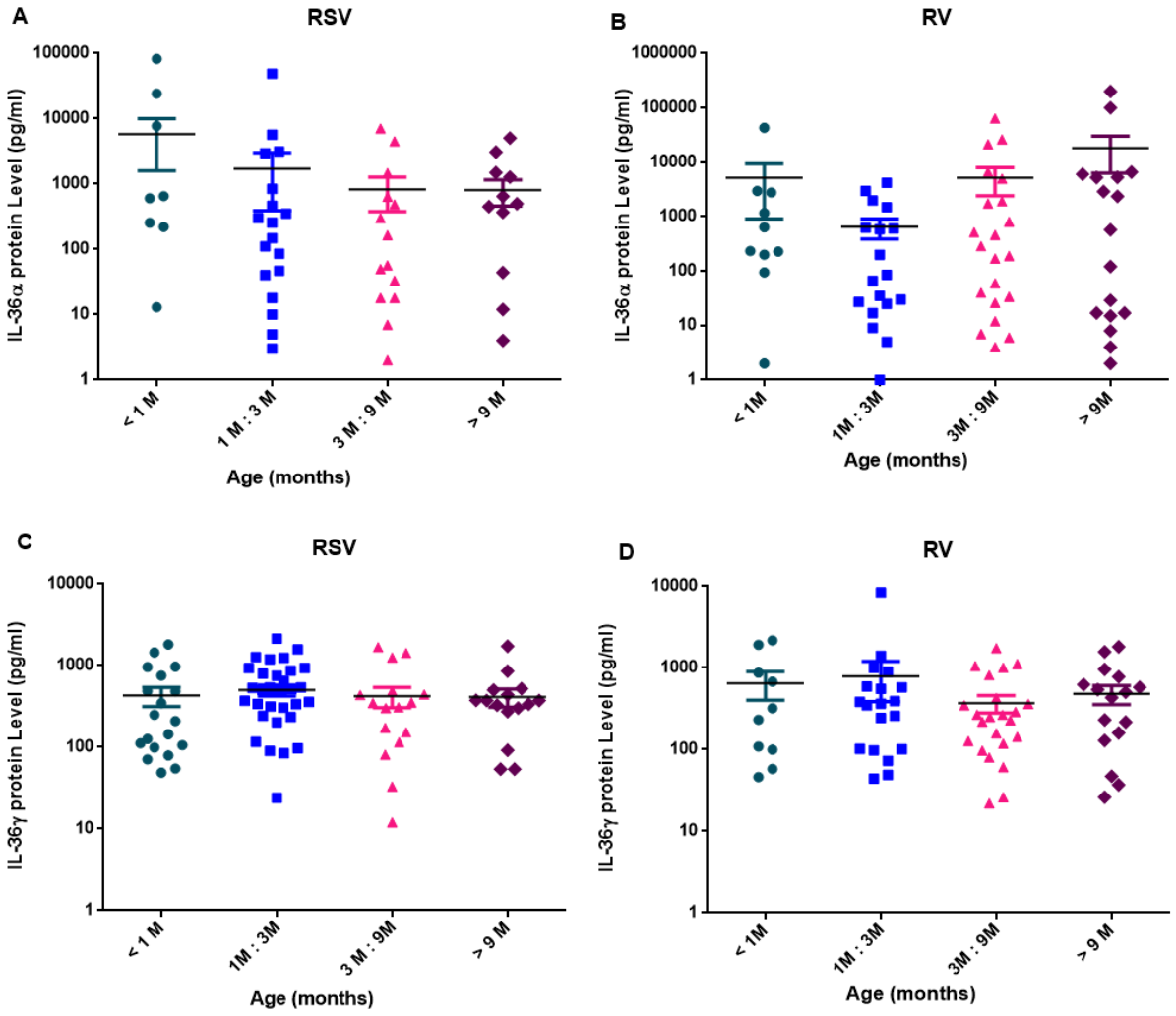

Figure 3.3.1.1. The relationships between IL36α and IL-36γ protein expression and RSV and RV patients' age. Concentrations of IL-36α in **A)** RSV patients (n=91) and **B)** RV patients (n= 73), patients in four groups of age. Results are presented as scatter plot with mean ± SEM, based on Kruskal-Wallis test: less than 1 month (n = 20, 10), patients between 1 to 3 months (n= 37, 20), 3 months to 9 months (n= 18, 25) and above of 9 months (n = 16, 18). Concentrations of IL-36γ in **C)** RSV patients (n=92) and **D)** RV patients (n= 73), patients age less than 1 month (n = 20, 10), patients between 1 to 3 months (n= 38, 20), 3 months to 9 months (n= 18, 25) and above of 9 months (n = 16, 18) in RSV and RV respectively. Dunn's multiple comparisons test between to compare the mean rank of group with the mean rank of every other group.

3.3.1.2 The relationship between Severity and Expression of IL-36α and IL-36γ Proteins in RSV and RV Patients

Across RSV cases, no significant differences in levels of IL-36α protein expression were observed between severity groups (p= 0.1). The mean value for IL-36α in the mild group was 2,223 pg/ml, (n= 31), while the moderate and severe groups had mean= 1,454 pg/ml (n= 45) and mean= 6,918 pg/ml (n= 15), respectively, based on the Kruskal-Wallis test and Dunn's multiple comparison test (Figure 3.3.1.2 A). Similarly, no significant differences in IL-36α expression were observed in patients with different severities of RV disease (p= 0.9). The mean value of IL-36α in the mild group was 2,026 pg/ml (n= 48), while the moderate and severe groups had mean= 4,298 pg/ml (n= 18) and mean= 7,122 pg/ml (n= 7) respectively (Figure 3.3.1.2 B).

There was, however, a significant difference in the levels of IL-36γ protein expression level across severity groups, at least in the RSV sample. The level of IL-36γ was significantly ~~higher~~ lower in the ~~mild~~ severe group in comparison to the mild ($p<0.05$) and moderate ~~severe~~ groups ($p<0.001$). The mean value for IL-36γ in the mild group was 332 pg/ml (n= 32), while the mean for the in moderate group was 615 pg/ml (n= 45) and that for the severe group was 68 pg/ml (n= 15) among the RSV patient sample (Figure 3.3.1.2 C). No significant differences in the levels of IL-36γ protein were seen between severity groups in RV patient samples, however: the mean value of IL-36γ in the mild group was 523 pg/ml (n= 48), with mean= 384 pg/ml (n= 18) in the moderate group and mean= 447 pg/ml (n= 8) in the severe group of RV patients (Figure 3.3.1.2 D). These results indicate that the levels of IL-36α and IL-36γ proteins in NPAs do not generally correlate with disease severity, based on the Kruskal-Wallis test and the Dunn's multiple comparison test, with the exception of IL-36γ's correlation with RSV disease severity.

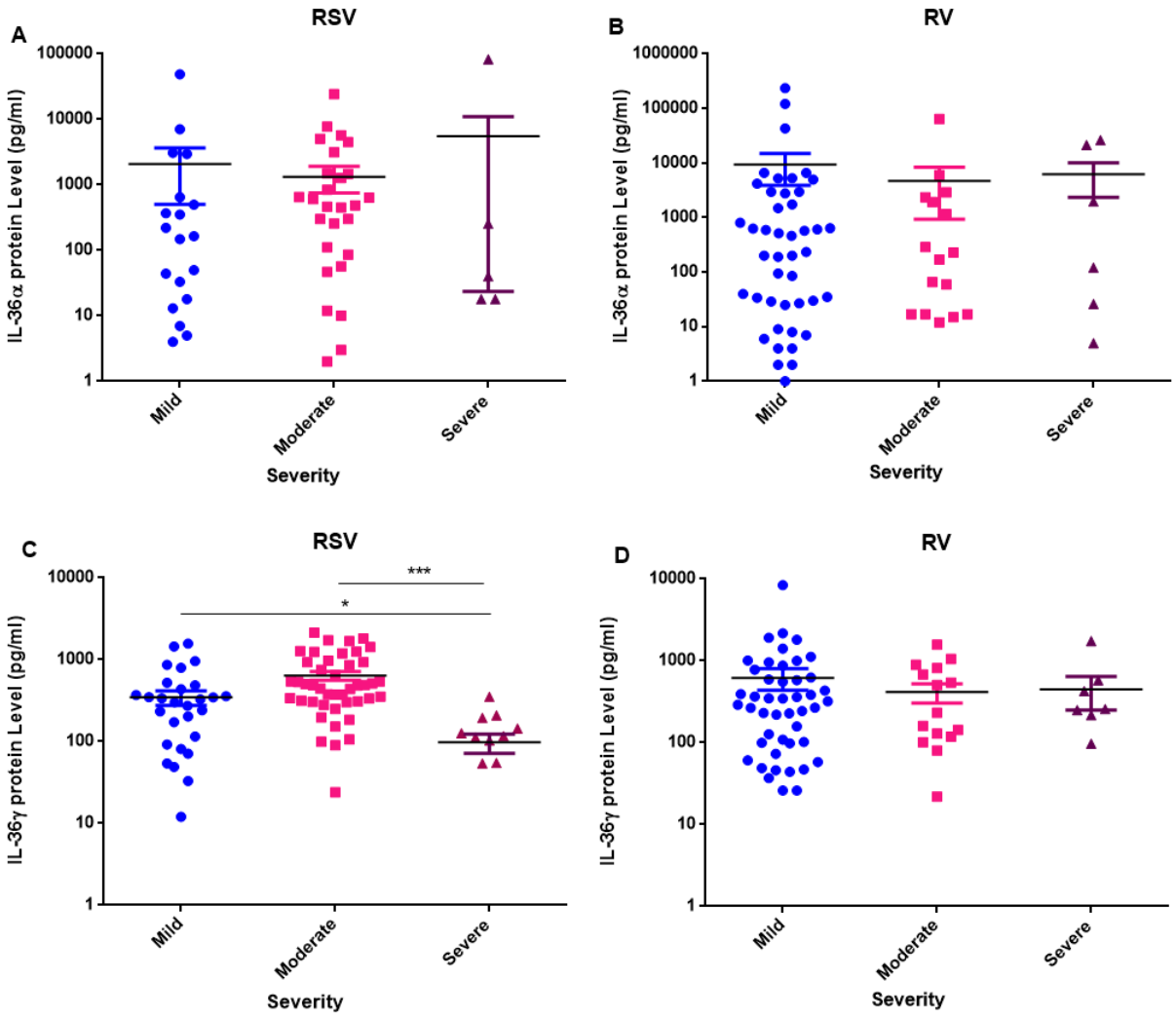

Figure 3.3.1.2. The relationship between IL-36α and IL-36γ protein expression and RSV and RV case severity. Concentrations of IL-36α in RSV patients (n= 91) **A)** and RV patients (n= 73), **B)** from mild patients (n= 31, 48), moderate patient (n= 45, 17) and severe patients (n= 15, 8). Concentrations of IL-36γ in RSV patients (n=92) **C)** and RV patients (n=73), **D)** from mild patients (n= 32, 48), moderate patient (n= 45, 17) and severe patients (n= 15, 8) respectively. Results presented as scatter plot with mean ± SEM, based on Kruskal-Wallis test between three groups of severity and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group, * $p< 0.05$ and ***$p<0.001$.

3.3.1.3 The relationship between patients oxygen need and supplementation and expression of IL-36α and IL-36γ proteins in RSV and RV patient samples

As the "severe" group in Figure 3.3.1.2 featured only a small number of patients, RSV and RV patient samples were divided more simply into groups based on receipt of oxygen supplementation. No significant differences in IL-36α protein levels were observed in relation to oxygen requirement in RSV patient samples (p= 0.9). The mean value for IL-36α protein in patients who did not require oxygen supplementation was 2,302 pg/ml (n= 28), while the mean value for IL-36α protein 2,820 pg/ml (n= 53) in patients who were supplied with oxygen supplementation (Figure 3.3.1.3 A). In addition, there were no significant differences in IL-36α protein levels in relation to oxygen requirements across the RV patient samples (p= 0.798). The mean value of IL-36α protein in patients who did not require oxygen supplementation was 1,933 pg/ml (n= 45), while the mean value for IL-36α protein in patients supplied with oxygen supplementation was 4,741 pg/ml (n= 29) (Figure 3.3.1.3 B).

Similarly, no significant differences in IL-36γ protein levels were observed in relation to oxygen requirements in RSV patients (p= 0.3, n= 81) (Figure 3.3.1.3 C). The mean value for IL-36γ protein in patients who did not require oxygen supplementation was 344 pg/ml (n= 28), while the mean value for IL-36γ protein in patients supplied with oxygen supplementation was 481 pg/ml (n= 53) (Figure 3.3.1.3 C). There were also no significant differences in IL-36γ protein levels in RV patients in relation to oxygen requirements (p= 0.8, n= 78). The mean value for IL-36γ protein in patients who did not require oxygen supplementation was 521 pg/ml (n= 47), while the mean value for patients with RV who were supplied with oxygen supplementation 404 pg/ml (n= 31) in (Figure 3.3.1.3 D). These results indicate that the IL-36α and IL-36γ proteins levels did not change alongside oxygen requirements (Mann-Whitney test).

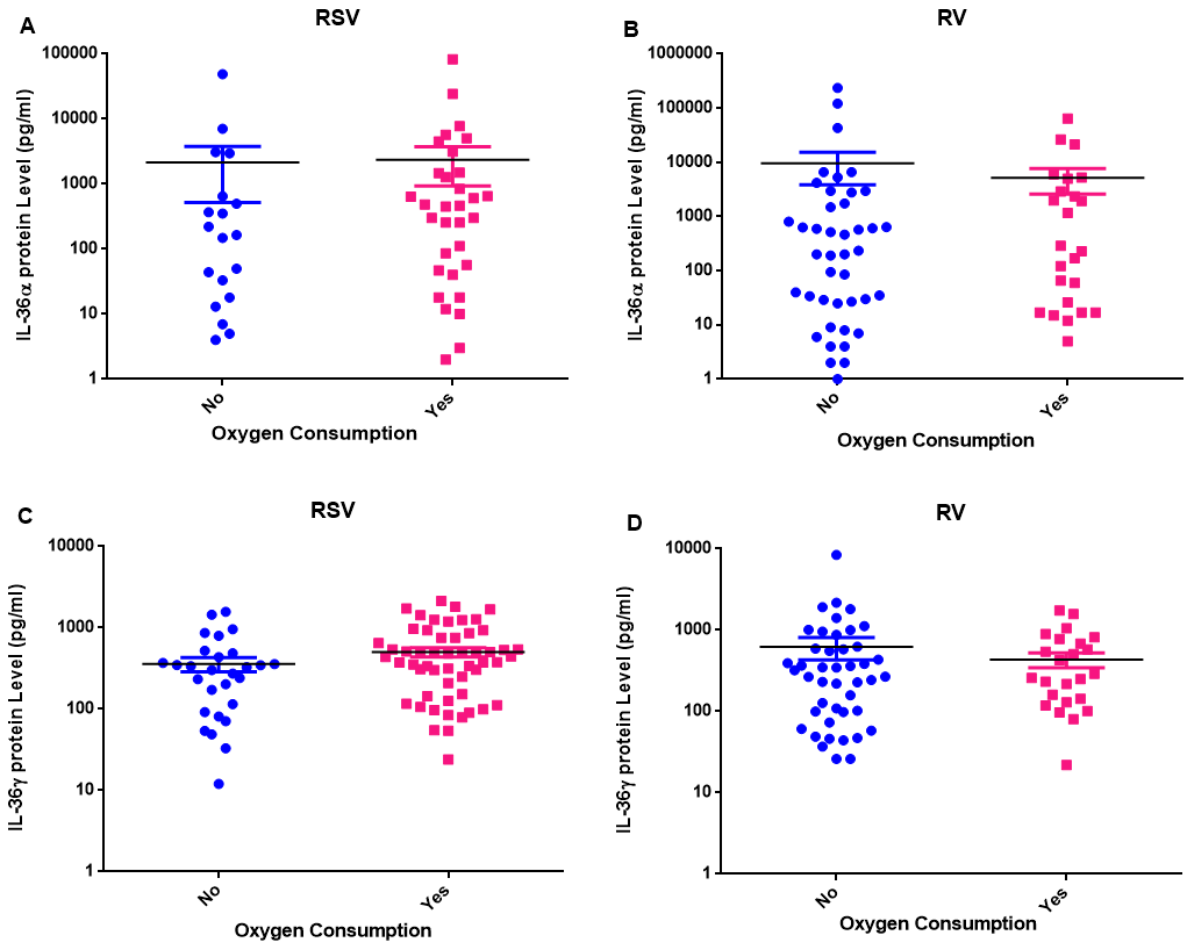

Figure 3.3.1.3. The relationships between IL-36α and IL-36γ expression and RSV and RV Patients' Oxygen Requirements. Concentrations of IL-36α in RSV patients (n= 81) **A)** and RV patients (n= 74) **B)** for patients without oxygen requirements (n= 28, 45), as compared with patients with oxygen requirements (n= 53, 29) Concentrations of IL-36γ in RSV patients (n= 81) **C)** and RV patients (n= 78) **D)** for patients with oxygen requirements (n= 53, 31), as compared with patients without oxygen requirements (n= 28, 47). (Results presented as scatter plots with mean ± SEM, using the Mann-Whitney t-test for two unpaired nonparametric test).

3.3.1.4. The relationship between Expression of IL-36α and IL-36γ Proteins in RSV and RV Patients

IL-36α and IL-36γ cytokines are highly related both structurally and functionally. To determine any correlation between observed IL-36α and IL-36γ protein levels across RSV or RV patients (NPAs), Spearman's rank-order analysis (two-tailed, n = number of XY pairs), was applied. The results are shown in Figure 3.3.1.4 A, and these indicate that there are no positive correlations between protein levels detected in RSV NPAs (r=0.2; P<0.05). In addition, there was no positive correlations was observed between IL-36α and IL-36γ in RV patient samples (r=0.5; P<0.001), (Figure 3.3.1.4 B).

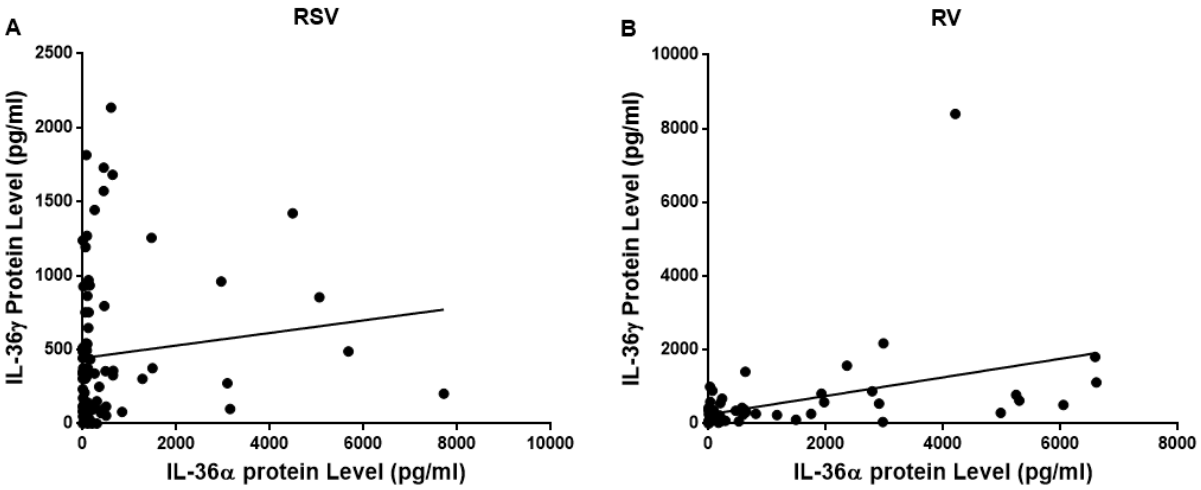

Figure 3.3.1.4. Correlations between protein levels: IL-36α against IL-36γ in RSV and RV NPAs. A) RSV patients (n= 88), **B)** RV patients (n=67). Correlations assessed using Spearman's rank-order analysis, (two-tailed, n = number of XY pairs). A statistically significant correlation was determined at p<0.05; p<0.0001.

3.3.2 Expression of IL-36 Cytokines and their receptors in HNAECs during RSV infections

Human Nasal Airways Epithelial Cells (HNAECs) were isolated and grown; the cultures were taken from healthy adult donors and developed in 96 well tissue culture plates. These HNAECs were infected with RSV A2 at MOIs of 0.1, 1 and 2.5 which were allowed to grow for 48 hours according to (McNamara *et al.,* 2012; Fonceca *et al.,* 2012; Alturaiki., 2014) studies. Palivizumab is a humanized monoclonal antibody that targets the F glycoprotein on the surface of the RSV virus (Teusink-Cross *et al.,* 2016), thereby preventing viral replication, in this study palivizumab was used with as an anti-RSV control at an MOI of 2.5. RNA was then extracted from cells using Qiagen and reverse transcribed, after which, mRNA expression was determined using TaqMan primer-probe assays: L32, IL-36α, IL36γ, IL-36Rα, and IL-36Rs (IL1RL2 and IL1RAP) were thus normalised to the housekeeping gene L32 (section 2.1).

3.3.2.1. RSV replication in HNAECs

RSV N gene expression was measured by using qPCR in human nasal airway epithelial cells to confirm infection. RSV N RNA was not detected in non-infected cells, but it was seen to increase significantly at MOIs of 1 ($p<0.001$) and 2.5 ($p<0.0001$) (Figure 3.3.2.1 A). Palivizumab, an anti-RSV monoclonal antibody that neutralises RSV and reduces infectivity, was used as a further control, which resulted in significantly decreased expression of RSV N RNA expression in RSV MOI 2.5 as compared to the sample without Palivizumab ($p<0.01$). Figure 3.3.2.1 B shows the RSV N RNA expression of individual donor HNAECS. RSV N RNA was increased as the amount of RSV increased (Figure 3.3.2.1. B), which indicates successful *in vitro* RSV infection and replication in HNAECs.

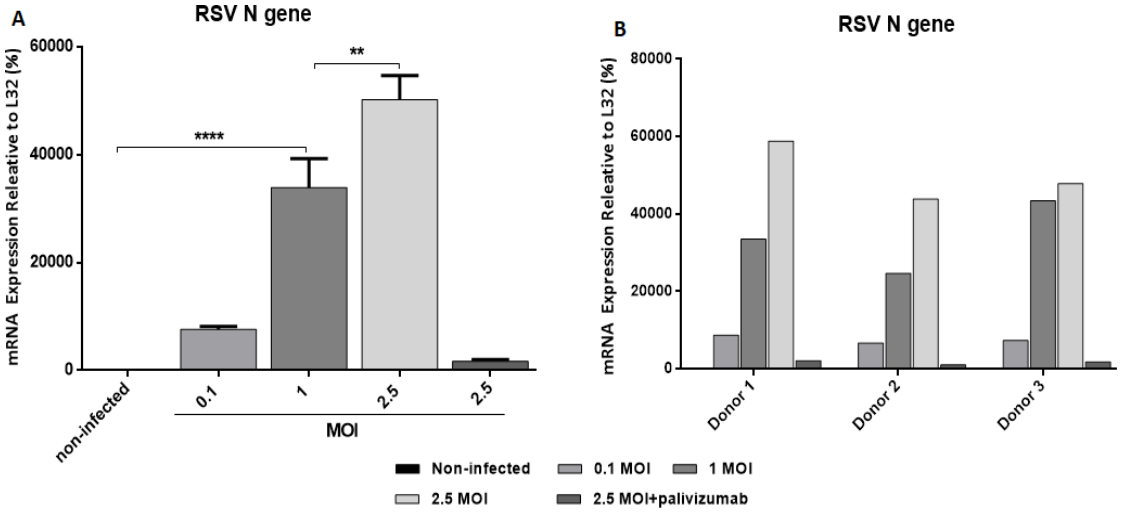

Figure 3.3.2.1. Expression of the RSV N gene in RSV A2 infected HNAECs. HNAECs were infected with RSV A2 at MOIs of 0.1, 1, and 2.5 for 48h. Control, non-infected, cultures were generated in the absence of RSV, and Palivizumab was used as a further anti-RSV control. **A)** RSV N gene (n=3), Data expressed as the mean ± SEM, based on fold expression relative to L32, Friedman test for four groups/Dunn's multiple comparison test to compare the mean rank of group with the mean rank of every other group (**p<0.01 and ****p<0.0001). **B)** Individual donor expression of RSV N gene. Data expressed as the mean ± SEM, based on fold expression relative to L32, RM-two-way ANOVA/Tukey's multiple comparisonstest.

3.3.2.2. IL-8 mRNA Expression in HNAECs in Response to RSV Infection

IL-8 gene expression was measured to confirm viral replication to check whether the HNAECs responded to the introduced challenge. IL-8 mRNA expression was significantly increased at MOI 2.5 ($p<0.0001$) in comparison to non-infected HNAECs (Figure 3.3.2.2 A), while Palivizumab anti-RSV control significantly decreased the expression of IL-8 mRNA between MOI 2.5 and MOI 2.5 plus Palivizumab ($p<0.001$). At MOI 2.5, IL-8 mRNA expression across all donors in infected HNAECs showed between ~6- to 14- fold higher expression than seen in non-infected HNAECs (Figure 3.3.2.2 A). Figure 3.3.2.2B shows that the IL-8 mRNA expression of individual donors all increased with increasing amounts of RSV being introduced. At MOI 2.5, donor 1 showed ~1.7-fold higher expression than donors 2 and 3, however (Figure 3.3.2.2 B). These results indicate successful *in vitro* RSV infection and replication in HNAECs more generally, as shown by assessing IL-8 mRNA.

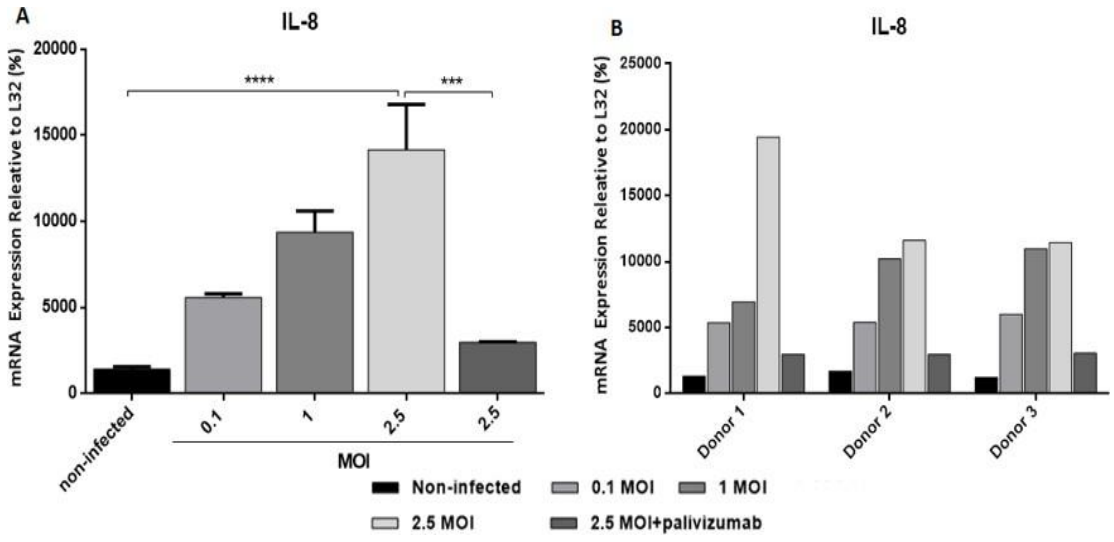

Figure 3.3.2.2. Expression of IL-8 mRNA in infected HNAECs. HNAECs were infected with RSV at MOIs of 0.1, 1, and 2.5 for 48 hours. Control non-infected cultures were cultured in the absence of RSV, and Palivizumab was used as an anti-RSV control. **A)** IL-8 mRNA expression (n=3) Friedman test for four groups/Dunn's multiple comparison test to compare the mean rank of group with the mean rank of every other group (***$p<0.001$ and ****$p<0.0001$). **B)** Individual donor expressions of IL-8 mRNA. Data expressed as the mean ± SEM, based on fold expression relative to L32, RM-two-way ANOVA/Tukey's multiple comparisonstest.

3.3.2.3 Human nasal epithelial cell expression of IL-36α mRNA following RSV infection: the effect of viral concentration

To determine the effect of RSV concentration on the expression of IL-36α mRNA, HNAECs were infected with increasing concentrations of RSV, namely MOI 0.1, 1, and 2.5, for 48 hours. Palivizumab was used with RSV at MOI 2.5 as an anti-RSV control. In infected HNAECs, IL-36α mRNA expression was significantly increased for MOI 2.5 ($p<0.01$) in comparison to non-infected HNAECs (Figure 3.3.2.3 A). However, no significant difference was seen between control and RSV infected HNAECs at MOIs 0.1 and 1. At MOI 2.5, infected HNAECs' IL-36α mRNA expression was seen to be from ~7 to 11-fold higher than that measured in non-infected donor HNAECs (Figure 3.3.2.3 A). The individual results shown in Figure 3.3.2.3 B indicate that IL-36α mRNA expression by each donor increased with increasing doses of RSV. At MOI 2.5, infected HNAECs from donor 3 showed a ~1.2 to 1.7-fold higher expression than those from donors 1 and 2 (Figure 3.3.2.3 B). However, the results indicate a significant increase in IL-36α mRNA expression during RSV infection more generally.

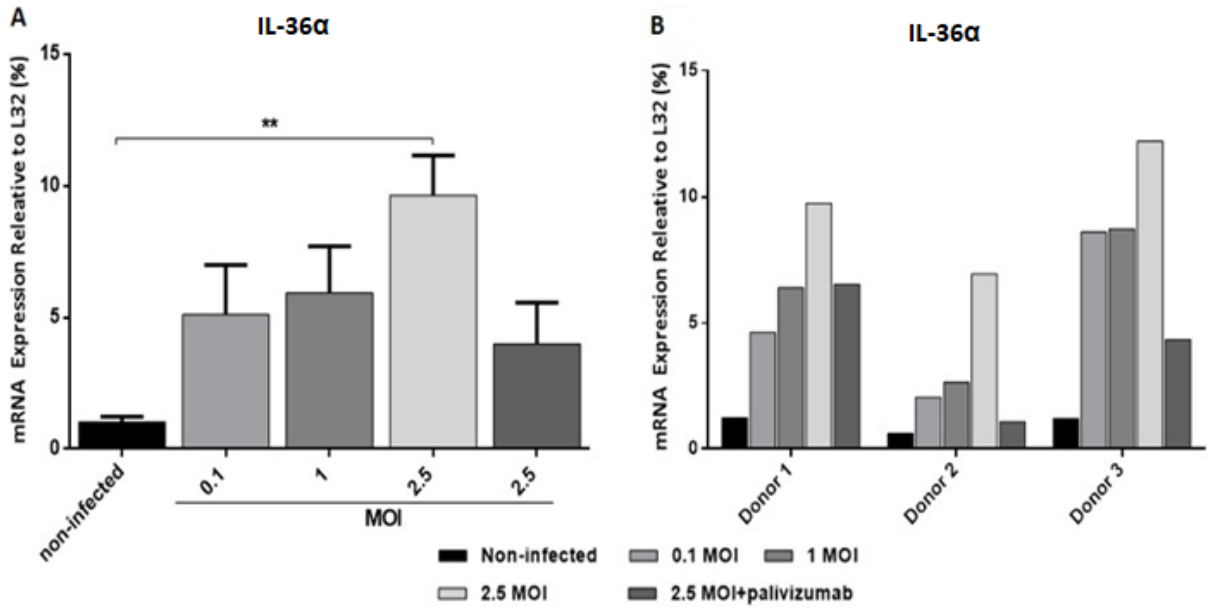

Figure 3.3.2.3. Expression of IL-36α in mRNA infected HNAECs. HNAECs were infected with RSV at MOIs of 0.1, 1, and 2.5 for 48 hours. Control non-infected cultures were cultured in the absence of RSV, with Palivizumab was used as an anti-RSV control. **A)** IL-36α mRNA (n=3), Friedman test for four groups/Dunn's multiple comparison test to compare the mean rank of group with the mean rank of every other group (**$p<0.01$). **B)** Individual donor expressions of IL-36α mRNA. Data expressed as the mean ± SEM, based on fold expression relative to L32, RM-two-way ANOVA/Tukey's multiple comparisonstest.

3.3.2.4 Human nasal epithelial cell expression of IL-36γ mRNA following RSV Infection: the effect of viral concentration

To determine the effects of RSV on the expression of IL-36γ mRNA, HNAECs were infected with increasing concentrations of RSV at MOI 0.1, 1, and 2.5 for 48 hours. Palivizumab was used with RSV at an MOI 2.5 as an anti-RSV control. IL-36γ mRNA expression was significantly increased at MOI 2.5 ($p<0.01$) in comparison to that seen in non-infected HNAECs (Figure 3.3.2.4 A). Figure 3.3.2.4 B further shows that IL-36γ mRNA expression in each donor was increased with the increasing dose of RSV. However, no significant difference was seen between control and RSV infected HNAECs at MOIs 0.1 and 1. At MOI 2.5, infected HNAECs' IL-36γ mRNA expression was from ~2.7 to 4.3-fold higher than in non-infected HNAECs from the same donors (Figure 3.3.2.4 A). The individual results shown in Figure 3.3.2.4 B indicate that IL-36γ mRNA expression by each donor was increased with the increasing dose of RSV. At MOI 2.5, infected HNAECs from donor 1 showed ~1.1 to2-fold higher expression than those for donors 2, 3, and 4 (Figure 3.3.2.4 B). The results overall indicate a significant increase in IL-36γ mRNA expression during RSV infection.

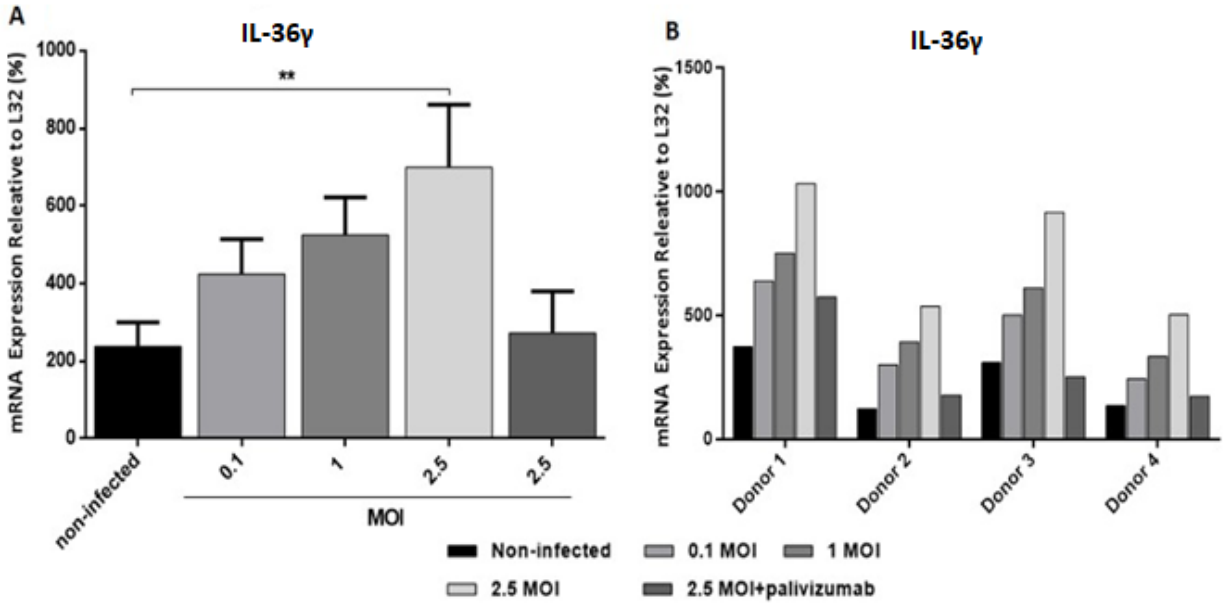

Figure 3.3.2.4. Expression of IL-36γ in mRNA infected HNAECs. HNAECs were infected with RSV at MOIs of 0.1, 1, and 2.5 for 48 hours. Control non-infected cultures were cultured in the absence of RSV, and Palivizumab was used as an anti-RSV control. **A)** IL-36γ mRNA expression (n=4), Friedman test for four groups/Dunn's multiple comparison test to compare the mean rank of group with the mean rank of every other group (**$p<0.01$). **B)** Individual donor expressions of IL-36γ mRNA. Data expressed as the mean ± SEM, based on fold expression relative to L32, RM-two-way ANOVA/Tukey's multiple comparisonstest.

3.3.2.5. Human nasal epithelial cell expression of IL-36Rα mRNA following RSV Infection: the Effect of viral concentration

To determine the effect of RSV on the expression of IL-36Rα mRNA, HNAECs were infected with increasing concentrations of RSV at MOI 0.1, 1, and 2.5 for 48 hours. Palivizumab was used with RSV at MOI 2.5 as an anti-RSV control. IL-36Rα mRNA expression was significantly increased at MOI 2.5 ($p<0.05$) in comparison to that seen in non-infected HNAECs (Figure 3.3.2.5 A). Figure 3.3.2.5 B indicates that IL-36Rα mRNA expression in each donor was increased with increasing doses of RSV (Figure 3.3.2.5 B). However, no significant difference was seen between the control and RSV infected HNAECs at MOIs 0.1 and 1. At MOI 2.5, infected HNAECs' IL -36Rα mRNA expression was from ~7 to 11 -fold higher than in non-infected HNAECs across donors (Figure 3.3.2.5 A). The individual results shown in Figure 3.3.2.5 B indicate that IL-36Rα mRNA expression in each donor was increased with the increasing dose of RSV. At MOI 2.5, the infected HNAECs for donor 4 showed ~1.3 to 1.7-fold higher expression than those for donors 1, 2, and 3 (Figure 3.3.2.5 B). The results overall indicate a significant increase in IL-36Rα mRNA expression during RSV infection, however.

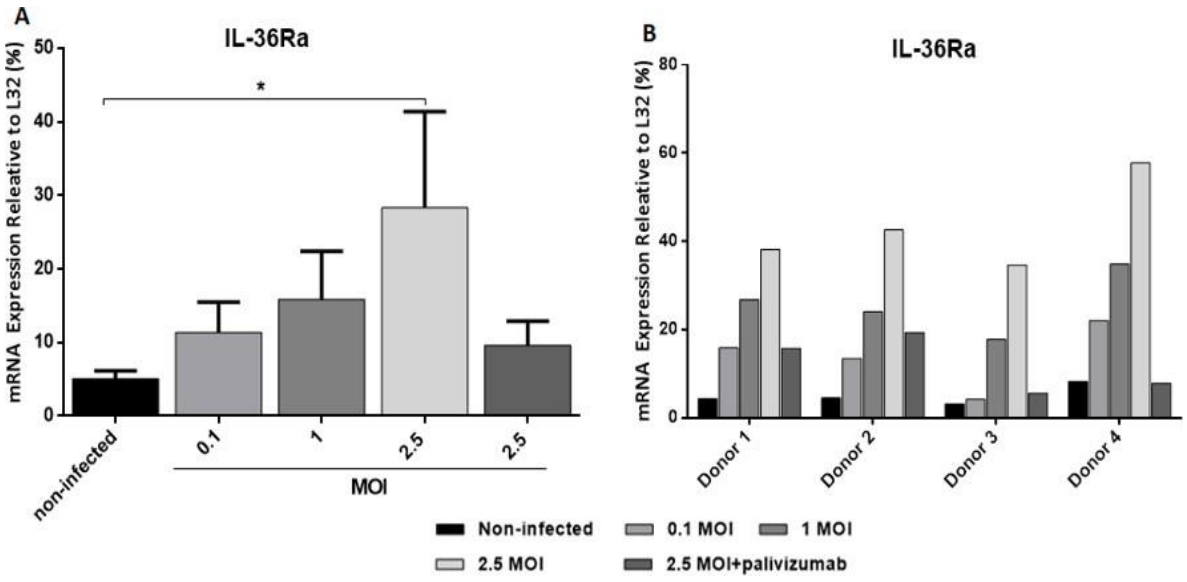

Figure 3.3.2.5. Expression of IL-36Rα mRNA infected HNAECs. HNAECs were infected with RSV at MOIs of 0.1, 1, and 2.5 for 48 hours. Control non-infected cultures were cultured in the absence of RSV, and Palivizumab was used as an anti-RSV control. **A)** IL-36Rα mRNA expression (n=4), Friedman test for four groups/Dunn's multiple comparison test to compare the mean rank of group with the mean rank of every other group (*$p<0.05$). **B)** Individual donor expression of IL-36Rα mRNA. Data expressed as the mean ± SEM, based on fold expression relative to L32, RM-two-way ANOVA/Tukey's multiple comparisonstest.

3.3.2.6. Human nasal epithelial cell expression of IL-36 receptor (IL-1RL2 and IL-1RAP) mRNA following RSV Infection: the effect of viral concentration

To determine the effect of RSV concentration on the expression of IL-1RL2 and IL-1RAP mRNA, HNAECs were infected with increasing concentrations of RSV at MOI 0.1, 1, and 2.5 for 48 hours. Palivizumab was used with RSV at an MOI 2.5 as an anti-RSV control.

There was no change in expression of IL-1RL2 mRNA observed during RSV infection as compared to that seen in non-infected HNAECs (Figure 3.3.2.6 A). Figure 3.3.2.6 B, showing the expression of IL-1RL2 mRNA in individual donors, indicates that IL-1RL2 mRNA expression by each donor showed no significant differences between control and RSV infected HNAECs at MOIs 0.1, 1, or 2.5. The mean levels of IL-1RL2 mRNA expression relative to L32 (%) were 6.3, 4.5, and 3.2 in MOIs 0.1, 1, and 2.5, respectively, as compared to 8 in non-infected HNAECs and MOI 2.5 plus Palivizumab 6 (Figure 3.3.2.6 A). The individual results shown in Figure 3.3.2.6 B indicate that IL-1RL2 mRNA expression showed no significant difference across the infected HNAECs for each donor (Figure 3.3.2.6 B). These results thus indicate that HNAECs are not a source of IL-1RL2 during RSV infection.

As shown in figure 3.3.2.6 C, there was a significant decrease in IL-1RAP mRNA expression at MOI 2.5 as compared to that seen in non-infected HNAECs. However, no significant difference was seen between control and RSV infected HNAECs at MOIs 0.1 and 1. At MOI 2.5, infected HNAECs' IL-1RAP mRNA expression was from ~1.8 to 3.4-fold lower than that seen in non-infected HNAECs (Figure 3.3.2.6 C). The individual results shown in Figure 3.3.2.6 D indicate that IL-1RAP mRNA expression by each donor decreased with increasing doses of RSV, and the results overall indicate a significant decrease in IL-1RAP mRNA expression during RSV infection.

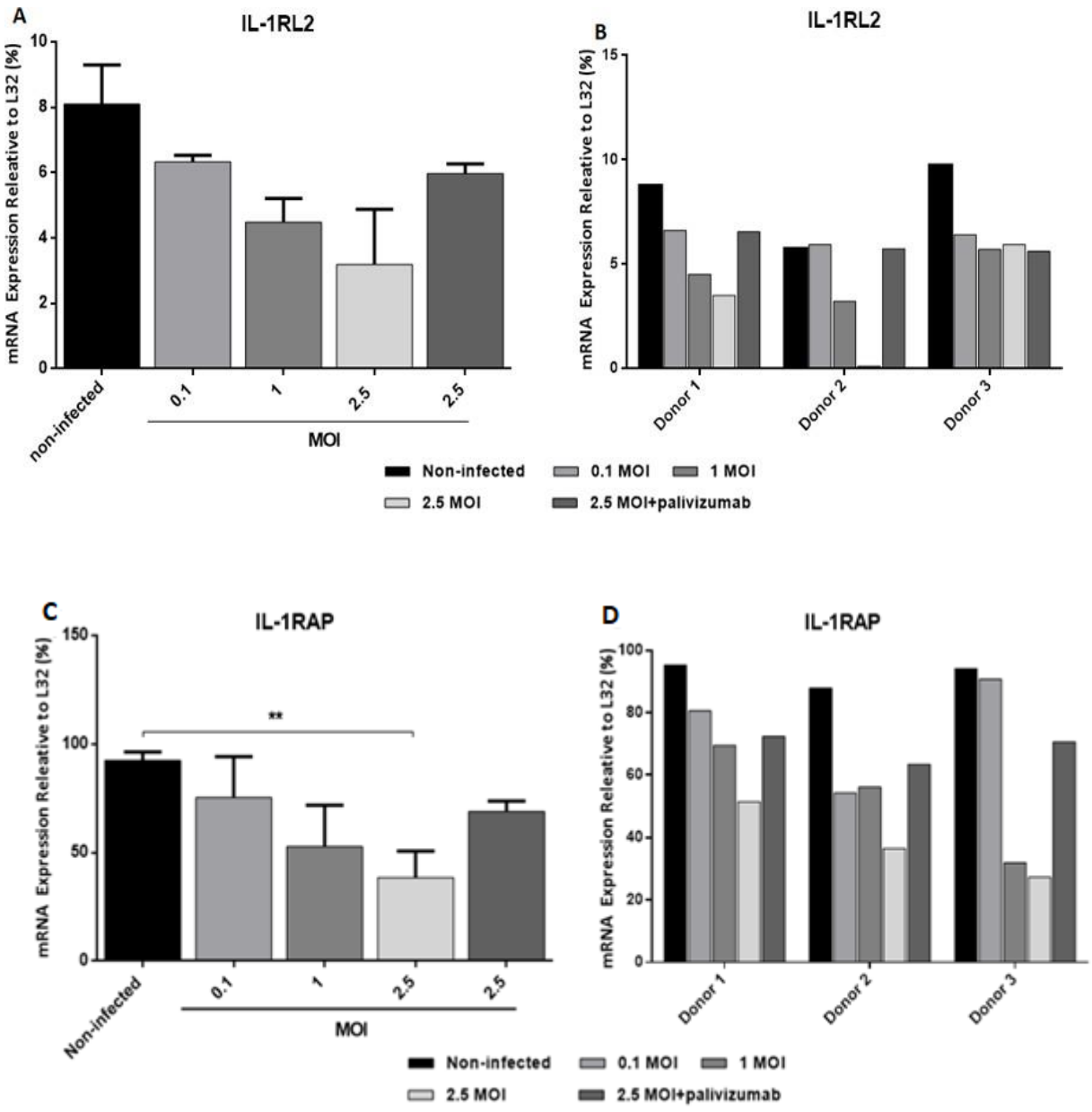

Figure 3.3.2.6. Expression of IL-1RL2 and IL-1RAP mRNA infected HNAECs. HNAECs were infected with RSV at MOIs of 0.1, 1, and 2.5 for 48 hours. Control non-infected HNAECs were cultured in the absence of RSV, and Palivizumab was used as an anti-RSV control. **A)** IL-1RL2 mRNA (n=3), **B)** individual donor expression of IL-1RL2 mRNA. **C)** IL-1RAP mRNA (n=3), **D)** individual donor expression of IL-1RAP mRNA. Friedman test for four groups/Dunn's multiple comparison test to compare the mean rank of group with the mean rank of every other group (*p<0.01) (Aand C). Data expressed as the mean ± SEM, based on fold expression relative to L32, RM-two-way ANOVA/Tukey's multiple comparisonstest (B and D).

3.3.2.7. IL-36 Protein Expression by Human Nasal Airway Epithelial Cells after RSV Infection

To confirm the effect of RSV on the expression of IL-36α, IL-36γ proteins in human nasal airways epithelial cells, HNAECs were infected by A2 RSV at MOIs 0.1, 1, and 2.5 for 48 hours. Palivizumab was used with RSV at an MOI 2.5 as an anti-RSV control. IL-36α, IL-36γ proteins were quantified by an IL-36 sandwich ELISA developed in the (Section 2.4), but there were no IL-36α, IL-36γ proteins detected. Then, this study use the commercial ELISA for both IL-36α and IL-36γ proteins (R&D system) and there were a significant has observed on the expression of IL-36α and IL-36γ proteins in the infected and non-infected HNAECs.

Then, according to Kovach *et al.,* (2016) study which suggest that IL-36γ protein was produced and packaged within membrane-bound vesicles. This study suggest that IL-36α, IL-36γ proteins could secreted in microparticles and this structure could prevent the actual level of IL-36 proteins. Therefore, culture medium from infected and non-infected HNAECs were collected and sonicated three times for 10 second to disrupt any membrane-bound structures by using the sonication instrument (section 2.1). IL36α, IL-36γ proteins were quantified in sonicated and non-sonicated of culture medium by ELISA. The results showed that, there were an extracellular IL-36α, IL-36γ proteins after sonication of conditioned medium in infected comparing to non-infected HNAECs but the level of these proteins were little, therefore this study sought to find another way to blebbing IL-36α and IL-36γ proteins of microparticles from the plasma membrane.

IL-36 cytokines belong to IL-1 family which not contain known signal sequences, and the mechanism of secretion is not well understood (Kumar *et al.,* 2000). Therefore, this study suggest that RSV stimulate the expression of IL-36 proteins which accumulate intracellularly and packaged in vesicles. Thus, the secretion mechanism of IL-36 proteins need another trigger to produce these proteins extracellularly. Several studies indicated that extracellular adenosine triphosphate (ATP) was a significant mediator for extracellular secretion of IL-36 cytokines (Aoyagi *et al.,* 2017; Lian *et al.,* 2012; Martin *et al.,* 2009). Therefore, this study examined whether the intracellular IL-36α and IL-36γ proteins was secreted from the RSV infected HNAECs in response to ATP stimulation *in vitro*. According to (Martin *et al.,* 2009; Kovach *et al.,* 2016) study, ATP (5 mM) was added on infected HNAECs for 30 minutes and the culture medium was collected after ATP stimulation and IL-36 proteins were assessed.

3.3.2.7.1 IL-36α protein expression by human nasal airway epithelial cells after RSV infection

To confirm the effect of RSV on the expression of IL-36α protein in HNAECs, these were infected with A2 RSV at MOIs of 0.1, 1, and 2.5 for 48 hours. Palivizumab was used with RSV at MOI 2.5 as an anti-RSV control. The observed IL-36α protein in the culture medium was upregulated significantly at MOI 1 ($p<0.05$) and MOI 2.5 ($p<0.001$) as compared to the results for non-infected HNAECs. Neutralising the virus before challenge using Palivizumab treatment resulted in significantly decreased expression of IL-36α protein at MOI 2.5 ($p<0.05$) (Figure 3.3.2.7.1 A). The expression of IL-36α protein was ~10 fold higher at MOI 1 and ~20 fold higher at MOI 2.5 as compared with that seen inn non-infected cells. In contrast, the expression was decreased ~5-fold for MOI 2.5 plus Palivizumab as compared with just MOI 2.5 (Figure 3.3.2.7.1 A).

There was a significant increase in expression of IL-36α protein after ATP treatment at MOI 2.5 ($p<0.01$) as compared with that seen for non-infected cells, while Palivizumab treatment resulted in significantly decreased expression of IL-36α protein in comparison to simple infection at MOI 2.5 ($p<0.01$) (Figure 3.3.2.7.1 B). The expression of IL-36α protein was ~10 fold higher at MOI 1 and ~20 fold higher at MOI 2.5 in infected HNAECs as compared to that seen in non-infected cells, whereas this expression decreased ~5 fold at MOI 2.5 plus Palivizumab as compared with the level for just MOI 2.5 (Figure 3.3.2.7.1 B).

As shown in Figure 3.3.2.7.1 C, the expression of IL-36α protein was increased significantly at MOIs 1 and 2.5 ($p<0.01$) in ATP-treated HNAECs as compared with the levels seen in HNAECs without ATP treatment. (Figure 3.3.2.7.1 C). Overall, the expression of IL-36α protein was ~1.2 to 2-fold higher in the ATP treatment groups compared to their equivalents without treatment.

The apparent induction in IL-36α protein expression in the supernatant following RSV infection was also reflected for protein expression in cell pellets by a significant increase in IL -36α protein at MOI 2.5 as compared to hat seen in non-infected HNAECs ($p<0.01$) and this expression was 2.6-fold higher at MOI 1 and 4.7-fold higher at MOI 2.5 as compared with observed intracellular IL-36α protein levels in non-infected cells (Figure 3.3.2.7.1 D).

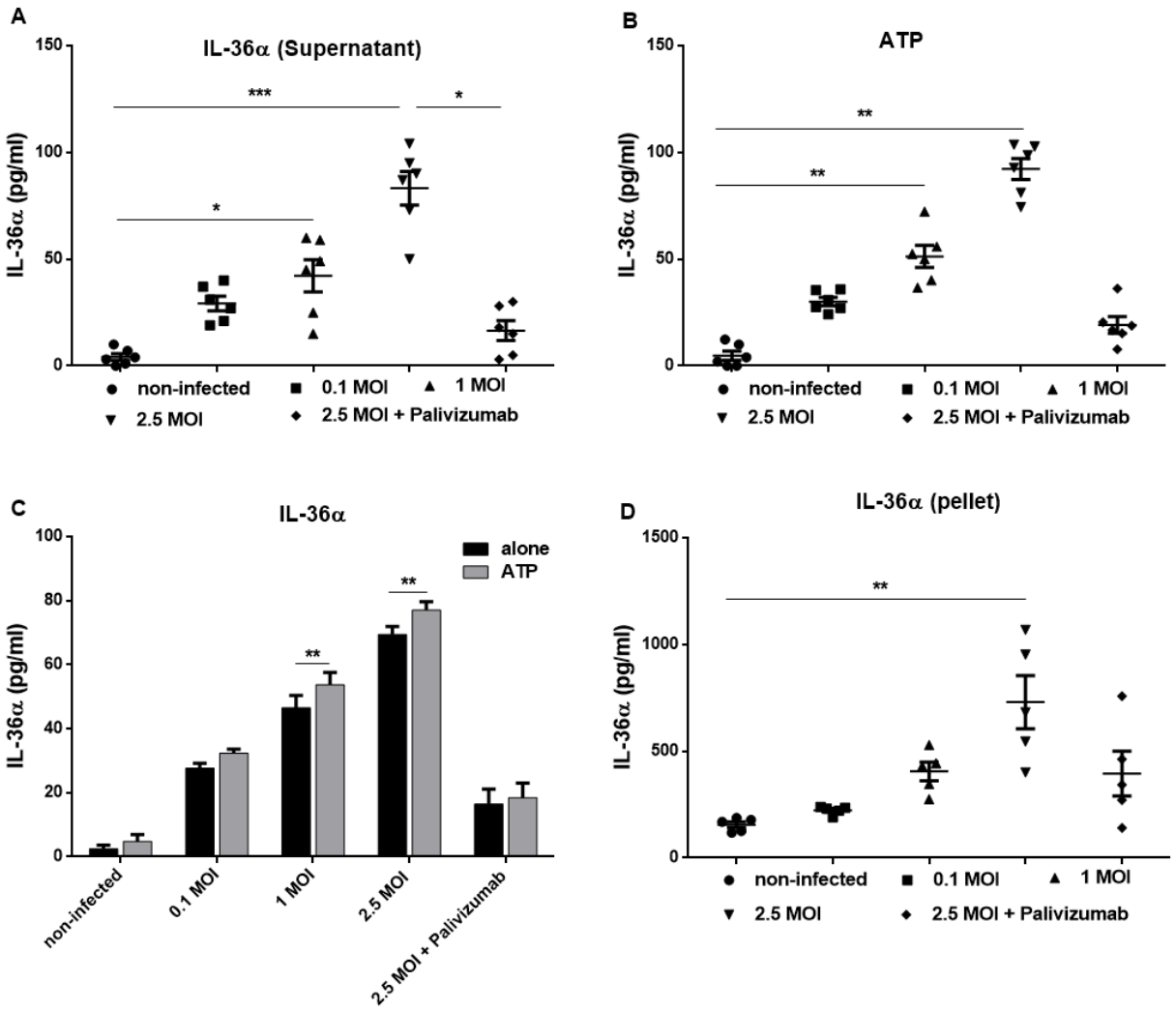

Figure 3.3.2.7.1 Expression of IL-36α protein in infected HNAECs. HNAECs were infected with RSV at MOIs of 0.1, 1, and 2.5 for 48 hours. Control non-infected HNAECs were cultured in the absence of RSV, and Palivizumab was used as an anti-RSV control. **A)** IL-36α protein in culture medium (n=6), **B)** IL-36α protein in culture medium after ATP treatment (n=6). **C)** Comparison between IL-36α protein expression in culture medium in ATP treatment group with the one group without any treatment (n=6). **D)** IL-36α protein in cell pellet (n=6). Data expressed as the mean ± SEM (foldexpression relative to non-infected cells, Friedman test for five groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group *p<0.05, **p<0.01, and ***p<0.001).

3.3.2.7.2 IL-36γ protein expression by human nasal airway epithelial cells after RSV infection

To confirm the effect of RSV on the expression of IL-36γ protein in human nasal airway epithelialcells, HNAECs were infected by A2 RSV at MOIs of 0.1, 1, and 2.5 for 48 hours. Palivizumab was also used with RSV at MOI 2.5 as an anti-RSV control.

IL-36γ protein expression in the culture medium was upregulated significantly at MOI 2.5 ($p<0.001$) as compared to that seen in non-infected HNAECs. The addition of Palivizumab as an anti-RSV control, however, led to significantly reduced IL-36γ protein expression at MOI 2.5 ($p<0.05$) (Figure 3.3.2.7.2 A). The expression of IL-36γ protein was ~6 fold higher at MOI 1 and ~9.5 fold higher at MOI 2.5 as compared with non-infected cells. In contrast, such expression was decreased ~3.8 fold for MOI 2.5 plus Palivizumab as compared with the level of IL-36γ seen at MOI 2.5 without Palivizumab (Figure 3.3.2.7.2 A).

Similarly, there was a significant increase in expression of IL-36γ protein after ATP treatment at MOI 2.5 ($p<0.01$) as compared with that seen in non-infected cells. Palivizumab treatment resulted in significantly decreased expression of IL-36γ protein in comparison to simple infection at MOI 2.5 ($p<0.05$) (Figure 3.3.2.7.2 B), while the expression of IL-36γ protein was ~4 fold higher at MOI 1 and ~7 fold higher at MOI 2.5 as compared with that in non-infected cells (Figure 3.3.2.7.2 B).

As shown in Figure 3.3.2.7.2 C, the expression of IL-36γ protein increased significantly at MOIs 0.1 ($p<0.001$), 1 ($p<0.01$), and 2.5 ($p<0.001$) in ATP treated HNAECs in comparison with that seen in HNAECs without ATP treatment (Figure 3.3.2.7.2 C). Overall, the expression of IL-36γ protein was ~1.5 to 2.5-fold higher in the ATP treatment group than in the one without any treatment.

The apparent induction in IL-36γ protein expression following RSV infection in the supernatant was also reflected in protein expression within the in-cell pellet, with a significant increase in IL-36γ protein at MOIs 1 ($p<0.5$) and 2.5 ($p<0.001$) as compared to that in non-infected HNAECs (Figure 3.3.2.7.2 D). The level of IL-36γ intracellular protein expression was thus 2.9-times higher at MOI 1 and 3.8-times higher at MOI 2.5 (Figure 3.3.2.7.2 D).

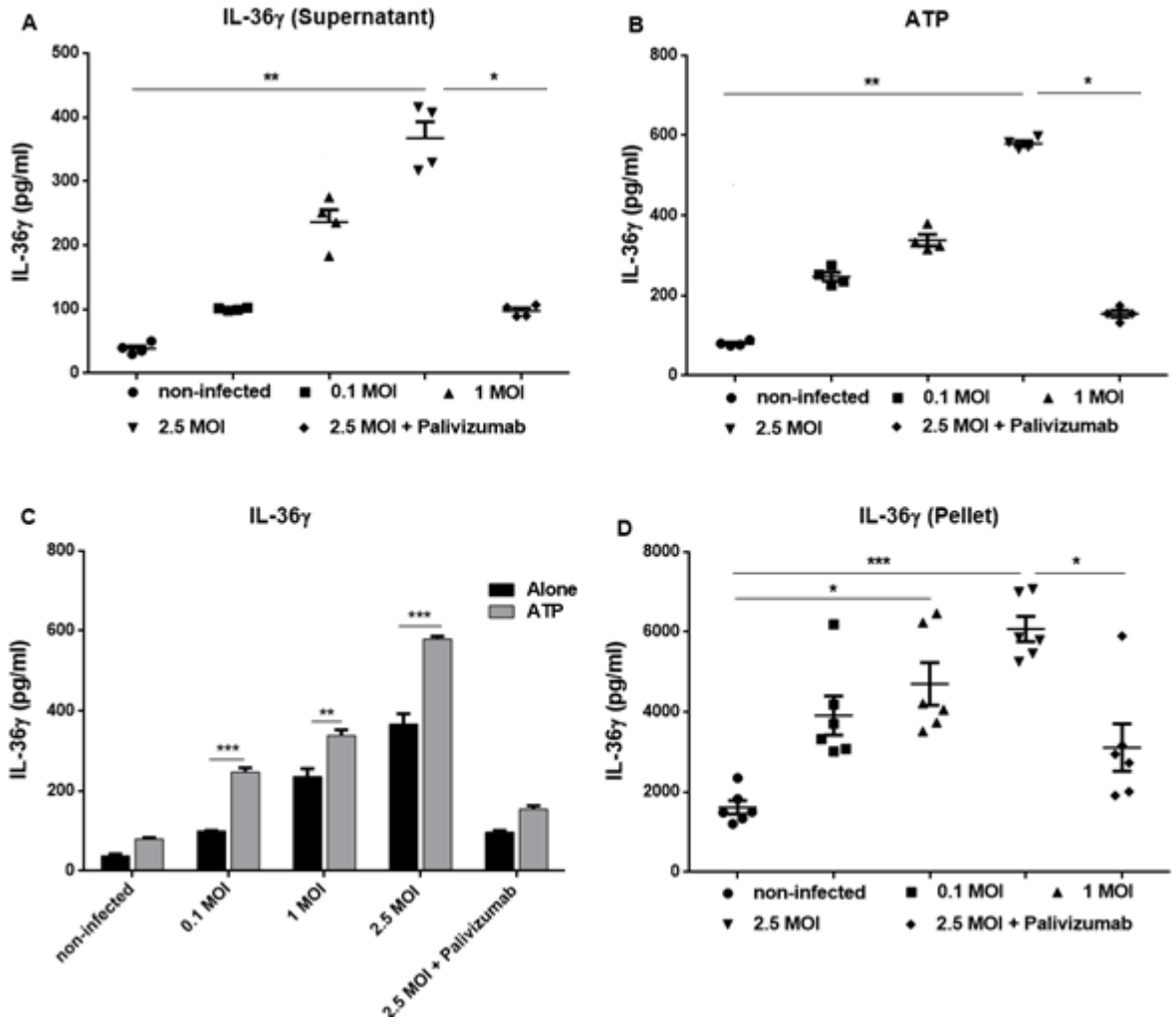

Figure 3.3.2.7.2 Expression of IL-36γ protein in infected HNAECs. HNAECs were infected with RSV at MOIs of 0.1, 1, and 2.5 for 48 hours. Control non-infected HNAECs were cultured in the absence of RSV, and Palivizumab was used as an anti-RSV control. **A)** IL-36γ protein in culture medium (n=6), **B)** IL-36γ protein in culture medium after ATP treatment (n=6). **C)** Comparison between IL-36γ protein expression in culture medium in ATP treatment group with the one group without any treatment (n=6). **D)** IL-36γ protein in cell pellet (n=6). Data expressed as mean ± SEM (fold expression relative to non-infected cells, Friedman test for five groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group *p<0.05, **p<0.01, and ***p<0.001).

3.3.2 Expression of IL-36 cytokines and their receptors by bronchial epithelial cell lines A549 and BEAS-2B following RSV infection

3.3.3.1 RSV replication in the bronchial epithelial cell lines A549 and BEAS-2B

A549 and BEAS-2B cells were grown in 12-well tissue culture plates until they were 80% confluent. Cultured A549 and BEAS-2B colonies were then infected with RSV A2 at MOIs of 0.1, 1, and 2.5 for 48 hours, with non- infected cells used as a control (McNamara *et al.,* 2012; Alturaiki, 2014). In addition, an A549 culture was infected with RSV A2 at MOI 2.5 for 4, 8, 24, and 48 hours. RNA was extracted from the cells using Qiagen and reverse transcribed. The resulting mRNA expression was the determined using TaqMan primer-probe assays, with L32, IL-36α, IL36γ, IL- 36Rα, and IL-36Rs (IL1RL2 and IL1RAP) normalised to the housekeeping gene L32 (section 2.5).

After infection, RSV N gene expression was measured using qPCR to confirm viral replication and to ensure that the A549 and BEAS-2B cells responded to the challenge. There was no RSV N RNA in the non-infected control cells, while RSV N RNA was induced significantly in both A549 and BEAS-2B Cells at 0.1 MOI ($p<0.01$ and $p<0.05$, respectively), 1 MOI ($p<0.01$ in both cell lines), and 2.5 MOI ($p<0.001$ and $p<0.01$, respectively) (Friedman test/Dunn's multiple comparison test) (Figure 3.3.3.1. A and B). RSV infection and replication in both A549 and BEAS-2B cells increased with increases in MOI.

Using the A549 cell line, RSV N RNA expression was assessed at various time points after infection. RSV N RNA was not detected in the non-infected control cells at any time point, while RSV N RNA was significantly increased at 24 hours and 48 hours ($p<0.001$) in infected cells as compared to non-infected cells (Figure 3.3.3.1 C). These results further indicate that RSV N RNA levels increase significantly with time after RSV infection in A549 cells (two-way RM ANOVA test).

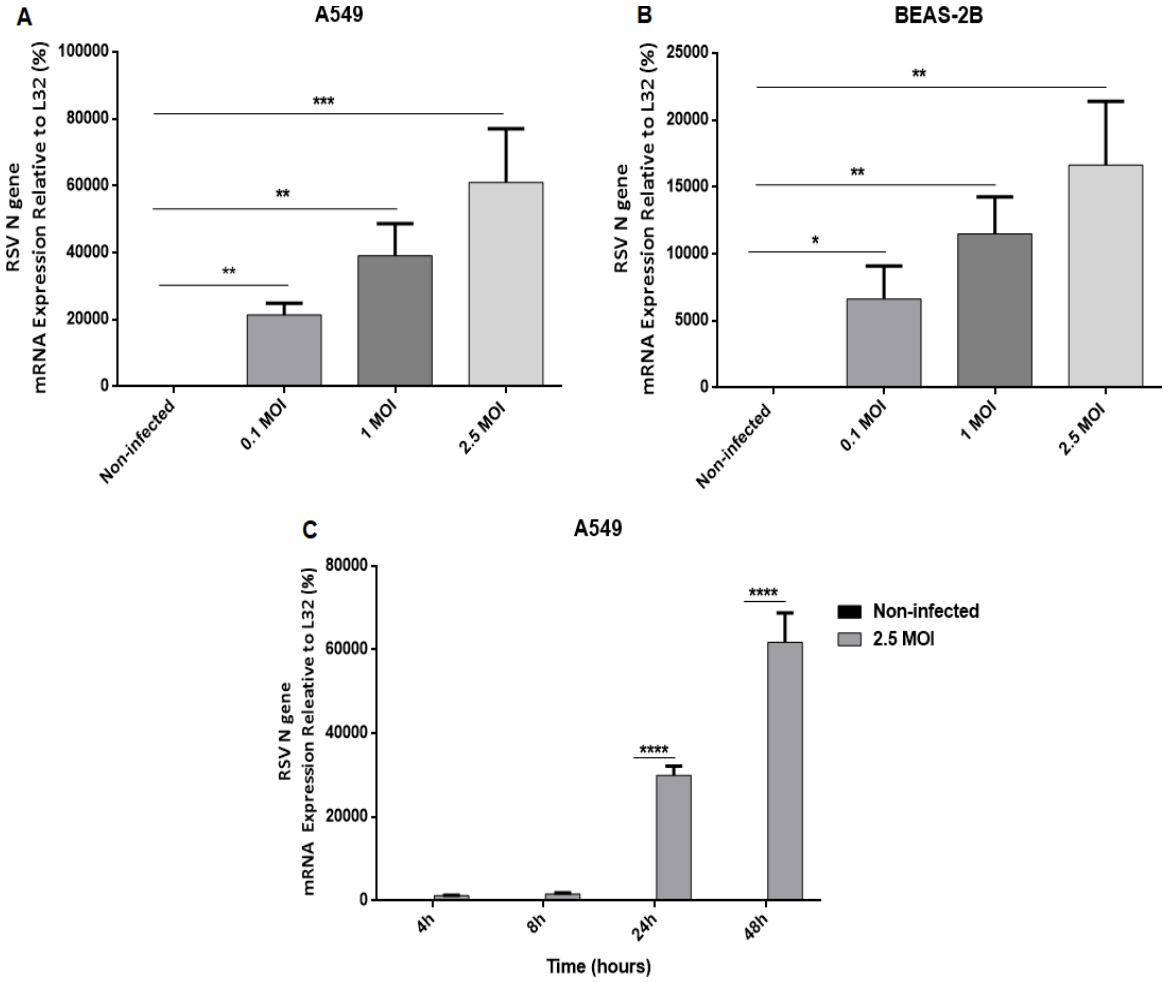

Figure 3.3.3.1. RSV N gene mRNA expression in RSV A2 in infected bronchial epithelial cells (A549 and BEAS-2B). A) A549 (n=4), and **B)** BEAS-2B cells (n=3), were infected with RSV A2 at different MOI (0.1, 1 and 2.5) for 48 hours. Non-infected cells were used as a control. **C)** A549 cell swere infected with RSV at a different time point (4h, 8h, 24h, and 48h) (n=3). Data expressed as Mean ± SEM (Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group (A and B) and Two-way RM ANOVA test (C) , * $p<0.05$, **$p<0.01$, *** $p<0.001$, and *** $p<0.0001$).

3.3.3.2 IL-8 mRNA expression in bronchial epithelial cells (A549 and BEAS-2B cells) in response to RSV infection

IL-8 gene expression was measured to confirm viral replication to ensure the A549 and BEAS-2B cells responded to the challenge. IL-8 mRNA expression was upregulated with an increasing dose of RSV in A549 cells at 0.1 MOI ($p<0.05$), 1 MOI ($p<0.05$) A549, and 2.5 MOI ($p<0.01$) (Friedman test/Dunn's multiple comparisons test) (Figure 3.3.3.2 A). The expression of IL -8 mRNA was 82-fold higher at MOI 0.1, 92-fold higher at MOI 1, and 115-fold higher at MOI 2.5 in infected A549 cells compared to non-infected cells (Figure 3.3.3.2 A). Increased MOI increased the IL-8 mRNA expression in the A549 cell.

IL-8 mRNA expression was upregulated with an increasing dose of RSV in BEAS-2B cells at 0.1 MOI ($p<0.05$), 1 MOI ($p<0.01$) in BEAS-2B cells, and 2.5 MOI ($p<0.01$) (Friedman test/Dunn's multiple comparisons test) (Figure 3.3.3.2 B). The expression of IL-8 mRNA was 41-fold higher at MOI 0.1, 63-fold higher at MOI 1, and 74-fold higher at MOI 2.5 in infected BEAS-2B cells compared to non-infected cells (Figure 3.3.3.2 B). An increased MOI increased the IL-8 mRNA expression in BEAS-2B cells.

In A549 cells, IL-8 mRNA expression was significantly increased at 8 hours ($p<0.05$), 24 hours ($p<0.0001$),and 48 hours ($p<0.0001$) compared to non-infected cells (Figure 3.3.3.2 C). These results indicate that the bronchial epithelial cell lines A549 respond to RSV infection by expressing IL-8 (Two-way RM ANOVA test).

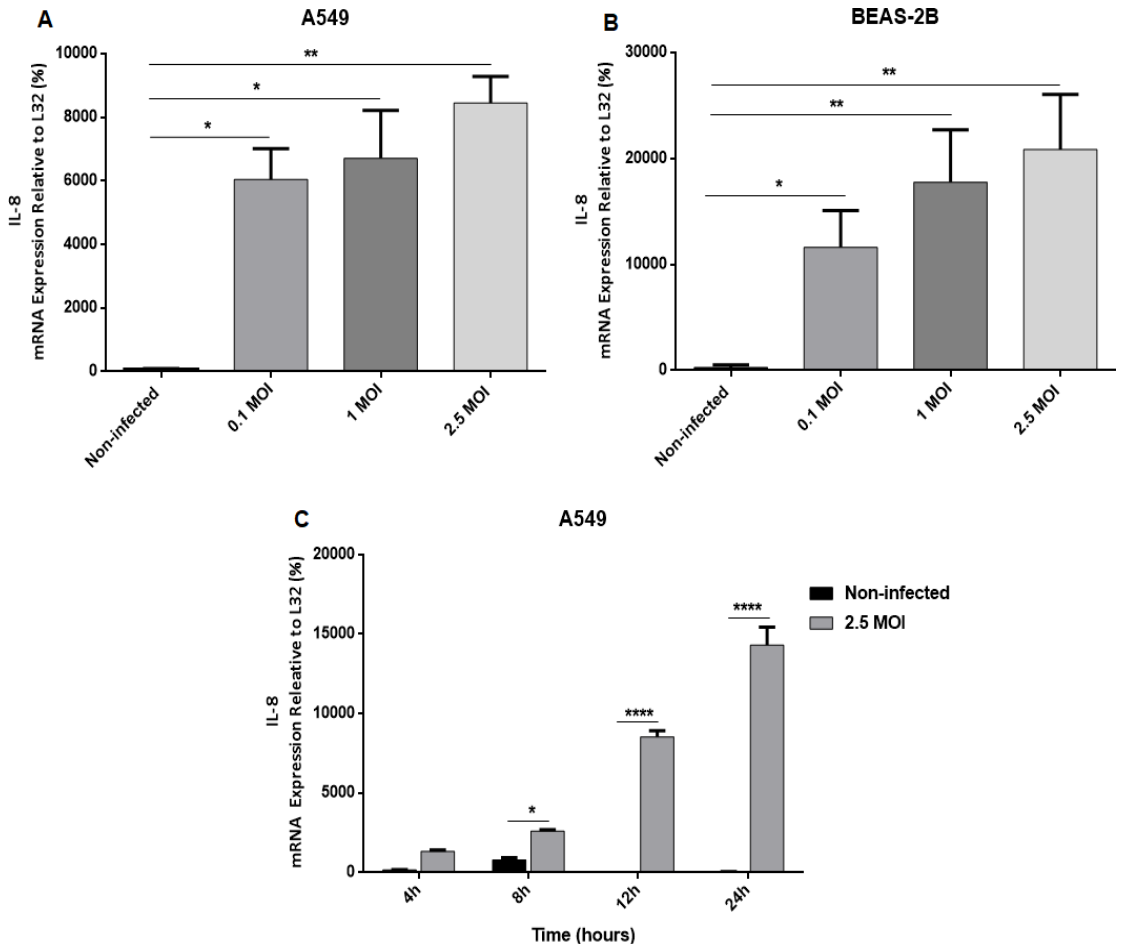

Figure 3.3.3.2. Expression of IL-8 mRNA in RSV A2 in infected bronchial epithelial cells (A549 and BEAS-2B). (A) A549 (n=4) and **(B)** BEAS-2B cells (n=4) were infected with RSV A2 at different MOI (0.1, 1 and 2.5) for 48 hours. Non-infected cells were used as a control. **(C)** A549 cells were infected with RSV at different time points (4h, 8h, 24h, and 48h) (n=3). Data are expressed as the Mean ± SEM ((Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group (A and B) and Two-way RM ANOVA test (C) , * p<0.05, **p<0.01, and **** p<0.0001).

3.3.3.3 IL-36α mRNA expression by the bronchial epithelial cell lines (A549 and BEAS-2B cells) after RSV Infection

To confirm the expression of IL-36α mRNA in infected A549 and BEAS-2B cells, the results indicate that there were significant differences observed between IL-36α mRNA expression in MOI 2.5 infected A549 cells in comparison with non-infected cells ($p<0.01$) (Friedman test/Dunn's multiple comparisons test) (Figure 3.3.3.3 A). IL-36α mRNA expression in infected A549 cells were increased by increasing the viral load of MO1. IL-36α mRNA expression was 12-fold higher at MOI 0.1, 43-fold higher at MOI 1, and 61-fold higher at MOI 2.5 of infected A549 cells compared with non-infected cells (Figure 3.3.3.3 A). The IL-36α mRNA expression in A549 cells increased by an increased MOI.

IL-36α mRNA expression in MOI 2.5 infected BEAS-2B cells in comparison with non-infected cells ($p<0.05$) (Friedman test/Dunn's multiple comparisons test) (Figure 3.3.3.3 B). IL-36α mRNA expression in infected BEAS-2B cells was increased by increasing the viral load of MO1. The expression of IL-36α mRNA was 7-fold higher at MOI 0.1, 10-fold higher at MOI 1, and 15-fold higher at MOI 2.5 of infected BEAS-2B cells in comparison with non-infected cells (Figure 3.3.3.3 B). The IL-36α mRNA expression in BEAS-2B cell increased by an increased MOI.

IL-36α mRNA was significantly increased in A549 cells at 8 hours ($p<0.01$), 24 hours ($p<0.0001$), and 48 hours ($p<0.0001$), respectively, compared to non-infected cells (Figure 3.3.3.3 C) (Two-way RM ANOVA test). These results indicate that bronchial epithelial cell IL-36α mRNA expression increases in response to RSV infection.

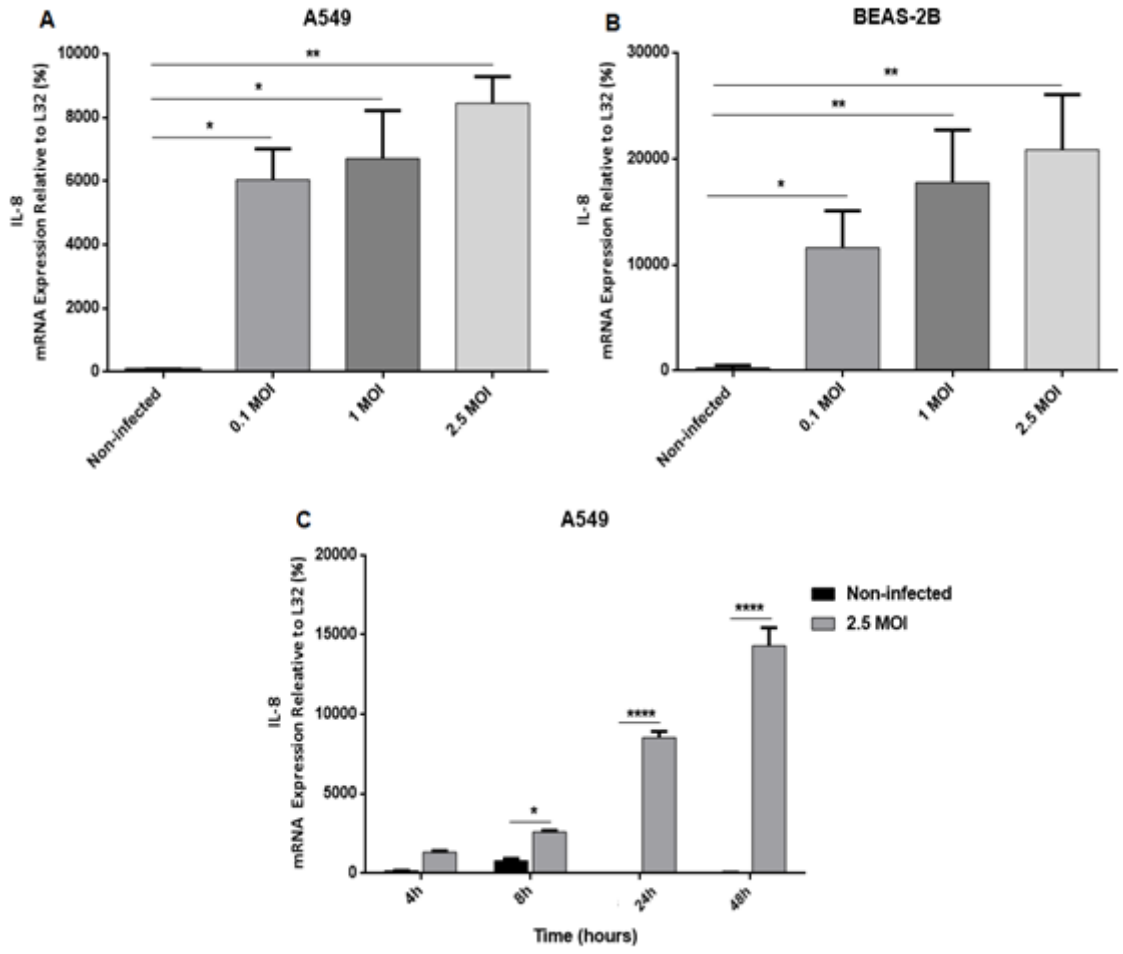

Figure 3.3.3.3. Expression of IL-36α mRNA in RSV A2 in infected bronchial epithelial cells (A549 and BEAS-2B). (A) A549 (n=4) and **(B)** BEAS-2B cells (n=4) were infected with RSV A2 at different MOI (0.1, 1 and 2.5) for 48 hours. Non-infected cells were used as a control. **(C)** A549 cells wereinfected with RSV at different time points (4h, 8h, 24h, and 48h) (n=3). Data are expressed as the Mean ± SEM ((Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group (A and B) and Two-way RM ANOVA test (C) , , * p<0.05, **p<0.01, and **** p<0.001).

3.3.3.4 IL-36γ mRNA Expression by the bronchial epithelial cell lines (A549and BEAS-2B cells) after RSV infection

To confirm the expression of IL-36γ mRNA in infected A549 and BEAS-2B cells, for infected A549 cells, significant differences in IL-36γ mRNA expression were observed between MOI 2.5 infected and non-infected cells ($p<0.01$) (Friedman test/Dunn's multiple comparisons test) (figure 3.3.3.4 A). IL-36γ mRNA expression by infected A549 cells was increased with increasing viral load. The expression of IL-36γ mRNA was 50-fold higher at MOI 0.1, 88-fold higher at MOI 1, and 110-fold higher at MOI 2.5 of infected A549 cells compared to non-infected cells (Figure 3.3.3.4 A).

In infected BEAS-2B cells, there was a significant difference in IL-36γ mRNA expression was observed between MOI 2.5 infected and non-infected cells ($p<0.01$) (Friedman test/Dunn's multiple comparisons test) (figure 3.3.3.4 B). IL-36γ mRNA expression by infected BEAS-2B cells was increased with increasing viral load. The expression of IL-36γ mRNA was 18-fold higher atMOI 0.1, 35-fold higher at MOI 1, and 47-fold higher at MOI 2.5 of infected BEAS-2B cells compared with non-infected cells (Figure 3.3.3.4 B).

IL-36γ mRNA expression was significantly increased in A549 cells at both 24 hours ($p<0.05$) and 48 hours ($p<0.0001$), as compared to that in non-infected cells (two-way RM ANOVA test) (Figure 3.3.3.4 C). These results indicate that IL-36γ mRNA expression by infected airway epithelial cells in response to RSV infection is dependent on both viral load and time.

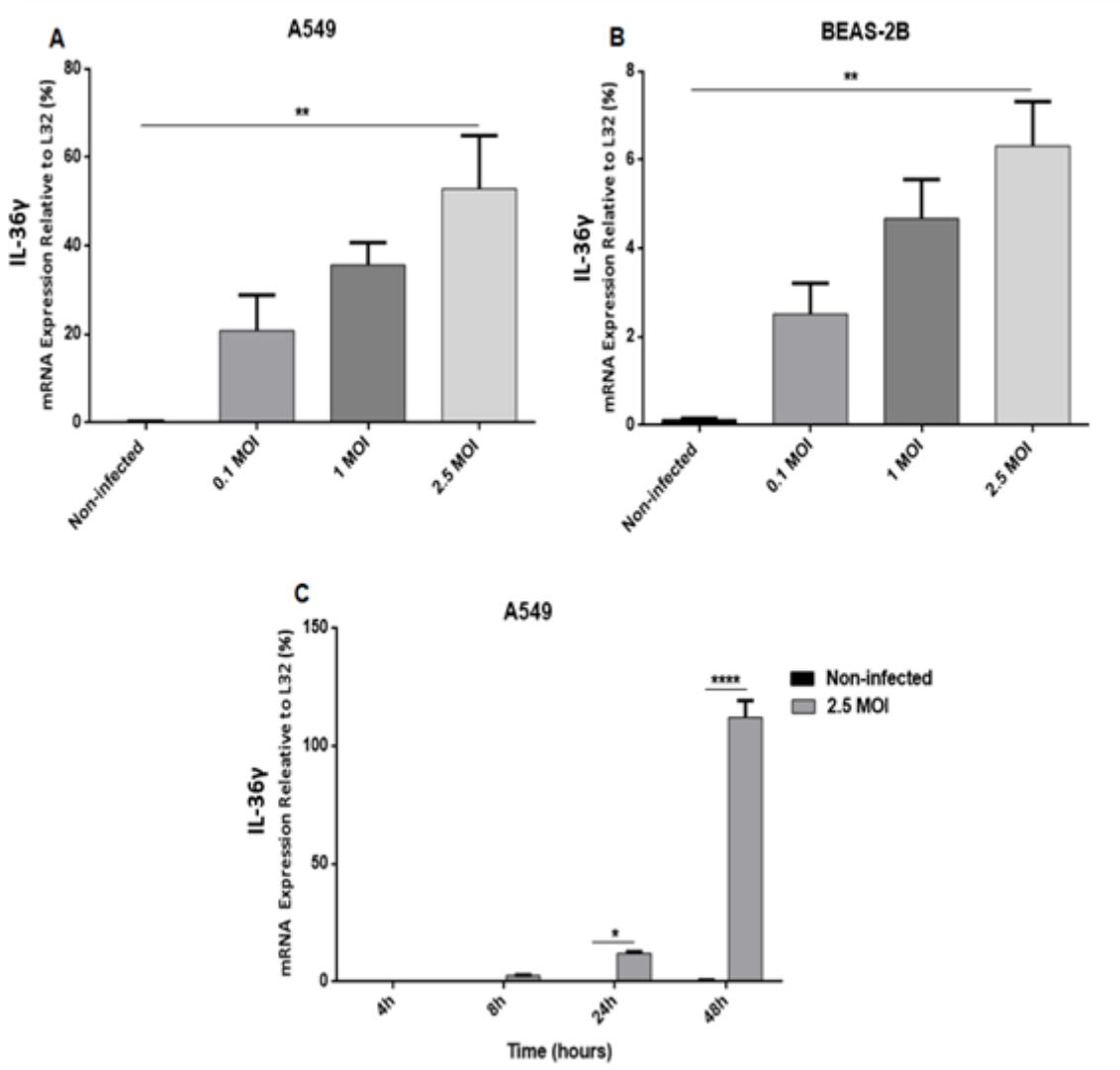

Figure 3.3.3.4. Expression of IL-36γ mRNA in RSV A2 in infected bronchial epithelial cells (A549 and BEAS-2B). A) A549 (n=4) and **B)** BEAS-2B cells (n=4) were infected with RSV A2 at different MOIs (0.1, 1 and 2.5) for 48 hours. Non-infected cells were used as a control. **C)** A549 cells were infected with RSV at different time points (4 h, 8 h, 24 h, and 48 h) (n=3). Data expressed as mean ± SEM ((Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group (A and B) and Two-way RM ANOVA test (C) , * p<0.05, **p<0.01, and **** p<0.001).

3.3.3.5 IL-36 receptor antagonist mRNA expression by bronchial epithelial celllines (A549 and BEAS-2B cells) after RSV infection

The results indicated that there were significant differences between IL-36Rα mRNA expression in MOI 2.5 infected A549 cells in comparison with that in non-infected cells ($p<0.05$) (fold expression relative to L32, Friedman test/Dunn's multiple comparison test) (figure 3.3.3.5 A). IL-36Rα mRNA expression by infected A549 cells was increased by increasing the viral load. The expression of IL-36Rα mRNA was 108-fold higher at MOI 0.1, 135-fold higher at MOI 1, and 306-fold higher at MOI 2.5 in comparison with levels seen in non-infected cells (Figure 3.3.3.5 A).

There were significant differences observed between IL-36Rα mRNA expression in MOI 2.5 infected BEAS-2B cells and that in non-infected cells ($p<0.01$) (fold expression relative to L32, Friedman test/Dunn's multiple comparison test) (figure 3.3.3.5 B). IL-36Rα mRNA expression by infected BEAS-2B cells was increased on increasing the viral load MOI. The expression of IL-36Rα mRNA was 7-fold higher at MOI 0.1, 9-fold higher at MOI 1, and 15-fold higher at MOI 2.5 as compared to the levels seen in non-infected cells (Figure 3.3.3.5 B).

IL-36Rα mRNA was further significantly increased in A549 at 48 hours ($p<0.0001$) as compared to the levels seen in non-infected cells (two-way RM ANOVA test) (Figure 3.3.3.5 C). These results indicate that the level of IL-36Rα mRNA expressed by infected airway epithelial cells in response to RSV infection is dependent on both viral load and time.

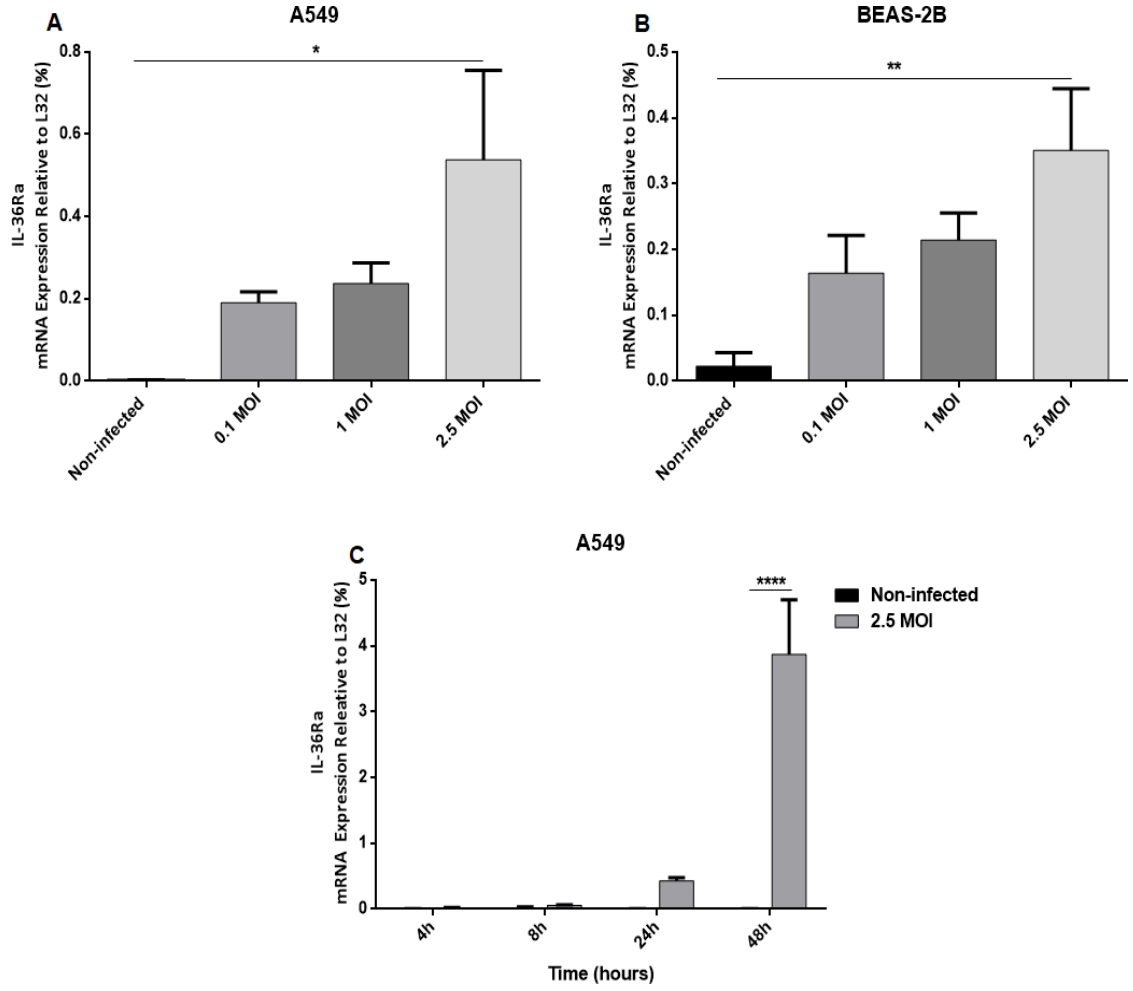

Figure 3.3.3.5. Expression of IL-36Rα mRNA in infected bronchial epithelial cells (A549 and BEAS-2B). A) A549 (n=3) and **B)** BEAS-2B cells (n=4) were infected with RSV A2 at different MOI (0.1, 1 and 2.5) for 48 hours. Non-infected cells were used as a control. **C)** A549 cells were infectedwith RSV at different time points (4h, 8h, 24h, and 48h) (n=3). Data expressed as the Mean ± SEM ((Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group (A and B) and Two-way RM ANOVA test (C) , * $p<0.05$, **$p<0.01$, and **** $p<0.001$).

3.3.3.6 Expression of IL1RL2 mRNA in RSV infection in bronchial epithelial cells (A549 and BEAS-2B)

The results indicated that there was no significant additional expression of IL-1RL2 mRNA in infected A549 cells as compared to that in non-infected cells (P=0.05) (Friedman test/Dunn's multiple comparison test) (Figure 3.3.3.6 A).

However, the expression of IL-1RL2 mRNA increased significantly in infected BEAS-2B cells at 1 and 2.5 MOI ($P<0.05$) in comparison with that seen in non-infected cells (Friedman test/Dunn's multiple comparison test) (Figure 3.3.3.6 B). The expression of IL-1RL2 mRNA was 4-time higher at MOI 0.1 and 6-time higher at MOI 2.5 as compared to non-infected cells (Figure 3.3.3.6 B).

There were no significant differences observed between IL-1RL2 mRNA expression in infected and non-infected A549 cells at any time point (two-way RM ANOVA test) (Figure 3.3.3.6 C). These results indicate overall that there was a significant difference between control and RSV infected BEAS-2B cells, but not between control and infected A549 cells.

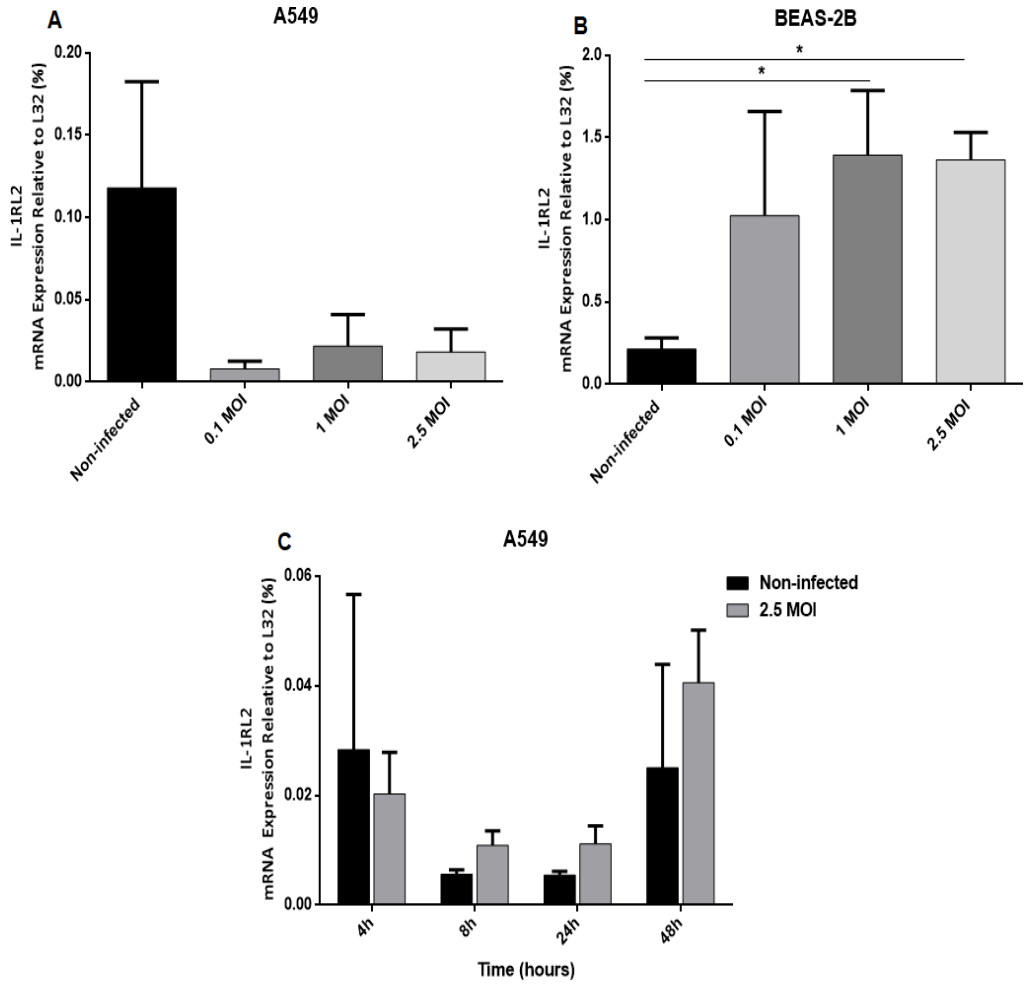

Figure 3.3.3.6 RSV infection induced IL-1RL2 mRNA expression in infected airway epithelial cells (A549 and BEAS-2B). A) A549 (n=4), and **B)** BEAS-2B cells (n=4), were infected with RSV A2 at different MOI (0.1, 1 and 2.5) for 48 hours. Non-infected cells were used as a control. **C)** A549 cells were infected with RSV at different time points (4h, 8h, 24h, and 48h) (n=3). Data expressed as the Mean ± SEM ((Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group (A and B) and Two-way RM ANOVA test (C) , * $p<0.05$, **$p<0.01$, and *** $p<0.001$).

3.3.3.7 Expression of IL-1RAP mRNA by RSV infection in bronchial epithelial cells (A549 and BEAS-2B cells)

The results in this work indicate that no significant increase in expression of IL-1RAP mRNA occurs in infected A549 cells as compared to that seen in non-infected cells (P= 0.1) (Friedman test/Dunn's multiple comparison test) (Figure 3.3.3.7 A), although the expression of IL-1RAP mRNA was 1.3-fold higher at MOI 0.1, 1.4-fold higher at MOI 1, and 0.5-fold higher at MOI 2.5 in infected A549 cells as compared to that in non-infected cells (Figure 3.3.3.7 A). Similarly, there was no significant increase in expression of IL-1RAP mRNA observed in infected BEAS-2B cells for any MOIs (P= 0.2) (Friedman test/Dunn's multiple comparisons test) (Figure 3.3.3.7 B), though the expression of IL-1RAP mRNA was 1.3-fold higher at MOI 0.1, 1.9-fold higher at MOI 1, and 1.6-fold higher at MOI 2.5 as compared to that in non-infected cells (Figure 3.3.3.7 B).

No significant difference were observed for any time point (Figure 3.3.3.7 C). These results indicate that no change in IL-1RAP mRNA expression occurs due to infected airways' epithelial cells' responses to RSV infection.

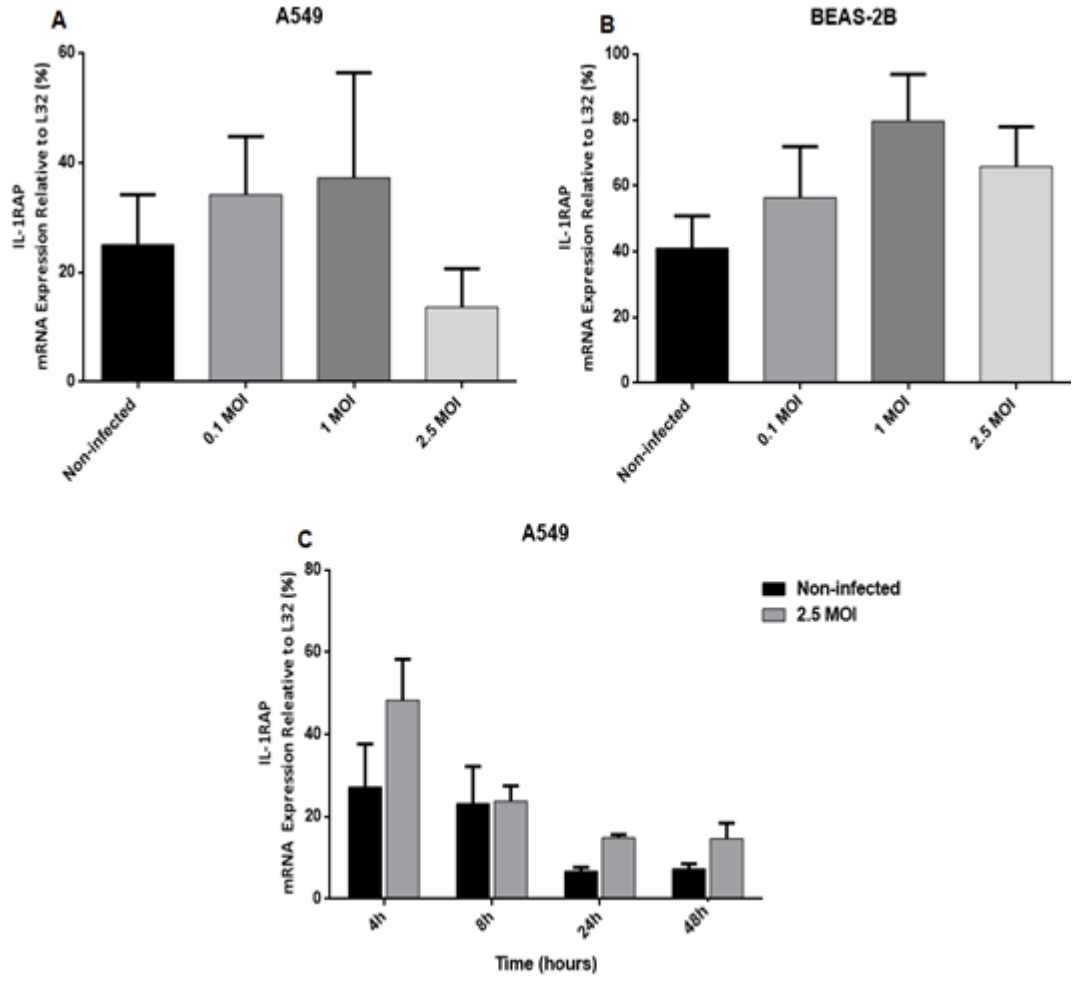

Figure 3.3.3.7. RSV infection induced IL-1RAP mRNA expression in infected bronchial epithelial cells (A549 and BEAS-2B). A) A549 (n=3) and **B)** BEAS-2B cells (n=3) were infected with RSV A2 at different MOI (0.1, 1 and 2.5) for 48 hours. Non-infected cells were used as a control. **C)** A549 cells were infected with RSV at different time points (4h, 8h, 24h, and 48h) for 48h (n=3). Data expressed as Mean ± SEM ((Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group (A and B) and Two-way RM ANOVA test (C) , * $p<0.05$, **$p<0.01$, and *** $p<0.001$).

3.3.3.8 Expression of IL-36α protein by human RSV infection in bronchial epithelial cells (A549 and BEAS-2B)

To confirm the effect of RSV on the expression of IL-36α protein in cultured A549 cells, these were infected with A2 RSV at MOIs 0.1, 1, and 2.5 for 48 hours; non-infected cells were used as a control.

IL-36α protein expression in the culture medium was upregulated significantly at MOI 2.5 ($p<0.05$) as compared to that seen in the non-infected control A549 cells (Friedman test/Dunn's multiple comparison test) (Figure 3.3.3.8a A). The mean levels of IL-36α protein expression were 28 pg/ml at MOI 0.1, 42 pg/ml at MOI 1, and 53 pg/ml at MOI 2.5 (Figure 3.3.3.8a A).

Similarly, there was a significant increase in expression of IL-36α protein after ATP treatment at MOI 2.5 ($p<0.05$) as compared with that seen in non-infected cells (Friedman test/Dunn's multiple comparison test) (Figure 3.3.3.8a B). The expression of IL-36α protein was ~5 fold higher at MOI 1 and ~6 fold higher at MOI 2.5 as compared to non-infected cells (Figure 3.3.3.8a B).

As shown in Figure 3.3.3.8a C, the expression of IL-36α protein increased significantly at MOIs 0.1 ($p<0.0001$), 1 ($p<0.0001$), and 2.5 ($p<0.0001$) in ATP treated A549 cells in comparison with that in A549 cells without any treatment (Figure 3.3.3.8a C). Overall, the expression of IL-36α protein was ~1 to 3-fold higher in the ATP treatment group.

The apparent induction of IL-36α protein expression following RSV infection in the supernatant was also reflected in protein expression within the in-cell pellet, with a significant increase in IL -36α protein at MOI 2.5 ($p<0.05$) as compared to that seen in non-infected A549 cells (Figure 3.3.3.8a D). The IL- 36α intracellular protein level was 6-times higher at MOI 1 and 10-fold higher at MOI 2.5 (Figure 3.3.3.8a D).

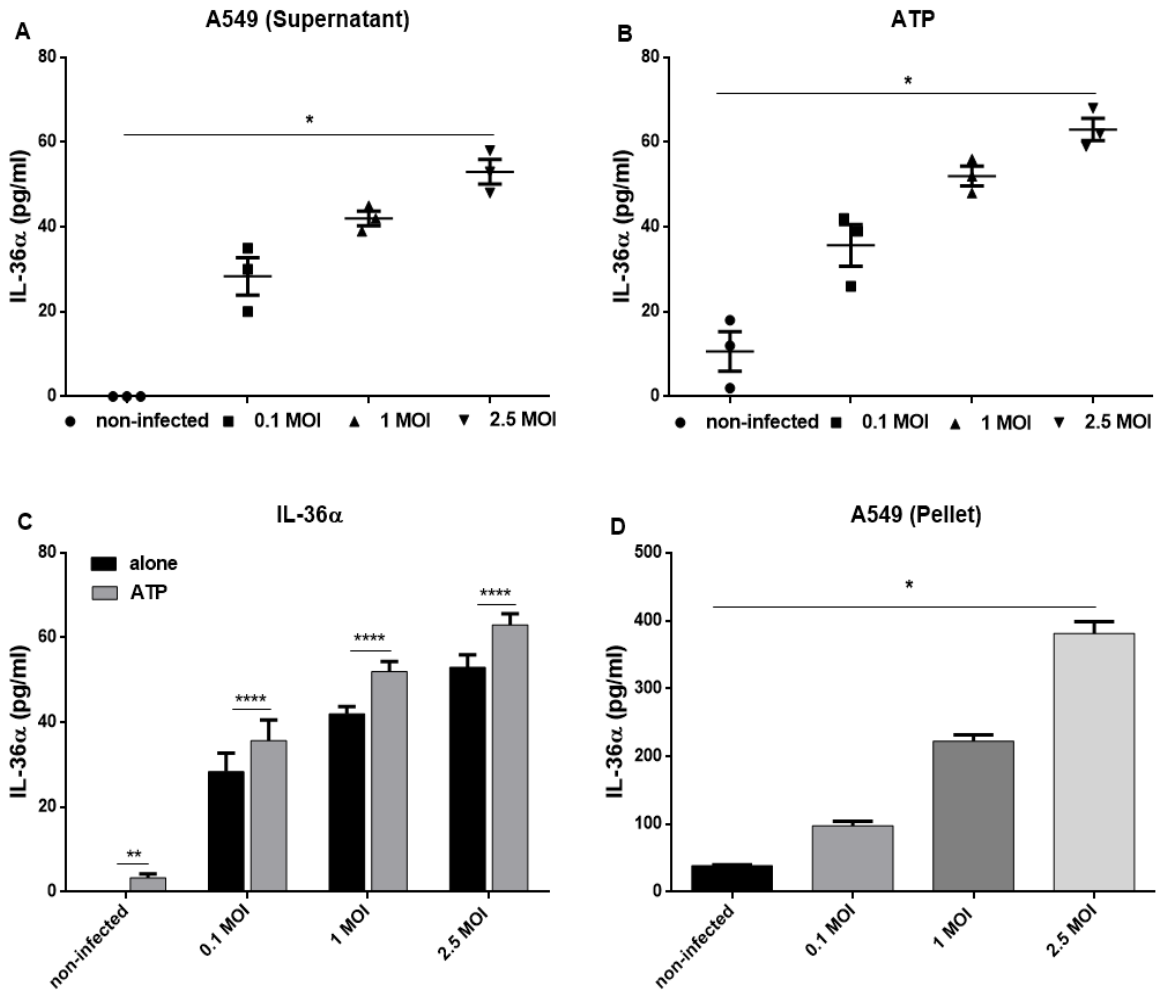

Figure 3.3.3.8a Expression of IL-36α protein in infected bronchial epithelial A549 cells. A549 cells were infected with RSV at MOIs of 0.1, 1, and 2.5 for 48 hours. Control non-infected HNAECs were cultured in the absence of RSV. **A)** IL-36α protein expression in culture medium (n=3), **B)** IL-36α protein expression in culture medium with ATP (n= 3), **C)** Comparison between IL- 36α protein expression in ATP treatment group and without any treatment group (n= 3). Data expressed as the mean ± SEM (Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group and Two-way RM ANOVA test, *p<0.05, **P<0.001, ***P<0.0001 and ****p<0.0001).

To confirm the effect of RSV on the expression of IL-36α protein in cultured BEAS-2B cells, such cells were infected with A2 RSV at MOIs 0.1, 1, and 2.5 for 48 hours; non-infected cells were used as a control.

IL-36α protein expression in the culture medium was upregulated significantly at MOI 2.5 ($p<0.05$) as compared to that seen in non-infected control BEAS-2B cells (Friedman test/Dunn's multiple comparison test) (Figure 3.3.3.8b A). The mean levels of IL-36α protein expression were 27 pg/ml at MOI 0.1, 43 pg/ml at MOI 1, and 54 pg/ml at MOI 2.5 for infected BEAS-2B cells (Figure 3.3.3.8b A).

Similarly, there was a significant increase in expression of IL-36α protein after ATP treatment at MOI 2.5 ($p<0.05$) as compared with that seen in non-infected cells (Friedman test/Dunn's multiple comparisons test) (Figure 3.3.3.8b B). The expression of IL-36α protein was ~5 fold higher at MOI 1 and ~6 fold higher at MOI 2.5 in comparison with non-infected cells (Figure 3.3.3.8b B).

As shown in Figure 3.3.2.8 C, the expression of IL-36α protein increased significantly at MOIs 0.1 ($p<0.001$), 1 ($p<0.001$), and 2.5 ($p<0.05$) in ATP treated BEAS-2B cells in comparison with the BEAS-2B cells without any treatment (Figure 3.3.3.8b C). Overall, the results show that the treatment of A549 cells with ATP alongside RSV infection increased the expression of IL-36α protein between ~1 to 2-times that seen in infected cells without ATP treatment.

The apparent induction in IL-36α protein expression following RSV infection in supernatant was also reflected in protein expression within the in cell pellet with a significant increase in IL-36αprotein at MOIs 2.5 ($p<0.05$) compared to non-infected BEAS-2B cells (Figure 3.3.3.8 D). The IL-36α intracellular protein was 5-fold higher at MOI 1 and 10-fold higher at MOI 2.5 (Figure 3.3.3.8b D).

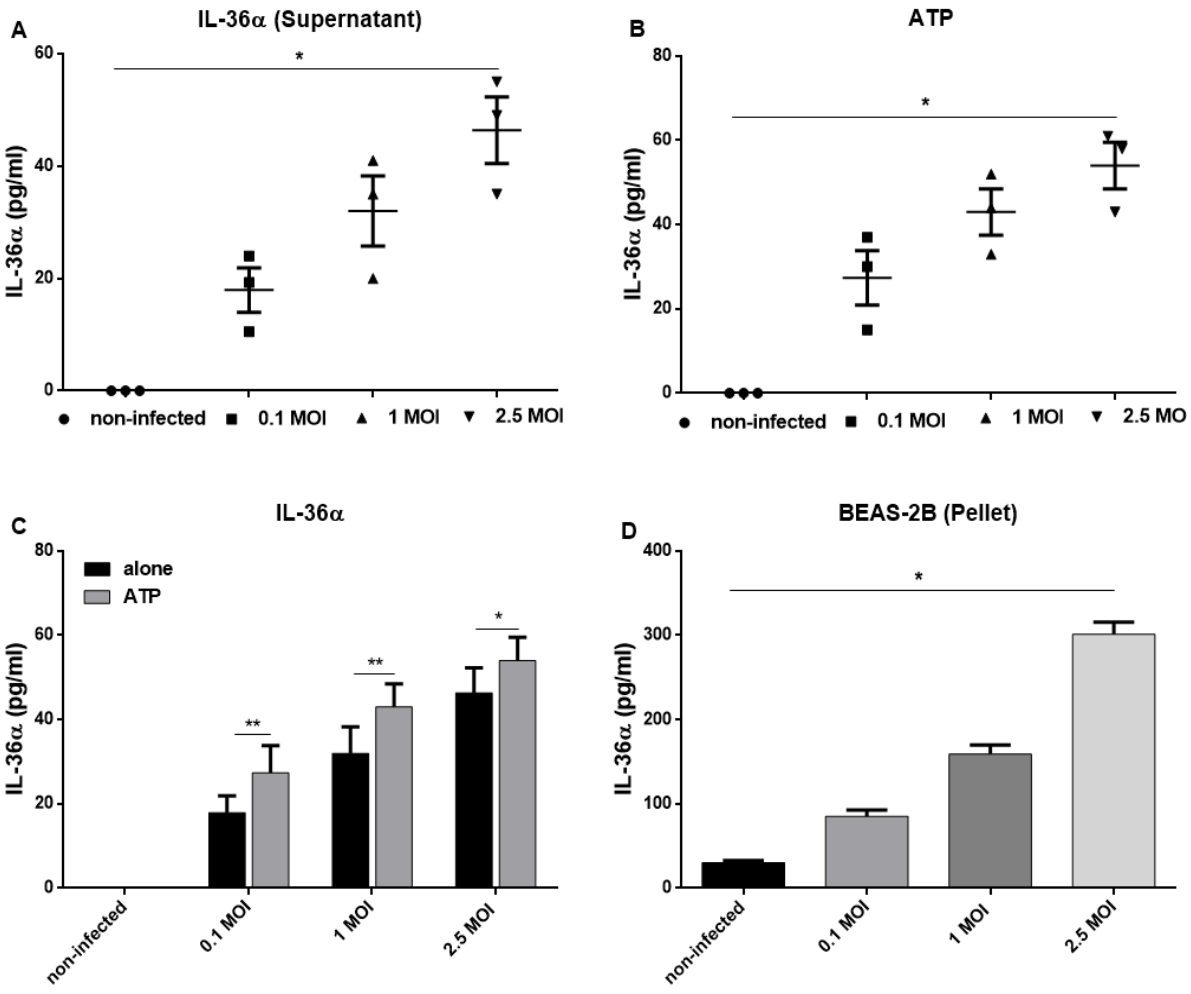

Figure 3.3.3.8b Expression of IL-36α protein in infected bronchial epithelial BEAS-2B cells. BEAS-2B cells were infected with RSV at MOIs of 0.1, 1, and 2.5 for 48 hours. Control non-infectedcultures were cultured in the absence of RSV. **A)** IL-36α protein expression in culture medium (n=3), **B)** IL-36α protein expression in culture medium with ATP (n= 3), **C)** Comparison between IL-36α protein expression in ATP treatment group and without any treatment group (n=3). Data expressed as the mean ± SEM (fold expression relative to non-infected cells, Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group and Two-way RM ANOVA test, *p<0.05 and **P<0.001).

3.3.3.9. Expression of IL-36γ protein after human RSV infection in bronchial epithelial cells (A549 and BEAS-2B)

To confirm the effect of RSV on the expression of IL-36γ protein in culture A549 cells, A549 cells were infected by A2 RSV at MOIs 0.1, 1, and 2.5 for 48 hours, and non-infected cells were used as a control.

IL-36γ protein expression in the culture medium was upregulated significantly at MOIs 1 ($p<0.05$) and 2.5 ($p<0.0001$) as compared to that seen in non-infected control A549 cells (Friedman test/Dunn's multiple comparison test) (Figure 3.3.3.9a A). The expression of IL-36γ protein was ~5 fold higher at MOI 1 and ~8 fold higher at MOI 2.5 than in non-infected cells (Figure 3.3.3.9a A).

Similarly, there was a significant increase in expression of IL-36γ protein after RSV infection plus ATP treatment at MOIs 1 ($p<0.05$) and 2.5 ($p<0.0001$) as compared with non-infected cells (Friedman test/Dunn's multiple comparison test) (Figure 3.3.3.9a B). IL-36γ protein expression was ~4.5 fold higher at MOI 1 and ~6.4 fold higher at MOI 2.5 as compared with that seen in non-infected cells (Figure 3.3.3.9a B).

As shown in Figure 3.3.3.9a C, the expression of IL-36γ protein increased significantly at MOIs 1 ($p<0.001$) and 2.5 ($p<0.0001$) in ATP treated A549 cells in comparison with the levels seen in A549 cells without such treatment (Figure 3.3.3.9a C). Overall, the results show that the treatment of A549 cells with ATP alongside RSV infection increases the expression of IL-36γ protein between ~1.1 to 1.5- times.

The apparent induction in IL-36γ protein expression following RSV infection in the supernatant was also reflected in protein expression within the in-cell pellet, with a significant increase in IL-36γ protein at MOI 2.5 ($p<0.05$) as compared to that seen in non-infected A549 cells (Figure 3.3.3.9a D). The level of IL- 36γ intracellular protein was 8-fold higher at MOI 1 and 11-fold higher at MOI 2.5 (Figure 3.3.3.9a D).

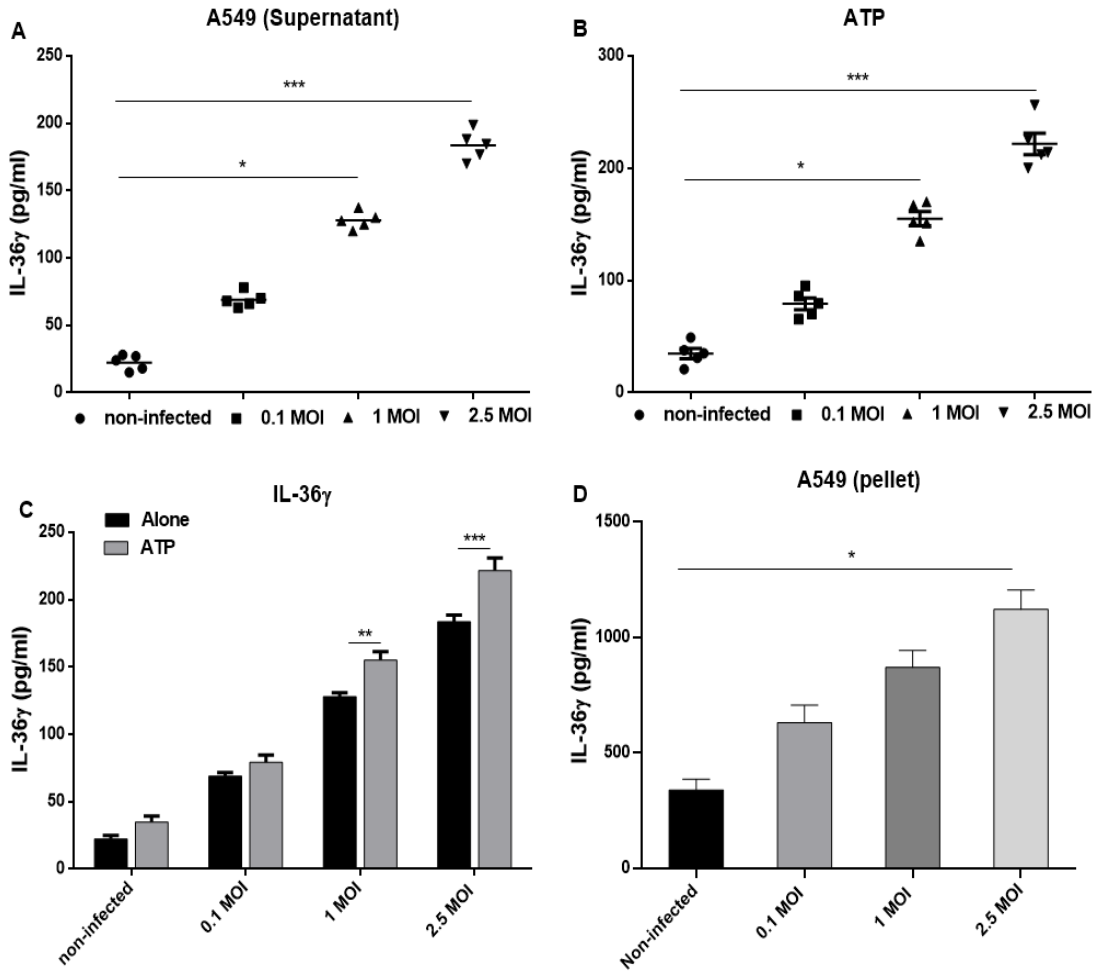

Figure 3.3.3.9a Expression of IL-36γ protein in infected bronchial epithelial A549 cells. A549 cells were infected with RSV at MOIs of 0.1, 1, and 2.5 for 48 hours. Control cultures were cultured in the absence of RSV. **A)** IL-36γ protein expression in culture medium (n=5), **B)** IL-36γ protein expression with ATP treated cells (n=5), **C)** Comparison between IL-36γ protein expression in ATP treatment group and in that without any treatment (n= 5). **D)** IL-36γ protein expression in cell pellet (n=3). Data expressed as mean ± SEM (fold expression relative to non-infected cells, Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group and Two-way RM ANOVA test, *p<0.05, **p<0.01, and ***p<0.001).

To confirm the effect of RSV on the expression of IL-36γ protein in cultured BEAS-2B cells, such cells were infected with A2 RSV at MOIs 0.1, 1, and 2.5 for 48 hrs, with equivalent non-infected cells used as a control.

IL-36γ protein expression in the culture medium was upregulated significantly at MOIs 1 ($p<0.01$) and 2.5 ($p<0.0001$) as compared to levels seen in the non-infected control BEAS-2B cells (Friedman test/Dunn's multiple comparison test) (Figure 3.3.3.9b A). The expression of IL-36γ protein was ~6 fold higher at MOI 1 and ~12 fold higher at MOI 2.5 in infected A549 as compared to non-infected cells (Figure 3.3.3.9b A).

Similarly, there was a significant increase in expression of IL-36γ protein after RSV infection plus ATP treatment at MOIs 1 ($p<0.05$) and 2.5 ($p<0.0001$) as compared with non-infected cells, based on a Friedman test or Dunn's multiple comparison test (Figure 3.3.3.9b B). IL- 36γ protein expression was ~5 fold higher at MOI 1 and ~9 fold higher at MOI 2.5 in infected BEAS-2B cells as compared with that seen in non-infected cells (Figure 3.3.3.9b B).

As shown in Figure 3.3.3.9b C, the expression of IL-36γ protein increased significantly at MOIs 0.1 ($p<0.00001$), 1 ($p<0.00001$), and 2.5 ($p<0.00001$) in ATP-treated BEAS-2B cells as compared to that seen in BEAS-2B cells without such treatment (Figure 3.3.3.9b C). Overall, the expression of IL-36γ protein was ~1.5 to 2 times higher in the ATP treatment group than in the group without such treatment.

The apparent induction of IL-36γ protein expression following RSV infection of the supernatant was also reflected in protein expression within the in-cell pellet, with a significant increase in IL-36γ protein at MOI 2.5 ($p<0.05$) as compared to levels seen in non-infected BEAS-2B cells (Figure 3.3.3.9b D). The IL-36γ intracellular protein level was seen to be 4-fold greater at MOI 1 and 7-fold greater at MOI 2.5 than in the non-infected cells (Figure 3.3.3.9b D).

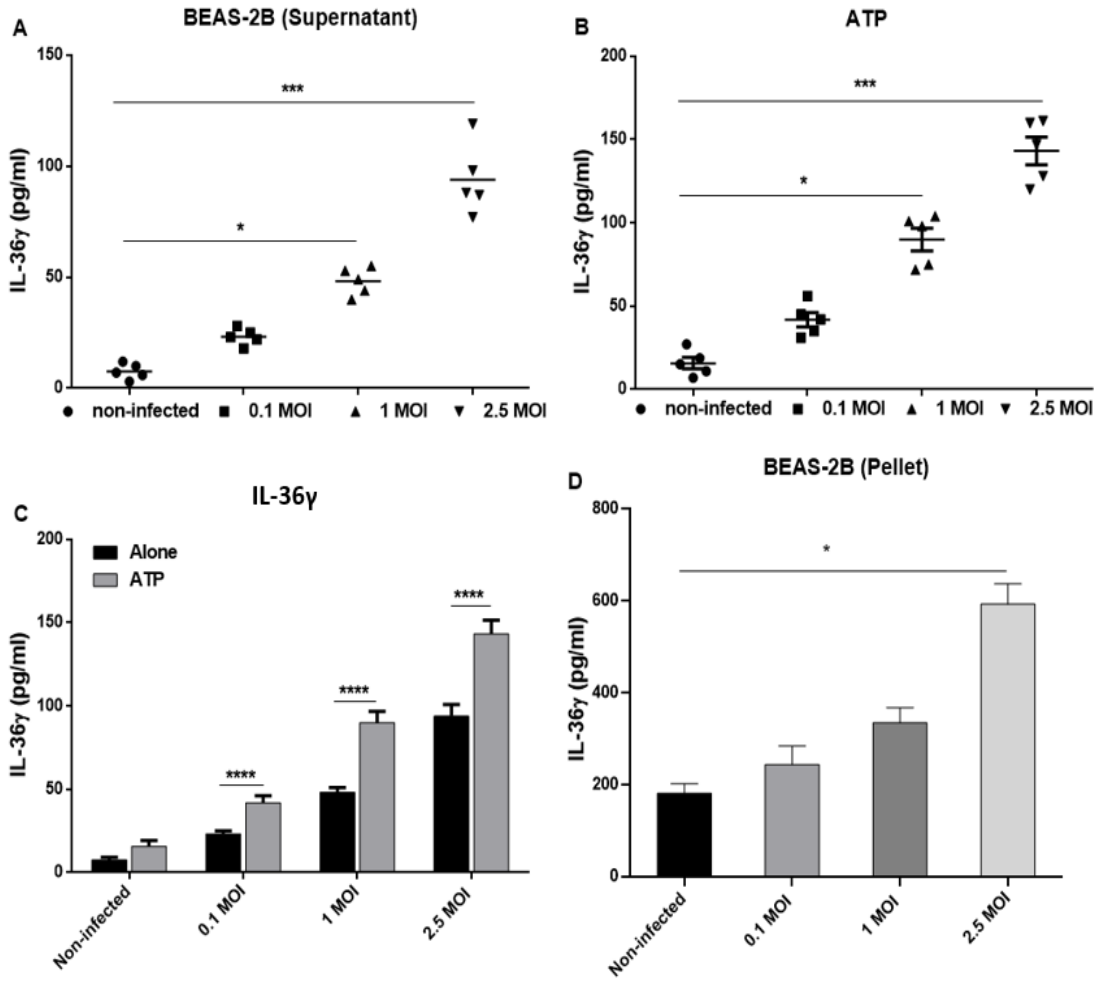

Figure 3.3.3.9b Expression of IL-36γ protein in infected bronchial epithelial BEAS-2B cells. BEAS-2B cells were infected with RSV at MOIs of 0.1, 1, and 2.5 for 48 hours. Control non-infected cultures were cultured in the absence of RSV. **A)** IL-36γ protein expression in culture medium (n=5), **B)** IL-36γ protein expression with ATP treated cells (n=5), **C)** Comparison between IL- 36γ protein expression in ATP treatment group and without any treatment (n= 5). **D)** IL-36γ protein expression in cell pellet (n=3). Data are expressed as the mean ± SEM (fold expression relative to non-infected cells, Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group and Two-way RM ANOVA test, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001).

3.4 Discussion

During infection, airway epithelial cells initiate and amplify immune responses by expressing cytokines, chemokines, and similar inflammatory mediators (Butler *et al.*, 2016). A few studies have investigated the role of IL-36 cytokines during airways infection, and, in general, IL-36 cytokines are assumed to serve as an amplifier of innate immunity as mediators of inflammation, thus potentially contributing to the activation of the adaptive immune response during immune diseases (Garlanda *et al.,* 2013). To date, however, no studies have investigated the role of IL-36 cytokines as a mediator or potentially modulator of airway epithelial cells during RSV infection. Moreover, the role and secretion mechanisms of IL-36 cytokines during infection in the lung are unknown. The main aim of this chapter was thus to focus on the role of IL-36 cytokines as proinflammatory factors and to investigate their receptors on airway epithelial cells during viral infection. To test these effects, this work investigated the expression of IL-36α and IL-36γ in NPAs from patients with RSV and RV disease.

Similarly, the role of IL-36 proteins in RSV and RV infection and their relationship to disease severity has not previously been established, and the findings of this investigation may thus be considered unique. The tests noted throughout this chapter were intended to establish the mediator and modulator roles of IL-36 cytokines cultured human airway epithelial cells (HNAECs) subject to RSV infection. Additionally, expression of IL-36α, IL-36γ, and IL-36Rα and their receptors in cultured epithelial cell lines during RSV infection was also examined. The final objective of this chapter was thus to measure the expression of IL-36α and IL-36γ proteins in HNAECs and culture cell line (A549 and BEAS-2B) cells during RSV infection and to thus determine whether the expression of these proteins was observable in the culture medium simply based on induction by RSV or whether another co-stimulatory factor was required.

3.4.1 Expression of IL-36α and IL-36γ proteins in NPAs from RSV and RV infected infants

In general, this study showed that the levels of protein expression for proinflammatory cytokines IL-36α and IL-36γ were high in both RSV and RV NPAs (Figure 3.3.1). Additionally, RV infection induced higher expression of both proteins in comparison to RSV (Figure 3.3.1 B), though the expression of IL-36α protein was higher than that of IL-36γ protein in both diseases ($p<0.05$) (Figure 3.3.1 D). In RSV NPAs, the level of IL-36α was higher than the expression of IL-36γ; however, this was not significant (Figure 3.3.1 C). These findings suggest that RV exceeds

RSV in terms of the capacity to trigger IL-36α/γ protein expression from various sources such as epithelial cells or other immune cells activated during airway infection. In addition, the results in this study indicate that levels of IL-36α protein are higher than those of IL-36γ protein in response to both RV and RSV, suggesting that there may be many different sources of IL-36α protein.

Although the levels of IL-36α protein were high in NPAs for RSV and RV patients, the results demonstrated no effect of age on these levels. The changes in levels of IL-36α protein in RSV ($p=0.3$) and RV ($p=0.09$) patients between age groups were in significant (Figures 3.3.1.1 A and B). Similarly, IL-36γ protein expression showed no significant differences in level between age groups in RSV ($p= 0.921$) and RV ($p= 0.656$) patients (Figures 3.3.1.1 C and D).

In contrast, there was a significant difference in the level of IL-36γ, though not IL-36α, protein expression levels between the severity groups in RSV patients. In the RSV cohort, the level of IL-36γ was increased significantly in the moderate group in comparison to that seen in the mild ($p<0.05$) and severe groups ($p<0.001$) (Figure 3.3.1.2). However, no significant differences in the levels of IL-36γ protein were seen between severity groups in RV patients. The Kruskal-Wallis test and Dunn's multiple comparison test also showed no significant difference in the levels of IL-36α protein expression across severity groups in RSV patient samples ($p= 0.188$) or among patients with different severity of RV disease ($p= 0.902$). The findings suggest that the level of IL-36α protein in NPAs does not correlate with disease severity.

The severe segment of the patient population was divided into two groups, though this sample was small in number. Again, no significant differences in IL-36α protein levels were observed concerning oxygen requirements in RSV patients ($p= 0.982$) or RV patients ($p= 0.798$). The IL-36α protein level thus did not change with oxygen requirement. A similar procedure produced the same result for IL-36γ protein levels in relation to oxygen requirements in RSV patients ($p= 0.311$) and RV patients ($p= 0.877$) (Figure 3.3.1.3).

The relationship between the patterns of expression of IL-36α and IL-36γ in RSV and RV patient samples were compared, and no positive correlations between cytokine levels and RSV NPAs were observed. In addition, no positive correlation coefficient was observed between IL-36α and IL-36γ in RV patient samples ($r= 0.5$) (Figure 3.3.1.4). This supports the fact reported in the literature that IL-36α and IL-36γ cytokines are highly structurally and functionally related.

Overall, the expression of both IL-36α/γ proteins may be involved in the immune response against viral respiratory infection based on inducing inflammation; alternatively, they may trigger other inflammatory cytokines and chemokines which could eventually limit the spread of these viruses.

3.4.2 Cultured HNAECs and cell lines (A549 and BEAS-2B) are a sources of IL-36 subfamily cytokines and are able to express these cytokines and their receptors in response to the RSV infection.

Successful *in vitro* RSV infection and replication in HNAECs was achieved as demonstrated by measuring RSV N gene RNA levels; these increased significantly at MOIs 1 ($p<0.001$) and 2.5 ($p<0.0001$). Palivizumab was used as a control measure, resulting in a significant decrease in expression as compared to RSV MOI 2.5 and MOI 2.5 plus Palivizumab ($p<0.01$). Similarly, IL-8 gene expression was measured *in vitro* RSV infection and replication in HNAECs. The IL-8 mRNA expression of individual donors increased with the increasing amount of RSV at MOIs 2.5 ($p<0.0001$), while the Palivizumab plus MOI 2.5 control significantly decreased the expression of IL-8 mRNA ($p<0.001$) (Figure 3.3.2.1 and 2).

Upon infecting HNAECs with increased concentrations of RSV (MOI 0.1, 1, and 2.5) for 48 hours, the expression of IL-36α mRNA expression was found to be significantly increased at MOIs 2.5 ($p<0.01$) in comparison to non-infected HNAECs. Similarly, IL-36γ and IL-36Rα mRNA expression were significantly increased at MOIs 2.5 ($p<0.01$) and ($p<0.05$) respectively, in comparison to that observed in non-infected HNAECs (Figure 3.3.2.3, 4 and 5). However, no effects of RSV were observed on the expression of IL-36 receptors' mRNA (IL-1RL2 and IL-1RAP) in HNAECs (Figure 3.3.2.6). Statistical analysis thus suggests that HNAECs are not a source of IL-1RL2 during RSV infection.

Regarding to the culture cell line (A549 and BEAS-2B) cells, viral expression and replication of RSV were achieved successfully in A549 and BEAS-2B cells at 0.1 MOI ($p<0.01$ and $p<0.05$, respectively), 1 MOI ($p<0.01$ in both cell lines), and 2.5 MOI ($p<0.001$ and $p<0.01$, respectively) (Figure 3.3.3.1 and 2). IL-8 gene expression was measured in the A549 and BEAS-2B cells responded to the challenge. IL-8 mRNA expression was upregulated with an increasing dose of RSV in A549 and BEAS-2B cells at 0.1 MOI ($p<0.05$), 1 MOI ($p<0.05$) A549 and ($p<0.01$) in BEAS-

2B, and 2.5 MOI ($p<0.01$) in both cell lines.

The results indicated significant differences between the IL-36α mRNA expression observed in MOI 2.5 infected A549 and BEAS-2B cells and that in non-infected cells ($p<0.05$). Two-way RM ANOVA test showed that IL-36α mRNA was significantly increased in A549 cells at 24hr and 48hr ($p<0.0001$) as compared to the levels in non-infected cells. These results indicate that bronchial epithelial cell IL-36α mRNA expression increases in response to RSV infection. In infected A549 and BEAS-2B cells, there was also a significant difference in IL-36γ mRNA expression between MOI 2.5 infected and non-infected cells ($p<0.01$) (Figure 3.3.3.3 and 4).

Regarding to the expression of IL-1RL2 mRNA in culture cell line (A549 and BEAS-2B) cells, IL-1RL2 mRNA expression was upregulated in BEAS-2B cells with an increasing dose of RSV in BEAS-2B cells at 0.1 MOI ($p<0.05$), 1 MOI ($p<0.05$) while there was no significant increase were observed in A549 cells (Figure 3.3.3.6). These different results suggest that because the source of A549 cell line are drieved from lung carcinoma tissue while BEAS2-B cell line were derived from human bronchial epithelium transformed by a hybrid adenovirus 12-SV40.

3.4.3 Can RSV induce the expression of IL-36α and IL-36γ proteins in infected HNAEC and cell line (A549 and BEAS-2B) cells?

According to the results of this study with respect to the expression of IL-36α and IL-36γ proteins in RSV and RV NPAs (section 3.3.1), high levels of these proteins were observed. In addition, the expressions of IL-36α and IL-36γ mRNA in cultured HNAECs and cell line (A549 and BEAS-2B) cells were upregulated significantly during RSV infection. The expressions of IL-36α and IL-36γ proteins in culture medium after RSV infection were assessed using a dedicated ELISA, prepared according to the outline in Kovach *et al.* (2016). However, the level of IL-36γ protein remained under the level of detection. Attempts were then made to detect the intracellular IL-36α/γ protein using a western blot test, which also offered no detection of this protein (data not shown). As shown above, although the expression of IL-36γ mRNA in particular was significantly higher in infected HNAECs and cell lines, but there was no protein can detected in the same cells. Therefore, the cell pellet were examined in infected HNAECs and cell lines which showed a significant increase in the intracellular of IL-36α/γ proteins in both cells (more details in section 3.4.4). This results suggest that IL-36 protein were expressed

in response to RSV infection but the mechanism of releasing them from the cells is unknown. According to Kovach *et al.* (2016) finding which suggest that, IL-36γ may be secreted in microparticles, which could prevent the actual measurement of IL-36γ protein level. A further attempt as thus made to measure the expression of IL-36γ protein by sonicating the culture medium of both the HNAECs and cell lines. Culture medium were collected at 48 hours after RSV infection, and centrifuged at 1,500 g for 30 minutes to remove apoptotic bodies and cellular debris, followed by ultracentrifugation at 25,000 g for 30 minutes to retrieve microparticles. Then, 3 times sonication for 10 seconds of culture medium of infected HNAECs and cell lines to breakdown the membrane and release the proteins from microparticles

The expression of IL-36α/γ protein were measured by sonicate culture medium of infected HNAECs and cell line. The expression of IL-36α/γ proteins in infected HNAECs were then seen to increase significantly at MOIs 1 ($p<0.05$) and 2.5 ($p<0.0001$) ($p<0.01$) (Figure 3.3.2.7 and 8), respectively in comparison to non-infected cells. Similarly, in A549 and BEAS-2B cell which show a significant increase of IL-36α/γ protein expression at MOI ($p<0.05$) and at MOI 2.5 ($p<0.001$) in comparison to non-infected cells (Figure 3.3.3.8 and 9).

Kovach *et al.* (2016) similarly demonstrated that the detection of extracellular IL-36γ protein was significantly greater after sonication of conditioned medium for KP and SP- infected pulmonary macrophages (PMs) in comparison to non-sonicated samples. This result thus suggests that a significant portion of secreted IL-36γ protein is packaged within membrane-bound vesicles and detected only by ultracentrifugation. Several further studies have also confirmed that the IL-36α protein is secreted in microparticles: Aoyagi *et al.* (2016) confirmed that IL-36α was induced in microparticles from airway epithelial cells and BAL fluid after influenza virus administration, while influenza virus infection in mice results in upregulation of IL-36α protein in the form of microparticles released by alveolar epithelial cells (Kovach *et al.,* 2016).

3.4.4 Is RSV infection able to induce the production of IL-36α and IL-36γ proteins extracellularly or need a second induction?

Many attempts to detect IL-36γ protein in culture media have produced detectable levels after sonication of the culture medium; however, the levels of the protein have not commonly

reflected the levels seen during RSV infection. This study thus focused on the expression of IL-36γ protein in infected cultures based on HNAECs and cell lines (A549 and BEAS-2B) as accumulated intracellularly in cell pellets after RSV infection, as shown in Figure 3.3.2.7 D and Figure 3.3.2.8 D. The levels of intracellular IL-36α protein observed in cell pellets suggested that there was a significant increase in IL-36α protein level at MOI 2.5 ($p<0.05$) in cultured HNAECs, A549, and BEAS-2B cells. Similarly the level of intracellular IL-36γ protein was upregulated significantly at MOI 1 ($p<0.05$) and at MOI 2.5 ($p<0.001$) in HNAECs in comparison to that seen in non-infected cells. Infected A459 and BEAS-2B cells also showed increases in intracellular IL-36γ protein that were significant for MOI 2.5 ($p<0.05$) in comparison to the results for non-infected cells.

As the levels of intracellular IL-36α and IL-36γ proteins in RSV infected HNAECs were significantly higher in comparison to those observed in equivalent non-infected cells, the results of this study suggest that the secretion of IL-36α/γ proteins requires some co-stimulation to be seen in the culture medium. In Martin *et al.* (2009), ATP was reported to play a critical role on the secretion of IL-36 cytokines, with the release of IL-36α achieved by treatment with LPS followed by ATP in bone marrow-derived macrophages (BMDMs). In addition, secretion of IL-36γ protein was enhanced by ATP from marine and human lung macrophages after bacterial infection. They also found that extracellular IL-36γ protein detection was markedly enhanced by sonication to disrupt membrane bound structures (Kovach *et al.,* 2016). In Liu *et al.,* (2020) study they found the influenza infected A549 cells were secrete a significant IL-36γ protein after treated with ATP for 2 hours (Liu *et al.,* 2020). This study thus used ATP on the culture cells to promote second signalling after to RSV infection in an attempt to trigger the release of IL-36α and IL-36γ proteins in the culture media of the HNAECs and cell line (A549 and BEAS-2B) cells.

The explanation of that may be because ATP-stimulated secretion is mediated by the P2X7R (Ferrari *et al.,* 1997; Ferrari *et al.,* 2006). ATP/ P2X7R signaling has been shown to induce plasma membrane blebbing and shedding of membrane components in MPs (Dubyak *et al.,* 2012; Verhoef *et al.,* 2003) and also promote the inflammatory facilitates inflammatory responses. Pro-IL-1β, which also lacks a secretory signal sequence, is secreted in response to ATP and is packaged in MP complexed with caspase-1, which is necessary for posttranslational processing of IL-1β (MacKenzie *et al.,* 2001). P2X7R is ion channel that present in nearly all tissues and organs (He *et al.*, 2013), with high expression shown in the immune cell types. Upon P2X7R

activated by extracellular ATP (Khadra *et al*., 2013) allow the Efflux of K+ and influx of Na^+ and Ca^{2+} through the P2X7R channel and also form a non-selective pore that allows movement of molecules up to ~900 Da through the cell membrane (Kopp *et al.,* 2019; Khadra *et al*., 2013). These findings suggest that IL-36 cytokines secretion could be occur by blebbing the microparticles which need another signal like ATP/P2X7R to release IL-36 proteins, still the mechanism of IL-36 protein is complex and poorly understood.

The results showed a significant increase in the expression of IL-36α protein in infected HNAECs after ATP treatment at MOIs 1 and 2.5 ($p<0.01$) as compared with that seen in non-infected cells. Neutralising the virus before challenge using Palivizumab treatment resulted in significantly decreased expression of IL-36α protein compared to that seen in standard infection at MOI 2.5 ($p<0. 05$), however. Using ATP in infected culture HNAECs resulted a significant higher level of IL-36α protein expression in comparison to that seen in equivalent infected cells without the addition of ATP at MOIs 1 and 2.5 ($p<0.01$) (Figure 3.3.2.7 and 8).

To confirm the impact of ATP on the secretion of IL-36α/γ proteins non-infected and infected HNAECs and cell lines were treated with ATP. There was a significant increase on the IL-36α protein in HNAECs at MOI 1 and 2.5 ($p<0.01$), whereas in infected A549 at MOI ($p<0.05$) and at MOI 1($p<0.05$), MOI 2.5 ($p<0.001$) in infected BEAS-2B cells in comparison to ATP treat non-infected cells. Similarly, there was a significant upregulate in IL-36γ protein from HNAECs at MOI 1 ($p<0.05$) and at MOI 2.5 ($p<0.01$), and in infected A549 cells at MOI 2.5 ($p<0.05$), whereas in infected BEAS-2B cells at MOI 1 ($p<0.05$) and 2.5 ($p<0.001$) in comparison to ATP treat non-infected cells (Figure 3.3.3.8 and 9).

To further confirm that ATP stimulation could be used as a second signal to release IL-36α/γ proteins *in vitro* from infected HNAECs and cell lines. There was a significant increase of IL-36α protein from infected HNAECs at MOI 1 and MOI 2.5 ($p<0.01$) and at MOI 0.1, 1 2.5 ($p<0.0001$) in A549 cells, whereas in BEAS-2B cells ($p<0.01$) at MOI 0.1, 1 and MOI 2.5 ($p<0.05$) in comparison to the same infected cells without ATP stimulation. Similarly, there was a significant upregulate in IL-36γ protein from HNAECs at MOI 0.1 and 2.5 ($p<0.001$) and at MOI 1 ($p<0.01$), and in infected A549 cells at MOI 1 ($p<0.01$) and MOI 2.5 ($p<0.001$), whereas in infected BEAS-2B cells at MOI 0.1, 1 and 2.5 ($p<0.0001$) in comparison to the same infected cells without ATP stimulation (Figure 3.3.2.7 and 8) and (Figure 3.3.3.8 and 9).

These results are consistent with previous studies demonstrating that IL-36α is induced in and secreted from alveolar epithelial cells during influenza virus infection (Aoyagi *et al.,* 2017). Moreover, Qin *et al.* (2019) found that IL-36α mRNA and protein in eosinophils in allergic rhinitis (AR) were both significantly upregulated as compared with levels seen in normal controls, especially in cases of AR with asthma. In agreement with this data, IL-36α gene expression was significantly and specifically elevated among ulcerative colitis (UC) patients compared with control patients in Russell *et al.* (2016). The current results are also in line with others, showing that IL-36α mRNA levels reach a peak 48 hours after influenza administration (Aoyagi, 2017). In contrast, however, Kovach *et al.* (2017) reported no induction observed in IL-36α mRNA in the whole lung after Streptococcus pneumoniae (SP), Klebsiella pneumoniae (KP), and bacterial Flagellin infection (Kovach e*t al.,* 2017). These findings suggests that IL-36α may play a potential role in the inflammatory response during RSV infection, but not in all related infections.

Multiple clinical studies have also reported that IL-36α and IL-36γ mRNA levels are significantly elevated in patients with allergic rhinitis (AR) as compared to those seen in normal controls (Qin *et al.,* 2019). Chu *et al.* (2015) also reported that IL-36α and IL-36γ levels in plasma concentrations were significantly higher in patients with active systemic lupus erythematosus (SLE) than in healthy controls, a finding confirmed by a later study (Mai *et al.,* 2018).

Collectively, these findings indicate that IL-36α and IL-36γ may be good biomarkers of, and contribute to, pathogenic immune response. Although several studies have confirmed the impact of respiratory infection viruses, few have specifically explored the role of IL-36 cytokines in immune response in infants with RSV bronchiolitis. Overall, these results thus indicate that airway epithelial cells are able to express the IL-36 subfamily cytokines, while IL-36γ in particular occurs in response to RSV infection. However, the trigger for the expression of IL-36α and IL-36γ proteins remains unknown, and may involve multiple sources of stimulation, further supporting the aggressive inflammatory role of these cytokines in initiating inflammation that may alarm other proinflammatory cytokines and activate other immune cells.

3.5 Summary

There were high levels of IL-36 α and IL-36γ proteins detected in the NPAs from children infected with RSV and RV, which suggests that these proteins could mediate these respiratory viral diseases. In addition, the levels of these proteins induced in response to RV where higher than those in RSV NPA samples. There was no significant difference in the levels of IL-36α between age groups across both RSV and RV patients, and there were no positive correlations between protein levels in either. There was a significant difference in the level of IL-36γ protein expression between the severity groups of RSV patients, which suggests that RSV disease induces moderated symptoms in general between children. A positive but weak correlation was also observed between IL-36α and IL-36γ in RV patients.

IL-36α, IL-36γ, and IL-36Rα mRNA were all upregulated significantly in RSV-infected cultured HNAECs and A594 and BEAS-2B cells, though the expression of IL-36γ mRNA was the highest among all IL-36 subfamily cytokines. HNAECs, A549, and BEAS-2B cells are not the source of IL-36 receptors (IL-1RL2 and IL-1RAP) during RSV infection, however, though IL-36α protein expression in the culture medium was upregulated significantly in infected A549 and BEAS-2B cells.

RSV may induce the expression of IL-36α and IL-36γ proteins; however, such expression accumulates intracellularly. As the mechanism triggering the release of these proteins is unknown, many attempts were made in this study to uncover its effects. Sonication of the culture medium allowed detection of IL-36α/γ proteins, which suggests that these proteins are released in microparticles in a way that could reduce the actual level of proteins released in the culture medium. This study thus suggests that IL-36α/γ proteins could act as aggressive proinflammatory cytokines, but that they need special conditions in order to be released and activated. This study also suggests that the expression of IL-36α/γ proteins requires the co-stimuli of ATP within the RSV infected culture cells, as this offered a second means of signalling the trigger for the cultured cells and released IL-36α and IL-36γ proteins in the culture medium for both HNAECs and cell line (A549 and BEAS-2B) cells.

Chapter 4. Induction of the IL-37 cytokine and IL-37 receptors by RSV infection in nasopharyngeal aspirate, human nasal epithelial cells and cultured airways epithelial cell lines

4.1 Introduction

Airway epithelial cells response at the site of infection and could have a pivotal role in regulating immune responses. These epithelial cells are responsible for detecting viral pathogens and then release cytokines and chemokines, therefore their response to infection has an important role in determining disease outcomes. This immune response consists of the expression of numerous of inflammatory and antiviral genes that stimulates subsequent innate and adaptive immunity. It has been found that a significant elevated plasma IL-37 in patients COVID-19, suppressing inflammatory responses and restraining the occurrence of cytokine storms (Li *et al.,* 2021). As described in Chapter 1, IL-37 has been identified as an anti-inflammatory cytokine that can inhibit the innate and adaptive immune response during inflammation by suppressing the excessive inflammatory cytokines production (section 1.4.2) (Alanazi *et al.,* 2019). Emerging evidence demonstrate that IL-37 is expressed at low levels in human cells and tissues but upregulated by inflammatory stimuli. Despite multiple emerging studies on the role of IL-37 as anti-inflammatory cytokine and its role in limiting the immune response in different inflammatory disease, no studies have investigated the role of IL-37 in airways during RSV infection. The work described in this chapter focusses on the ability of epithelial cells to expressed IL-37 and IL-37 receptors (IL-18R1 and IL-1R8) in response to RSV infection. Also, this work aims to analyse the expression of IL-37 isoforms by airways epithelial cells during RSV infection. To then further understand how the airway epithelium in response to RSV infection may express and activate IL-37 protein by investigating the localization of IL-37 protein in BEAS-2B cells.

Hypothesis: The inhibitory cytokine IL-37 and IL-37 receptors may highly expressed by airway epithelial cells in response to RSV infection.

4.2 Objectives

1) Investigate if IL-37 is present during RSV or RV disease by measuring expression in nasopharyngeal aspirate samples from infants.
2) Ascertain whether RSV infection of human nasal and airway epithelial cells (A549 and BEAS-2B) results in the release of IL-37 by measuring mRNA and protein expression following *in vitro* infection of HNAECs.
3) Determine if RSV infection of BEAS-2B cultured airway epithelial cells results in the release of IL-37 isoforms (IL-37, IL-37a, b and c) by measuring mRNA expression following *in vitro* infection of BEAS-2B cell lines.
4) Examine human nasal epithelial cells for the presence of IL-37 receptors (IL-1R8 and IL-18R1), and test whether airway epithelial cells (A549 and BEAS-2B) could respond to IL-37 cytokine.
5) Identify the localisation of intracellular IL-37 protein during RSV infection.

4.3 Results

4.3.1. Detection of IL-37 protein in nasopharyngeal aspirate samples from children with RSV and RV infection

4.3.1.1 The relation between age and expression of IL-37 protein in RSV and RV patient samples

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4.3.4 Immunofluorescent localization of IL-37 protein in airways epithelial cell lines (BEAS-2B cells)

4.3.5 Immunofluorescent localization of IL-37 protein in RSV infected airways epithelial cell lines (BEAS-2B cells)

4.3.1 Detection of IL-37 protein in nasopharyngeal aspirate samples from children with RSV and RV infection

The first aim of the work described in this chapter was to measure expression of IL-37 in nasopharyngeal aspirate samples (NPAs) from RSV infected infants and from RV infected children. NPAs samples were collected as routine sample for viral infection diagnosis and the remaining sample material subsequently made available for use in this study.

A total of 163 NPAs from RSV (n=90) and RV (n=73) infected infants were collected, using ELISA to measure the concentration of IL-37 protein. Section (2.7) details the selection criteria for the NPAs used, they were divided into two groups according to viral disease. Group 1: patients with RSV infection (n= 90, range: 0 to 4691pg/ml) and group 2: patients with rhinovirus (n= 68, range: 0 to 2679 pg/ml). Protein expression was compared by:

1) Patient age by group, less than one-month-old (<1M), one to three months old (1-3M), three months to nine months old (3M-9M), and more than 9 months.

2) Patient disease severity: mild (no oxygen required), moderate (oxygen required), and severe (oxygen and stay in paediatric critical care unit).

3) Whether patients required oxygen. No protein values followed a normal distribution.

In RSV NPAs, the mean of IL-37 protein level in RSV patient samples was 282 pg/ml, median= 131 pg/ml, range 0- 4691 pg/ml (n=90), whereas the mean value of IL-37 protein level in RV patient samples was 707 pg/ml, median= 365 pg/ml, range from 0- 6120 pg/ml (n=73), $p< 0.001$. The average measure of IL-37 protein in NPAs of patients with RV infections was substantially greater than those with RSV (Mann Whitney test-unpairs signed rank test, $p< 0.001$) (Figure 4.3.1).

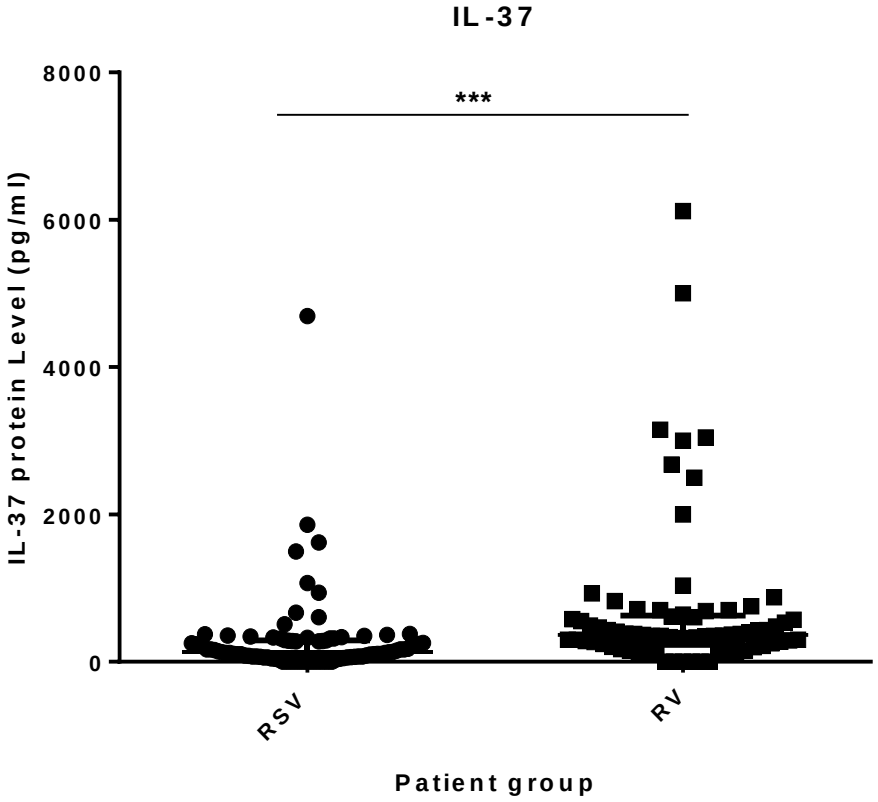

Figure 4.3.1 IL-37 protein expression in nasopharyngeal aspirate samples (NPAs) of RSV and RV patients. All samples measured the concentration of IL-37 protein by ELISA. NPAs were divided into two groups according to virus disease. Group 1: patients with RSV infection (n= 90, Median= 131 pg/ ml) and group 2: patients with rhinovirus (n= 73, Median= 365 pg/ml). Data expressed in the graph as the median with interquartile range, (Mann Whitney test-unpairs signed rank test, *** p< 0.001).

4.3.1.1 The relationship between age and expression of IL-37 protein in RSV and RV patient samples

A test to determine if age affects the production of IL-37 protein utilised ELISA to measure the levels of ninety RSV and seventy-three RV patient samples divided into four age groups:

less than one month (RSV, n= 20, Median= 113 pg/ml; RV, n= 14, Median= 316 pg/ml), between one to three months (RSV, n= 38, Median= 188 pg/ml; RV, n=19, Median= 289 pg/ml), between three months to nine months (RSV, n= 18, Median= 210 pg/ml; RV, n=22, Median= 388 pg/ml) and above of nine months (RSV, n= 14, Median= 76 pg/ml; RV, n=18, Median= 234 pg/ml). As shown in (Figure. 4.3.1.1 A, B), no significant difference in IL-37 protein expression was observed between age groups in RSV (p= 0.4) and RV (p=0.1) (Kruskal-Wallis test). These results indicate no association between age groups and IL-37 protein level in NPAs.

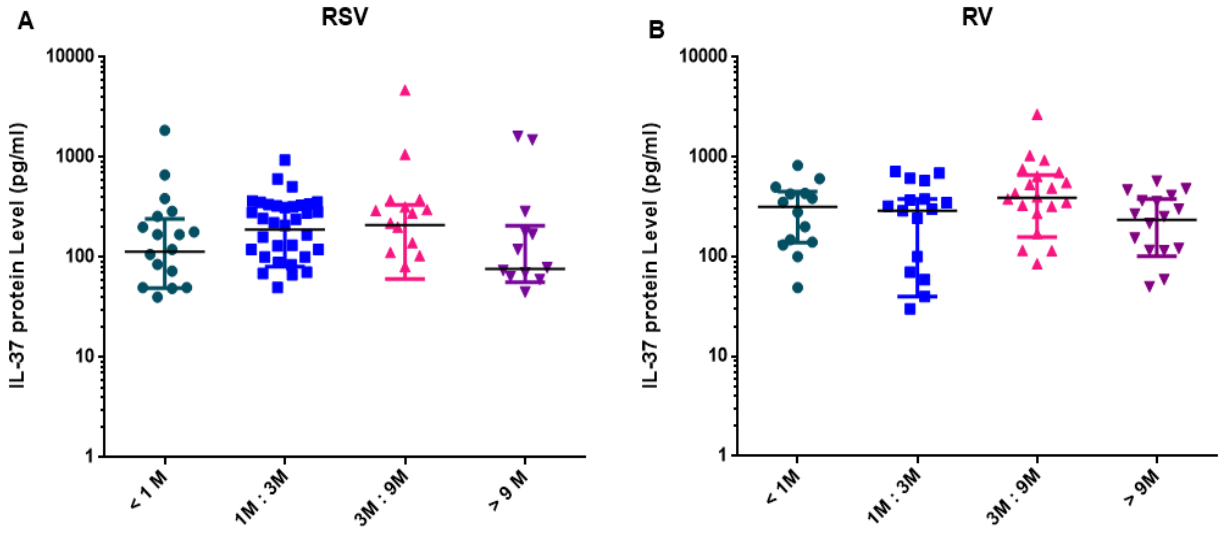

Figure.4.3.1.1. The relationship between age groups and expression of IL-37 protein in RSV and RV patient samples. Concentrations of IL-37 in **A)** RSV patients (n= 90) and **B)** RV patients (n= 73), patients in four groups of age. Results are presented as scatter plot with mean ± SEM, based on Kruskal-Wallis test: less than 1 month (n = 20, 14), patient who between 1 to 3 Months (n= 38, 19), between 3 months to 9 months (n= 18, 22) and above of 9 months (n = 14, 18) respectively. Dunn's multiple comparisons test between to compare the mean rank of group with the mean rank of every other group.

4.3.1.2 The relationship between severity and expression of IL-37 protein in RSV and RV patient samples

A study of NPAs with RSV infection showed that the level of IL-37 in the severe group was considerably lower than that of the mild and moderate groups ($p<0.05$).

The median value of IL-37 in mild group was (median= 169 pg /ml, n= 32), (median= 209 pg /ml, n= 43) in moderate group and (median= 60 pg /ml, n= 15) in severe group of RSV patient samples (Figure 4.3.1.2 A). However, no significant differences in the level of IL-37 protein were seen between severity groups in RV patient samples. The median value of IL-37 in mild group was (median= 328 pg /ml, n= 45), (median= 110 pg /ml, n= 16) in moderate group and (median= 185 pg /ml, n= 12) in severe group of RV patient samples (Figure 4.3.1.2 B) (Kruskal-Wallis test/ Dunn's multiple comparisons test, p= 0.3). These results indicate that the level of IL-37 protein in NPAs does not correlate with RV disease severity.

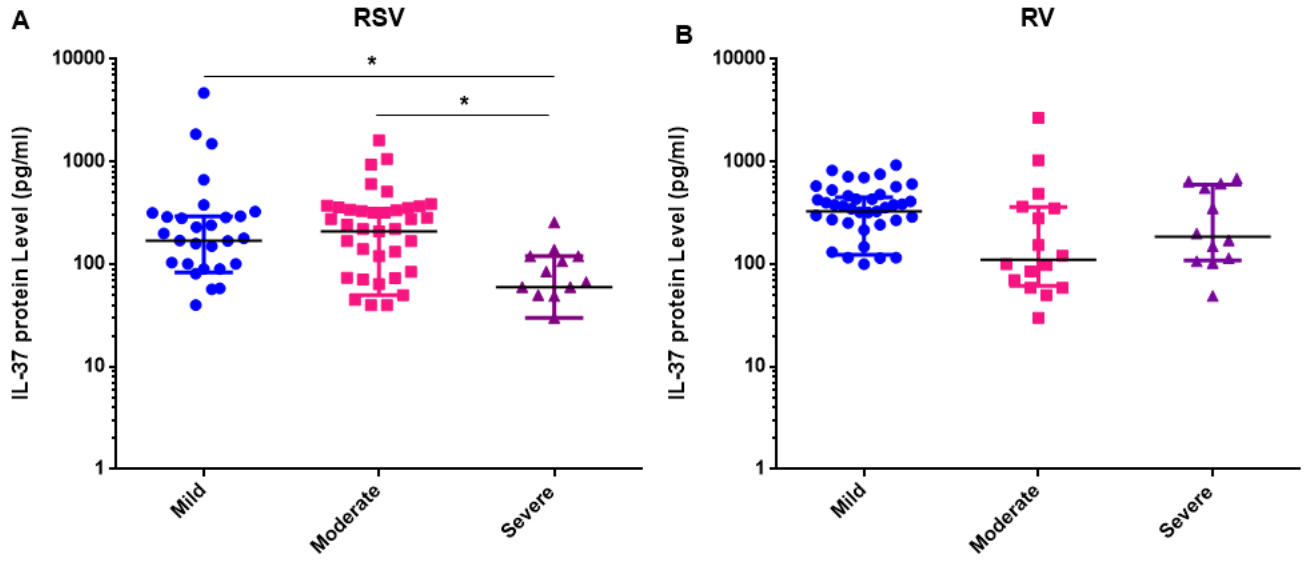

Figure 4.3.1.2. The relationship between severity and expression of IL-37 protein expression between RSV and RV patients. Concentrations of IL-37 in **A)** RSV patients (n= 90), and **B)** RV patients (n= 73), from mild patients (n = 32, 45), moderate patient (n= 43, 16) and severe patients (n= 15, 12) respectively. Results presented as scatter plot, median with interquartile range, statistical test were use Kruskal-Wallis test between three groups of severity and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group, *P<0.05)

4.3.1.3 The relationship between oxygen and expression of IL-37 protein in RSV and RV patient samples

To investigate whether IL-37 concentrations differ in children who receive and do not receive oxygen supplementation, NPAs with RSV and RV infection were measured by ELISA.

As shown in Figure. 4.3.1.3 A, in RSV patient's samples (n= 90), there was no significant difference observed in the level of IL-37 in relation to oxygen requirement (median= 120 pg/ml, n= 59) in comparison to the samples without oxygen requirement (median= 170 pg/ml, n= 31) (Mann Whitney test, p= 0.4). Similarly, in RV patient samples (n= 73), there was no significant difference observed in the level of IL-37 in relation to oxygen requirement (median= 162 pg/ml, n= 28) in comparison to the samples without oxygen requirement (median= 320 pg/ml, n= 45) (Mann Whitney test, p= 0.5) (Figure. 4.3.1.3 B). These results indicate that the IL-37 protein level did not change significantly with oxygen supplementation.

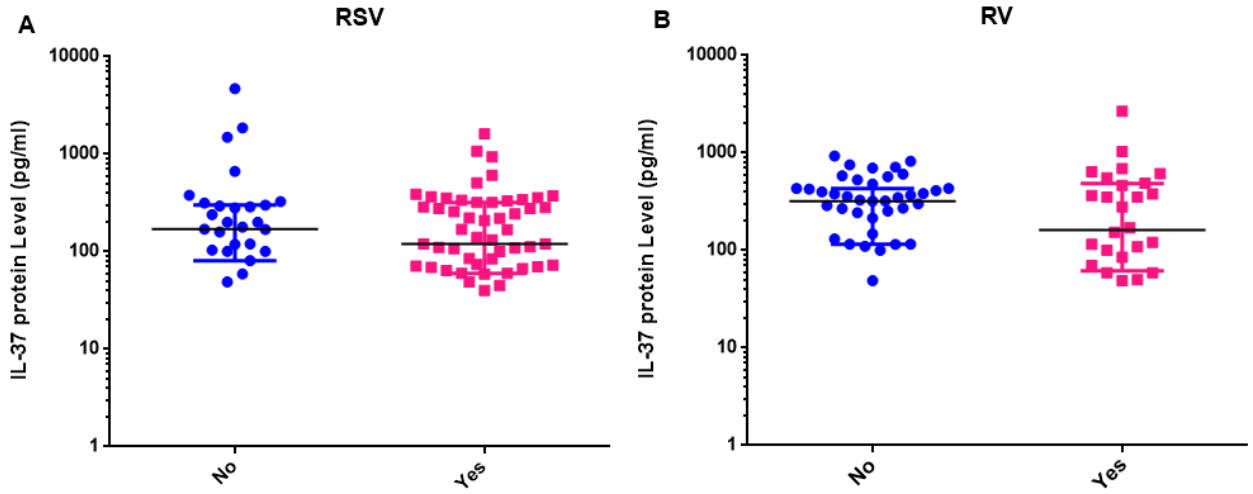

Figure 4.3.1.3. The relationship between oxygen supplement and expression of IL-37 in RSV and RV patients. Concentrations of IL-37 in **A)** RSV patients (n= 90), and **B)** RV patients (n= 73), from patients who supplied by oxygen (n = 59, 28), comparing with patients without oxygen supplement (n = 31, 45) respectively. Results presented as scatter plot with median with interquartile range using the Mann-Whitney t-test for two unpaired nonparametric test).

4.3.1.4 The relationship between expression of IL-36α and IL-37 protein in RSV and RV patient samples

To determine if there is any correlation between the observed in IL-36α and IL-37 protein level in each patients of RSV or RV patient samples. NPAs from 83 RSV patients and 70 RV patients were measured by ELISA. Results are shown in Figure (4.3.1.4) that there were no correlations between protein levels detected in RSV and RV NPAs samples (Spearman's rank order analysis, P= 0.3, 0.8; r=-0.09, r=-0.1) respectively, (Figure. 4.3.1.4 A, B).

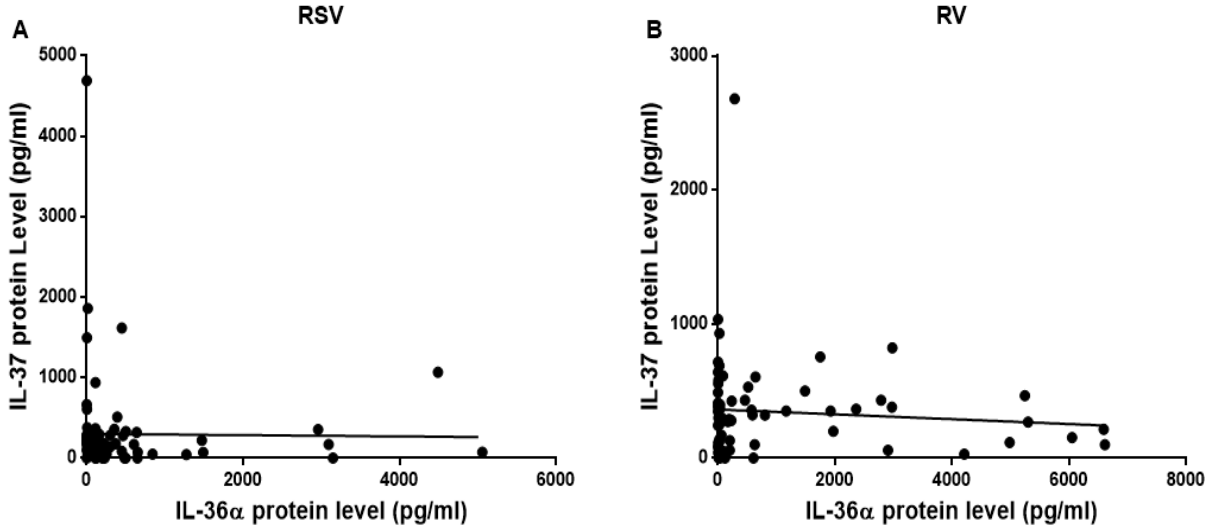

Figure 4.3.1.4 Correlations between protein levels of IL-36α against IL-37 in RSV NPAs from children. A) Correlation between IL-36α and IL-37 in RSV patients (n= 83), **B)** correlation between IL-36α and IL-37 in RV patients (n= 70). Correlations were presented by Spearman's rank order analysis, P two-tailed, n = number of XY pairs).

4.3.1.5 The relationship between expression of IL-36γ and IL-37 protein in RSV and RV patient samples

NPAs from eighty-three RSV-infected children and seventy-two RV-infected children were measured by ELISA to ascertain any connection between the IL-36γ and IL-37 protein levels observed in patient samples.

The results shown in Figure 4.3.1.5 reveal that there was no a positive correlation between IL-36γ and IL-37 protein levels detected in both RSV NPAs (Spearman's rank-order analysis, P< 0.05, r= 0.2) (Figure. 4.3.1.5 A) and the RV patient samples (Spearman's rank-order analysis, P=0.7, r= 0.03) (Figure. 4.3.1.5 B).

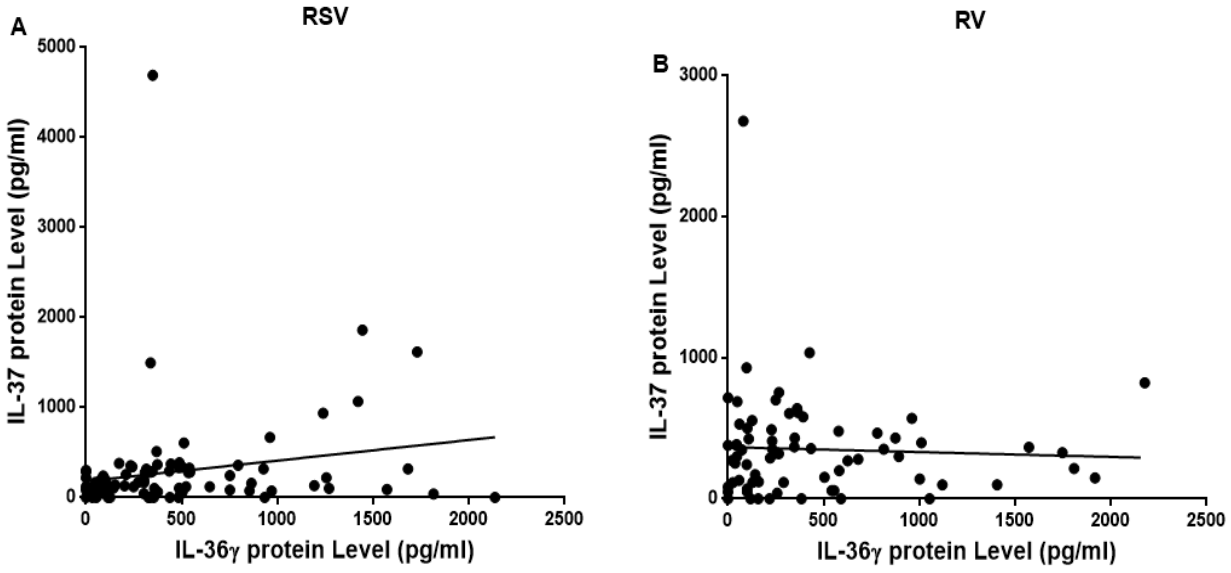

Figure 4.3.1.5 Correlations between protein levels of IL-36γ against IL-37 in RSV NPAs from children. A) Correlation between IL-36γ and IL-37 in RSV patients (n= 83), **B)** Correlation between IL-36γ and IL-37 in RV patients (n= 72). Correlations were presented by Spearman's rank order analysis, P two-tailed, n = number of XY pairs.

4.3.2 Expression of IL-37 cytokines and their receptors in human nasal epithelial cells during RSV infection

4.3.2.1 Human nasal epithelial cell expression of IL-37 mRNA following RSV infection: the effect of viral concentration

This study surveyed the production of IL-37 cytokines and receptors within human nasal epithelial cells (HNAECs) during RSV infections. Healthy test subjects donated HNAECs, separated and cultivated in ninety-six well tissue culture plates.

HNAECs were infected with RSV at MOIs of 0.1, 1 and 2.5 for 48 hours. Palivizumab was used with RSV at an MOI 2.5 as an anti-RSV control.

RNA were extracted from cells using Qiagen and reverse transcribed. mRNA expression was determined using Taq Man primer probe assays: L32, IL-37, IL-1R8 and IL-18R1, normalised to thehousekeeping gene L32.

The study found a significant elevation of IL-37 mRNA in infected HNAECs at MOI 2.5 ($p<0.01$, Mean= 1.02) in comparison to non-infected HNAECs. However, the MOI 2.5+ palivizumab group displayed decreased IL-37 mRNA expression but did not reach a significant point. Conversely, no cogent upregulation in IL-37 mRNA was shown in the MOI 0.1 (Mean= 0.2) or MOI 1 (Mean= 0.3) groups compared to non-infected cells (Mean= 0.1) or RSV palivizumab (Mean= 0.7) (fold expression relative to L32, Friedman test/Dunn's multiple comparisons test) (Figure 4.3.2.1 A). Figure (4.3.2.1 B) shows that IL-37 mRNA expression of an individual donor advanced with the increasing dose of RSV. In donor 4, IL-37 mRNA expression at 2.5 MOI of infected HNAECs was two-thirds higher than in donors 1 and 2 (Figure 4.3.2.1. B).

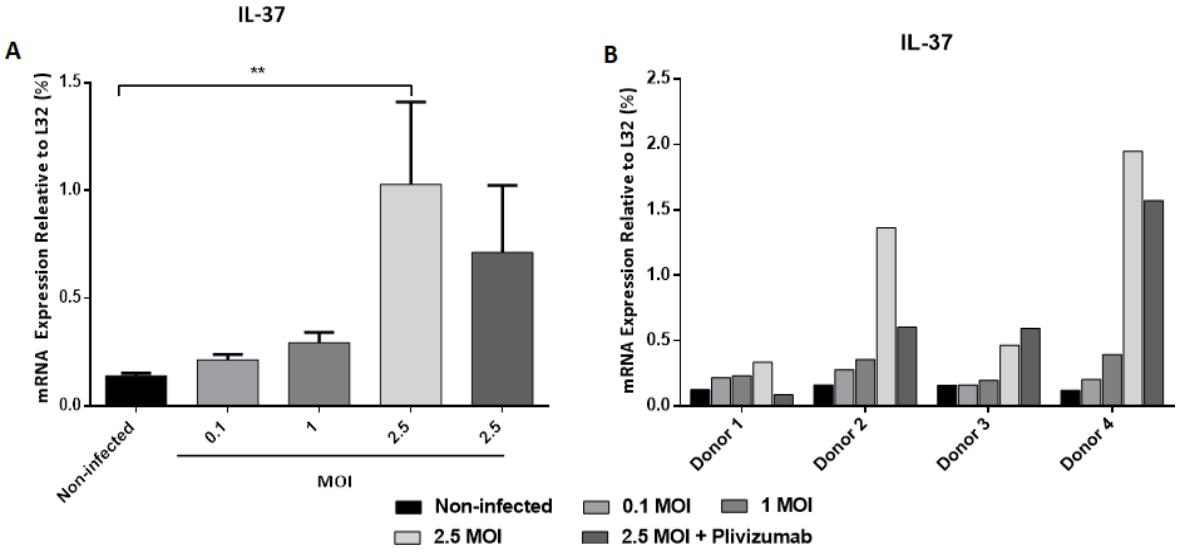

Figure 4.3.2.1. Expression of IL-37 mRNA by infected HNAECs in response to RSV infection. HNAECs were infected with RSV at MOIs of 0.1, 1 and 2.5 for 48 hours. Control non-infected cultures were cultured in the absence of RSV and Palivizumab used as an anti-RSV control. **A)** IL-37 mRNA (n=4), Friedman test for four groups/Dunn's multiple comparison test to compare the mean rank of group with the mean rank of every other group (**p<0.01). **B)** individual donor expression of IL-37 mRNA (n= 4), data expressed as the mean ± SEM, based on fold expression relative to L32, RM-two-way ANOVA/Tukey's multiple comparisonstest.

4.3.2.2 Expression of IL-1R8 mRNA by human RSV infection in human nasal epithelial cells

IL-1R8 is a negative regulator of IL-1 signalling (Molgora *et al.,* 2017). To evaluate the influence of RSV infection on expression of IL-1R8 mRNA, HNAECs were infected with increasing concentrations of RSV MOIs: 0.1, 1, 2.5 and 2.5+ palivizumab for 48 hours. IL-1R8 mRNA was measured in infected HNAECs in comparison to non-infected control cells and MOI 2.5+ palivizumab. There was no meaningful change in expression of IL-1R8 mRNA observed in infected HNAECs at MOI 0.1 (Mean= 13), MOI 1 (Mean= 7.1), MOI 2.5 (Mean= 5.2) compared to non-infected HNAECs (Mean= 13.7) or MOI 2.5+ palivizumab (Mean= 18.7) (fold expression relative to L32, Friedman test/Dunn's multiple comparisons test) (Figure 4.3.2.2 A). Figure (4.3.2.2 B) shows the IL-1R8 mRNA expression of individual donors during RSV infection.

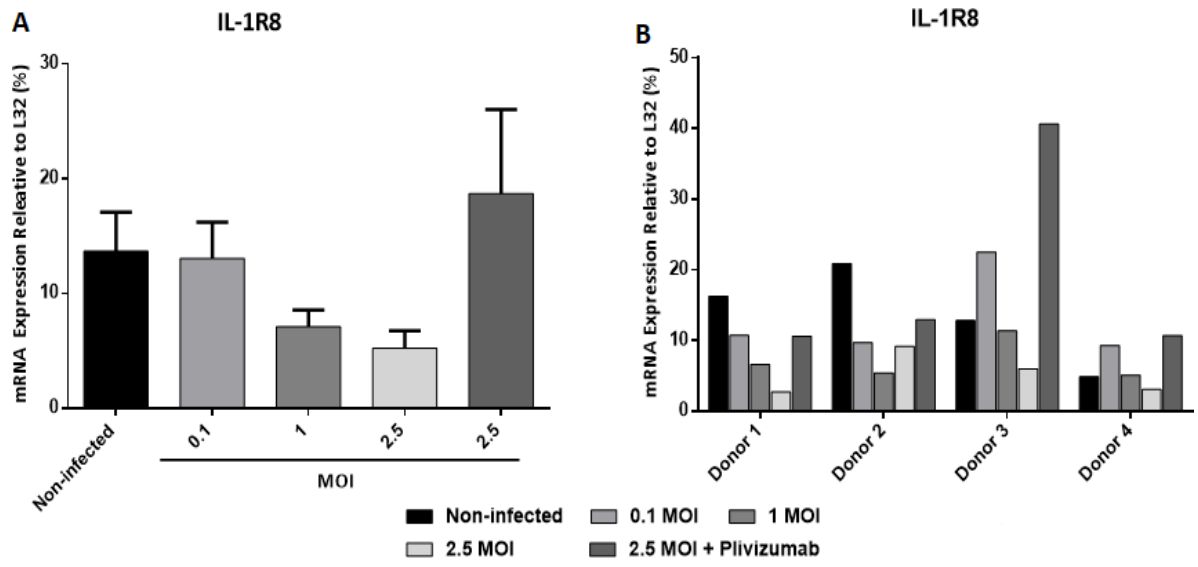

Figure 4.3.2.2 Expression of IL-1R8 mRNA by infected HNAECs during RSV infection. HNAECs were infected with RSV at MOIs of 0.1, 1 and 2.5 for 48 hours. Control non-infected cultures were cultured in the absence of RSV and Palivizumab used as an anti-RSV control. **A)** IL-1R8 mRNA (n= 4), Friedman test for four groups/Dunn's multiple comparison test to compare the mean rank of group with the mean rank of every other group. **B)** individual donor expression of IL-1R8 mRNA (n= 4), data expressed as the mean ± SEM, based on fold expression relative to L32, RM-two-way ANOVA/Tukey's multiple comparisonstest.

4.3.2.3 Expression of IL-18R1 mRNA by human RSV infection in human nasal epithelial cells

To determine the effect of RSV on expression of IL-18R1 mRNA, HNAECs were infected with different concentrations of RSV MOI: 0.1, 1, 2.5 and 2.5+ palivizumab for 48 hours. The study compared IL-18R1 mRNA levels between infected HNAECs and non-infected control cells and RSV+ palivizumab.

The results found a significant elevation of IL-18R1 mRNA in infected HNAECs at MOI 2.5 ($p<0.05$, Mean= 25.1) when compared to non-infected HNAECs (Mean= 10.9). However, infected HNAECs at MOI 0.1 (Mean= 17.5) and MOI 1 (Mean= 20.9) displayed no significant upregulation when compared to non-infected HNAECs or MOI 2.5+ palivizumab (Mean= 11.6) (Figure 4.3.2.3 A) (fold expression relative to L32, Friedman test/Dunn's multiple comparisons test). Figure (4.3.2.3 B) shows thatIL-18R1 mRNA expression of individual donors increased in accordance with higher levels of RSV. During RSV infection, donor 1 had the greatest expression of IL-18R1 mRNA levels in his group at 2.5 MOI in infected HNAECs (Figure 4.3.2.3 B).

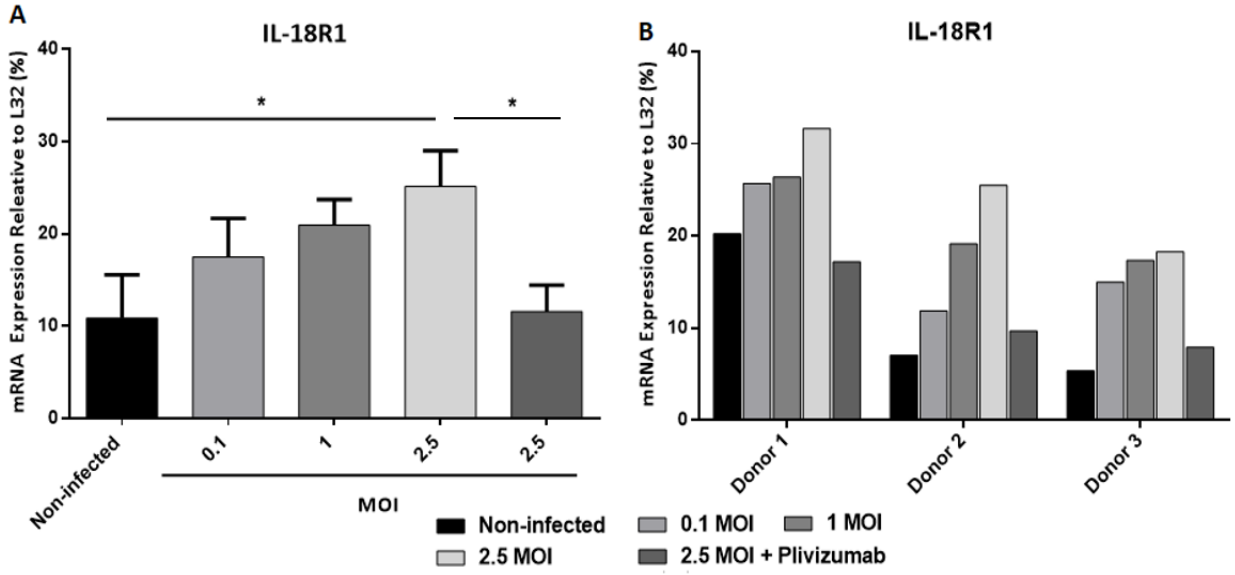

Figure 4.3.2.3 Expression of IL-18R1 mRNA by infected HNAECs in response to RSVinfection. HNAECs were infected with RSV at MOIs of 0.1, 1 and 2.5 for 48 hours. Control non- infected cultures were cultured in the absence of RSV and Palivizumab used as an anti-RSV control. **A)** IL-18R1 mRNA (n=3), Friedman test for four groups/Dunn's multiple comparison test to compare the mean rank of group with the mean rank of every other group. **B)** Individual donor expression of IL-18R1 mRNA. , (n= 3), data expressed as the mean ± SEM, based on fold expression relative to L32, RM-two-way ANOVA/Tukey's multiple comparisonstest.

4.3.2.3 Expression of IL-37 protein in human nasal airway epithelial cells during RSV infection

This study investigated the effect of RSV infection on the generation and exudation of IL-37 proteins in human nasal airway epithelial cells (HNAECs). Healthy test subjects donated HNAECs, separated and cultivated in ninety-six well tissue culture plates. HNAECs were infected with RSV at MOIs of 0.1, 1 and 2.5 for 48 hours. Palivizumab was used with RSV at MOI 2.5 as an anti-RSV control. ELISA calculated the extracellular and intracellular IL-37 protein expression in culture media and cell pellets, then compared with a control of non-infected cells.

The results in Figure 4.3.2.4 A for the culture supernatant show a significant increase of extracellular IL-37 protein levels in infected cells compared to non-infected cells (Friedman test/Dunn'smultiple comparisons test, $p< 0.05$, $n= 6$). Conversely, the cell pellet shows the average IL-37 protein level in each group was: (Mean= 6891.5 pg/ml) at MOI 0.1, (Mean= 8079.7) at MOI 1, (Mean= 6761.6 and 10158.7 pg/ml) at MOI 2.5 and MOI 2.5+ palivizumab, respectively. Furthermore, there was no significant difference in IL-37 protein levels between the infected cell groups and the non-infected control cells group (Friedman test/Dunn's multiple comparisons test, $p=0.2$)(Figure 4.3.2.4 B).

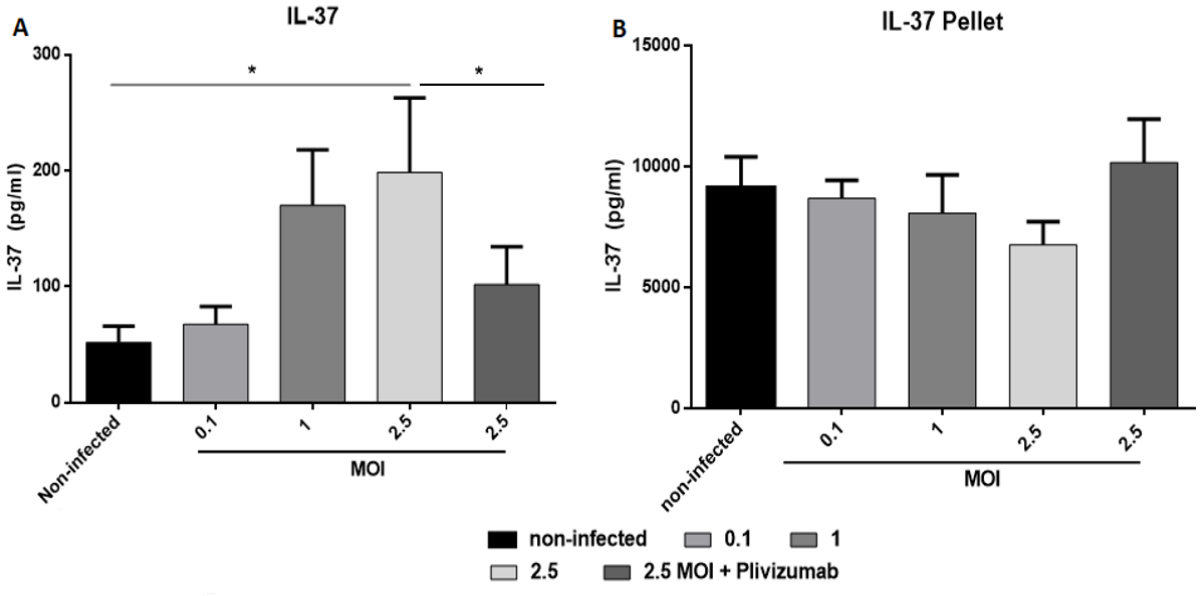

Figure 4.3.2.4 Expression of IL-37 protein in human nasal airway epithelial cells during RSV infection. Nasal cells were infected with RSV (MOI 2.5) for 48 hours and measured by ELISA. Non-infected cells used as a control. IL-37 protein expression in **A)** Culture supernatant, **B)** Cell pellet. Values are shown as IL-37 protein expression in comparison to non-infected cells. Data expressed as the mean ± SEM (foldexpression relative to non-infected cells, Friedman test for five groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group , * $p<0.05$, n= 6).

4.3.3 Expression of IL-37 cytokines and IL-37 receptors in bronchial epithelial cell lines (A549 and BEAS-2B) during RSV infection

This study examined whether RSV infection induced the formulation of IL-37 cytokines and IL-37 receptors in airway epithelial cell lines. Firstly, airways epithelial cells A549 and BEAS-2B cells were grown on 12-well plates until 80% confluent. Cultures A549 and BEAS-2B cells were infected with RSV at a multiplicity of infection MOI of 0.1, 1 and 2.5, with non-infected cells used as control at 48 hours. To confirm theoptimize time for IL-37 mRNA expression, A549 and BEAS-2B cells were infected with RSV (2.5MOI) and RNA were extract at 4, 8, 24 and 48 hours post infection. IL-37 mRNA isoforms (IL-37 all,IL-37a, IL-37b and IL-37c) expression was measured by RT-PCR. Values are shown as fold expression in comparison to L32. Data is expressed as the Mean ± SEM (Friedman test / Dunn'smultiple comparisons test, * $p<0.05$ and ** $p<0.01$).

4.3.3.1 Expression of IL-37mRNA cytokines in airways epithelial cell lines (A549 and BEAS-2B) during RSV infection

To assess the influence of RSV infection on the expression of IL-37 mRNA in culture airway epithelial cells line, A549 and BEAS-2B cells were infected with RSV at MOI 0.1, 1 and 2.5. Results observed significant differences in IL-37 mRNA presence at MOI 2.5 ($p< 0.001$, $p< 0.05$ and Mean= 0.3 and 3, respectively) in comparison with non-infected cells (Mean= 0.02 and 0.8, respectively).

Contrastingly, for both A549 and BEAS-2B cell culture, there was no significant expression observed in infected cells at MOI 0.1 (Mean= 0.1 and 1.5) and MOI 1 (Mean= 0.2 and 2.1) (fold expression relative to L3, Friedman test/Dunn’s multiple comparisons test) (Figures 4.3.3.1 A and B). Testers achieved supplemental IL-37 mRNA production in infected A549 and BEAS-2B cells by increasing the viral load of MOI.

Identification of the prime timing for IL-37 mRNA expression pinpointed a significant increase in the BEAS-2B cells at the 48-hour interval ($p<0.05$, Mean= 2.8) compared to non-infected cells at the same time (Mean= 1). There were no significant changes in IL-37 mRNA observed at 4 hours (Mean= 0.7, 0.6), 8 hours (Mean= 1.1, 0.6) and 24 hours (Mean= 1.7, 0.9) for either infected or non-infected cells (fold expression relative to L32 Friedman test/Dunn's multiple

comparisons test) (Figure 4.3.3.1 C). These results indicate that IL-37 mRNA expression is highest in RSV A2-infected airway epithelial cells after 48 hours.

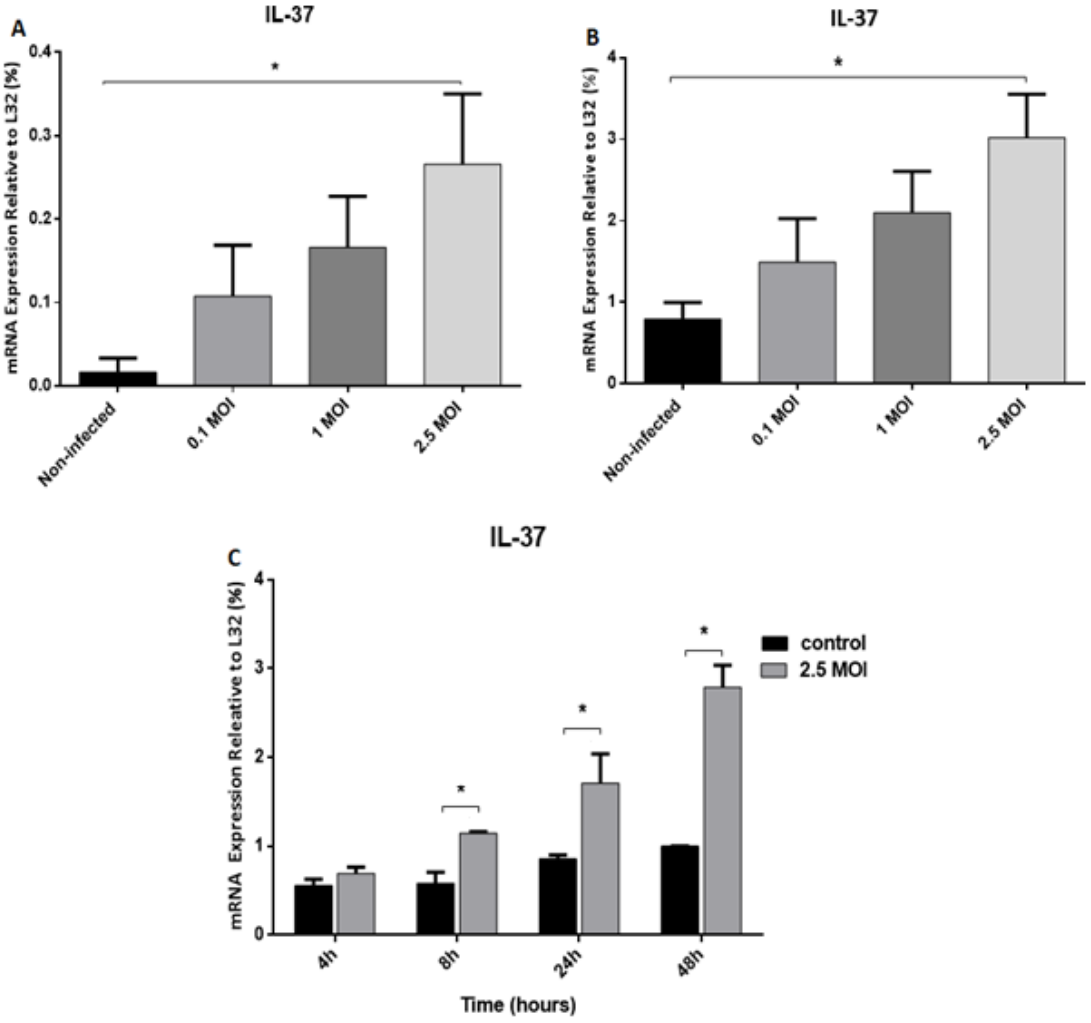

Figure 4.3.3.1 RSV infection induced IL-37 mRNA expression infected bronchial epithelial cells (A549 and BEAS-2B). A549 and BEAS-2B cells were infected with RSV A2 at different MOI (0.1, 1 and 2.5) for 48 hours. **A)** A549, **B)** BEAS-2B. Non-infected cells used as a control (n=3). **C)** BEAS- 2B cells were infected with RSV at time intervals (4h, 8h, 24h and 48h) (n=3). Values are shown as fold expression in comparison to L32. Data is expressed as Mean ± SEM (Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group (A and B) and Two-way RM ANOVA test (C) , * $p<0.05$).

4.3.3.2 Expression of IL-37 Isoforms mRNA by human RSV infection in culture epithelial cells (BEAS-2B) cells

This test assessed an RSV infection's influence on the creation of IL-37 isoforms in culture airway epithelial cell lines (BEAS-2B). BEAS-2B epithelial cells were infected with RSV at MOIs 0.1, 1 and 2.5 over 48 hours.

IL-37 isoforms mRNA expression was significantly upregulated in infected BEAS-2B cells at MOI 2.5 ($p< 0.05$ and Mean= 2.9) in comparison with non-infected cells (Mean= 0.6). In contrast, there was no significant expression observed in infected BEAS-2B cells at MOI 0.1 (Mean= 1.6) and MOI 1 (Mean= 2.8) (fold expression relative to L32, Friedman test/Dunn's multiple comparisons test) (Figure 4.3.3.2 A).

However, there was no cogent increase on the expression of IL-37A mRNA at any MOI of infected cells in comparison to the control non-infected cells. The expression of IL-37A mRNA was MOI 0.1 (Mean= 0.05), MOI 1 (Mean= 0.05) and MOI 2.5 (Mean= 0.1) compared to non-infected cells (Mean= 0.02) (Friedman test/Dunn's multiple comparisons test, $p= 0.1$) (Figure 4.3.3.2 B).

IL-37B mRNA expression was significantly elevated in infected BEAS-2B cells at MOI 1 ($p<0.05$, Mean= 1.2) and 2.5 ($p< 0.05$ and Mean= 1.4) in comparison with non-infected cells (Mean= 0.3), whereas there was no significant expression observed in infected cells at MOI 0.1 (Mean=0.7) (fold expression relative to L3, Friedman test/Dunn's multiple comparisons test) (Figure 4.3.3.2 C).

In addition, IL-37C mRNA expression was significantly upregulated in infected BEAS-2B cells at MOI 1 ($p<0.05$, Mean= 0.3) and 2.5 ($p< 0.05$ and Mean= 0.3) in comparison with non-infected cells (Mean= 0.1), whereas there was no significant expression observed in infected cells at MOI 0.1 (Mean= 0.2) (fold expression relative to L32, Friedman test/Dunn's multiple comparisons test) (Figure 4.3.3.2 D).

These results indicates that IL-37B and IL-37C mRNA are the activated isoforms of IL-37 during RSV infection.

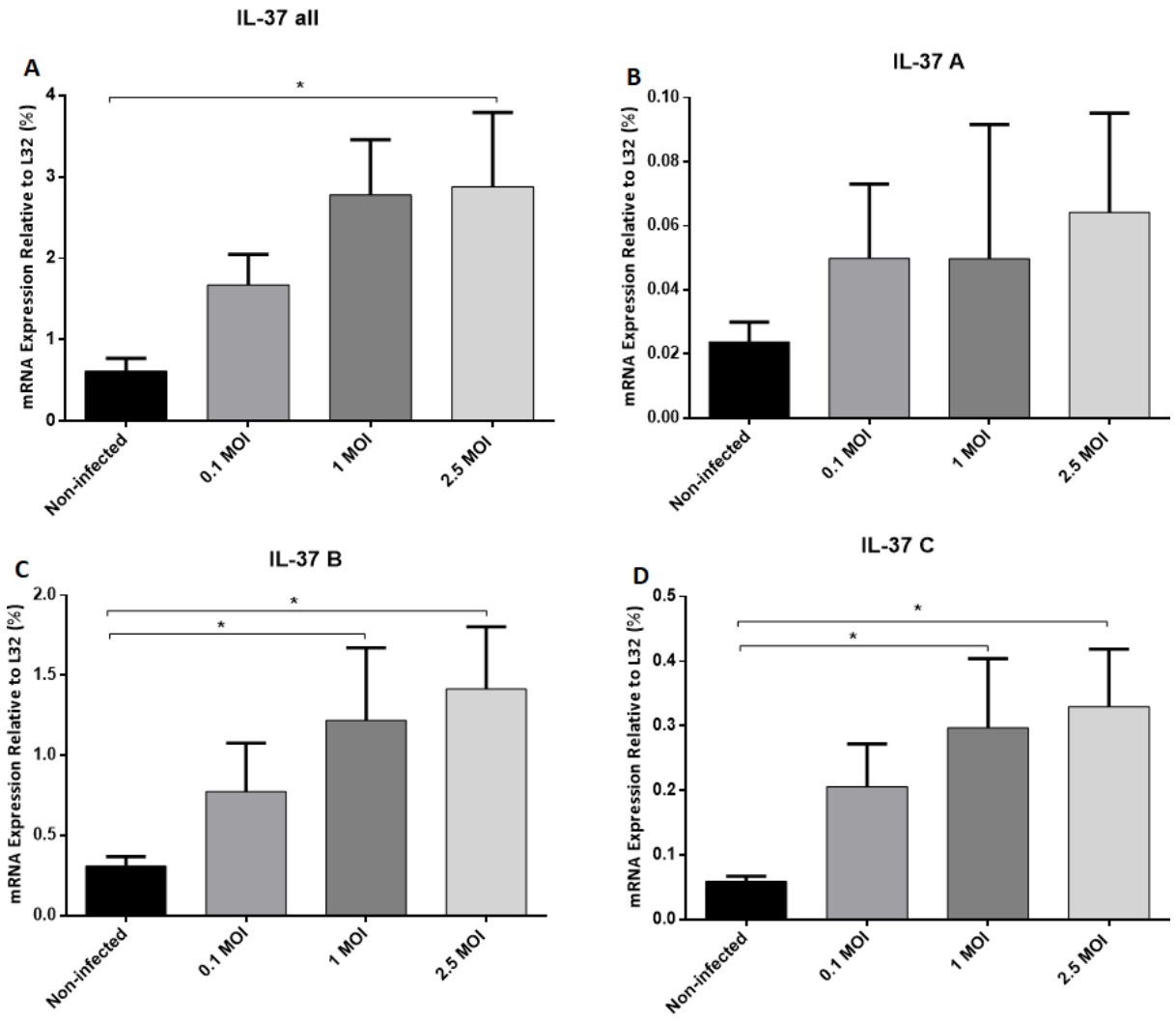

Figure 4.3.3.2 RSV infection induced IL-37 Isoforms mRNA expression in BEAS-2B cells. BEAS-2B cells were infected with RSV at different MOI (0.1, 1 and 2.5) for 48 hours **A)** IL-37 mRNA all, **B)** IL-37a mRNA, **C)** IL-37b mRNA and **D)** IL-37c mRNA. Non-infected cells used as a control. Values are shown as fold expression in comparison to L32. Data is expressed as the Mean with ± SEM (Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group, * $p<0.05$, n=3).

4.3.3.3 Expression of IL-1R8 mRNA by human RSV infection in bronchial epithelial cells (A549 and BEAS-2B) cells

To assess the influence of RSV infection on the expression of IL-1R8 mRNA in culture airway epithelial cell lines, A549 and BEAS-2B cells were infected with RSV at MOI 0.1, 1 and 2.5. The results indicate significant differences in IL-1R8 mRNA expression of infected BEAS-2B cells at MOI 1 ($p<0.05$, Mean= 0.3).

Infected A549 and BEAS-2B cells both showed significant increases of IL-1R8 mRNA at MOI 2.5 ($p< 0.05$, Mean= 0.9 and 0.4) in comparison with non-infected cells (Mean= 0.3 and 0.1, respectively).

However, there was no significant expression observed in infected A549 and BEAS-2B cell cultures at MOI 0.1 (Mean= 0.4 and 0.2, respectively) and MOI 1 (Mean= 0.7) in A549 cells (fold expression relative to L32, Friedman test/Dunn's multiple comparisons test) (Figure 4.3.3.3 A and B). IL-1R8 mRNA expression in infected A549 and BEAS-2B cells was increased by boosting the viral load of MOI.

There was no significant increase in IL-1R8 mRNA expression in infected BEAS-2B cells at the indicated time intervals ($p= 0.8$), 4 hours (Mean= 0.07, 0.08), 8 hours (Mean= 0.3, 0.2), 24 hours (Mean= 0.4, 0.3) and 48 hours (Mean= 0.7, 0.6), in comparison to non-infected cells (fold expression relative to L32 Friedman test/Dunn's multiple comparisons test)(Figure 4.3.3.3 C). These results indicate IL-1R8 mRNA proliferation in infected airway epithelial cells in response to RSV infection.

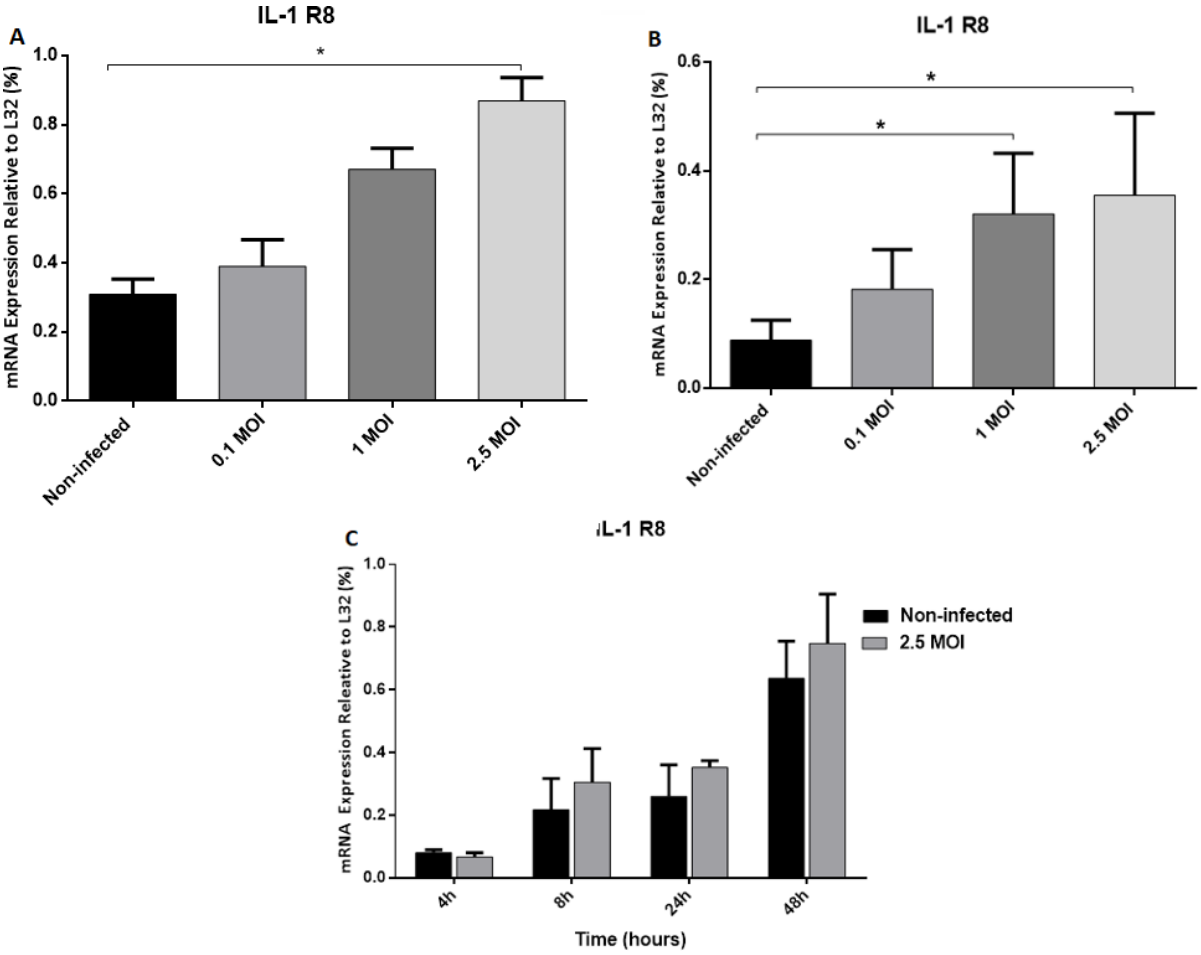

Figure 4.3.3.3 RSV infection induced IL-1R8 mRNA expression infected bronchial epithelial cells (A549 and BEAS-2B). A549 and BEAS-2B cells were infected with RSV A2 at different MOI (0.1, 1 and 2.5) for 48 hours. **A)** A549, **B)** BEAS-2B. Non-infected cells used as a control (n=3). **B)** BEAS-2B cells were infected with RSV at separate times (4h, 8h, 24h and 48h) (n=3). Values are shown as fold expression in comparison to L32. Data is expressed as the Mean ± SEM (Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group (A and B) and Two-way RM ANOVA test (C), * $p<0.05$).

4.3.3.4 Expression of IL-18R1 mRNA by human RSV infection in bronchial epithelial cells (A549and BEAS-2B) cells

To assess the influence of RSV infection on the expression of IL-18R1 mRNA in culture airway epithelial cell lines, A549 and BEAS-2B cells were infected with RSV at MOI 0.1, 1 and 2.5. The results indicate there were significant differences observed in IL-18R1 mRNA expression of infected A549 and BEAS-2B cells at MOI 2.5 ($p< 0.01$, Mean= 15.5 and 2.3) in comparison with non-infected cells (Mean= 4.7 and 0.2, respectively). In contrast, there was no significant expression observed in infected cells at MOI 0.1 (Mean= 5.5 and 1.1) and MOI 1 (Mean= 9.6 and 2.1) in both A549 and BEAS-2B cell cultures, respectively (fold expression relative to L32, Friedman test/Dunn's multiple comparisons test) (Figure 4.3.3.4 A and B). IL-18R1 mRNA expression in infected A549 and BEAS-2B cells was boosted by increasing the viral load of MOI.

Regarding the effects of time, there was no significant increase in IL-18R1 mRNA expression in infected BEAS-2B cells at specific time intervals ($p= 0.7$) of 4 hours (Mean= 1.3, 3), 8 hours (Mean= 1.5, 3.8), 24 hours (Mean= 1.7, 5.7) and 48 hours (Mean= 3.9, 7.8) in comparison to non-infected cells (fold expression relative to L32 Friedman test/Dunn's multiple comparisons test)(Figure 4.3.3.4 C). These results indicate that IL-18R1 mRNA grows in infected airway epithelial cells in response to RSV infection.

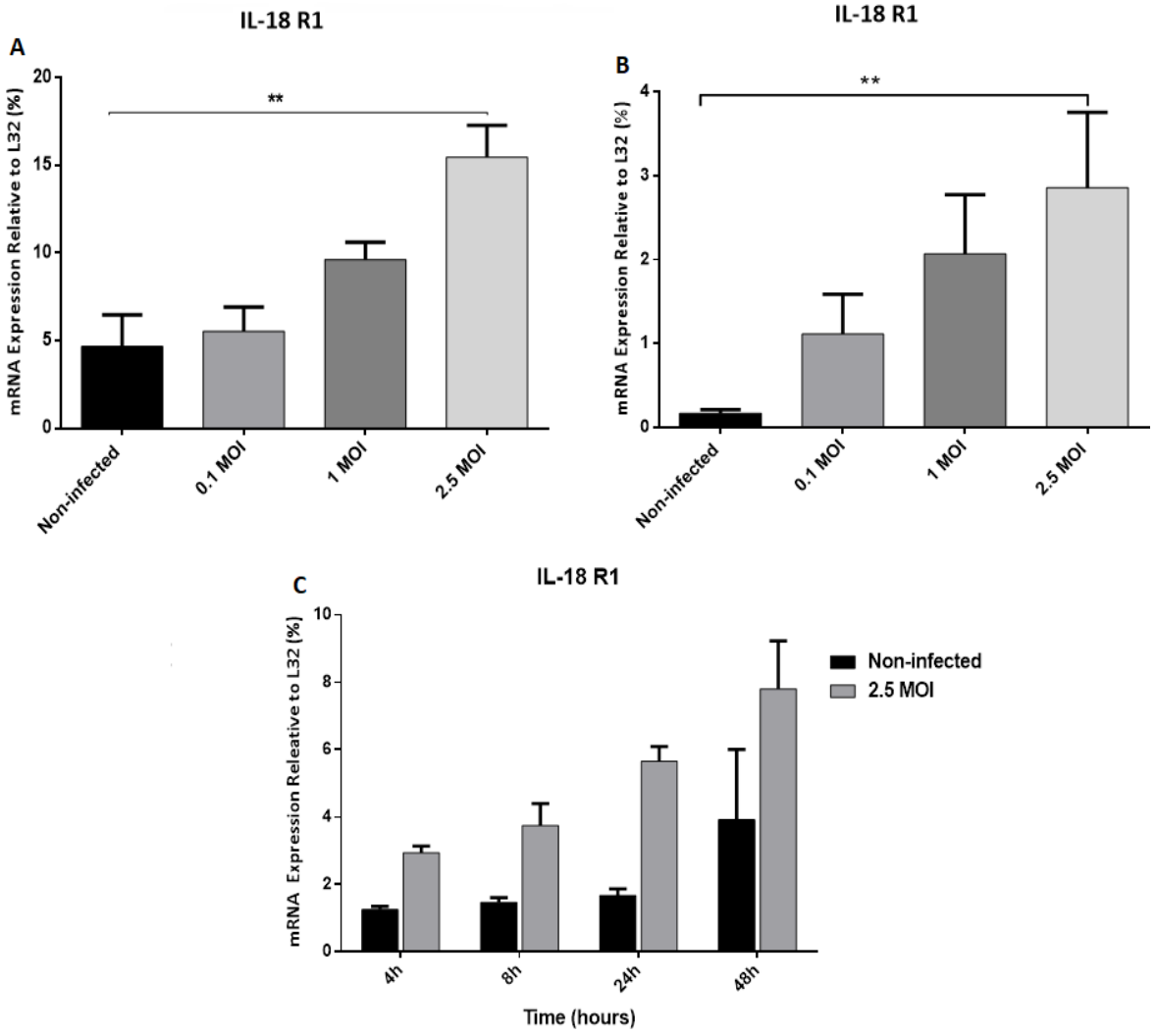

Figure 4.3.3.4 RSV infection induced IL-18R1 mRNA expression infected bronchial epithelial cells (A549 and BEAS-2B). A549 and BEAS-2B cells were infected with RSV A2 at different MOI (0.1, 1 and 2.5) for 48 hours. **A)** A549, **B)** BEAS-2B. Non-infected cells used as a control (n=3). **B)** BEAS-2B cells were infected with RSV at separate times (4h, 8h, 24h and 48h) (n=3). Values are shown as fold expression in comparison to L32. Data is expressed as the Mean ± SEM ((Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group (A and B) and Two-way RM ANOVA test (C), ** $p<0.01$).

4.3.3.5 Expression of IL-37 protein by human RSV infection in bronchial epithelial cells (A549 and BEAS-2B) cells

To analyse the ability of culture airways epithelial cells (A549 and BEAS-2B) to the produce and secrete IL-37 protein in response to RSV infection, A549 and BEAS-2B cells were grown in culture 12- well plate. A549 and BEAS-2B were infected with RSV at MOIs of 0.1, 1 and 2.5 for48hr. The expression of extracellular and intracellular IL-37 protein in culture media and cell pellet were measured by ELISA and compare that to non-infected cells which used as a control.

In culture supernatant, ELISA detected no significant difference in IL-37 protein production in either infected or non-infected A549 and BEAS-2B cells (p= 0.6, 0.07) (Figure 4.2.3.5 A, B).
The mean levels of IL-37 protein were akin in both A549 and BEAS-2B cell groups: (Mean= 212, 65 pg/ml) at 0.1, (Mean= 227, 71 pg/ml) at MOI 1, (189, 85 pg/ml) at 2.5 and (Mean= 205, 65 pg/ml) in non-infected cells (Friedman test/Dunn's multiple comparisons test, p= 0.001, n= 3) (Figure 4.3.3.5 A, B).

Likewise, the cell pellets displayed no significant disparity in IL-37 protein expression during RSV infection (p= 0.7) in either A549 or BEAS-2B cell groups (Figure 4.2.3.5 C, D). The mean level of IL-37 protein presence was similar in all groups: (Mean= 5044, 3040 pg/ml) at 0.1, (Mean= 4720, 2721 pg/ml) at MOI 1, (4906, 2904 pg/ml) at 2.5 and (Mean= 5630, 3632 pg/ml) in control cells (Friedman test/Dunn's multiple comparisons test, p= 0.001, n=3) (Figure 4.3.3.5 C, D).

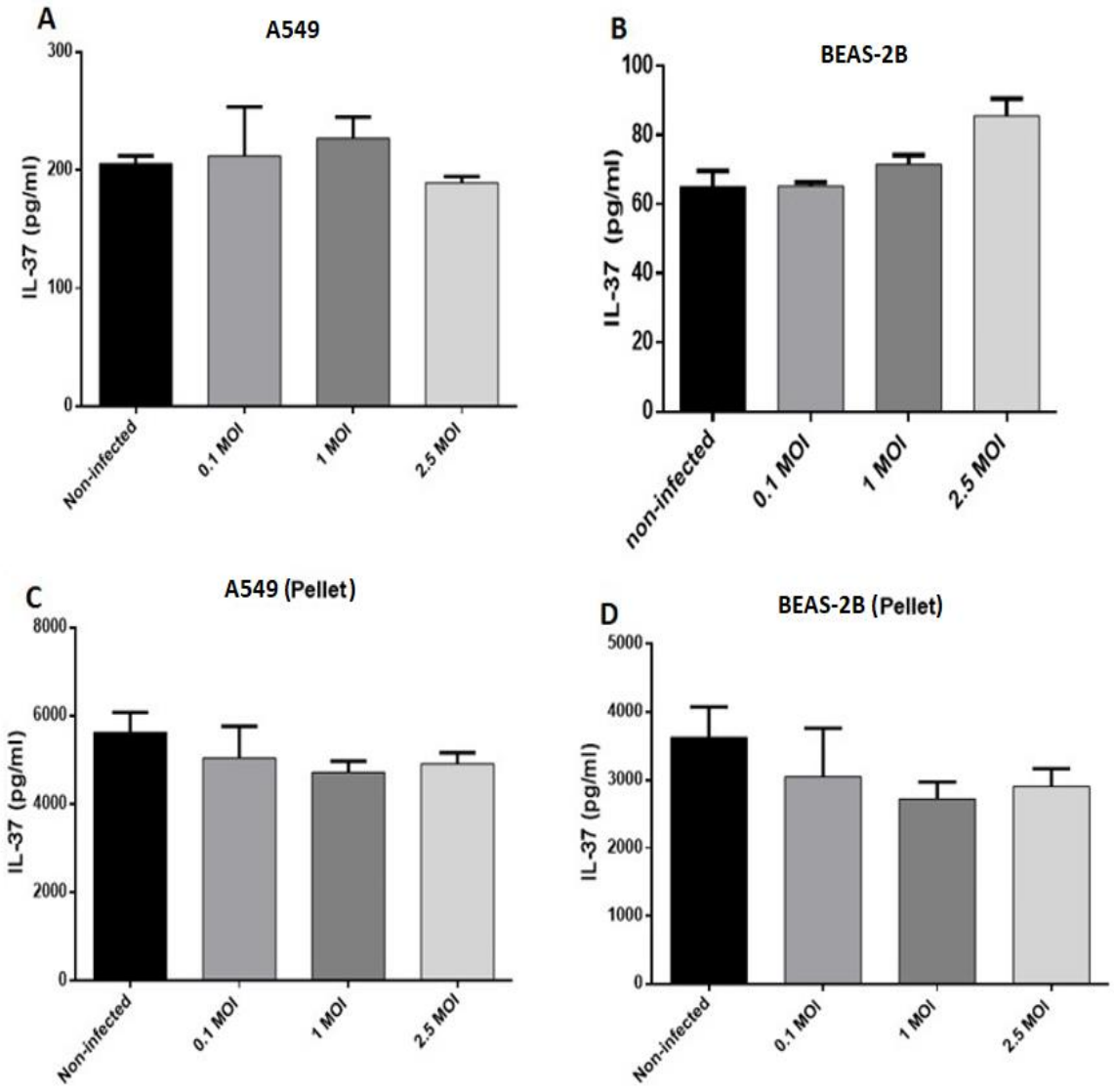

Figure 4.3.3.5 IL-37 protein expression in infected bronchial epithelial cells (A549 and BEAS-2B). A549 and BEAS-2B cells were infected with RSV A2 at different MOI (0.1, 1 and 2.5) for 48 hours. **IL-37** protein expression in A549 cell culture supernatant **(A)**, BEAS-2B cell culture supernatant **(B)**, A549 cell pellet **(C)** and BEAS-2B cell pellet **(D)**. Non-infected cells used as a control (n=3). Values are shown as fold expression in comparison to L32. Data is expressed as the Mean ± SEM (Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group (A and B) and Two-way RM ANOVA test (C), * $p<0.05$)

4.3.4.1 Immunofluorescent localization of IL-37 protein in bronchial epithelial cell lines (BEAS-2B) cells

IL-37 protein is synthesized as a precursor which is capable of secreting into the extracellular space. The precursor of IL-37 is also capable of being processed intracellularly into mature form by Caspase-1. In the cytosol, mature IL-37 binds to the phosphorylated form of the Smad3 factor (pSmad3). Apparently, The IL-37 / Smad3 complex is able to translocate into the nucleus and inhibit the transcription of pro-inflammatory genes. To determine the localization and expression of IL-37 during RSV infection, Firstly, airways epithelial cell BEAS-2B were grown on 12-well plates until 80% confluent. Cultures BEAS-2B cells were infected with RSV at MOI 1 with non-infected cells used as control at 12, 24 and 48 hours. Concurrently, BEAS-2B cells were divided into three groups, one group were used as Isotype control of infected and non-infected BEAS-2B cells (panel A), second group were used as control in both infected or non-infected BEAS-2B cells (panel B), third group infected and non-infected BEAS-2B cells were treated with 100ng /ml of LPS for 18 hours which used as a positive control of IL-37 protein (panel C). In addition, LPS has been found to enhance the stability of IL-37 mRNA and therefore express more level of IL-37 protein (Bufler *et al.,* 2004; Boraschi *et al.,* 2011). IL-37 protein has been stained with specific monoclonal antibody anti-IL-37 (green) and matched isotype controls followed by secondary antibody before immobilisation onto glass slides. Nuclei are stained in blue (DAPI). Images were obtained using confocal microscopy at 40x magnification (section 2.6). No staining was observed with the isotype control.

Figure 4.3.4.1a reveals IL-37 protein in BEAS-2B cells after 12 hours as being localised around the nucleus. No comparable staining was visible with isotype control (Figure 4.3.4.1a, A). IL-37 protein was localised around the nucleus (Figure 4.3.4.1a, B). Of note, stimulation by LPS enhance expression of IL-37 which appears as strong positive staining and could be secreted around the nucleus in BEAS-2B cells (Figure 4.3.4.1a, C).

Figure 4.3.4.1b shows the localisation of IL-37 protein in non-infected BEAS-2B cells after 24 hours around the nucleus. No comparable staining was visible with isotype control (Figure 4.3.4.1b, A). IL-37 protein was localised around the nucleus (Figure 4.3.4.1b, B). Of note, stimulation by LPS enhanced expression of IL-37, which appeared as strong positive staining

and could be secreted around the nucleus in non-infected cells (Figure 4.3.4.1b, C).

Figure 4.3.4.1c shows the localisation of IL-37 protein in BEAS-2B cells after 48 hours. No comparable staining was evident with isotype control (Figure 4.3.4.1c, A). There was no IL-37 protein staining observed around the nucleus in non-infected cells (Figure 4.3.4.1c, B). In addition,no IL-37 protein expression staining was localised around the nucleus in BEAS-2B cells after LPS stimulation (Figure 4.3.4.1c, C).

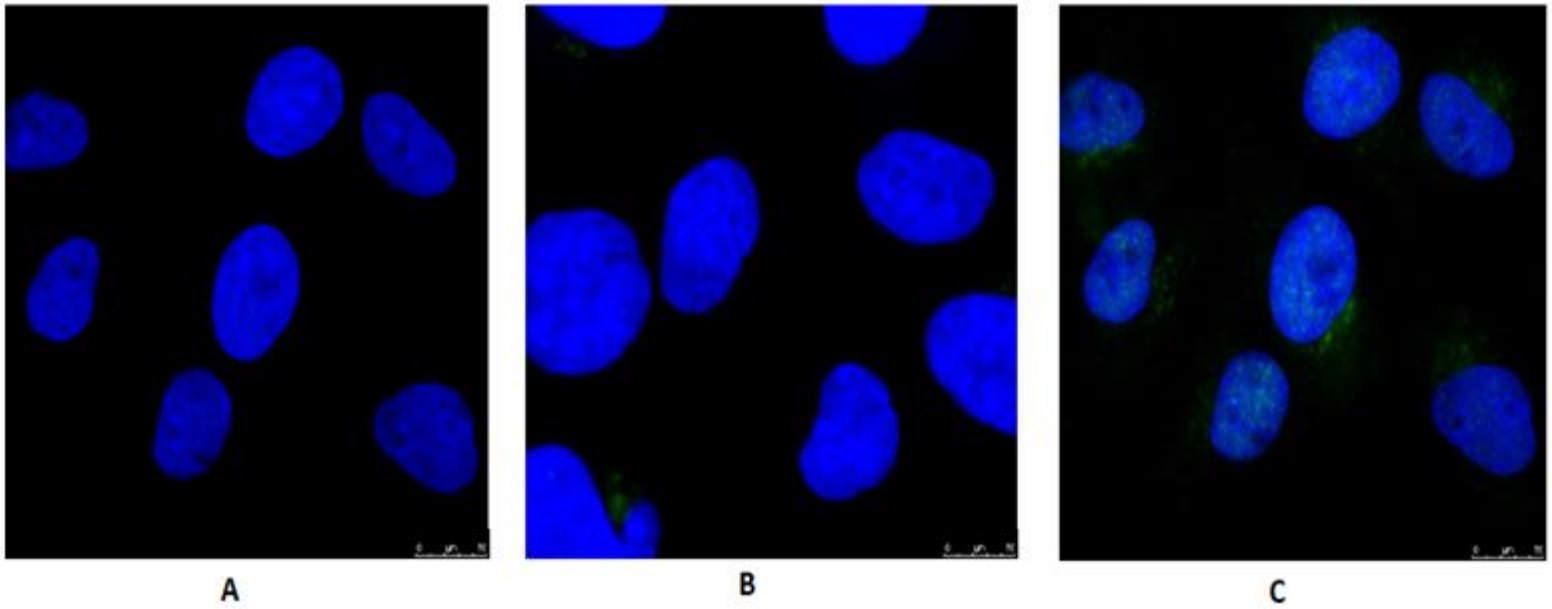

Figure 4.3.4.1a Immunofluorescence detection of IL-37 in BEAS-2B cells (12hr). Control non-infected cultures cells were cultured in the absence of RSV. **(A)** Isotype control of BEAS-2B cells. **(B)** IL-37 protein was observed in BEAS-2B cells. **(C)** Culture cell were stimulated with (100ng/ml) of LPS for 18hrs in BEAS-2B cells. Cells were stained with IL-37 antibody (green) and matched isotype controls followed by secondary antibody before immobilisation onto glass slides. Nuclei are stained in blue (DAPI). Images were obtained using confocal microscopy at 40x magnification (section 2.6).

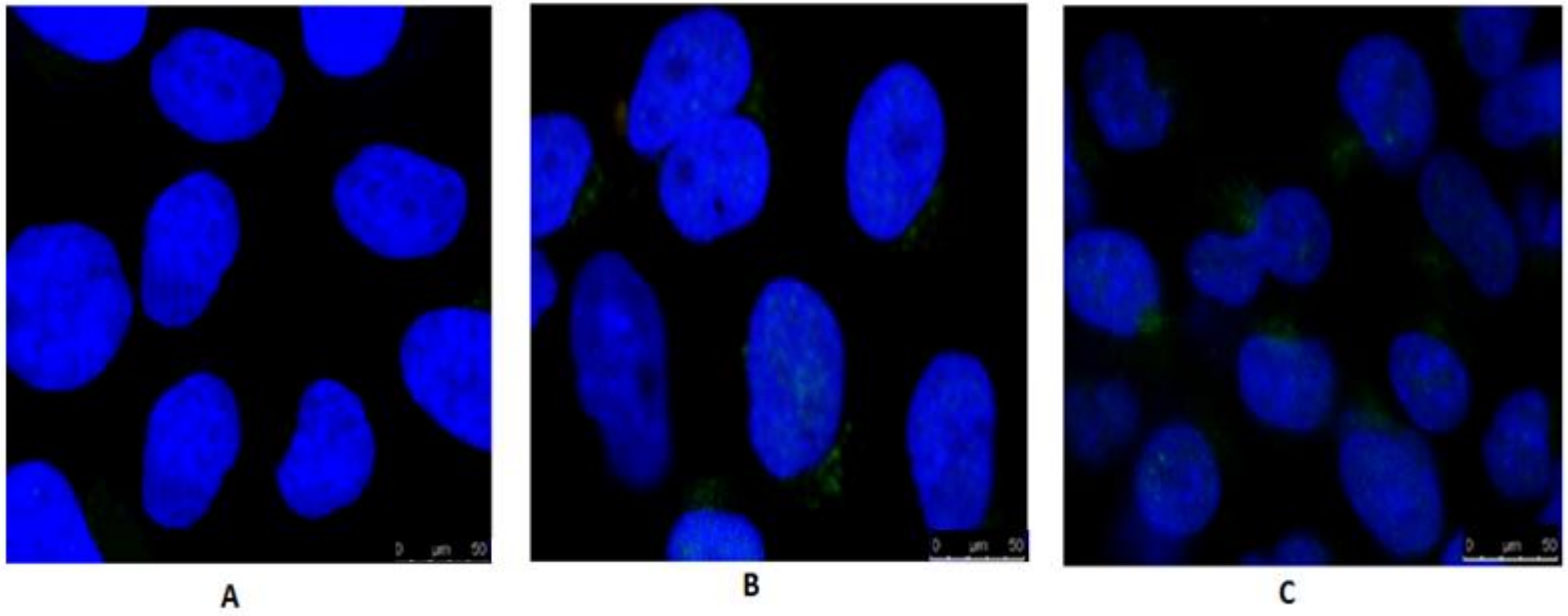

Figure 4.3.4.1b Immunofluorescence detection of IL-37 in BEAS-2B cells (24hr). Control non-infected cultures cells were cultured in the absence of RSV. **(A)** Isotype control of BEAS-2B cells. **(B)** IL-37 protein was observed in BEAS-2B cells. **(C)** Culture cell were stimulated with (100ng/ml) of LPS for 18hrs in BEAS-2B cells. Cells were stained with IL-37 antibody (green) and matched isotype controls followed by secondary antibody before immobilisation onto glass slides. Nuclei are stained in blue (DAPI). Images were obtained using confocal microscopy at 40x magnification (section 2.6).

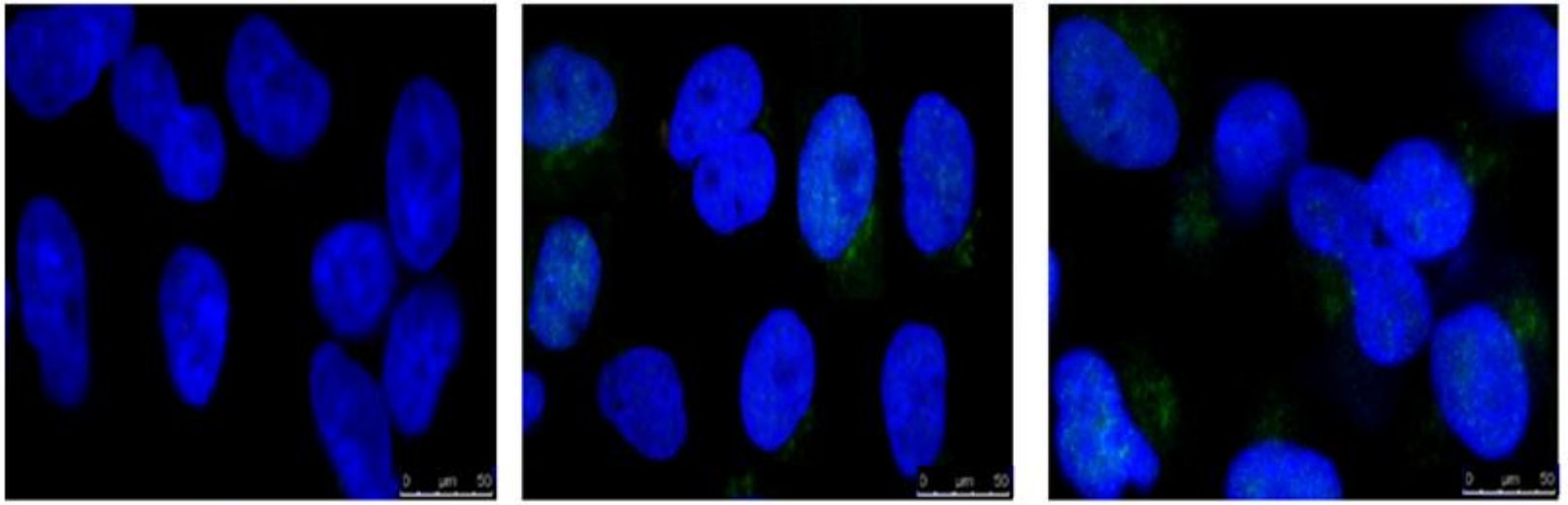

Figure 4.3.4.1c Immunofluorescence detection of IL-37 in BEAS-2B cells (48hr). Control non-infected cultures cells were cultured in the absence of RSV. **(A)** Isotype control of BEAS-2B cells. **(B)** IL-37 protein was observed in BEAS-2B cells. **(C)** Culture cell were stimulated with (100ng/ml) of LPS for 18hrs in BEAS-2B cells. Cells were stained with IL-37 antibody (green) and matched isotype controls followed by secondary antibody before immobilisation onto glass slides. Nuclei are stained in blue (DAPI). Images were obtained using confocal microscopy at 40x magnification (section 2.6).

4.3.4.2 Immunofluorescent localization of IL-37 protein in infected bronchial epithelial cell lines (BEAS-2B)

This study sought to establish the localisation of IL-37 proteins in BEAS-2B cells during RSV A2 infection. Cells were infected with MOI 1 RSV at 12, 24 and 48-hour intervals and compared with non-infected control cells. One group was treated with 100ng/ml of LPS for 18 hours and used as a positive control of IL-37 protein in both infected and non-infected cells. IL-37 protein was stained with a specific monoclonal antibody anti-IL-37 (green). No staining was visible with the isotype control.

Figure 4.3.4.2a shows the localisation of IL-37 protein in RSV A2-infected BEAS-2B cells after 12 hours as being situated around the nucleus. No comparable staining was observed with the isotype control in infected cells (Figure 4.3.4.2a, A). IL-37 protein was localised around the nucleus (Figure 4.3.4.2a, B). Of note, stimulation by LPS enhanced the expression of IL-37 which appeared as strong positive staining and could be secreted around the nucleus in infected cells (Figure 4.3.4.2a, C).

Figure 4.3.4.2b shows the localisation of IL-37 protein in RSV A2-infected BEAS-2B cells at 24 hours is situated around the nucleus. No comparable staining was visible with isotype control (Figure 4.3.4.2b, A). IL-37 protein was localised around the nucleus (Figure 4.3.4.2b, B). Notably, stimulation by LPS enhanced the production of IL-37, appearing as strong positive staining and could be secreted around the nucleus in infected cells (Figure 4.3.4.2b, C).

Figure 4.3.4.2c shows the localisation of IL-37 protein in RSV A2-infected BEAS-2B cells at 48 hours; no comparable staining was observed with isotype control in infected cells (Figure 4.3.4.2c, A). There was no IL-37 protein staining visible around the nucleus in infected cells (Figure 4.3.4.2c,B). In addition, no IL-37 protein expression staining was localised around the nucleus in BEAS-2B cells after LPS stimulation (Figure 4.3.4.2c, C).

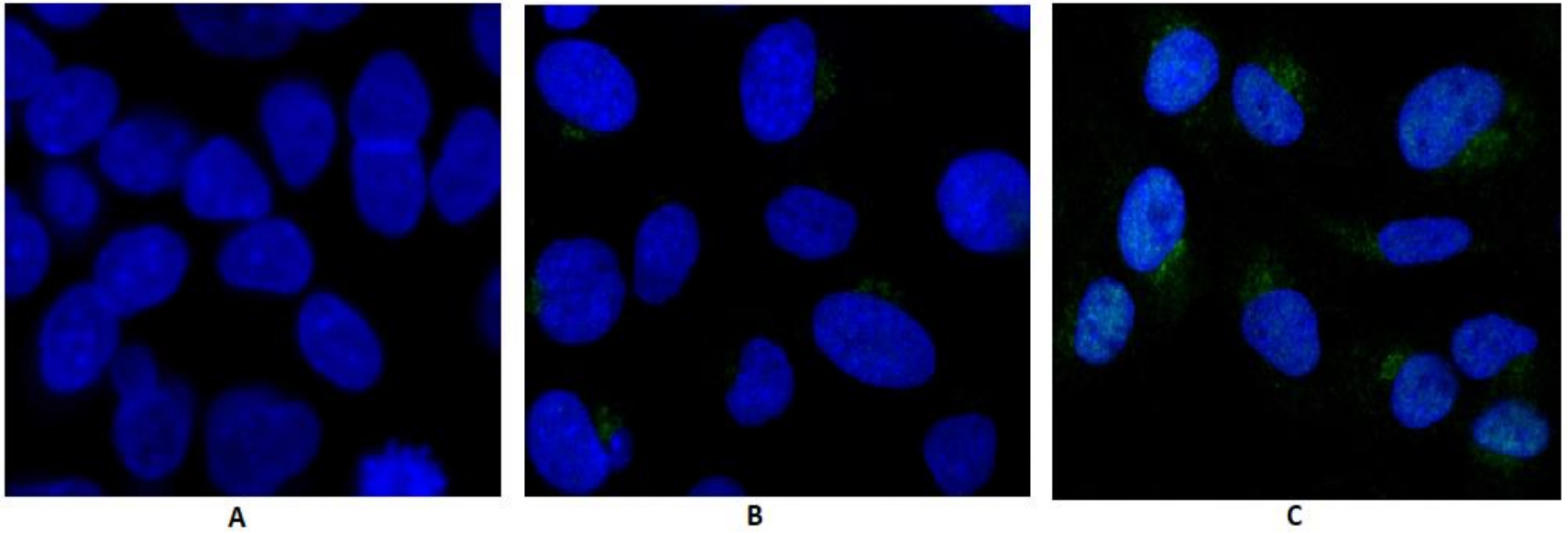

Figure 4.3.4.2a Immunofluorescence detection of IL-37 in RSV A2 infected BEAS-2B cells (12hr). BEAS-2B cells were infected with RSV A2 at MOI 1 for 12hr. **(A)** Isotype control of infected cells. **(B)** IL-37 protein was observed in infected cells **(C)** Culture cell were stimulatedwith (100ng/ml) of LPS for 18hrs in infected cells. Cells were stained with IL-37 antibody (green) and matched isotype controls followed by secondary antibody before immobilisationonto glass slides. Nuclei are stained in blue (DAPI). Images were obtained using confocal microscopy at 40x magnification (section 2.6).

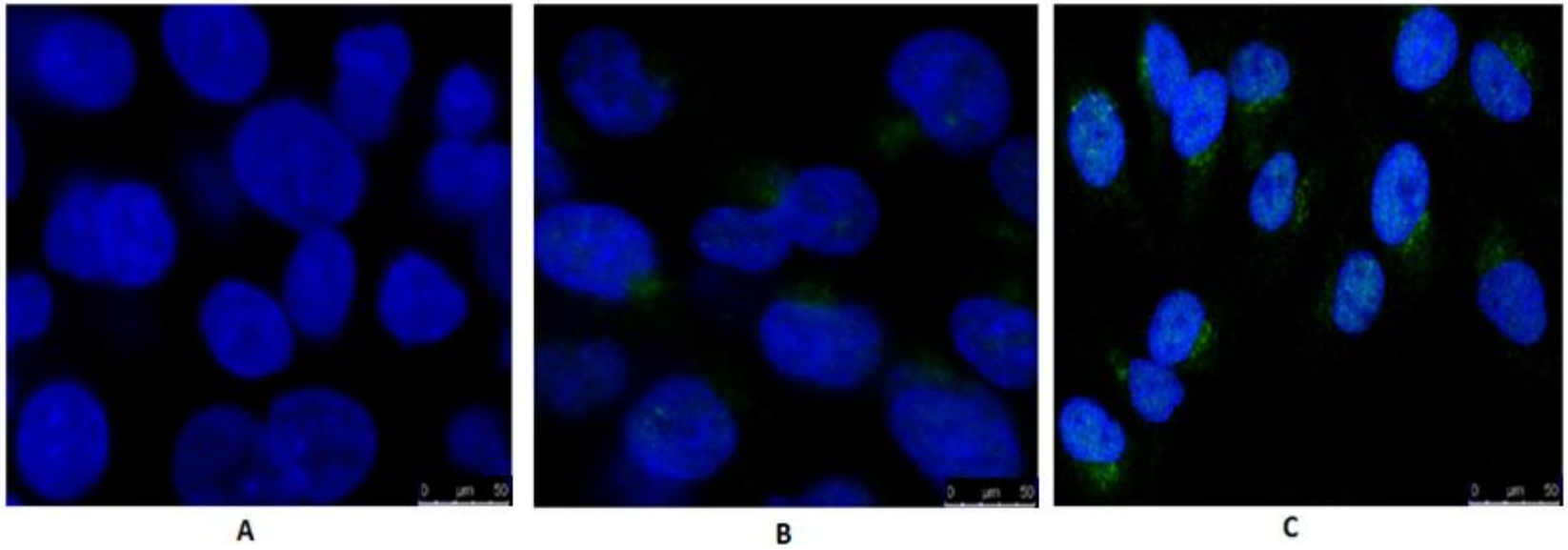

Figure 4.3.4.2b Immunofluorescence detection of IL-37 in RSV A2 infected BEAS-2B cells (24hr). BEAS-2B cells were infected with RSV A2 at MOI 1 for 24hr. (A) Isotype control of infected cells. (B) IL-37 protein was observed in infected cells (C) Culture cell were stimulated with (100ng/ml) of LPS for 18hrs in infected cells. Cells were stained with IL-37 antibody (green) and matched isotype controls followed by secondary antibody before immobilisation onto glass slides. Nuclei are stained in blue (DAPI). Images were obtained using confocal microscopy at 40x magnification (section 2.6).

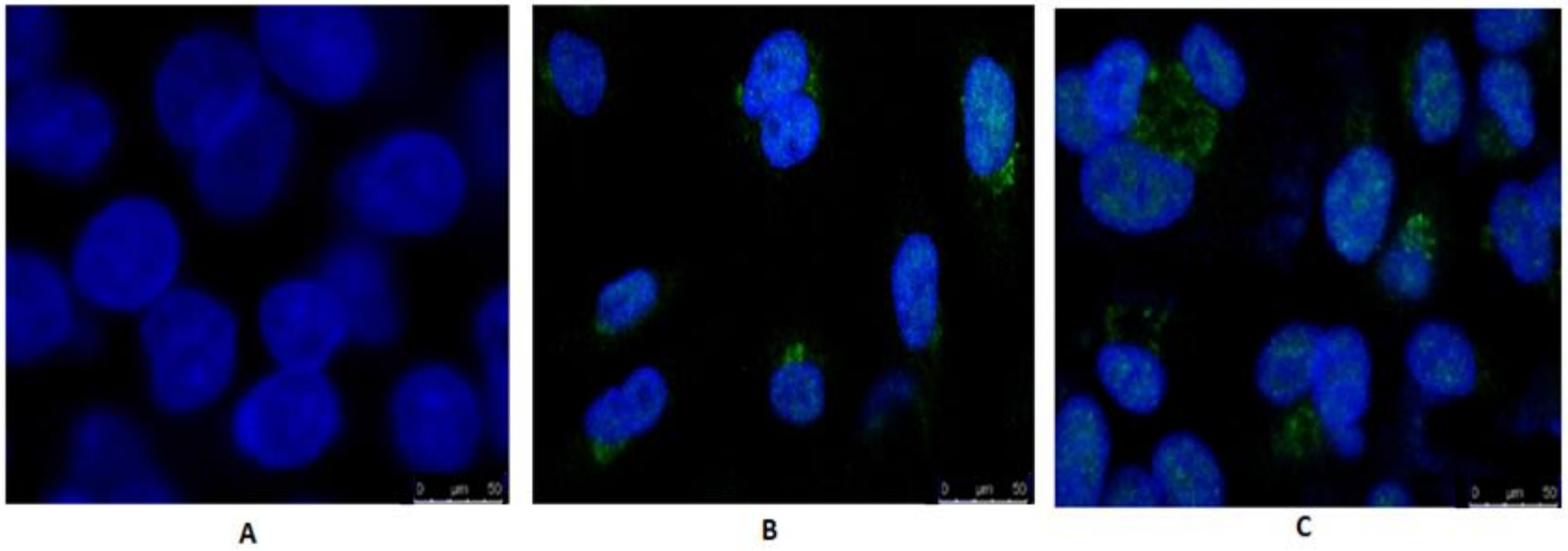

Figure 4.3.4.2c Immunofluorescence detection of IL-37 in RSV A2 infected BEAS-2B cells (48hr). BEAS-2B cells were infected with RSV A2 at MOI 1 for 48hr. **(A)** Isotype control of infected cells. **(B)** IL-37 protein was observed in infected cells **(C)** Culture cell were stimulatedwith (100ng/ml) of LPS for 18hrs in infected cells. Cells were stained with IL-37 antibody (green) and matched isotype controls followed by secondary antibody before immobilisationonto glass slides. Nuclei are stained in blue (DAPI). Images were obtained using confocal microscopy at 40x magnification (section 2.6).

4.4 Discussion

IL-37 plays a pivotal role in the pathogenesis of several infectious diseases. This chapter focused on the role of IL-37 in the immunopathogenesis of airway viral diseases. As mentioned in the introduction, few studies investigated the role of IL-37 cytokines during airway infection and their work to limit inflammation and protect lung tissue from viral infection. This chapter investigated the ability of RSV to induce the expression of IL-37 by human airway epithelial cells. The overexpression of cytokines and respiratory inflammation are known as critical pathogenesis mechanisms during respiratory viral infection (Wang *et al.,* 2017) and IL-37 is a potent suppressor against inflammation; therefore, this chapter rationalised that IL-37 may play a protective role during RSV infection and should be explored and considered for use in treatment. This study reported that among 163 patients with RSV and RV, elevated IL-37 responses probably favour the balance of innate responses and thus maintain internal homeostasis during infection. The investigators measured the level of IL-37 protein in nasopharyngeal aspirate infants with RSV and RV infection. HNAECs and bronchial epithelial cell line (A594 and BEAS-2B) cells were cultured *in vitro* and infected with increasing RSV at the indicated times. Then, the analysis assessed the response of HNAECs and cultured bronchial epithelial cell line (A594 and BEAS-2B) cells to RSV infection by expressing IL-37 cytokine and its receptors.

The NPAs of patients with RV diseases had significantly enhanced levels of IL-37 protein compared to patients with RSV diseases (Figure 4.3.1). An analysis of the age groups taking part in the study showed no significant difference in IL-37 protein levels between RSV ($p= 0.35$) and RV patient samples ($p= 0.09$) (Figure 4.3.1.1). The IL-37 count showed minimal overall deviation across all the age groups in both sample types. The severity levels of the RSV and RV diseases were analysed to ascertain the differences in IL-37 protein expression. The RSV severe group ($p<0.05$) recorded significantly lower expression in severe group in comparison to mild and moderate groups, which were of a similar level (Figure 4.3.1.2 A). The RV groups showed no significant difference in IL-37 levels. ($P= 0.9$) (Figure 4.3.1.2 B). The study analysed the connection between IL-37 protein production and oxygen administration (Figure. 4.3.1.3 A, B) in RSV and RV patient samples. These results revealed elevated levels of IL-37 protein in the moderate group in RSV patient samples, significantly higher than in the severe group,

suggesting that IL-37 can work as an immune regulatory cytokine in mild and moderate cases but not severe ones. Comparison of samples with oxygen requirement and without showed no significant difference in IL-37 expression (p= 0.8 and p= 0.6, respectively). In both RSV and RV patients' samples, there were no positive correlations between IL-37 protein and IL-36α (4.3.1.4 A, B). Similarly, there was no positive correlation between the level of IL- 37 protein and IL-36γ in RSV patient samples (P= 0.1, r= 0.2) (Figure. 4.3.1.5 A).

This study is the first to examine the relationship between RSV infection and IL-37 expression. Few studies focus on the role of IL-37 cytokines within respiratory viral infection. Although there are various microarray studies showing the role of RSV in inducing the expression of cytokines, IL-37 cytokine is not mentioned which suggests that there was no relationship between RSV infection and expression of IL-37 cytokines. In 2020, Li *et al.* demonstrated that SARS-CoV-2 infection causes elevation of plasma IL-37; higher early IL-37 responses correlated with earlier viral RNA negative conversion. They also showed that higher IL-37 was associated with lower IL-6 and IL-8 and higher IFN-α responses. In addition, they observed that IL-37 administration attenuated lung inflammation and alleviated respiratory tissue damage in human angiotensin-converting enzyme 2–transgenic mice infected with SARS-CoV-2. These results indicated that IL-37 is a robust suppressor of inflammation and may play a protective role during SARS-CoV-2 infection by restraining the occurrence of cytokine storms and should, therefore, be explored for treatment. In addition, Qi *et al.* (2019) confirmed that treating BALB/c mice infected with influenza A (H1N1) with IL-37 increases the survival rate and body weight, reduces the pulmonary index, impairs lung injury and decreases the production of pro-inflammatory cytokines in the BALF and lung tissue. They also found that IL-37 administration enhances the percentage of macrophages, suggesting that IL-37 treatment can ameliorate influenza pneumonia by attenuating cytokine production, especially by macrophages. Thus, IL-37 might serve as a promising new solution for the treatment of influenza A-induced pneumonia.

RSV induces the expression of IL-37 mRNA in human nasal airway epithelial cells (HNAECs) at levels 0.2-1% of the L32 housekeeping gene. In this study, the administration of RSV resulted in significant upregulation of IL-37 mRNA in HNAECs only at MOI 2.5 ($p<0.01$) in comparison with

non-infected nasal cells (Figure 4.3.2.1 A). Similarly, the bronchial epithelial cell line showed a significant increase in IL-37 mRNA expression at levels ~0.1-0.3% in A549 and ~1-3% in BEAS-2B cells of the L32 housekeeping gene only at MOI 2.5 ($p<0.05$), respectively (Figure 4.3.3.1 A, B). In the BEAS-2B cell line, RSV stimulated IL-37 mRNA expression, which reached its peak at 48 hours ($p<0.05$) in comparison with non-infected cells (Figure 4.3.3.1 C).

These results reflect that RSV infections activate the signalling pathways of IL-37 cytokine in airway epithelial cells and result in the expression of host genes such as antiviral interferons (IFN-α/β) and pro-inflammatory cytokines such as IL-6 and TNF-α (Ramaswamy *et al.,* 2004). Thus, the pro-inflammatory stimuli triggered by pro-inflammatory cytokines, can induce the expression of IL-37 cytokine, which is a self-protective mechanism against uncontrolled inflammation and excessive tissue damage. In addition, IL-37 is constitutively expressed in several types of human tissue and cells, which may help in the maintenance of the immune homeostasis. Although IL-37 constitutive expression is relatively low, the inducible expression of IL-37 can exert a powerful anti-inflammatory effect or immune regulatory role (Su and Tao *et al.,* 2021).

The results are consistent with previous studies demonstrating that IL-37 levels in the sera and PBMCs of patients infected with Influenza A virus (IAV) were higher than those of healthy subjects. The expression of IL-37 mRNA and protein in IAV-infected A549 cells and PBMCs showed upregulation, and IL-37 protein was able to inhibit the replication of IAV RNA (Zhou *et al.,* 2019). Moreover, RAW macrophages transfected with human IL-37 (RAW 264.7) could express IL-37 mRNA after stimulation with S. pneumoniae (Schauer *et al.,* 2017). Therefore, IL-37 could be used as a therapeutic agent in decreasing the pulmonary damage caused by microorganism infection. In addition, IL-37 mRNA expression significantly increased in PBMCs compared with non-infected patients, and the steady-state level of IL-37 mRNA in PBMCs positively correlated with HIV viral load (Hojen *et al.,* 2015). It has been found that IL-37 suppresses HIV replication, which suggests the potential role of IL-37 in treating HIV infection (Hojen *et al.,* 2015).

There was no significant observable difference in expression of IL-1R8 mRNA during RSV infection in human nasal airways epithelial cells (Figure 4.3.2.2 A). Conversely, In A549 cells, the expression of IL-1R8 mRNA was significantly upregulated at MOI 2.5 ($p<0.05$) and in BEAS-2B cells was at 1 and 2.5 MOI ($p<0.05$) in comparison with non-infected nasal cells (Figure 4.3.3.3 A, B). IL-18R1 mRNA levels in HNAECs displayed significant upregulation at MOI 2.5 ($p<0.05$) (Figure 4.3.2.3 A). Similarly, in A549 and BEAS-2B cells, there was a significant increase in IL-18R1 mRNA ($p<0.01$) compared with non-infected cells (Figure 4.3.3.4 A, B).

These finding indicate that there was an increased expression of IL-1R8 in culture cells in response to RSV infection but they were minimal. These results align with previous studies on pulmonary aspergillosis in mice, whereby IL-37 cannot trigger its anti-inflammatory effect on NALP3 inflammasomes in TIR-8/SIGIRR deficient mice (Moretti *et al.* 2014). Nold *et al*. (2003) report that in combination with IL-18Rα, IL-37 can exert its anti-inflammatory effect, whereas the anti-inflammatory effect of IL-37 disappeared in SIGIRR knockout mice (IL-37tg-SIGIRR-KO mice). Nold-Petry *et al.* (2014) discovered that IL-1R8 and IL-18Rα were required for IL-37 to show anti-inflammatory effects *in vivo* and *in vitro*. Therefore, the result in this study shows that IL-1R8 production may be dependent on the expression of IL-37 cytokine and suggests that the low level of IL-1R8 in culture cells is due to a deficiency of IL-37 protein. Additionally, the low expression of IL-37 cytokine may be because the stability of IL-37mRNA is weak and easy to degrade. Furthermore, when IL-37 is created in response to an RSV infection and secreted into the IL-37 precursors and the mature forms, the precursor is less efficient in binding to the receptor (Bulau *et al.,* 2014).

Several studies suggest that IL-1R8 may play a potential role in the inflammatory response during Mycobacterium tuberculosis infection. Garland's study shows an aggressive inflammatory response, identified by induced macrophage and neutrophil lung infiltration and enhanced inflammatory cytokines, which increased mortality in IL-1R8-deficient mice (Garlanda *et al.,* 2007). In P. aeruginosa lung infections, the level of mortality was increased with bacterial load and the level of pro-inflammatory cytokines (Veliz *et al.,* 2012). Moreover, in Aspergillus fumigatus infections, there was increased mortality and susceptibility to lung infections in IL-1R8-deficient mice (Bozza *et al.,* 2008).

This study recorded a significant increase in extracellular IL-37 protein levels in RSV-infected cells compared to non-infected cells of HNAECs supernatant ($p< 0.05$) (Figure 4.3.2.4 A). Conversely, no significant increase was observed in the level of IL-37 protein in A549 and BEAS-2B culture cells during RSV infection in either supernatant or cell pellet (Figure 4.3.3.5 A, B). In contrast, there was no significant expression on the level of IL-37 protein in the cell pellet of HNAECs during RSV infection (Figure 4.3.2.4 B). Similarly, there was no significant increase in IL-37 protein level between infected cells groups compared to non-infected control cells in A549 and BEAS-2B cell pellets (Figure 4.3.3.5 C, D).

The study results revealed a cogent increase of IL-37 protein in primary HNAECs but not in A549 or BEAS-2B culture cells during RSV infection. These results may be because the primary HNAECs are natural cells that can respond to the RSV compared to the culture cells. A previous study demonstrated that IL-37 treatment increases the survival rate and body weight, reduces the pulmonary index, impairs lung injury, and decreases the production of pro-inflammatory cytokines in the BALF and lung tissue in influenza-infected mice (Qi *et al*., 2019). The results suggest that the pro-inflammatory cytokines expressed in post-RSV infection could stimulate the expression of IL-37 during RSV infection. In addition, TLRs activated upon the airway cells infected by RSV can also induce the expression of IL-37, which can work as a negative feed loop and downregulate the overexpression of pro-inflammatory cytokines in airway epithelial cells.

With regard to the immunofluorescence staining of RSV-infected BEAS2B cells after 12 and 24 hours, it was noticed that IL-37 protein was expressed and localised around the nucleus in contrast to non-infected cells (Figures 4.3.4.1a and b, Figure 4.3.4.2a and b). Conversely, no IL-37 protein staining was observed around the nucleus in infected and non-infected cells at 48 hours (Figure 4.3.4.1c, Figure 4.3.4.2c). Notably, stimulation by LPS enhances more expression of IL-37, which appears as strong positive staining and could be secreted around the cells in both infected and non-infected cells at 12 hours (Figure 4.3.4.1c, C) and 24 hours (Figure 4.3.4.2c, C).

The results showed that IL-37 protein around the nucleus and the amount of IL-37 protein was about the same in both infected and non-infected cells. LPS treatment induces a stronger stain of IL-37 protein than in the group without stimulation, but this stain is still the same in both infected and non-infected cells. Indeed, LPS can enhance the stability of IL-37 mRNA and therefore express more levels of IL-37 protein (Bufler *et al*., 2004; Boraschi *et al.,* 2011). The results have a limitation: IL-37 could break down quickly. IL-37 protein localises around the nucleus suggesting that it does not translocate to the nucleus, and this may be due to the culture cells, which are not primary cells; about 25% of the mature form of IL-37 binds with SMAD-3 in the cytoplasm, translocates to the nucleus, and inhibits transcription of genes for several pro-inflammatory cytokines and chemokines (Bulau *et al.,* 2015). Another limitation of these results is the stain of RSV, which cannot indicate the effect of the virus on the cells. In addition, the translocation of IL-37 protein to the nucleus, as the second signal to be produced and secreted, may need more induction. Furthermore, it could be argued that there was no difference between infected and non-infected cells; it may be that IL-37 protein is breaking down quickly or was never translated. In conclusion, the *in vitro* and *in vivo* experiments confirmed that RSV can upregulate the expression of IL-37 and that IL-37 could conversely inhibit the replication of RSV. This finding could help to understand the pathogenesis of RSV and the development of antiviral therapies

4.5 Summary

The expression of IL-37 protein was higher in RV than in RSV patients' samples. In age groups and oxygen requirements of NPAs, there was no significant difference in the level of IL-37 protein in both RSV and RV patient samples. For severity groups, NPA samples from infants infected with RSV show significantly lower IL-37 protein levels in the severe group compared with mild and moderate groups. In addition, no positive correlation was observed between either IL-36α or IL-36γ with IL-37 in both RSV and RV patient samples.

RSV infection of HNAECs induces the expression of IL-37 mRNA. There was a significant expression of IL-37 receptor IL-18R1 but not IL-1R8 in HNAECs. In addition, there was a significant increase in the IL-37 protein level supernatant of cell culture.

RSV infection of the culture airway epithelial cell line (A549 and BEAS-2B) induces the expression of IL-37 mRNA. There was a significant expression of IL-37 in both culture cell lines (A594 andBEAS-2B) at 48 hours after RSV infection. For IL-37 Isoforms, there was a significant increase in IL-37 mRNA expression for IL-37b and IL-37c but not IL-37a isoforms. Regarding IL-37 receptors, there was a significant increase in IL-1R8 and IL-18R1 mRNA expression in both cell cultures (A549 and BEAS-2B). In contrast to HNAECs, no significant increase in the IL-37 protein level was observed in both culture cells (A549 and BEAS-2B).

After RSV infection, IL-37 protein was expressed and localised around the nucleus in infected and non-infected cells. The propensity of BEAS-2B cells to produce more IL-37 protein stain was observed at 12 and 24 hours but not at 48 hours. Overall, the results demonstrate that RSV infection stimulates airway epithelial cells to express IL-37 cytokines in HNAECs and culture cells.

Chapter 5: The Effect of Other Cytokines on Expression of IL-36 gamma (IL-36γ)

5.1 Introduction

As Chapter 3 shows, during RSV infection the expression of IL-36 cytokines and IL-36γ is selectively induced in epithelial cells, as induced in A549 cells more than BEAS-2B cells. Currently, there are few reports regarding the induction of IL-36 cytokines by other inflammatory cytokines in pulmonary airways. Among the various cytokines, proinflammatory cytokines such as TNF-α and IL-1β play a primary role in the infection and expressed in epithelial cells and the most of innate immune cells such as neutrophils, macrophages, NK cells and dendritic cells (Kany *et al.,* 2019). Previous study identified how IL-1β is a strong inducer of IL-36γ in colonic myofibroblasts (Takahashi *et al.*, 2015). Meanwhile, Kovach *et al.* (2016) reported that the expression of IL-36γ mRNA was upregulated significantly when stimulating pulmonary macrophages with IL-1β and TNF-α cytokines stimulation. Carrier *et al*. (2011) also found that IL-1β and TNF-α cytokines induce the expression of IL-36 cytokines' mRNA in keratinocytes. Furthermore, IL-1β has been shown to induce the expression of IL-36γ in human colonic myofibroblasts (Takahashi *et al.,* 2015) thus suggesting that the IL-1β cytokine could be involved in the expression of IL-36γ cytokines. On the other hand, TNF-α is a central mediator of airway inflammation, inflammatory cell infiltration response, vascular permeability, and chemokine release, and may play a role in the immunopathogenesis of RSV infection (Choi *et al.,* 2010). Boutet *et al.,* study observed that a positive correlation between the expression of IL-36 α, IL-36γ and IL-36Rα, and IL-1β and Th17 cells cytokines (IL-17A, IL-22, IL-23, CCL20) in human psoriasis (Boutet *et al.,* 2016). In human keratinocytes, it has been found that TNF-α, IL-17A and IL-22 induce the expression of IL-36α and IL-36γ while, IFNγ only induce the expression of IL-36β cytokine (Carrier *et al.,* 2011). Taken together, their findings prompted me to examine whether this combination could be a good stimulant for expressing IL-36γ in airway epithelial cells as a source of IL-36γ.

The aims of this chapter was to investigate whether primary inflammatory cytokines such as TNF-α and IL-1β are good stimulators of IL-36γ in the airway epithelial cells. Additionally, this chapter also focused on the expression of IL-36γ by A549 cells resulting from the induction from Th1, Th2 and Th17 cells cytokines including IL-17, IL-22, IFNγ, IFNβ and IL-4 either

separately or combined with the primary cytokines IL-1β and TNF-α. Nonetheless, exactly how inflammation in airway epithelial cells is affected by IL-1β and TNF-α is still unascertained.

Hypothesis: That regarding the induction of IL-36 expression, a key role is played by proinflammatory cytokines such as IL-1β and TNF-α. The combination of either IL-1β or TNF-α with other Th1, Th2 and Th17 cytokines such as IFNγ, IL-4, IL-17 and IL-22 cytokine could also stimulate the expression of IL36γ during RSV infection.

5.2 Objectives

- To determine the influence of the inflammatory cytokines including IL-1β, TNF-α, IL-17, IL-22, IFNγ, IFNβ and IL-4 cytokines on expression of IL-36γ by A549 cells, alongside assessing the optimum time and concentration of each cytokine.

- To investigate the optimum time (3hrs, 6hrs, and 24hrs) and concentration (1, 5, 10, 50, 100 and 200 ng/ml) of IL-1β cytokine required to stimulate the expression of IL-36γ.

- To determine whether the combination of IL-1β cytokines with TNF-α, IL-17, IL-22, IFNγ, IFNβ, and IL-4 cytokines in addition to LPS could induce the expression of IL-36γ mRNA and protein by A549 cell lines.

- To determine whether the synergistic of TNF-α cytokines with IL-17, IL-22, IFNγ, IFNβ, and IL-4 cytokines in addition to LPS could induce the expression of IL-36γ mRNA and protein by A549 cell lines.

- To investigate the synergistic effect of stimulation by IL-1β cytokines with TNF-α, IL-17, IL-22, IFNγ, IFNβ, and IL-4 cytokines in addition to LPS could induce the expression of IL-36γ mRNA and protein by infected A549 cell lines.

- To determine whether the synergistic effect of stimulation by TNF-α cytokines with IL-17, IL-22, IFNγ, IFNβ, and IL-4 cytokines in addition to LPS could induce the expression of IL-36γ mRNA and protein by infected A549 cell lines.

5.3 Results

5.3.1 Effect of pro-inflammatory cytokines (L-1β, TNF-α, IL-17, IL-22, IFNγ, IFNβ, IL-4) and LPS on the expression of IL-36γ in A549 cells (Dose/ time) depend manner

5.3.2 Effect of IL-1β on expression of IL-36γ mRNA: IL-1β (dose/ Time) dependent manner

5.3.3 The effect of cytokines combination on expression of IL-36γ mRNA in airways epithelial cells

5.3.3.1 The effect of IL-1β with other cytokines and LPS combination on expression of IL-36γ mRNA in A549 cells

5.3.3.2 The effect of TNF-α with other cytokines and LPS combination on expression of IL-36γ mRNA in A549 cells

5.3.4 The effect of cytokines combination on expression of IL-36γ mRNA in RSV infected airways epithelial cells

5.3.4.1 The effect of IL-1β with other cytokines and LPS combination on expression of IL-36γ mRNA in RSV infected A549 cells

5.3.4.2 The effect of TNF-α with other cytokines and LPS combination on expression of IL-36γ mRNA in RSV infected A549 cells

5.3.5 The effect of cytokines combination on expression of IL-36γ protein in airways epithelial cells

5.3.5.1 The effect of IL-1β with other cytokines and LPS combination on expression of IL-36γ protein in A549 cells

5.3.5.2 The effect of TNF-α with other cytokines and LPS combination on expression of IL-36γ protein in A549 cells

5.3.6 The effect of cytokines combination on expression of IL-36γ protein in RSV infected airways epithelial cells

5.3.6.1 The effect of IL-1β and other cytokines combination on expression of IL-36γ protein in infected A549 cells

5.3.6.2 The effect of TNF-α and other cytokines combination on expression of IL-36γ protein in infected A549 cells

5.3.1 Effect of pro-inflammatory cytokines (L-1β, TNF-α, IL-17, IL-22, IFNγ, IFNβ, IL-4) and LPS on the expression of IL-36γ in A549 cells (Dose/ time dependant)

A549 cells were incubated with 200 ng/ml per cytokine for varying times – as presented in 5.3.1 – to assess how the expression of IL-36γ mRNA in cultured airway epithelial cells was influenced by different cytokines' stimulations. Subsequently, A549 cells were stimulated with IL-1β, TNF-α, IL-17, IL-22 IFNγ, IFNβ, IL-4 and LPS for different lengths of time (3, 6, 12, 24, and 48hrs). IL-1β stimulation significantly induced IL-36γ mRNA expression in a time dependent manner at 3hrs ($P<0.0001$), 6hrs ($P<0.0001$), 12hrs ($p<0.001$) and 24hrs ($p<0.05$). Furthermore, the expression of IL-36γ mRNA reached high levels at 3hrs which increased 139-fold relative to the control, and 116-fold relative to the control at 6hrs ($P<0.0001$), 81-fold at 12hrs ($p<0.01$), and 60-fold at 24hrs ($p<0.05$).However, this expression was not significant relative to the control – as presented in Figure 5.3.1A – when increased 12-fold to forty-eight hours. Additionally, the stimulation of A549 cells by TNF-α only significantly induced the expression of IL-36γ mRNA at 6hrs, increasing to 31-fold higher relative to the control ($P<0.001$) (Figure 5.3.1 B).

Aside from this, there was no significant change on expression of IL-36γ mRNA when stimulating A549 cells with IL-17 ($p=0.1$) (Figure 5.3.1 C), IFNγ ($P= 0.2$) (Figure 5.3.1 D), or IL-22 ($P=0.08$), with the exception of 48hrs of incubation with IL-22 cytokines, which significantly reduced ($p<0.05$) (Figure 5.3.1 E). Moreover, there was no significant change on the expression of IL-36γ when stimulating the cells with LPS, IFNβ, or IL-4 ($p=0.09$) (Figure 5.3.1 F, G, H), (Two-way RM ANOVA) (Figure 5.3.1).

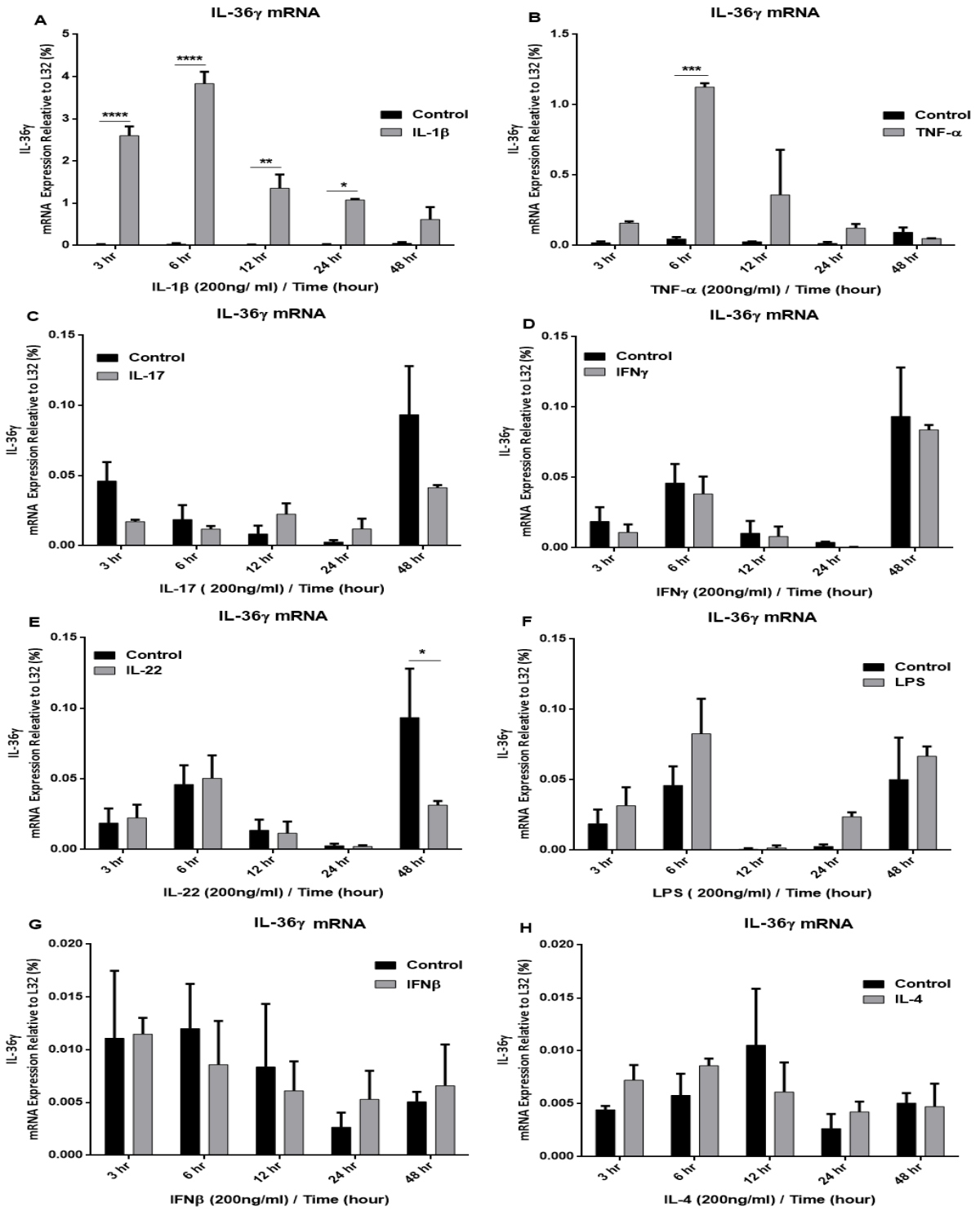

Figure 5.3.1 Effects of cytokines on expression of IL-36γ mRNA by airways epithelial cells. A549 cells were incubated with various cytokines and LPS at 3hrs, 6hrs, 12hrs, 24hrs and 48hrs, **A)** IL-1β, **B)** TNF-α, **C)** IL-17, **D)** IFNγ, **E)** LPS, **F)** IL-22, **G)** IFNβ, and **H)** IL-4 utilised in comparison to unstimulated control cells. IL-36γ mRNA expression was expressed relative to L32 mRNA level (mean ± SEM from three different experiments), facilitated by the use of two-way RM ANOVA between groups and Sidak's multiple comparisons test to determine the statistical significance, and evaluate adjusted P value, represented as *p<0.05, **p<0.001, ***p<0.0001 and ****P<0.00001).

5.3.2 Effect of IL-1β on expression of IL-36γ mRNA and IL-1β (dose/ Time dependent)

The expression of IL-36γ mRNA through A549 cells owing to the ability of IL-1β cytokines to induce has been revealed in previous studies. In order to determine the optimum concentration of IL-1β cytokine required to induce IL-36γ mRNA expression, A549 cells were stimulated with IL1-β at concentrations of 1 ng/ml, 5 ng/ml, 10 ng/ml, 50ng/ml, 100 ng/ml and 200 ng/ml for 3 hrs, 6hrs, and 24hrs. Furthermore, significant expression of IL-36γ mRNA (Figure 5.3.2) was determined in each concentration with different time points. Subsequently, compared to IL-36γ mRNA expression without stimulation, with stimulation there is increased expression after 3 hours ($P<0.0001$) and 6 hours ($P<0.05$), following the stimulation of A549 cells with IL-1β at a concentration of 1 ng/ml. Moreover, IL-36γ mRNA expression was 112- and 31-fold higher at 3hrs and 6hrs respectively, whereas there was no significant increase of IL-36γ mRNA after 24hrs of incubation compared to the expression of IL-36γ mRNA at the same time point without any stimulation (Two-way RM ANOVA/Sidak's multiple comparisons test) (Figure 5.3.2 A).

Meanwhile, the stimulation of A549 cells with 5ng/ml of IL-1β concentration saw IL-36γ mRNA expression increase significantly after 6hrs ($P<0.05$) and 24hrs ($p<0.0001$) compared to the expression of IL-36γ mRNA with no stimulation (two-way RM ANOVA/Sidak's multiple comparisons test) (Figure 5.3.2 B). Subsequent to stimulation of A549 cells with IL-1β at 10ng/ml concentration, IL-36γ mRNA expression increased significantly after 3hrs ($P<0.0001$) and 6hrs ($p<0.0001$) compared to the expression of IL-36γ mRNA without any stimulation (two-way RM ANOVA/Sidak's multiple comparisons test) (Figure 5.3.2 C). Furthermore, stimulation with 50ng/ml resulted in IL-36γ mRNA expression being increased significantly after 3hrs ($P<0.0001$) and 6hrs ($p<0.0001$) compared to the expression of IL-36γ mRNA without stimulation (Two-way RM ANOVA/Sidak's multiple comparisons test) (Figure 5.3.2 D).

Compared to the expression of IL-36γ mRNA with no stimulation, expression was increased significantly at the 3hr ($P<0.0001$) and 6hr ($p<0.0001$) points as a result of A549 cells' stimulation with 100ng/ml of IL-1β concentration. Indeed, IL-36γ mRNA expression was 86- and 87-fold higher at 3hrs and 6hrs respectively, compared to the expression of IL-36γ mRNA at the same time point without stimulation (two-way RM ANOVA and Sidak's multiple comparisons test) (Figure 5.3.2 E). Furthermore, Figure 5.3.2 F demonstrates that the

stimulation of A549 cells with 200ng/ml of IL-1β concentration saw IL-36γ mRNA expression increase significantly at 6hrs ($p<0.05$) compared to the expression of IL-36γ mRNA without stimulation. However, as evidenced through the two-way RM ANOVA and Sidak's multiple comparisons test (Figure 5.3.2.F), there was no significant stimulation observed after either three or twenty-four hours.

To conclude, the stimulation of A549 cells with 100ng/ml of IL-1β was the optimum concentration, especially after six hours, ultimately stimulating a high level of IL-36γ mRNA; additionally, this occurred at the 3hr point when stimulating A549 cells with 1ng/ml of IL-1β.

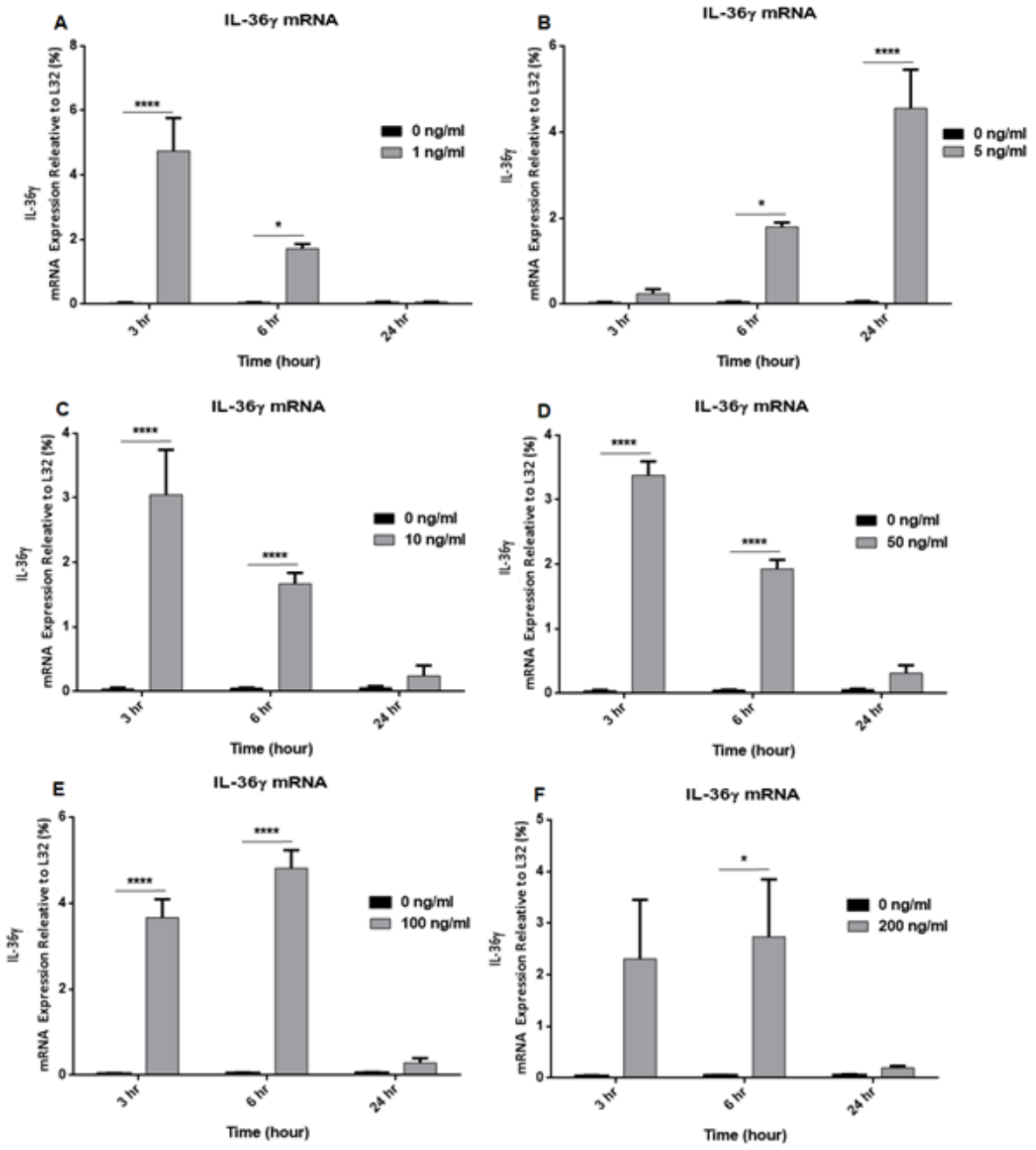

Figure 5.3.2 Effects of IL-1β concentration on expression of IL-36γ mRNA by airways epithelial cells. A549 cells were incubated for 3hrs, 6hrs and 24hrs with increasing concentrations of IL-1β, **A)** 1ng/ml, **B)** 5ng/ml **C)** 10ng/ml, **D)** 50ng/ml, **E)** 100ng/ml, **F)** 200ng/ml, compared to unstimulated cells. Subsequently, IL-36γ mRNA expression was expressed relative to L32 mRNA expression (mean ± SEM from three different experiments), facilitated by the use of two-way RM ANOVA between groups and Sidak's multiple comparisons test to determine the statistical significance, and evaluate adjusted P value, represented as *p<0.05, **p<0.001, ***p<0.0001, and ****P<0.00001).

5.3.3 The effect of cytokines combination on expression of IL-36γ mRNA in airways epithelial cells

The highest level of A549 cells' IL-36γ mRNA expression was induced by the IL1β and TNF-α cytokines, as presented in the results in section 5.3.1. To determine if these cytokines can stimulate a higher level of IL-36γ mRNA expression when combined with other cytokines, A549 cells were stimulated with various cytokines or LPS in combination at a 100 ng/ml concentration per cytokine and LPS. Specifically, the combinations used in this section were either IL-1β or TNF-α alone, or with IL17, IL-22, IFNγ, IFNβ, IL-4 and IL-1β with LPS for 6 hours. In each combination of the cytokines, it was determined that IL-36γ was expressed significantly (Figure 5.3.3).

5.3.3.1 The effect of IL-1β with other cytokines and LPS combination on the expression of IL36γ mRNA in A549 cells

There was no significant increase when stimulating A549 cells with the combination of IL-1β and TNF-α cytokines compared to the same cells when stimulated with either IL-1β alone ($p > 0.9$) or with TNF-α alone ($p = 0.2$) (Figure 5.3.3.1 A). However, Figure 5.3.3.1 B indicates a significant increase in the expression of IL-36γ mRNA when stimulating A549 cells with IL-1β and IL-17 cytokines compared to the same cells when stimulated with IL-17 cytokine alone ($p < 0.05$). Additionally, the combination of IL-1β with IL-22 demonstrates that there was a significant increase on IL-36γ mRNA expression by A549 cells ($p<0.05$) compared to the expression of the same cells with IL-22 cytokine alone, but *not* with IL-1β cytokine alone (Figure 5.3.3.1 C). Furthermore, IL-36γ mRNA expression was increased when stimulated by A549 cells with a combination of IL-1β and IFNγ, INFβ or IL-4 cytokines compared to the cells stimulated by IFNγ, IFNβ or IL-4 cytokine alone ($p < 0.05$) (Figures 5.3.3.1 D, F, and G). Finally, there was a significant increase on IL-36γ mRNA expression when stimulated by A549 cells with the combination of IL-1β and LPS compared to the expression of IL-36γ mRNA with LPS alone (Figure 5.3.3.1 E). This was facilitated by the use of one-way ANOVA, the Friedman statistic test, and Dunn's multiple comparisons test. In conclusion, there were a significant increases of IL-36γ mRNA when stimulated by A549 cells through a combination of IL-1β with other cytokines, including IL-17, IL-22, IFNγ, IFNβ, and IL-4.

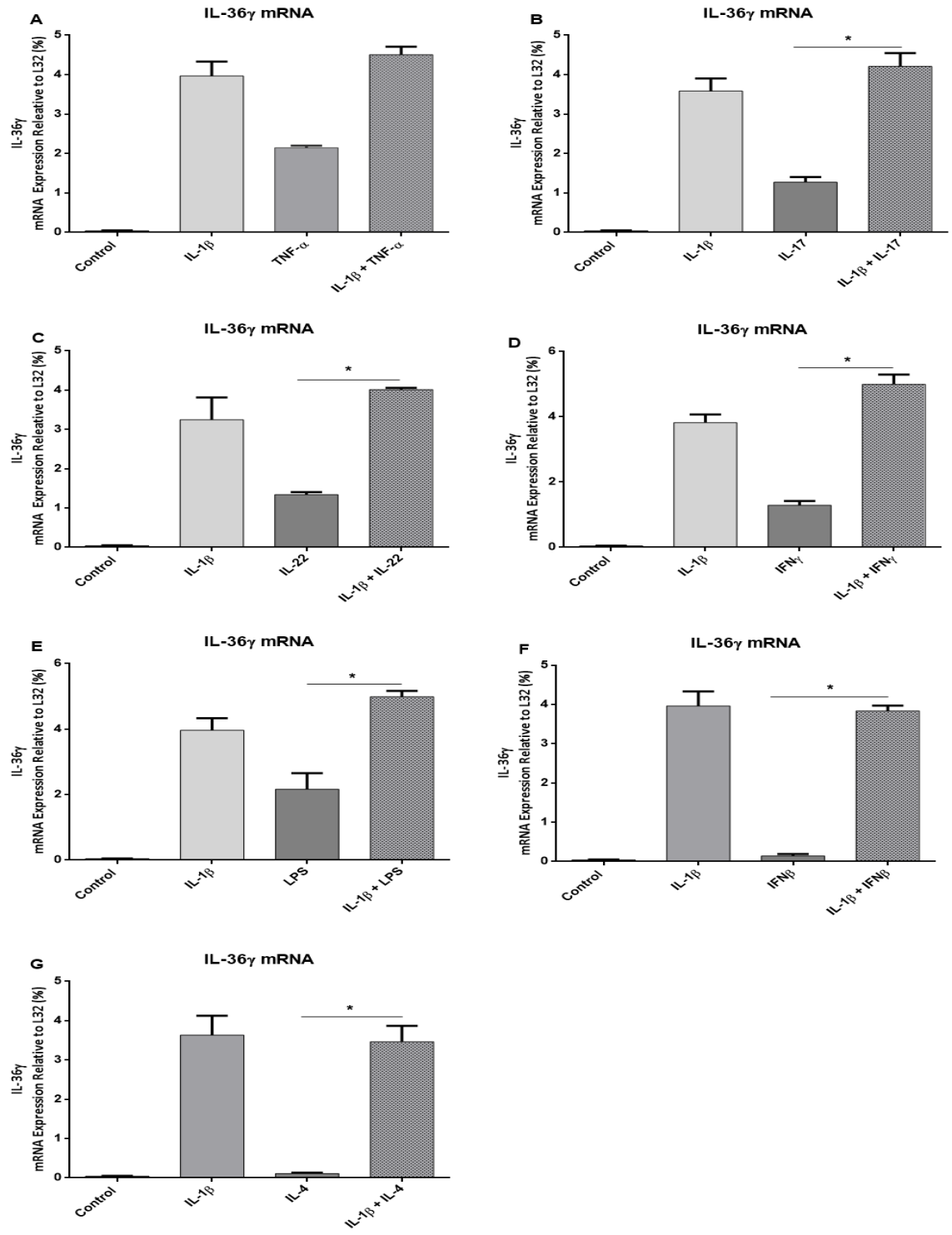

Figure 5.3.3.1 The effect of the combination of IL-1β with pro-inflammatory cytokines and LPS on expression of IL-36γ mRNA by airways epithelial cells. For six hours, various cytokine combinations of 100ng/ml were utilised to stimulate the A549 cells. These comprised IL-1β with **A)** TNF-α, **B)** IL-17, **C)** IL-22, **D)** IFNγ, **E)** LPS, **F)** IFNβ and **G)** IL-4 compared to unstimulated cells. IL-36γ mRNA expression was expressed relative to L32 mRNA expression (mean ± SEM from three different experiments, facilitated by the use of one-way ANOVA, Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group, *$p<0.05$).

5.3.3.2 The effect of TNF-α with other cytokines and LPS combination on the expression of IL-36γ mRNA in A549 cells

Previous results in Figure 5.3.1 demonstrated the ability of IL-1β and TNF-α cytokines to induce the expression of IL-36γ mRNA through A549 cells. Specifically, A549 cells were stimulated with 100ng/ml per cytokine for six hours in order to identify which combination of other cytokines are required to induce IL-36γ mRNA expression by those A549 cells. The A549 cells were stimulated with a combination of TNF-α with IL-17, IL-22, IFNγ, INFβ, IL-4, and LPS. Furthermore, as presented in Figure 5.3.3.2, in each combination of cytokines with TNF-α, a significant IL-36γ mRNA expression was identified. Specifically, Figure 5.3.3.2A revealed that there was a significant increase in IL-36γ expression when stimulating A549 cells with TNF-α and IL-17, compared to the expression of IL-36γ mRNA by A549 cells with the stimulation of IL-17 cytokine alone ($p< 0.05$). Similarly, the stimulated A549 cells with TNF-α combined with IL-22 cytokines revealed a significant increase in their expression of IL-36γ mRNA in A549 cells, compared to the expression of IL-36γ mRNA with IL-22 cytokine alone ($p< 0.05$) (Figure 5.3.3.2 B). Nonetheless, there was no significant increase on the expression of IL-36γ mRNA by TNF-α and IFNγ stimulated A549 cells ($p< 0.2$) (Figure 5.3.3.2 C). Meanwhile, IL-36γ mRNA expression was change when stimulating A549 cells with the combination of TNF-α and LPS, TNF-α with INFβ, or TNF-α with IL-4-compared to the expression of IL-36γ mRNA stimulated by LPS, INFβ and IL-4 cytokines alone through the A549 cells ($p< 0.05$) (Figure 5.3.3.2 D, E, F) (one-way ANOVA, the Friedman statistic test and Dunn's multiple comparisons test). Finally, the TNF-α cytokine alone can stimulate the expression of IL-36γ mRNA through A549 cells, and this expression was increased when boosting TNF-α with IL-17, IL-22, IFNβ, or IL-4 but not with IFNγ.

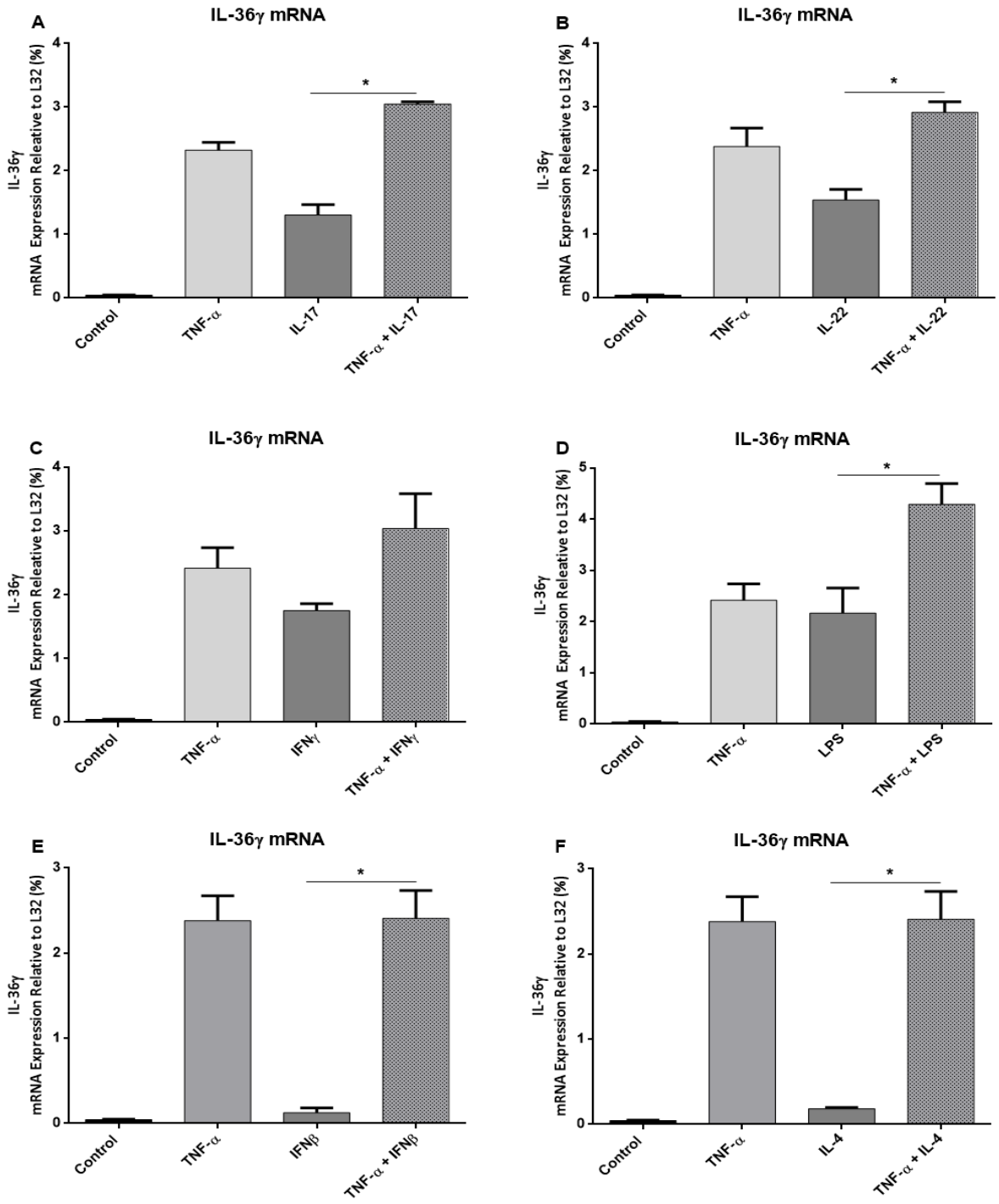

Figure 5.3.3.2 The effect of the combination of TNF-α, IL-17, IL-22, IFNγ, INFβ, IL-4 cytokines and LPS on the expression of IL-36γ mRNA by airways epithelial cells. For six hours, various cytokine combinations of 100ng/ml were utilised to stimulate the A549 cells. These comprised TNF-α with **A)** IL17, **B)** IL-22, **C)** IFNγ, **D)** LPS **E)** IFNβ, and **F)** IL-4, compared to unstimulated cells. Additionally, IL-36γ mRNA expression was expressed relative to L32 mRNA expression (mean ± SEM from three different experiments, facilitated by the use of one-way ANOVA, Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group, *p<0.05).

5.3.4 The effect of cytokines combination on expression of IL-36γ mRNA in RSV infected airways epithelial cells

Evidently, IL-36γ mRNA expression can be induced by RSV, as presented by the findings in section 3.3.3. Furthermore, as seen in section 5.2.3 IL-1β and TNF-α cytokines can also induce expression of IL-36γ mRNA through A549 cells. To determine if cytokines can stimulate IL-36γ mRNA expression either alone or in combination during an RSV infection of A549 cells, the A549 cells were infected with 1 MOI of RSV for twenty-four hours, and subsequently stimulated with various cytokines or LPS combined at a concentration of 100 ng/ml per cytokine and LPS. Specifically, the combinations used were both IL-1β and TNF-α, or together with IL-17, IL-22, IFNγ, INFβ, IL-4, and LPS for six hours. Notably, a significant expression of IL-36γ mRNA was determined in each combination of cytokines.

5.3.4.1 The effect of IL-1β with other cytokines and LPS combination on the expression of IL-36γ mRNA in RSV infected A549 cells

A549 cells were infected with 1 MOI of RSV for twenty-four hours to ascertain whether IL-36γ expression through infected A549 cells can be induced by cytokines. A549 cells were then stimulated with proinflammatory cytokines or LPS either alone or combined. The concentration used for each cytokine was 100 ng/ml for 6 hours, comprising IL-1β with TNF-α, IL-17, IL-22, IFNγ, INFβ, IL-4, and LPS. Subsequently, compared to the untreated group, each cytokine combination displayed significant IL-36γ mRNA expression. Meanwhile, there was no significant increase in IL-36γ mRNA expression when stimulating A549 cells with a combination of IL-1β and TNF-α compared to the expression of IL-36γ mRNA by IL-1β ($p= 0.6$) or TNF- ($p= 0.1$) stimulated A549 cells alone (Figure 5.3.4.1 A). Furthermore, Figure 5.3.4.1B showed that during RSV infection there was no significant difference observed on the expression of IL-36γ mRNA when stimulating A549 cells with IL-1β ($p= 0.6$) and IL-17 ($p= 0.4$) cytokines alone or combined. Likewise, there was no significant difference on the expression of IL-36γ mRNA when stimulating the infected A549 cells with IL-1β and IL-22 cytokines compared to the same cells when stimulated with IL-1β ($p= 0.6$) or IL-22 ($p= 0.4$) cytokines alone (Figure 5.3.4.1 C).

Moreover, no difference was observed in the combination of IL-1β and IFNγ cytokines, and IL-1β (p= 0.6), or IFNγ cytokine alone (p= 0.4) on the expression of IL-36γ mRNA through A549 cells during RSV (Figures 5.3.4.1 D). Regarding the stimulation of infected A549 cells with LPS, there was no change to the expression of IL-36γ mRNA with either LPS alone (p= 0.1) or combined with IL-1β (Figure 5.3.4.1 E). However, there was asignificant increase on expression of IL-36γ mRNA when stimulate A549 cells with IL-1β combined with INFβ cytokines in comparison to IL-1β or INFβ cytokines alone (p< 0.05) (Figure 5.3.4.1 F). However, no difference was observed in the combination of IL-1β and IL-4 cytokines, and IL-1β (p> 0.9), or IL-4 cytokine alone (p= 0.4) on the expression of IL-36γ mRNA through A549 cells during RSV (Figures 5.3.4.1 G). This was facilitated by the use of one-way ANOVA, the Friedman statistic test and Dunn's multiple comparisons test.

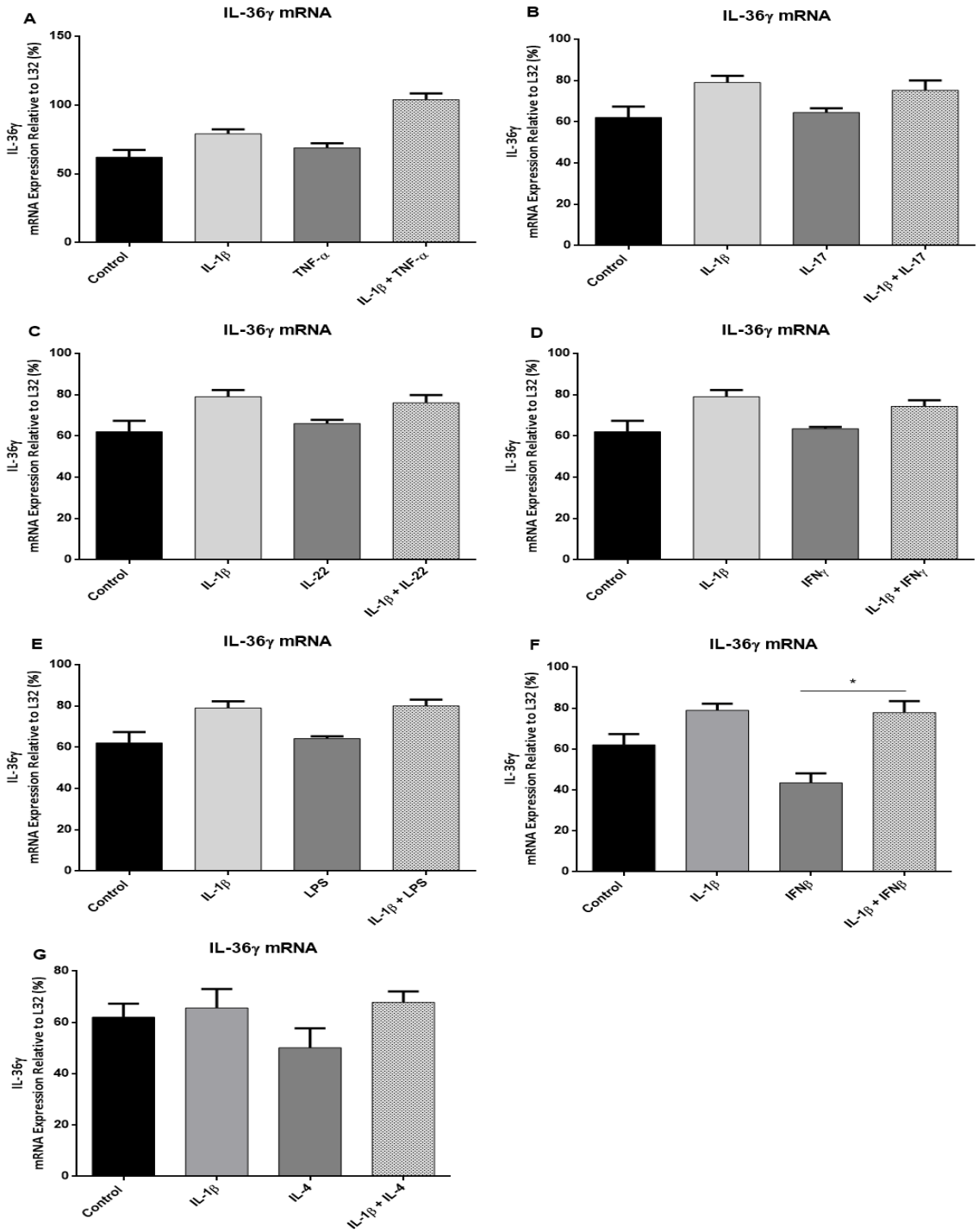

Figure 5.3.4.1 The effect of the combination of IL-1β, with other cytokines and LPS on IL36γ mRNA expression by infected airways epithelial cells. A549 cells were infected with 1 MOI of RSV for 48hr and then stimulated with various cytokines combinations at (100 ng/ml) for each cytokines at 6hr, IL-1β was combined with **A)** TNF-α, **B)** IL-17, **C)** IL-22, **D)** IFNγ, **E)** LPS **F)** IFNβ, and **G)** IL-4 in comparison to unstimulated cells. IL-36γ mRNA expression was expressed relative to L32 mRNA expression (mean ± SEM from three different experiments, facilitated by the use of one-way ANOVA, Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group, *p<0.05).

5.3.4.2 The effect of TNF-α with other cytokines and LPS combination on expression of IL-36γ mRNA in RSV infected A549 cells

For twenty-four hours the A549 cells were infected with MOI of RSV to ascertain whether IL-36γ mRNA expression could be induced by cytokines. Either alone or in combinations, they were subsequently stimulated with both proinflammatory cytokines and LPS alone. The concentration used for each cytokine was 100 ng/ml for six hours, comprising combinations of TNF-α with IL-17, IL-22, IFNγ, IFNβ, IL-4, and LPS.

Subsequently, significant expression of IL-36γ mRNA was determined in each combination of cytokines compared to the untreated control group. Furthermore, there was no significant increase of IL-36γ mRNA expression when stimulating the infected A549 cells with the combination of TNF-α with IL-17, compared to the same cells when stimulated with either TNF-α ($p > 0.9$) or IL-17 alone ($p = 0.1$) (Figure 5.3.4.2 A). Additionally, there was no significant increase in IL-36γ mRNA expression when stimulating the infected A549 cells with the combination of TNF-α and IL-22 cytokines compared to stimulation with either TNF-α ($p = 0.4$) or IL-22 ($p = 0.1$) cytokines alone (Figure 5.3.4.2 B). Meanwhile, IL-36γ mRNA expression from infected A549 cells cannot be increased from a combination of TNF-α and IFNγ cytokines, as presented in Figure 5.3.4.2 C). Likewise, the stimulation of infected A549 cells with the combination of TNF-α and LPS demonstrated that there was no change to the expression of IL-36γ mRNA when stimulating the same cells with either TNF-α ($p = 0.4$) or LPS alone ($p = 0.1$) (Figure 5.3.4.2 D). However, IL-36γ mRNA expression from infected A549 cells increased from a combination of TNF-α with IFNβ or TNF-α with IFNβ cytokines alone ($p < 0.05$), as presented in Figure 5.3.4.2 E). Meanwhile, IL-36γ mRNA expression from infected A549 cells cannot be increased from a combination of TNF-α ($p > 0.9$) and IL-4 cytokines ($p = 0.1$), as presented in Figure 5.3.4.2 F). This was facilitated through the use of one-way ANOVA, the Friedman statistic test and Dunn's multiple comparisons test.

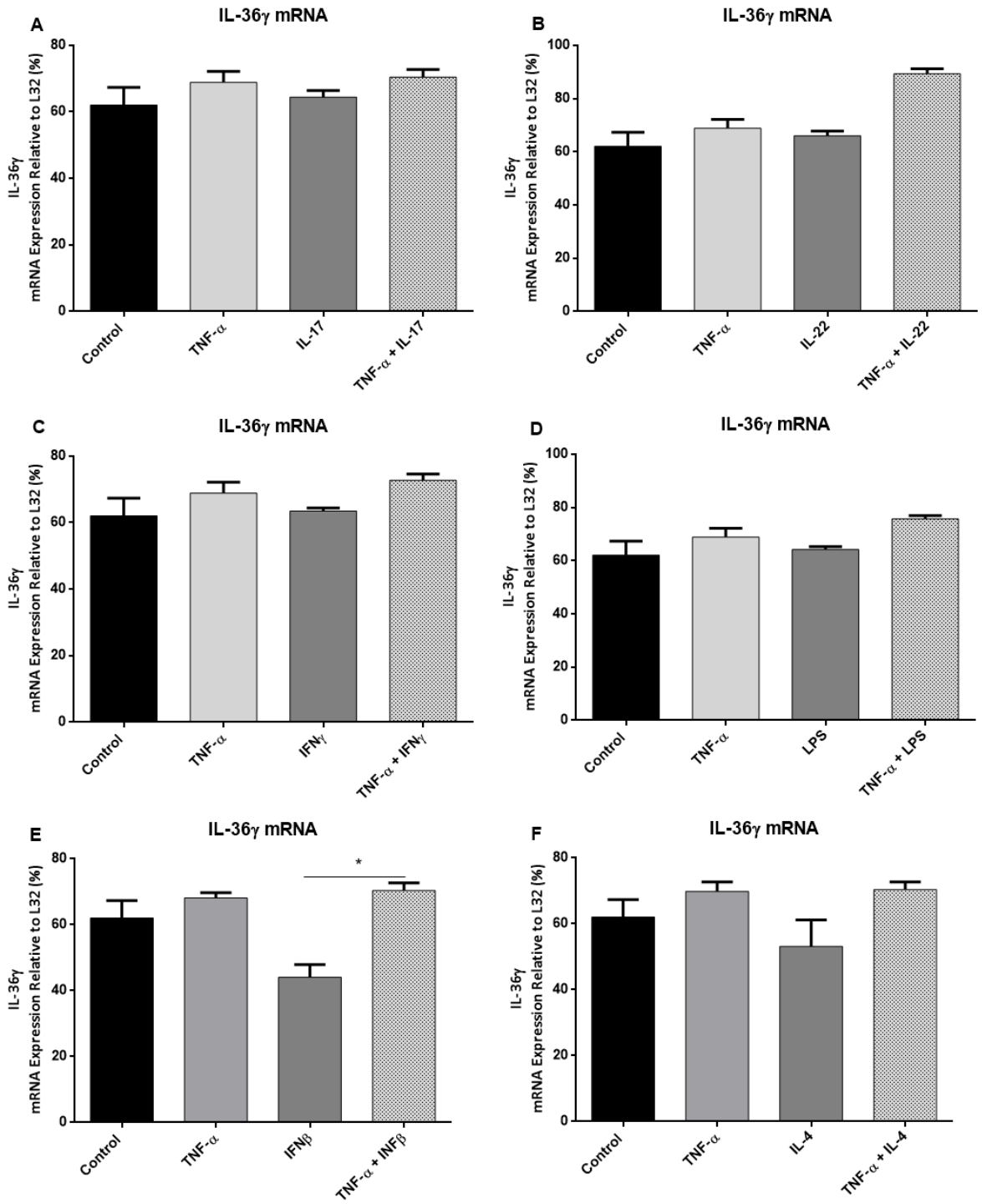

Figure 5.3.4.2: The effect of the combination of TNF-α with other cytokines and LPS on IL36γ mRNA expression through infected airway epithelial cells. For six hours, various cytokine combinations of 100ng/ml were utilised to stimulate A549 cells that had been infected with 1 MOI of RSV for forty-eight hours. Specifically, IL-1β was combined with **A)** IL-17, **B)** IL-22, **C)** IFNγ, **D)** LPS, **E)** IFNβ, and **F)** IL-4 compared to unstimulated cells. IL-36γ mRNA expression was expressed relative to L32 mRNA expression (mean ± SEM from three different experiments, facilitated by the use of one-way ANOVA, Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group, *p<0.05).

5.3.5 The effect of cytokines combination on expression of IL-36γ protein in airways epithelial cells

In addition to mRNA through epithelial cells, either cytokines alone or in combinations were utilised to stimulate A549 cells in order to determine which cytokine combinations could induce IL-36γ protein expression. Cytokines and LPS were utilised at 100 ng/ml per cytokine for twenty-four hours. Moreover, significant detection of IL-36γ protein was measured in supernatant and cell pellets (Figure 5.3.5).

5.3.5.1 The effect of IL-1β with other cytokine and LPS combinations on the expression of IL-36γ protein in A549 cells

IL-36γ protein expression was increased but did not reach a significant point when A549 cells were stimulated with the combination of IL-1β and TNF-α, compared to the expression of IL-36γ protein with either IL-1β ($p= 0.6$) or with TNF-α-stimulated A549 cells ($p= 0.1$) (Figure 5.3.5.1a A). Indeed, there was no significant increase in the expression of IL-36γ protein when stimulating A549 cells with IL-1β and IL-17 combined, compared to the expression of IL-36γ protein through A549 cells when stimulated with either IL-1β ($p> 0.9$) or IL-17 ($p= 0.2$) cytokines alone (Figure 5.3.5.1a B). Furthermore, the stimulation of A549 cells with IL-1β and IL-22, IFNγ cytokines revealed no significant increase of IL-36γ protein expression compared to the expression of IL-36γ protein with either IL-1β ($p= 0.6$), IL-22 ($p= 0.1$) or IFNγ alone ($p= 0.1$) (Figure 5.3.5.1a C and D). Additionally, the expression of IL-36γ protein also showed no significant increase in A549 cells when stimulated with IL-1β and IFNβ or with IL-4 ($p> 0.9$), in comparison to IFNβ ($p= 0.2$) or IL-4 cytokines alone ($p= 0.4$) (Figures 5.3.5.1a F, and G). Regarding LPS stimulation, the results indicated a significant increase on the expression of IL-36γ protein when stimulating A549 cells with IL-1β and LPS compared to the same cells when stimulated with either IL-1β or LPS alone ($p< 0.05$) (Figure 5.3.5.1a E).

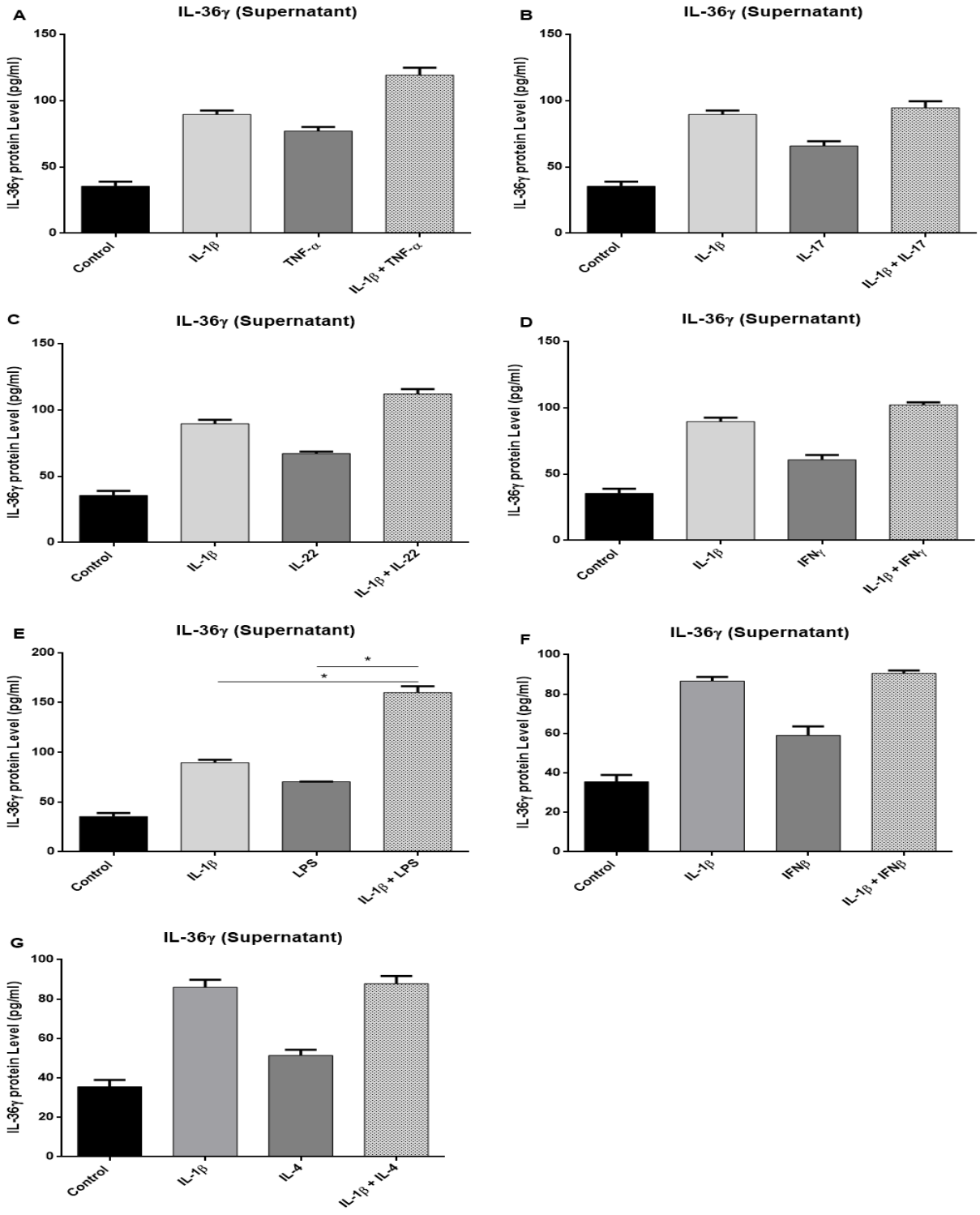

Figure 5.3.5.1a: The effect of the combination of IL-1β with other cytokines and LPS on IL36γ protein expression by airway epithelial cells. For twenty-four hours, each cytokine was utilised with various cytokine combinations of 100ng/ml to stimulate A549 cells. Specifically, IL-1β was combined with **A)** TNF-α, **B)** IL-17, **C)** IL-22, **D)** IFNγ, **E)** LPS, **F)** IFNβ, and **G)** IL-4, compared to unstimulated cells. IL-36γ protein expression was expressed relative to unstimulated cells (mean ± SEM from three different experiments, facilitated by the use of one-way ANOVA, Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group, *p<0.05).

IL-36γ protein expression in cell pellet was increased but did not reach a significant point when A549 cells were stimulated with the combination of IL-1β and TNF-α, compared to the expression of IL-36γ protein with either IL-1β (p= 0.6) or with TNF-α-stimulated A549 cells (p= 0.1) (Figure 5.3.5.1b A). Indeed, there was no significant increase in the expression of IL-36γ protein when stimulating A549 cells with IL-1β and IL-17 combined, compared to the expression of IL-36γ protein through A549 cells when stimulated with either IL-1β (p> 0.9) or IL-17 (p= 0.2) cytokines alone (Figure 5.3.5.1b B). Furthermore, the stimulation of A549 cells with IL-1β and IL-22, IFNγ cytokines revealed no significant increase of IL-36γ protein expression compared to the expression of IL-36γ protein with either IL-1β (p= 0.6), IL-22 (p= 0.1) or IFNγ alone (p= 0.1) (Figure 5.3.5.1b C and D). Additionally, the expression of IL-36γ protein also showed no significant increase in A549 cells when stimulated with IL-1β and IFNβ or with IL-4 (p> 0.9), in comparison to IFNβ (p= 0.2) or IL-4 cytokines alone (p= 0.4) (Figures 5.3.5.1b F, and G). Regarding LPS stimulation, the results indicated a significant increase on the expression of IL-36γ protein when stimulating A549 cells with IL-1β and LPS compared to the same cells when stimulated with either IL-1β or LPS alone (p< 0.05) (Figure 5.3.5.1b E).

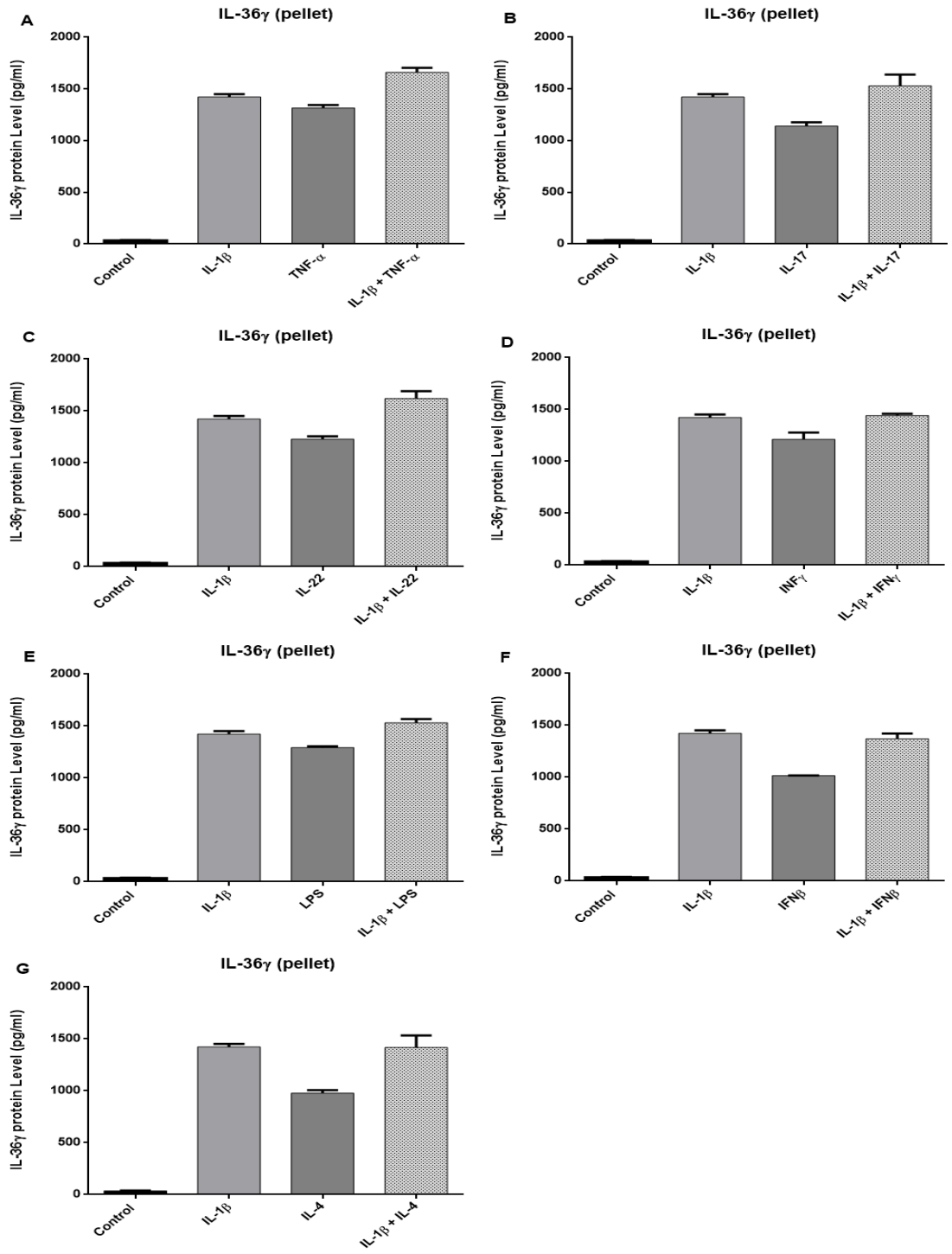

Figure 5.3.5.1b: The effect of the combination of IL-1β with other cytokines and LPS on IL36γ protein (Cell pellet) expression by airway epithelial cells. For twenty-four hours, each cytokine was utilised with various cytokine combinations of 100ng/ml to stimulate A549 cells. Specifically, IL-1β was combined with **A)** TNF-α, **B)** IL-17, **C)** IL-22, **D)** IFNγ, **E)** LPS, **F)** IFNβ, and **G)** IL-4, compared to unstimulated cells. IL-36γ protein expression was expressed relative to unstimulated cells (mean ± SEM from three different experiments, facilitated by the use of one-way ANOVA, Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group).

5.3.5.2: The effect of TNF-α with other cytokine and LPS combinations on the expression of IL-36γ protein in A549 cells

A549 cells were stimulated with 100ng/ml of particular combinations per cytokine in order to ascertain what TNF-α and cytokine combinations are required to induce IL-36γ protein production alongside mRNA through epithelial cells expression. Specifically, TNF-α was combined with IL-17, IL-22, and IFNγ, and also with LPS for twenty-four hours. Significant detection of IL-36γ protein was determined in each combination of cytokines in supernatant and cell pellets (Figure 5.3.5). Furthermore, IL-36γ protein expression was not significantly increased when stimulating A549 cells with either TNF-α alone or combined with IL-17 cytokines compared to the expression of IL-36γ protein when stimulated with TNF-α ($p= 0.4$) or IL-17 ($p= 0.2$) cytokines alone (Figure 5.3.5.2A). Additionally, IL-36γ protein expression did not change when stimulating A549 cells with the combination of TNF-α and IL-22 compared to the expression of IL-36γ protein with TNF-α ($p= 0.6$) or IL-22 ($p= 0.1$) cytokine alone (Figure 5.3.5.2B). The combination of TNF-α and IFNγ cytokines revealed no significant increase in the expression of IL-36γ protein through A549 cells compared to the expression of IL-36γ protein when stimulating A549 with TNF-α ($p= 0.6$), IFNγ ($p= 0.1$), IFNβ ($p= 0.2$), or IL-4 ($p= 0.4$) cytokines alone (Figures 5.3.5.2 C, E, and F). Likewise, compared to IL-36γ protein expression through A549 cells stimulation using only LPS ($p= 0.1$), when stimulating them with TNF-α and LPS combined there was no increase in the protein expression. This was facilitated through the use of one-way ANOVA, the Friedman statistic test and Dunn's multiple comparisons test).

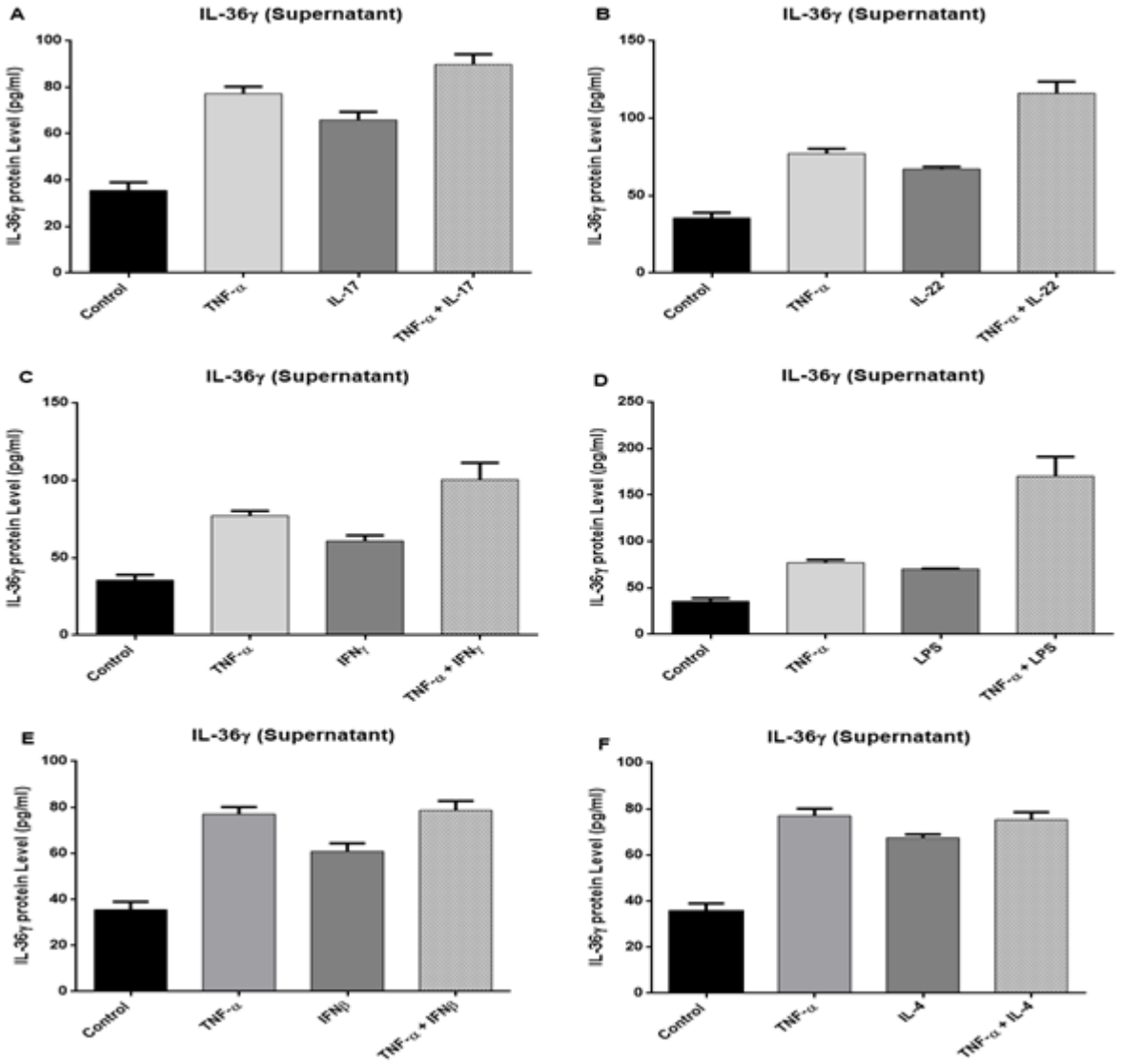

Figure 5.3.5.2a: The effect of the combination of TNF-α with other cytokines and LPS on IL36γ protein expression by airway epithelial cells. For twenty-four hours, various cytokine combinations of 100ng/ml were utilised to stimulate the A549 cells. Specifically, IL-1β was combined with **A)** IL-17, **B)** IL-22, **C)** IFNγ, **D)** LPS **E)** IFNβ and **F)** IL-4, compared to unstimulated cells. IL-36γ protein expression was expressed relative to unstimulated cells (mean ± SEM from three different experiments, facilitated by the use of one-way ANOVA, Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group).

In the cell pellet, there was no significant change on the expression of IL-36γ protein when stimulating A549 cells with the combination of TNF-α and IL-17, IL-22, IFNγ, IFNβ, and IL-4 compared to the expression of IL-36γ protein when stimulated by the same cytokines separately (Figure 5.3.5.2). A549 cells were stimulated with the combination of TNF-α, and IL-17 compared to the expression of IL-36γ protein with either TNF-α (p= 0.1) or with IL-17 stimulated A549 cells (p= 0.4) (Figure 5.3.5.2b B). Indeed, there was no significant increase in the expression of IL-36γ protein when stimulating A549 cells with TNF-α and IL-22 combined, compared to the expression of IL-36γ protein through A549 cells when stimulated with either TNF-α (p> 0.9) or IL-22 (p> 0.9) cytokines alone (Figure 5.3.5.2b B). Furthermore, the stimulation of A549 cells with TNF-α and IFNγ cytokines revealed no significant increase of IL-36γ protein expression compared to the expression of IL-36γ protein with either TNF-α (p= 0.6), or IFNγ alone (p= 0.1) (Figure 5.3.5.2b C). Additionally, the expression of IL-36γ protein also showed no significant increase in A549 cells when stimulated with TNF-α and IFNβ or with IL-4 (p= 0.6), in comparison to IFNβ (p= 0.1) or IL-4 cytokines alone (p= 0.1) (Figures 5.3.5.2b E, and F). Regarding LPS stimulation, the results indicated a significant increase on the expression of IL-36γ protein when stimulating A549 cells with TNF-α and LPS compared to the same cells when stimulated with either TNF-α (p= 0.2) or LPS alone (p= 0.4) (Figure 5.3.5.2b D).

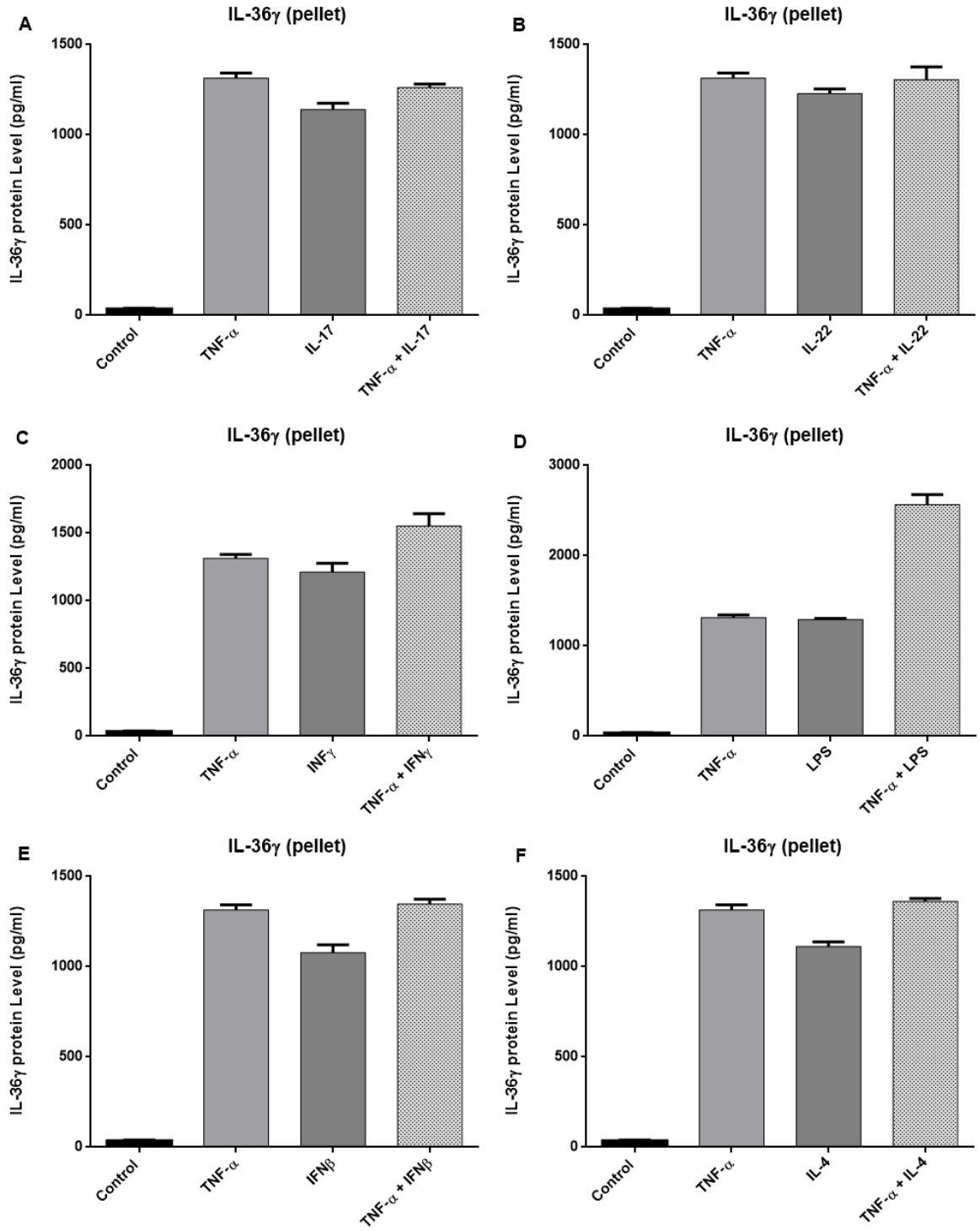

Figure 5.3.5.2b: The effect of the combination of TNF-α with other cytokines and LPS on IL36γ protein (cell pellet) expression by airway epithelial cells. For twenty-four hours, various cytokine combinations of 100ng/ml were utilised to stimulate the A549 cells. Specifically, IL-1β was combined with **A)** IL-17, **B)** IL-22, **C)** IFNγ, **D)** LPS **E)** IFNβ and **F)** IL-4, compared to unstimulated cells. IL-36γ protein expression was expressed relative to unstimulated cells (mean ± SEM from three different experiments, facilitated by the use of one-way ANOVA, Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group).

5.3.6 The effect of cytokine combinations on the expression of IL-36γ protein in RSV-infected airway epithelial cells

In addition to mRNA through epithelial cells, cytokines either alone or in combinations were utilised to stimulate A549 cells that had been infected with 1 MOI of RSV for twenty-four hours, in order to determine which cytokine combinations could induce IL-36γ protein production. The cytokines and LPS were utilised at 100 ng/ml per cytokine for twenty-four hours. Significant detection of IL-36γ protein was measured by supernatant volume (Figure 5.3.6).

5.3.6.1 The effect of IL-1β and other cytokine combinations on the expression of IL-36γ protein in infected A549 cells

There was no significant increase in the expression of IL-36γ protein when stimulating A549 cells with IL-1β and TNF-α combined compared to the expression of IL-36γ protein by A549 cells when stimulated with either IL-1β (P= 0.6) or TNF-α (p= 0.1) cytokines alone (Figure 5.3.6.1 A). Additionally, there was no significant increase in the expression of IL-36γ protein when stimulating A549 cells with IL-1β (p> 0.9) and IL-17 (p= 0.2) combined compared to the expression of IL-36γ protein by either IL-1β or IL-17 stimulated A549 cells during RSV infection (Figure 5.3.6.1 B). Furthermore, the stimulation of A549 cells with a combination of IL-1β and IL-22 cytokines demonstrated no significant increase on the expression of IL-36γ protein compared to the expression of IL-36γ protein by either IL-1β (p= 0.6) or IL-22 (p= 0.1) stimulated A549 cells during RSV infection (Figure 5.3.6.1 C). Moreover, there was no significant expression of IL-36γ protein in A549 cells when stimulated with IL-1β (p= 0.6) and IFNγ (p= 0.1), IFNβ (p= 0.2), or IL-4 (p= 0.4) cytokines and LPS (p = 0.1) compared to the same cells when stimulated with IL-1β or other cytokines alone (Figures 5.3.6.1 D, E, F, and G). This was facilitated through the use of one-way ANOVA, the Friedman statistic test and Dunn's multiple comparisons test.

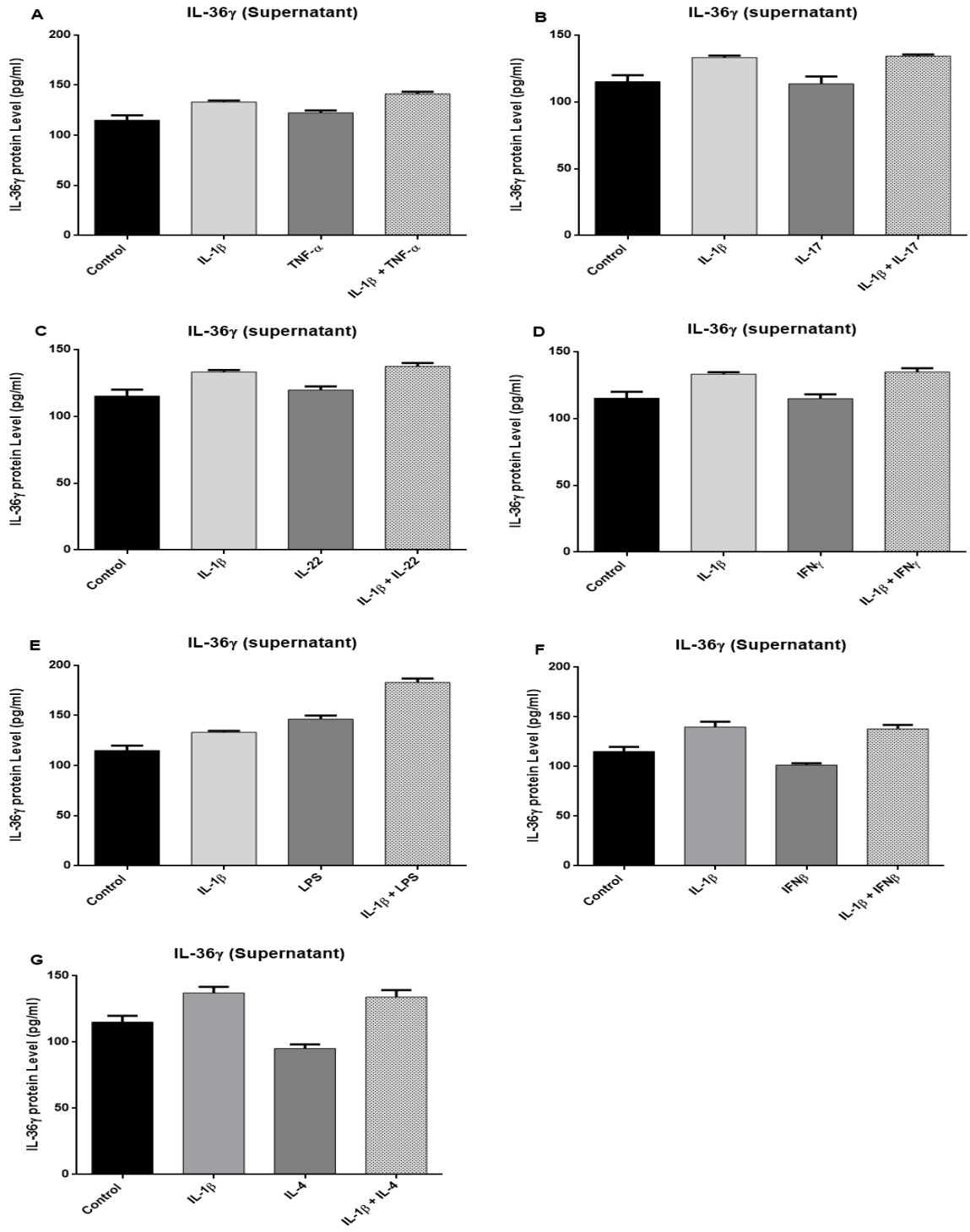

Figure 5.3.6.1: The effect of the combination of IL-1β with other cytokines and LPS on IL36γ protein expression by infected airway epithelial cells. For twenty-four hours, various cytokine combinations of 100ng/ml were utilised to stimulate A549 cells that had been infected with 1 MOI of RSV for forty-eight hours. Specifically, IL-1β was combined with **A)** TNF-α, **B)** IL-17, **C)** IL-22, **D)** IFNγ, **E)** LPS **F)** IFNβ, and **G)** IL-4 compared to unstimulated cells. IL-36γ protein expression was expressed relative to unstimulated cells (mean ± SEM from three different experiments), facilitated by the use of one-way ANOVA, the Friedman statistic test and Dunn's multiple comparisons test, *p<0.05).

5.3.6.2 The effect of TNF-α and other cytokine combinations on the expression of IL-36γ protein in infected A549 cells

A549 cells were infected with 1 MOI of RSV for twenty-four hours and subsequently stimulated with 100ng/ml of various combinations per cytokine. This was done to ascertain how both mRNA and IL-36γ protein production via epithelial cells was affected by the combination of TNF-α and other cytokines. TNF-α was combined with IL-17, IL-22, and IFNγ, in addition to LPS for twenty-four hours. Furthermore, significant detection of IL-36γ protein was determined in the supernatant of each cytokine combination (Figure 5.3.6).

Compared to the IL-36γ protein expression through either TNF-α or IL-17-stimulated A549 cells with RSV infection (Figure 5.3.6.2), when these were stimulated with either TNF-α alone (p= 0.4) or IL-17 (p= 0.2) cytokines, there was no increase in IL-36γ protein expression. Moreover, during RSV infection the combination of TNF-α and IL-22 indicated no significant different on the expression of IL-36γ protein in A549 cells compared to the expression when stimulating the same cells with either TNF-α (p= 0.6) or IL-22 alone (p= 0.1) (Figure 5.3.6.2 B). Additionally, there was no significant increase in IL-36γ protein when stimulating A549 cells with the combination of TNF-α (p= 0.6) and IFNγ (p= 0.1), IFNβ (p= 0.2), or IL-4 (p= 0.4) cytokines, compared to stimulating the same cells with either TNF-α or other cytokines alone (Figures 5.3.6.2 C, E, and F). Meanwhile, IL-36γ protein expression was not increased when stimulating A549 cells with either TNF-α alone or with TNF-α and LPS combined, compared to the expression of IL-36γ protein by either TNF-α (p= 0.6) or LPS-stimulated A549 cells during RSV infection (p= 0.1) (Figure 5.3.6.2D). This was facilitated by the use of one-way ANOVA, the Friedman statistic test and Dunn's multiple comparisons test).

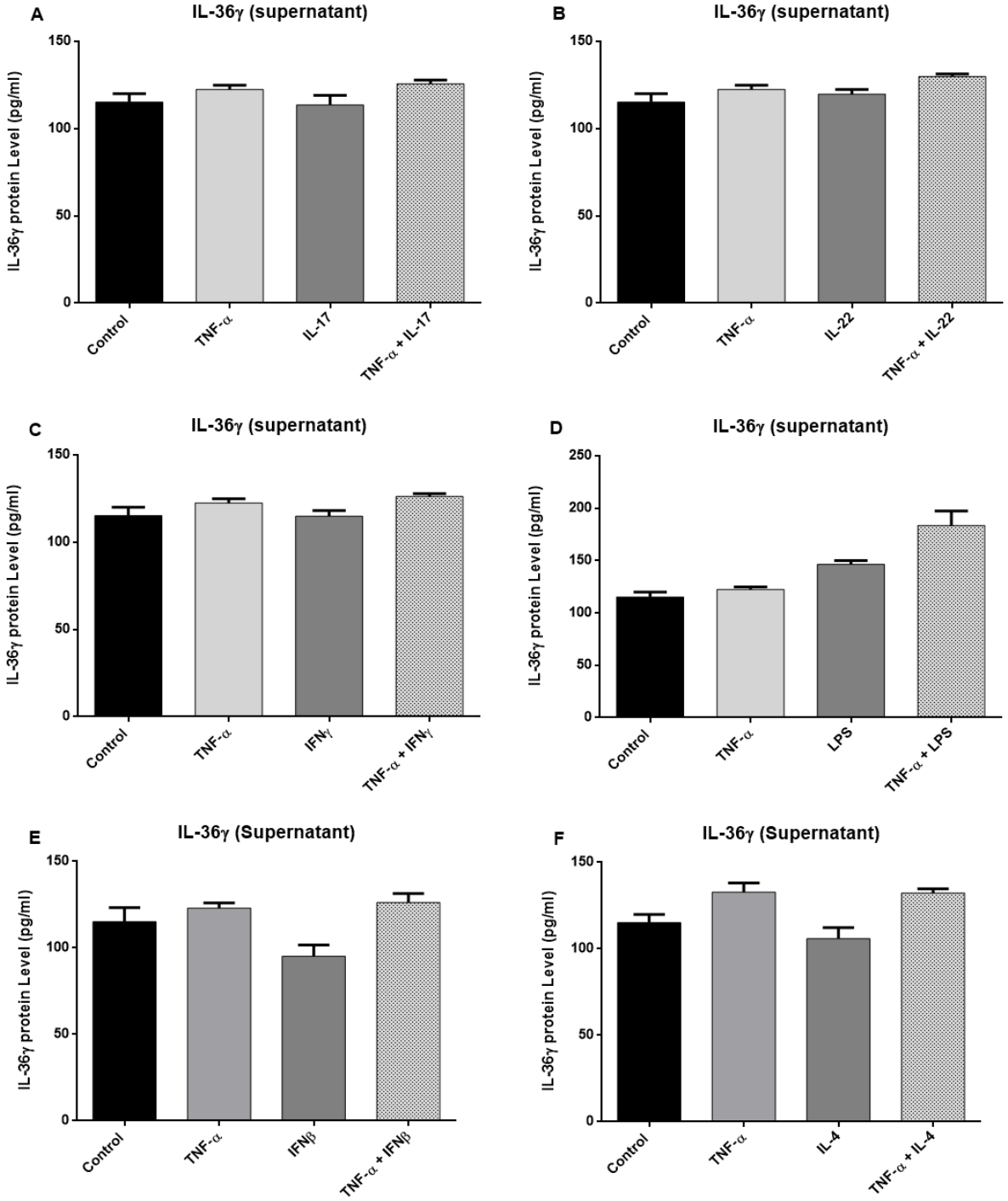

Figure 5.3.6.2: The effect of the combination of TNF-α with other cytokines and LPS on IL36γ protein expression in infected airway epithelial cells. For twenty-four hours, various cytokine combinations at 100ng/ml were utilised to stimulate A549 cells that had been infected with 1 MOI of RSV. Specifically, TNF-α was combined with **A)** IL-17, **B)** IL-22, **C)** IFNγ, **D)** LPS, **E)** IFNβ, and **F)** IL-4 compared to unstimulated cells. IL-36γ protein expression was expressed relative to unstimulated cells (mean ± SEM from three different experiments, facilitated by the use of one-way ANOVA, Friedman test for groups and Dunn's multiple comparisons test to compare the mean rank of group with the mean rank of every other group).

5.4 Discussion

As outlined by Carrier *et al.* (2011) and Takahashi *et al.* (2015), inflammatory diseases such as inflammatory bowel disease (IBD) and psoriasis have a pathogenesis that is affected by IL-36 cytokines. However, there are no reports concerning the role of IL-36 cytokines in lung inflammation. The purpose of this chapter was to investigate whether primary inflammatory cytokines such as TNF-α and IL-1β play a role and are good stimulators of IL-36γ in the airway epithelial cells. Additionally, this chapter also focused on the expression of IL-36γ from A549 cells resulting from other inflammatory cytokines including IL-17, IL-22, IFNγ, IFNβ, IL-4, and LPS either separately or combined with the primary cytokines IL-1β and TNF-α. Furthermore, IL-1β has been shown to induce the expression of IL-36γ in human colonic myofibroblasts (Takahashi *et al.,* 2015) thus suggesting that the IL-1β cytokine could be involved in the expression of IL-36γ cytokines. On the other hand, TNF-α is a central mediator of airway inflammation, inflammatory cell infiltration response, vascular permeability, and chemokine release, and may play a role in the immunopathogenesis of RSV infection (Choi *et al.,* 2010). Nonetheless, exactly how inflammation in airway epithelial cells is affected by IL-1β and TNF-α is still unascertained. In this chapter, the optimum time and concentration of different inflammatory cytokines were examined to observe their effect on IL-36γ cytokine expression. Specifically, I measured the expression of IL-36γ after stimulating A549 cells with IL-1β and TNF-α and other inflammatory cytokines, including IL-17, IL-22, IFNγ, IFNβ, IL-4, and LPS in both non-infected and infected A549 cells.

5.4.1 The effect of IL-1β and TNF-α either alone or combined with other inflammatory cytokines on the expression of IL-36γ mRNA and protein in RSV-infected and non-infected A549 cells

This study sought the optimum times (3, 6, 12, 24, or 48hrs) and concentrations (1, 5, 10, 50, 100, and 200ng/ml) of these cytokines required to induce the high level of IL-36γ mRNA expression when incubating cultures of airway epithelial cells (A549) with these cytokines (Figures 5.3.1 and 5.3.2). Another aim was to determine the induction of IL-36γ from both RSV-infected and non-infected A549 cells through these cytokines either alone or in combination (Figures 5.2.3 and 5.2.4). Consequently, there was a significant upregulation of IL-36γ mRNA expression when incubating A549 cells with 200ng/ml of IL-1β cytokines in a time dependent manner, ultimately increasing 139-fold after three hours ($P<0.0001$), 116-fold after six hours ($P<0.0001$), 81-fold after twelve hours ($p<0.001$), 60-fold after twenty-four hours ($p<0.05$), and 12-fold higher after forty-eight hours ($p<0.01$) – all these being relative to the control group (Figure 5.3.1 A). The results also

demonstrate a significant expression of IL-36γ mRNA when incubating A549 cells with TNF-α at 6hr ($p<0.001$), whereas there was no significant increase of IL-36γ mRNA expression observed at other times relative to the control (Figure 5.3.1B). Furthermore, the combination of IL-1β with other cytokines - including IL-17, IL-22 and IFNγ, IFNβ, IL-4 cytokines, and LPS - were shown to not enhance IL-36γ mRNA expression in cultured A549 cells compared to the expression of IL-36γ mRNA with the same cytokines alone (Figure 5.3.3.1). One theory for this is that in order to stimulate airway epithelial cell IL-36γ expression, a specific condition may be required for the inflammatory cytokines. Moreover, these cytokines may fail to bind with their receptors, the receptors may not be expressed from A549 cells, or the signalling of the cytokines failed at specific points in the A549 cells.

Among the various cytokines, one previous study identified how IL-1β is a strong inducer of IL-36γ in colonic myofibroblasts, with the combination of IL-1β and TNF-α exerting a remarkable effect on IL-36γ mRNA. Indeed, in colonic myofibroblast blot tests, the intracellular IL-36γ protein level was ascertained. Meanwhile, Kovach *et al.* (2016) reported that the expression of IL-36γ mRNA was upregulated significantly when stimulating pulmonary macrophages with 100 ug/ml of IL-1β and TNF-α cytokines four hours after stimulation. Carrier *et al*. (2011) also found that IL-1β and TNF-α cytokines induce the expression of IL-36 cytokines' mRNA in keratinocytes. Therefore, these findings indicate that IL-36γ expression is mediated primarily by IL-1β and that TNF-α is also an important factor through its synergistic effect with IL-1β. Taken together, their findings prompted me to examine whether this combination could be a good stimulant for expressing IL-36γ in airway epithelial cells as a source of IL-36γ. Collectively, two major proinflammatory cytokines IL-1β and TNF-α evidently play a pivotal role in the induction of IL-36γ in airway epithelial cells.

Equally, these results have illustrated the effect the incubation of other inflammatory cytokines has on IL-36γ mRNA expression through A549 cells, with no significant change on the expression of IL-36γ mRNA when stimulating A549 cells with IL-17, IFNγ, IL-22, IFNβ, IL-4, and LPS (Figures 5.3.1 C, D, E, F, G, and H). In this study there was increased expression of IL-36γ when stimulating A549 cells with TNF-α alone, however there was no change when combining the other inflammatory cytokines with TNF-α *in vitro*. The IL-β and TNF-α cytokines were selected dependent upon the previous finding by Coward *et al.* (2004), whose study found that TNF-α correlated with the Th1 cytokines IFNγ and IL-8. They identified that IL-8 and TNF-α were increased in allergen-activated eosinophils. Furthermore, other studies also reported that IFNγ and IL-8 were increased in RSV-infected cells in animal models (Jafri *et al.,* 2004). Therefore, regarding eosinophilic airway

inflammation in RSV-related bronchiolitis, it is evident that TNF-α cooperated with both IFNγ and inflammatory cytokines. Moreover, Carrier *et al* found a significant expression of IL-36γ mRNA when stimulating keratinocytes with the combination of TNF-α and IL-17, but *not* when stimulating the same cells with IFNγ (Carrier *et al,* 2011). Additionally, the level of IL-36γ protein was increased when stimulating the keratinocyte with the combination of either TNF-α and IL-22, or IL-17A (Carrier *et al,* 2011). Accordingly, understanding how IL-36γ expression is affected by Th1, Th2, and Th17 inflammatory cytokines was an important objective of this study. Finally, another study found that IL-36γ mRNA expression was upregulated when stimulating pulmonary macrophages with LPS (Kovach *et al,* 2016).

Further to this, since TNF-α is a primary cytokine with the role of amplifying the immune response during infection, this study suggests that TNF-α could stimulate the expression of IL-36γ directly from airway epithelial cells. Certainly, it is known that RSV is able to induce many kinds of cytokines from airway epithelial cells, but this study also investigated whether the combination of pro-inflammatory cytokines could stimulate the expression of IL-36γ mRNA or proteins in RSV-infected cells. Indeed, previous results revealed that TNF-α levels were significantly increased in the RSV group compared to the control group (Choi *et al.,* 2010). Considering that TNF-α is known to be an important inflammatory cytokine in the pathophysiology of asthma and the pathogenesis of RSV bronchiolitis (Choi *et al.,* 2010), it is argued that TNF-α activates the immune response and could involve the release of proinflammatory cytokines including IL-36γ against RSV infection. Several studies have demonstrated that RSV is able to stimulate other proinflammatory cytokines including TNF-α, IFNγ, Th2, and Th17 (Christiaansen *et al.,* 2014). However, research by Christiaansen *et al.* (2016) demonstrated that RSV induces IFNγ and TNF-α in RSV-infected infants under years of age, with a significant increase of IL-36γ mRNA expression when synergising TNF-α with IL-22 cytokines, whereas there was no significant increase observed when boosting TNF-α with IFNγ. Moreover, IL-1β exhibited the greatest increase in RSV-infected infants, and as such this finding agreed with this study indicating that the addition of IL-1β either alone or with a combination of other cytokines did not show any effective action on the expression of IL-36γ mRNA, as observed in A549 cells without RSV infection (section 5.3.3.1).

These findings indicate that the combination of IL-1β cytokines with other proinflammatory cytokines such as TNF-α, IL-17, IFNγ, and IL-22 does not affect IL-36γ expression. With a vital role in IL-36γ induction played by the two major proinflammatory cytokines IL-1β and TNF-a, collectively airway epithelial cells are demonstrated to be a cellular source of IL-36γ. Overall, the data

demonstrates that the addition of inflammatory cytokines either alone combined with cells did not affect the expression of IL-36γ mRNA or protein. Meanwhile, LPS only has an effect with IL-1β or TNF-α in producing the expression of IL-36γ protein in RSV-infected cells.

5.4.2 Effect of IL-1β on the expression of IL-36γ mRNA: IL-1β (dose/time dependent)

Among the other cytokines in A549 cells, the biggest inducer of IL-36γ mRNA expression was shown to be IL-1β, as evidenced by the previous study's findings. Accordingly, this study examined the induction's specifics by determining the optimum concentration and incubation time with IL-1β cytokines in A549 cells (Figure 5.3.2). Subsequently, cultured A549 cells were stimulated with IL-1β at concentrations of 1 ng/ml, 5 ng/ml, 10 ng/ml, 50ng/ml, 100 ng/ml and 200 ng/ml for three hours, six hours, and twenty-four hours. As presented in Figure 5.3.2A, A549 cells were incubated with 1ng/ml of IL-1β cytokine, thereby identifying that the expression of IL-36γ mRNA was increased significantly at 3hrs ($P<0.0001$), and 6hrs ($p<0.05$) compared to the expression of IL-36γ mRNA without any stimulation. However, the findings show that the incubation (5ng/ml) of IL-1β concentration is able to increase the expression of IL-36γ mRNA significantly at 6hrs ($P<0.05$) and ($p<0.0001$) 24hrs compared to the expression of IL-36γ mRNA without stimulation. Furthermore, the stimulation of A549 cells with 10, 50 or 100 ng/ml of IL1-β concentration saw IL-36γ mRNA expression increase significantly after three and six hours ($P<0.0001$), compared to the expression of IL-36γ mRNA at the same time point without stimulation (Figures 5.3.2 C, D, and E). However, the results reveal that expression of IL-36γ mRNA was increased significantly only at 6hrs when incubating A549 cells with 200ng/ml of IL-1β cytokine (Figure 5.3.2F). Overall, therefore, in order to induce the greatest IL-36γ mRNA expression through A549 cells, the optimum time/dose combination of IL-1B cytokines was shown to be 100ng/ml after six hours.

Consistent with previous studies, these results confirm that stimulating human bronchial epithelial cells with either IL-1β or TNF-α cytokines significantly induces IL-36γ mRNA, beginning three hours post stimulation and lasting for twenty-four hours, reaching a peak level at the 6hr point. However, stimulation with IL-1β or TNF-α at the 24hr point also induced the expression of IL-36γ mRNA, but was not as strong compared with six hours of stimulation (Chustz *et al*, 2011). Meanwhile, Takahashi *et al.* (2015) observed a significant increase of IL-36γ mRNA expression when induced by IL-1β dose-dependence at 0.05ng/ml ($p<0.05$), 0.1ng/ml ($p<0.01$), 1ng/ml ($p<0.01$), and 100ng/ml, twenty-four hours after stimulation in

myofibroblasts. Regarding the time dependence, From the research of Takahashi *et al.* (2015), as early as three, twelve, and twenty-four hours after IL-1β stimulation IL-36γ mRNA induction was observed, thereby demonstrating that this expression was indeed time-dependently induced. Moreover, Kovach et al identified that expression of IL-36γ mRNA was significantly upregulated when stimulating pulmonary macrophages with 100ug/ml of IL-1β cytokines at the 4hr point, but *not* at eighteen hours after stimulation (Kovach *et al,* 2016).

5.5 Summary

As shown by the evidence, IL-1β stimulation significantly induced IL-36γ mRNA expression in a time-dependent manner ranging from three to twenty-four hours. Furthermore, the stimulation of A549 cells by TNF-α significantly induced the expression of IL-36γ mRNA at the 6hr point only. However, when utilising IL-17, IFNγ, and IL-22 cytokines separately and at any time to stimulate the A549 cells, IL-36 mRNA expression was not significantly affected. The highest level of IL-36γ mRNA expression was at 6hrs when stimulating A549 cells with 100ng/ml of IL-1β cytokines, whereas this level peaked at the 6hr point after stimulating the cells with TNF-α. IL-1β cytokines were a strong inducer for IL-36γ expression, with this expression not increasing when the other Th1 cytokines TNF-α and IFNγ were added. Additionally, TNF-α cytokines also induce the expression of IL-36γ mRNA, and this did not change when the Th1 cytokine IFNγ and Th17 cytokine IL-22 were added. Indeed, the combination of LPS with either IL-1β or TNF-α appeared to stimulate the expression of IL-36γ mRNA and protein but did not reach a significant level. Furthermore, the combination of IL-1β with TNF-α, IL-22, or IFNγ cytokines did not induce the expression of IL-36γ protein in either the culture medium or cell pellets. Moreover, the combination of TNF-α with IL-22 and IFNγ did not increase the expression of IL-36γ protein. LPS is also unable to increase the expression of IL-36γ protein when combined with IL-1β or TNF-α in A549 cells. The combination of either IL-1β or TNF-α with other cytokines in RSV-infected cells does not increase the expression of IL-36γ mRNA and protein. Collectively, the collaboration between other cytokines and LPS cannot stimulate IL-36γ protein expression.

Chapter 6. General discussion and future work

Infection of airway epithelial cells by RSV increases the production of a variety of inflammatory and immune response mediators. This dissertation investigated the notion that RSV-infected AECs may express IL-36α, IL-36γ, IL-36Rα, and IL-36 receptors. The data mentioned in Chapter 3 indicate that NPAs are considerably higher in patients with RSV and RV. *In vivo* investigations find IL-36α and IL-36γ proteins in the NPAs of RSV and RV illness patients. Both RSV and RV disease-specific NPAs demonstrated that the IL-36α protein level was greater than the IL-36γ protein level. In Chapter 3, *in vitro* investigations reveal that in response to RSV infection, HNAECs, A549, and BEAS-2B cells expressed IL-36α, IL-36γ, and IL-36Rα. (Figure 3.3.2 and 3.3.3). In HNAECs, A549, and BEAS-2B cells, IL-36γ mRNA was expressed at a greater level than other IL-36 ligands. IL-36 receptor expression was exceedingly low in HNAECs, A549, and BEAS-2B cells infected with RSV. IL-36α and IL-36γ proteins were shown in RSV-infected HNAECs, A549 and BEAS-2B cells (Figures 3.3.2.7 and 3.3.2.8). (Figures 3.3.3.8 and 3.3.3.9). In RSV-infected HNAECs, A549, and BEAS-2B cells, co-stimulating with ATP induces the release of IL-36α and IL-36γ proteins into the culture medium.

The aim of this study was also to determine whether AECs could release IL-37 cytokines in response to RSV infection. Chapter 4 examined NPAs with RSV and RV disease and found elevated IL-37 protein levels. *In vitro,* this study also assessed HNAECs, A549 cells, and BEAS-2B cells' expression of IL-37 cytokine and its receptors in response to RSV infection. Figures 4.3.2.1 and 4.3.3.1 indicate that RSV-infected cells expressed IL-37 mRNA. With regard to the immunofluorescence staining of RSV-infected BEAS2B cells, IL-37 protein was expressed and localised around the nucleus and stimulation by LPS enhances more expression of IL-37 in both infected and non-infected cells at 12 hours (Figure 4.3.4.1c, C) and 24 hours (Figure 4.3.4.2c, C).

This research explores which inflammatory cytokines may trigger the production of IL-36γ in A549 cells in Chapter 5. The goal of this work was to see whether additional inflammatory cytokines, such as IL-1β and TNF-α, had a role in the induction of IL-36γ by A549 cells. The findings revealed that IL-1β and TNF-α were effective IL-36γ stimulators. However, there was

no effect of the expression of IL-36γ when combine theses cytokines with (IL-1β or TNF-α) with Th1, Th2 and Th17 cytokines (IL-4, IL-22, IL17, IFNβ and IFNγ) (Section 5.3.3, 5.3.5).

6.1 RSV infection induces IL-36 cytokines in nasopharyngeal aspirate samples (NPAs), HNAECS and cell lines

This research looks on the expression of IL-36 cytokines by airway epithelial cells after RSV infection. These results in Chapter 3 show that these cytokines were found at increased levels in nasopharyngeal aspirates from children with RSV and RV illness (section 3.3.1). *In vitro* tests in patients with RSV and RV illness validated these results (Figure 3.3.1 A and B). NPAs with RV illness had greater levels of IL-36α and IL-36γ proteins than NPAs with RSV disease. RV generates more IL-36 cytokines than RSV, suggesting that RV is more sensitive to airway epithelial cells. Fibroblasts, dendritic cells, and T cells expressing IL-36α and IL-36γ show a response to RV. Furthermore, ELISA analysis revealed that the amount of IL-36α protein in patient samples with both RV and RSV was greater than the level of IL-36γ protein *in vivo*. These findings imply that the process of IL-36α protein synthesis is simpler than that of IL-36γ protein, or that there are several sources of cells that may create IL-36α protein in different ways in response to viral infection.

IL-36α and IL-36γ protein levels were significantly elevated in NPAs from patients with RSV and RV illness. These findings imply that IL-36, as a pro-inflammatory cytokine, might boost the immune response during RSV and RV infection. This work was expanded in chapter 3 to look at the expression of IL-36α, IL-36γ and IL-36Rα *in vitro* in RSV infected HNAECs, A549, and BEAS-2B cells (section 3.3.2 and 3.3.3). At MOI 2.5, the expression of IL-36α and IL-36γ mRNA was considerably elevated in infected HNAECs, A549, and BEAS-2B cells (section 3.3.2 and 3.3.3). In contrast to NPAs, the level of IL-36γ was the highest among IL-36 cytokines *in vitro*, indicating that only airway epithelial cells are the source of IL-36 cytokines, whereas *in vivo*, different types of cells and immune cells participate in the production of IL-36 cytokines, resulting in a higher level of IL-36α than the level of IL-36γ. The findings imply that IL-36α and IL-36γ cytokines are especially crucial in initiating and amplifying the immune response to RSV infection by activating other cells or immune cells to generate additional pro-inflammatory cytokines and chemokines to clear and inhibit viral replication. These cytokines are linked to

both viral clearance and host problems, such as virus-induced asthma. These two molecules are members of the IL-1 family. It is generally known that members of the IL-1 family play an important role in innate immune response and inflammation, but IL-36 may also activate other immunological systems to produce adaptive immune responses.

As depicted in figure 6.1, RSV is recognised by TLR in airway epithelial cells, which cause NFkaaB to activate a complex of signalling cascades. Signalling cascades were induced resulting in the expression of several pro-inflammatory cytokines. As an example, CXCL-8 is a chemoattractant of neutrophils (section 3.3.2.2) and (section 3.3.3.2). In addition, IL-36γ was expressed significantly in RSV infected cells and they need the neutrophil proteases to convert into the active form and get a full of bioactivity. In addition, when RSV infection occurs, IL-36 appears to amplify the immune response by attracting neutrophils to the inflammation site. In addition, the active form of IL-36γ stimulates airway epithelial cells to induce an immune response during RSV infection. A variety of macrophages, including alveolar macrophages, can also be stimulated by IL-36. Researchers suggest that IL-36 expression during RSV infection may also induce DCs, a crucial link between innate and adaptive immunity. DCs can respond to IL-36 by expressing IL-36R, IL-6, IL-12, IL-1β, IL-23 and TNF-α (Figure 6.1). IL-36 cytokines may also be significantly expressed during RSV infection due to their ability to stimulate the activation of Th1 and Th17 by enhancing the expression of their cytokines and chemokines. The IL-36 response during RSV infection and consequent control of RSV replication in the cells (Figure 6.1) indicates that IL-36 can trigger an adaptive immune response and control the replication of the virus.

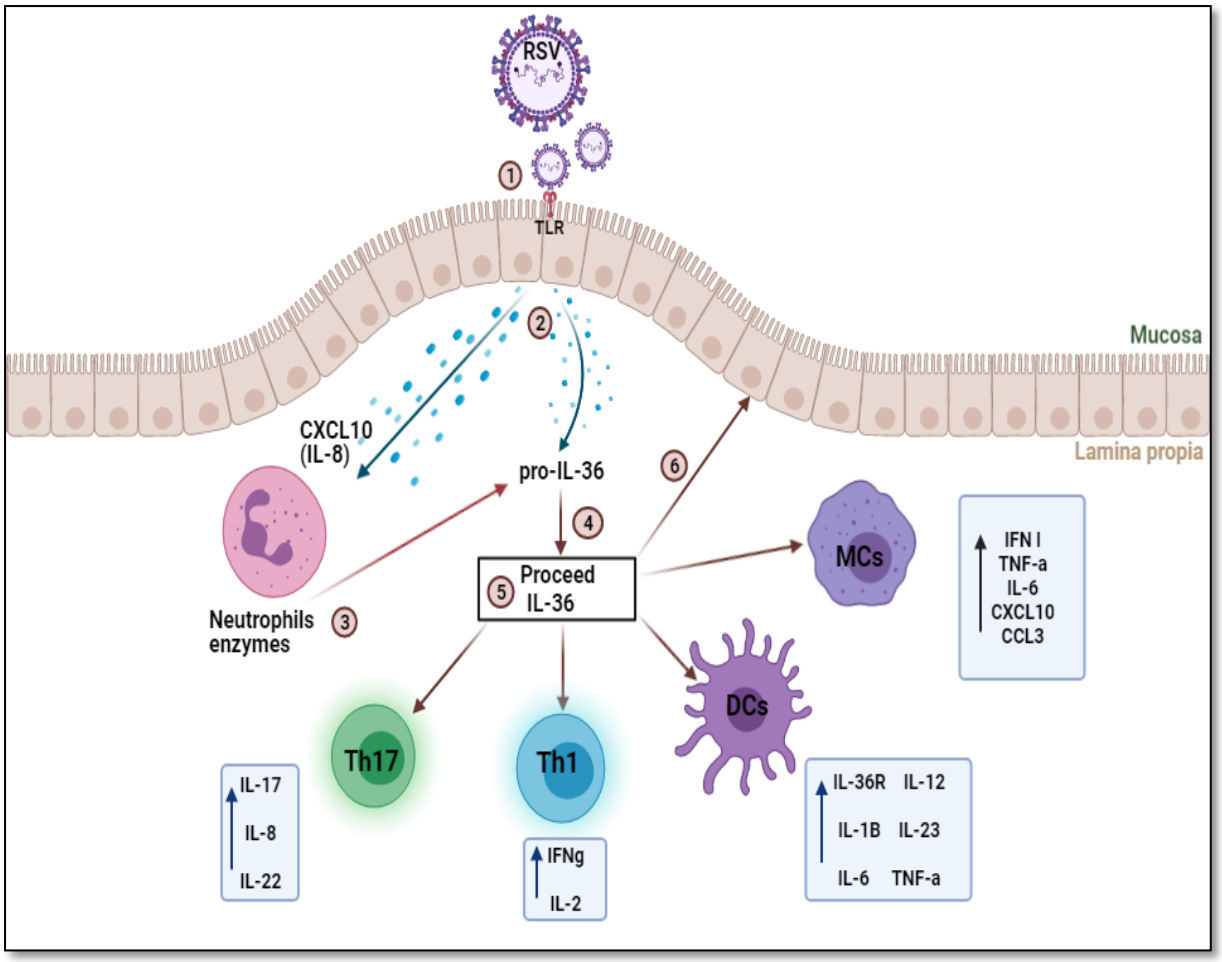

Figure 6.1 The functions of IL-36 cytokines and their role in immune response during RSV infection. 1) RSV binds to AEC TLRs, activating a series of signalling cascades. Initiates a complex of signalling cascades leading to expression of several of pro-inflammatory cytokine genes. **2)** AECs express neutrophils that chemoattract CXCL-8. **3)** Neutrophils proteases activate the pro-IL-36γ which expressed from AECs in response to RSV into the active form. **4)** IL-36 can amplify the immune response by attract the neutrophils to the inflammation site which is thought to happen during RSV infection. **5)** Continue to generate IL-36 from AECs and so impose a greater immunological response during RSV infection. **6)** IL-36 stimulates the expression of IFNI, IL-6 and TNF-α from alveolar macrophages. **7)** IL-36 induces DCs to express IL-6, IL-12, IL-1β, IL-23 and TNF-α. **8)** IL-36 cytokines enhance the activation of Th1 to produce (IFNγ and IL-2) and Th17 to express (IL-8, IL-17 and IL-22) which suggests that IL-36 may activate the adaptive immune response during RSV infection, limiting RSV replication in cells.

6.2 The secretion of IL-36 cytokines in HNAECS and cell lines (A549 and BEAS-2B) cells

In comparison to the NPAs, the expression of IL-36α and IL-36γ proteins in RSV-infected culture cells was relatively low. The quantities of IL-36α and IL-36γ proteins in the culture supernatant of infected HNAECs, A549, and BEAS-2B cells were extremely low, although the expression of IL-36α and IL-36γ mRNA suggested a considerable rise in response to RSV. Protein secretion pathways are still being researched. The mechanism of IL-36 secretion, like that of the IL-1 family, remains unknown. Several investigations have shown that microparticles produce IL-36 cytokines. As a result, it seems that IL-36 cytokines are initially undetectable in culture medium. The goal of this research was to figure out how to identify the protein in the most effective way possible, particularly when it is located in a cell pellet but not in culture conditions. Because IL-36, like other members of the IL-1 family, is released in microparticles, IL-36 proteins are not detectable in culture media. Sonication of microparticles may break their membranes and cause cytokines to be released, although they cannot be detected. According to Kovach (2016), sonication of conditioned media of KP and SP-infected pulmonary macrophages (PMs) significantly increased the identification of extracellular IL-36γ protein compared to non-sonicated samples (Kovach *et al.* 2016). Furthermore, Aoyagi *et al* (2016) revealed that IL-36α was produced in microparticles collected from respiratory cells in the airways and in BAL fluid after influenza virus vaccination.

According to earlier research, our study discovered that stimulation with ATP treated cells in addition to RSV stimulates the release of IL-36α and IL-36γ protein at all viral load concentrations (sections 3.3.2 and 3.3.3 - pages 108, 110, 126 and 136). This might be because IL-36 cytokines are produced in microparticles during RSV infection, but these proteins are packed in microparticles, and ATP must break down the membrane to excite the P2X7 receptor. In one study in 2012, Dubyak, *et al*. found that stimulating the P2X7 receptor induced the plasma membrane to bleb and shed membrane components of microparticles, enabling proteins to be released.

Accordingly, although IL-36 activation is poorly understood, this study confirms a pivotal role for IL-36 in the immune response to RSV infection. Several studies have documented the relationship between respiratory infection viruses and IL-36 cytokines. However, few studies have investigated IL-36 role in immune response in infants with RSV. Overall, these data show

that IL-36 ligands, particularly IL-36γ, are induced in bronchial epithelial cells following RSV infection and suggest a potential role of IL-36γ in mediating immune response against RSV.

6.3 RSV infection induces IL-37 cytokines in nasopharyngeal aspirate samples (NPAs), HNAECS and cell lines

In the second section of this dissertation, this study examined whether AECs infected with RSV could produce IL-37 cytokines and their receptors. NPAs were used *in vivo* for the examination of RSV and RV samples. A significant amount of the IL-37 protein is expressed in nasopharyngeal aspirates from children with RSV and RV, as reported in Chapter 4. Nasopharyngeal aspirates of patients with RSV and RV diseases were observed to contain high levels of IL-37 protein (Figure 4.2.1). When compared with RSV patient samples, RV patient samples had a significantly higher level of IL-37 protein expression. This can be explained by the presence of IL-37 protein in airway cells as well as the fact that RVare more responsive to airway cells than RSV. The expression of IL-37 mRNA and protein was evaluated in human nasal cells infected with IL-37 as well as in A549 and BEAS-2B epithelial cells cultivated *in vitro*. As described in sections 4.2.2 and 4.2.3, this research discovered substantial increases in IL-37 mRNA expression in infected HNECs and culture cells (A549 and BEAS-2B) at a multiplicity of infection (MOI) of 2.5 compared to non-infected HNAECs (section 4.2.2 and 4.2.3). This was notably true for the production of mRNA for the IL-37 gene at a multiplicity of infection (MOI) of 2.5. Moreover, the amount of IL-37 protein was considerably elevated in infected HNAECs relative to non-infected cells in the culture supernatant (Figure 4.2.2). In contrast, there were no significant differences in IL-37 protein expression between infected A549 and BEAS-2B cells and uninfected cells (Figure 4.2.3.5 A, B). During RSV infection, the expression of IL-37 isoforms mRNA was very low, but it rose dramatically with time. It is interesting that RSV infection had no effect on the expression of IL-37a mRNA. When RSV stimulates an airway epithelial cell, it generates cytokines and chemokines to begin an immune response against the virus. Despite its anti-inflammatory effects, IL-37 expression was substantial both *in vivo* and *in vitro* during RSV infection. During RSV infection, the IL-37 cytokine may decrease inflammation and prevent the overproduction of pro-inflammatory cytokines. In addition to IL-37, additional pro-inflammatory cytokines generated in response to RSV infection may increase IL-37 synthesis.

Furthermore, it has been proven that TLRs, which are activated by RSV infection, also trigger the production of IL-37 (Rudloff *et al.,* 2016).

The objective of Chapter 4 is to establish if IL-37 is expressed and localised in BEAS-2B cells during RSV infection. There were no variations between infected and uninfected cells in the position of the IL-37 protein surrounding the nucleus, as determined by this investigation. LPS treatment of BEAS-2B cells boosted IL-37 expression, as indicated by significant positive staining, and surrounding the nucleus in both infected and non-infected cells, especially 24 hours after stimulation because LPS can prevent the degradation of IL-37 (Figure 4.2.4.2B, C). Clearly, IL-37 protein translocation did not occur during RSV infection based on our results. In certain instances, it has been discovered that IL-37 degrades fast or does not reach the nucleus due to its inability to enter the nucleus.

During a viral infection, TLR4 on the cell surface may generate IL-37 by interfering with intracellular adapters to activate NF-KB, which increases the transcription of proinflammatory cytokine genes (Boraschi *et al.,* 2011). Thus, both TLRs and pro-inflammatory cytokines stimulate the production of IL-37. IL-37's anti-inflammatory effect suppresses infection-induced inflammation via a variety of routes (Figure 6.2). This is accomplished by binding to the IL-18R and IL-1R8 (SIGIRR) receptors (Nold-Petry *et al.,* 2015). The IL-37 binding sites on macrophages, dendritic cells, and peripheral blood mononuclear cells are part of a tripartite complex (IL-37–IL-1R8–IL-18R). IL-1R8 suppresses IL-1 and TLR-dependent inflammation, resulting in a broad suppression of cytokine production and lower expression of pro-inflammatory genes, including a reduction in IL-6 production, IFN response, and Th17 response. IL-37 may induce IL-10 and TGF- production in Treg cells (Wiercinska-Drapalo *et al.,* Garba *et al.,* 2002; An *et al.,* 2017). Through its interaction with Smad3, IL-37 has been shown to suppress intracellular inflammation (Nold *et al.,* 2010). Smad3 is a recognised transcriptional regulator that binds IL-37 and translocates to the nucleus. IL-37/Smad3 is a functional combination that decreases the inflammatory route of infection and promotes the production of anti-inflammatory cytokines (Figure 6.2).

IL-37 cytokines are recognised as anti-inflammatory cytokines; however, it is becoming apparent that the regulatory cytokine may regulate and minimise the danger of preterm newborns developing catastrophic symptoms due to RSV infection. Considering these results, IL-37 may be a viable treatment for RSV or, at the very least, lessen the unfavourable symptoms

associated with RSV-induced immune responses. When airway epithelial cells are exposed to RSV, they produce a considerable quantity of IL-37. The presence of IL-37 indicates that it can regulate the immune response and may serve as a cytokine with an immuno-regulatory function by inhibiting the overexpression of pro-inflammatory cytokines, suggesting that IL-37 may function as a negative feedback loop in the overexpression of pro-inflammatory cytokines.

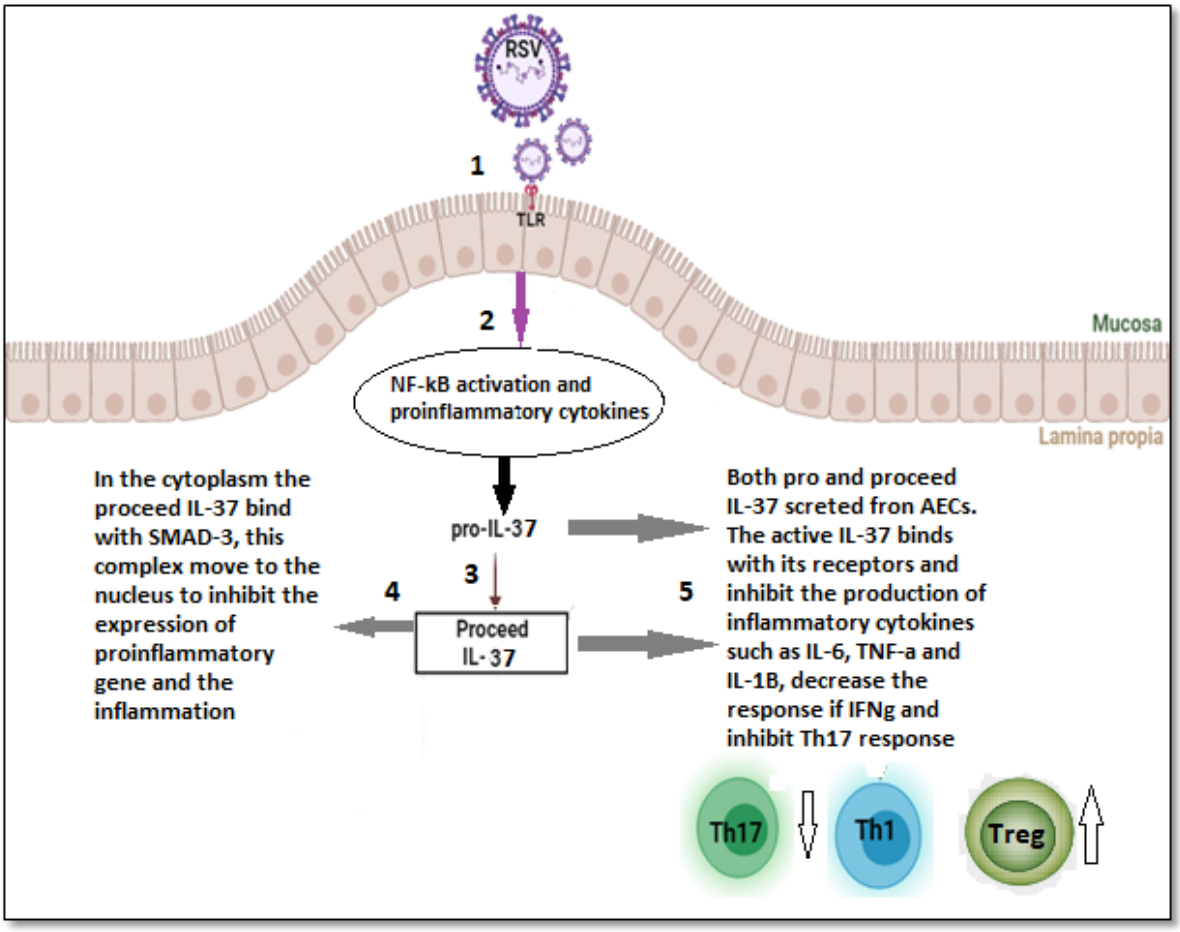

Figure 6.2 The functions of the IL-37 cytokine and their role in the immune response during RSV infection. 1) Viral infections induce Toll-like receptors (TLRs). **2)** TLRs activate NF-kB and inflammatory mediators such as IL-18 and IL-1β causing inflammation. **3)** As a result of inflammation and overproduction of TLRs, NF-kB, IL-18 and IL-1β lead to up-regulation of IL-37. Inflammation caused by infection can be inhibited through various pathways by IL-37. **4)** The extracellular pathways involved formation of the tripartite complexes: IL-37–IL-18Ra–IL-1R8. As a result of NF-kB activation reduction, this complex inhibits infection-induced inflammatory responses. The production of anti-inflammatory cytokines (IL-10 and TGF-b) is increased, and IL-6 production is downregulated. **5)** The intracellular IL-37 can inhibit inflammation through interacting with a transcription factor (Smad3) producing a functional complex (IL-37/Smad3) which reduces the pro inflammatory pathway and increases production of anti-inflammatory cytokines.

6.4 Could IL-1β and TNF-α be able to induce the expression of IL-36γ mRNA or protein, from A549 cells?

The major objective of this study is to determine how the IL-36 cytokine responds to other pro-inflammatory cytokines, since the IL-36 cytokine was the most heavily expressed in response to RSV in Chapter 5. Like other members of the IL-1 family, IL-1β is a vital inflammatory mediator and plays a significant function in cell biology.

The result found that, IL-1β cytokine is a strong inducer of IL-36γ cytokine in A549 cells. Regarding the time overall, the expression of IL-36γ mRNA was increased and reached to the peak early post stimulation with IL-1β from 3hr to 6hr (Figure 5.3.1 A) with TNF-α at 6hr (Figure 5.2.1 B). Although the IL-36γ mRNA and protein levels were induced after cytokines stimulation (section 5.3.3.1), there was no synergistic increase in IL-36γ mRNA or protein levels in RSV infected cells when treated with the combination of IL-1β with other cytokines including IL-17, IL-22, IFNγ, IFNβ and IL-4 cytokines (Figure 5.3.4.1). In addition, there was no significant increase of IL-36γ protein when stimulating the infected A549 cells with the combination of IL-1β with LPS in comparison to the expression of IL-36γ protein with the same cytokines alone (Figure 5.3.6.1). This result may be explained by the fact that the cytokine combination or the binding of the cytokine to the cell surface did not work well on the cell line A549 and required other factors. This research demonstrated that IL-1β and TNF-α are the most potent inducers of IL-36 in airway epithelial cells among other cytokines. In bronchial epithelial cells, TNF-α has been shown to generate IL-36γ (Chustz *et al.,* 2010). IL-36 expression was not induced by the combination of IL-1β, IL-17, IL-22, IFNγ, IFNβ, IL-4, and LPS. TNF-α also shown that there was no significant rise when paired with IL-17, IL-22, IFNγ, IFNβ, IL-4, and LPS. The expression of IL-36γ is mediated by IL-1β and TNF-α, according to these studies. Other cytokines, such as IL-17, IL-22, IFNγ, IFNβ and IL-4, also play an important role. However, synergistic effects with IL-1β and TNF-α were unable to alter IL-36 expression. After RSV infection, it has been suggested that monocytes expressed a large amount of IL-1β (Sone *et al.,* 1997). Additionally, IL-1β generated in the respiratory tract may serve as one of the primary inflammatory cytokines during the acute phase of an infection, in part through activating other inflammatory cytokines (Akira *et al.,* 1990). In addition, TNF-α is a key modulator of airway inflammation and chemokine production, and it may play a role in the immunopathogenesis of RSV infection. Patients with RSV bronchiolitis have elevated levels of TNF-α in their bronchoalveolar lavage

(BAL) fluid (Brightling *et al.,* 2008; Jafri *et al.,* 2004). TNF-α levels were considerably greater in the RSV bronchiolitis group compared to the control group, according to reports (Jungi *et al.,* 2010). Collectively, these data imply that IL-1β and TNF-α, two of the most important pro-inflammatory cytokines, may play a crucial role in the induction of IL-36γ by activating the signalling necessary for the production or activation of IL-36γ.

6.5 Limitations and implications of an *in vitro* airway epithelial cells

A significant weakness of this thesis is that none of the NPAs' information indicates when they originally became ill or when they were at their most ill before admission. In addition, there are no control groups to compare this research to others.

The disparity between *in vivo* and *in vitro* IL-36 protein levels was a possible drawback of our investigation. In addition, there are few research focusing on IL-36 release during viral respiratory infections. These mutations did not demonstrate whether these amounts originate from epithelial cells or other sources *in vivo*. This indicates that various stimuli may cause the development of IL-36 cytokines, while *in vitro* research only demonstrate the expression of IL-36 cytokines in RSV-infected cell cultures. Several variables *in vivo* may contribute to the production of increased IL-36 protein levels, including neutrophil proteinases that convert the inactive version of IL-36 into the active form. The active form of IL-36 enhances the production of IL-36 receptors and IL-36 cytokines from AECs. In addition, the production of IL-36 may boost other immune cells, such as DCs and macrophages, which subsequently activate other adaptive cells, such as Th1 and Th17, so increasing the quantity of IL-36 cytokines from several sources, not only AECs. In addition, samples from NPAs of children with RSV and RV illnesses were collected from healthy individuals, while samples from HNAECs were collected from children. Moreover, the impact of live RSV virus *in vivo* is more potent than the effect of frozen RSV virus *in vitro*. In addition, the culture of HNAECs was restricted to two passages each experiment, making it challenging to study several cytokines. A possible limitation of this research was that the cultured cell line did not exhibit the same flawless performance as *in vivo* animal models or primary cells.

The experiments presented in this thesis are limited by the lack of knowledge of the mechanism of IL-36 protein secretion and the lack of studies demonstrating its expression

during RSV infection. Since the secretion of IL-36 cytokines is still under investigation, this study cannot determine the actual level of IL-36 cytokines after infection with RSV, nor can it determine whether stimulation of IL-36 receptors results in the expression of airway epithelial cells. There was no evidence of IL-36 in the ELISA analysis. In addition to activating another pathway, co-stimulating with ATP may result in inaccurate measurement of IL-36. The effects of other cytokines on airway epithelial cells in vitro may need other factors to trigger the expression of IL-36γ from airways epithelial cells. Variable expression of IL-1RL2 mRNA in cultivated cell lines is another limitation of this research. BEAS-2B cells exhibited a considerable rise in IL-1RL2 mRNA expression, but A549 cells exhibited no significant increase (Figure 3.3.3.6). BEAS2B cells are derived from human bronchial epithelium that was transformed by hybrid adenovirus 12-SV40, while A549 cells are derived from lung carcinomas.

The fact that the site of IL-37 did not differ across groups suggests that a second induction is required. Immunofluorescence did not reveal any RSV staining in infected cells. In addition, there is no quantification in this work, and the quantities of mRNA vary across cells. Despite the use of LPS stimulation, the IL-37 level in this research did not accurately reflect the real IL-37 level since IL-37 levels are very low and decay rapidly after being produced.

The addition of inflammatory cytokines to induce the synthesis of IL-36 in A549 cells may not have interacted properly with their receptors, as shown in Chapter 5. In contrast to *in vivo* tests, the expression of IL-36 did not reveal the true function of these cytokines alone or in combination with other cytokines.

6.6. Future directions

In response to RSV infection, the results of this study reveal the capacity of airway epithelial cells to generate and express IL-36 and IL-37 cytokines and their receptors. In Chapter 3 *in vivo*, IL-36 and were highly expressed in NPAs, but RSV-infected HNAECs, A549, and BEAS-2B cells expressed but did not produce these proteins. In the future, it will be advantageous to comprehend the process by which IL-36 cytokines are released and their function as inflammatory cytokines during viral respiratory infection. Airway epithelial cell IL-36 secretion routes and IL-36/IL-36R complexes are required to analyse how IL-36 will induce immune responses. AECs produce IL-36γ protein mostly in response to lung infection, although little is known about the expression of this protein during infection. The further research should study this protein's antiviral properties in more depth. Additionally, its influence on the production of other inflammatory cytokines against viral infection in the lungs should be investigated. Collectively, IL-36 cytokines generate robust pro-inflammatory responses and may promote both innate and adaptive immune responses; their involvement in airway inflammatory illness should be explored further.

Chapter 4 examines IL-37 cytokine and its function as an anti-inflammatory cytokine, as well as its capacity to control the immune response, restrict the production of inflammatory cytokines, and protect infected tissues from harm. IL-37, a new cytokine, may be beneficial in the treatment of inflammatory illnesses. It is feasible to see IL-37 as an anti-inflammatory cytokine, which might mitigate the harsh symptoms of lung inflammatory illnesses.

IL-36γ levels rose during RSV infection and IL-1β and TNF-α activation, as described in Chapter 5. Neutralizing IL-1β / TNF-α or inhibiting their receptors will be used to determine whether IL-36γ expression was mediated by IL-1β / TNF-α. In addition, the function of IL-36γ in regulating other inflammatory cytokines during RSV infection should be investigated.

These investigations provide light on the inflammatory responses of AECs to various dosages of RSV infection and the production of novel inflammatory cytokines including IL-36 and IL-37. In the lungs, these cytokines function as therapeutic agents and may help alleviate the viral infection.

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Appendix

Nasopharyngeal Aspirate Samples (NPAs) with RSV Disease

	Sample ID	IL-36α Pg/ml	IL-36γ pg/ml	IL-37 pg/ml	Oxygen? Yes/No	Severity	Age
1	2845	1748	97	366	Yes	Moderate	2M 3D
2	2855	200	1816	40	Yes	Moderate	17D
3	2866	805	484	890	Yes	Moderate	13D
4	2870	0	0	215	Yes	Severe	1M
5	2877	7083	33	0	No	Mild	5M 18D
6	2883	50	302	0	No	Mild	3M 15D
7	2888	254	112	466	Yes	Severe	24D
8	2892	82689	126	701	Yes	Severe	13D
9	3147	486	354	1200	Yes	Moderate	7M 7D
10	3148	1925	1088	221	Yes	Moderate	1M 3D
11	3152	170	927	209	Yes	Moderate	1M 13D
12	3157	34	90	84	Yes	Moderate	1M 12D
13	3162	1925	115	0	No	Mild	7M 1D
14	3173	7715	249	490	Yes	Moderate	24D
15	3174	0	79	171	Yes	Moderate	21D
16	3183	498	54	0	No	Mild	11M 6D
17	2894	24227	488	386	Yes	Moderate	17D
18	2913	300	152	140	Yes	Moderate	3M 27D
19	2924	462	0	430	Yes	Severe	1M 21D
20	2941	352	795	159	No	Mild	2M 12D
21	2947	0	427	0	Yes	Severe	1M 12D
22	2949	4208	349	4691	No	Mild	8M 4D
23	2955	464	543	319	Yes	Moderate	1M 9D
24	2969	605	106	169	Yes	Moderate	24D
25	3189	107	209	257	Yes	Severe	25D
26	3191	638	1682	319	Yes	Moderate	3M 9D
27	3206	164	435	294	No	Mild	4M 21D
28	3211	230	0	240	No	Mild	2M 19D

29	3217	0	202	281	No	Mild	2M 1D
30	3232	0	49	169	No	Mild	9D
31	3235	4489	1421	1069	Yes	Moderate	3M 4D
32	2975	221	71	243	No	Mild	15D
33	2982	48770	241	100	No	Mild	2M 22D
34	2988	365	304	0	Yes	Moderate	1M 10D
35	2995	40	961	667	No	Mild	22D
36	3000	1054	266	59	No	Mild	3M 15D
37	3002	1120	0	0	No	Mild	2M 2D
38	2118	548	2136	168	Yes	Moderate	2M 9D
39	3146	250	144	107	Yes	Severe	20D
40	3240	0	487	341	Yes	Moderate	1M 13D
41	3244	13	1444	1860	No	Mild	9D
42	3245	301	338	0	Yes	Moderate	2M 24D
43	3254	148	1573	316	No	Mild	1M 12D
44	3272	4	337	1497	No	Mild	11M 12D
45	3275	7	173	380	No	Mild	5M 4D
46	3276	86	503	332	Yes	Moderate	2M 28D
47	3294	3	512	283	Yes	Moderate	1M 14D
48	3302	0	315	336	Yes	Moderate	1M 22D
49	3405	1011	540	607	Yes	Moderate	1M 21D
50	3408	0	347	289	No	Mild	23D
51	3434	57	308	277	Yes	Moderate	3M 4D
52	3472	5	357	327	No	Mild	1M 27D
53	3494	257	1269	510	Yes	Moderate	2M 15D
54	3495	315	369	0	No	Mild	2M 29D
55	3496	111	1193	354	Yes	Moderate	1M 3D
56	2704	18	116	610	Yes	Severe	1M 22D
57	2432	2958	233	380	No	Mild	1M 4D
58	2439	12	375	530	Yes	Moderate	1Y 2M
59	2457	10	752	930	Yes	Moderate	1M 15D
60	2489	3092	273	115	No	Mild	1Y
61	2739	1471	1256	555	Yes	Moderate	5M 21D
62	2613	18	81	320	No	Mild	3M 15D
63	2722	9725	727	701	Yes	Mild	10M 6D
64	2783	842	84	431	Yes	Moderate	2M 19D
65	2785	5678	335	171	Yes	Moderate	2M 25D

66	2793	0	0	85	Yes	Mild	1Y 3M
67	2797	44	522	379	No	Mild	1Y 1M
68	2801	47	24	260	Yes	Moderate	1M 6D
69	1976	449	1730	116	Yes	Moderate	11M 29D
70	1978	370	1757	480	No	Mild	1Y
71	1981	5058	854	115	Yes	Moderate	1Y 2M
72	2025	651	970	73	Yes	Moderate	12D
73	732027	1275	302	45	Yes	Moderate	2Y 7M
74	274000	649	327	0	No	Mild	1Y
75	207504	0	99	190	Yes	Moderate	23D
76	20170	185	55	2670	Yes	Severe	21D
77	2015	2	443	373	Yes	Moderate	3M 21D
78	2019	0	54	59	Yes	Severe	1Y 11M
79	2030	18	0	150	Yes	Severe	8M 23D
80	2042	40	250	369	Yes	Severe	1M 14D
81	1996	33	12	716	No	Mild	4M 19D
82	1994	1491	374	380	Yes	Moderate	2Y 3M
83	1983	24	28	300	No	Severe	3M 9D
84	1553	0	487	289	No	Moderate	3M 29D
85	1561	3149	934	300	Yes	Mild	2M 14D
86	1587	228	1239	100	No	Severe	1M 5D
87	1592	143	535	530	Yes	Moderate	2M 12D
88	1608	231	752	60	No	Moderate	18D
89	1809	780	438	328	No	Mild	9M 12D
90	1759	59	495	755	Yes	Severe	1M 17D
91	1772	625	503	930	Yes	Moderate	1Y 11M
92	1774	542	647	115	No	Moderate	2M 29D
93	1776	578	864	320	No	Moderate	2M 13D
94	1778	0	373	702	Yes	Severe	1Y
95	2041	13	915	1034	Yes	Moderate	3Y

Nasopharyngeal Aspirate Samples (NPAs) with RV Disease

	Sample ID	IL-36α Pg/ml	IL-36γ Pg/ml	IL-37 Pg/ml	Oxygen?	Severity	Age
1	3066	229	680	280	Yes	Moderate	29D
2	3309	43109	1918	148	No	Mild	17D
3	3323	200	58	131	No	Mild	19D
4	3352	2	46	384	Yes	Moderate	27D
5	3397	1167	232	352	No	Mild	16D
6	3646	95	100	49	No	Mild	14D
7	3984	234	109	425	No	Mild	12D
8	3727	2788	875	431	No	Mild	25D
9	3257	641	320	606	No	Mild	18D
10	4286	2981	2178	823	No	Mild	27D
11	2327	1972	581	689	Yes	Severe	2M 19D
12	3072	25	1002	6120	No	Mild	2M 13D
13	3371	1489	102	322	No	Mild	1M 3D
14	3381	629	1406	581	No	Mild	2M 6D
15	3662	27	49	200	No	Mild	1M 4D
16	3677	17	101	70	Yes	Moderate	1M 3D
17	3804	592	244	322	No	Mild	2M 3D
18	3929	9	393	243	No	Mild	2M
19	3142	5	257	40	Yes	Severe	1M
20	4332	607	385	0	No	Mild	1M 20D
21	4358	1	73	348	No	Mild	2M 3D
22	4369	0.779	98	243	No	Mild	1M 24D
23	3785	85	365	613	No	Mild	1M 14D
24	4331	4207	8398	716	No	Mild	1M 4D
25	2865	200	555	59	No	Mild	1M 11D
26	2965	30	591	0	Yes	Mild	1M 22D
27	3175	35	347	369	No	Mild	1M 1D
28	2053	0	0	716	Yes	Moderate	1M 12D
29	2650	2972	44	380	Yes	Mild	5M 8D
30	2631	66	893	300	Yes	Moderate	4M 27D
31	3076	191	220	289	No	Mild	4M 25D
32	3230	63989	1054	0	Yes	Moderate	7M
33	3306	6612	1120	100	No	Mild	4M 4D
34	3333	516	61	530	No	Mild	4M 3D
35	3363	40	158	0	No	Mild	6M 15D
36	3367	21435	1747	328	Yes	Severe	3M 27D
37	3675	1748	267	755	No	Mild	5M 3D
38	3677	26	97	930	Yes	Moderate	1M 3D

39	3957	4	26	115	No	Mild	3M
40	3985	805	266	320	No	Mild	8M 13D
41	4036	6	127	555	No	Mild	3M 17D
42	4101	12	118	0	Yes	Moderate	7M 4D
43	4235	26298	250	701	Yes	Severe	8M 20D
44	4298	462	349	431	No	Mild	6M 5D
45	3771	0	427	1035	Yes	Severe	5M 23D
46	3884	1925	814	352	Yes	Moderate	3M 2D
47	3791	34	1011	398	No	Mild	6M 21D
48	2840	0	361	641	No	Mild	7M 19D
49	3436	0	228	490	No	Mild	4M 23D
50	2631	60	143	171	Yes	Moderate	4M 27D
51	2650	4983	291	116	Yes	Mild	5M 8D
52	2685	170	22	272	Yes	Moderate	6M 10D
53	2053	0	0	85	Yes	Moderate	1M 12D
54	2788	7	0	379	No	Mild	5M 17D
55	1934	287	80	2679	Yes	Moderate	4M 8D
56	2413	17	129	7046	Yes	Moderate	9M 2D
57	3332	100000	578	480	No	Mild	11M 2D
58	3400	2	26	115	No	Mild	11M 27D
59	3679	4	0	0	No	Mild	10M 2D
60	3962	572	434	357	No	Mild	10M 24D
61	3871	200000	961	570	No	Mild	10M 17D
62	3117	0	231	410	No	Mild	11M 6D
63	3760	5243	780	466	Yes	Mild	10M 22D
64	2632	17	160	121	Yes	Moderate	3Y 2M
65	2664	5299	625	269	No	Mild	3Y 4M
66	2735	6048	504	153	Yes	Moderate	3Y 6M
67	2738	6590	1808	215	No	Mild	1Y 2M
68	1998	121	217	0	Yes	Severe	2Y 3M
69	2796	2360	1572	365	Yes	Moderate	3Y 1M
70	1916	29	37	253	No	Mild	10M 25D
71	2001	2906	542	59	Yes	Moderate	1Y
72	2033	15	0	50	Yes	Moderate	11M 15D
73	2634	8	47	300	No	Mild	3Y 10M