The role of airway epithelial cell and natural killer cell interactions during RSV infection

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by

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Abstract

Little is known about the early interactions that occur between respiratory syncytial virus (RSV) infected airway epithelium and NK cells. This includes airway epithelial cell (AEC) surface expression of IL-15/IL-15Rα complex, which could be a potent inducer of NK cell activation via trans-presentation. The work here describes the hypothesis that AECs after infection can activate NK cells which in turn influence immune and inflammatory responses including AEC activity.

Human bronchial epithelium cell line, BEAS-2B, and human nasal airway epithelial cells (HNAECs) were used *in vitro* to characterise the expression of IL-12, IL-15, IL-18 and IL-15Rα during RSV infection. IL-12 protein was not expressed. RSV infection significantly increased expression of cell surface IL-15Rα on BEAS-2B cells and HNAECs. BEAS-2B cells also expressed cell surface IL-15 indicating complex expression. IL-15 protein was only detected in infected BEAS-2B cell culture supernatants and not HNAECs. Soluble IL-18 protein was only expressed by HNAECs and significantly increased with infection. The RSV infected airway epithelium has the potential to activate NK cells with IL-15 signalling predominantly occurring through the IL-15/IL-15Rα complex.

A co-culture model was then established to examine AEC-NK cell interaction. Co-culture of peripheral blood NK cells with RSV infected BEAS-2B cells or HNAECs increased expression of IFN-γ from NK cells. IFN-γ expression was dependent on direct cell-to-cell contact with infected BEAS-2B cells. To further characterise AEC-NK cell interactions and how this may influence the wider inflammatory response to RSV, expression of AEC-derived CXCL9, CXCL10, CXCL11, TARC and BAFF during co-culture was characterised. CXCL10 was significantly increased from infected AEC-NK cell co-cultures compared to those without NK cells. BAFF mRNA was significantly increased in BEAS-2B cell-NK cell co-cultures however, soluble protein remained the same. Treatment of non-infected BEAS-2B cells with IFN-γ significantly increased cell surface expression of IL-15 and IL-15Rα and this response was not observed after infection. NK cells co-cultured with Th2 cytokine pre-treated and infected BEAS-2B cells had significantly increased IFN-γ expression compared to those from co-cultures with no cytokine or IFN-γ treatment pre-treatment.

Nasopharyngeal aspirates from RSV and rhinovirus (RV) infected infants were analysed for IL-15, IL-15/IL-15Rα complex and IL-18 to add *in vivo* relevance to the *in vitro* findings. IL-15 and IL-18 protein levels were significantly higher in NPAs from RSV infected infants who required oxygen treatment compared to those who did not. IL-15 and IL-18 were significantly higher in NPAs from those with RV infection compared to RSV.

Collectively, the *in vivo* relevance of these findings suggest that RSV infected AECs can alone activate NK cells. This is dependent on direct cell-to-cell contact of which the IL-15/IL-15Rα complex may be an essential method of NK cell activation. Activated NK cells may then aid in an increase in AEC-derived CXCL10 protein expression and possibly membrane-bound BAFF during contact with infected airway epithelium. NK cells may therefore contribute to adaptive immune responses through enhancing airway epithelial responses.

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. Specifically, flow cytometric analysis of intracellular perforin and IFN-γ expression was carried out by Liz Van Erp (National Institute for Public Health and the Environment (RIVM), Amsterdam).

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A549	Adenocarcinomic human alveolar basal epithelial cell line
AEC	Airway epithelial cell
APC	Allophycocyanin
B cell	B lymphocyte
BAFF	B cell activation factor
BAL	Bronchoalveolar lavage
BEAS-2B	Human bronchial epithelium cell line
BEGM	Bronchial epithelium growth medium
BSA	Bovine Serum Albumin
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CD56	Natural killer cell marker
CD3	T cell marker
cDNA	Complimentary deoxyribonucleic acid
CXC	C-X-C motif chemokine
CX3CR	The CXC chemokine receptor
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
EDTA	Ethylene diamine tetra acetate
ELISA	Enzyme-linked immunosorbent assay
F	Fusion protein
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
G	Glycoprotein
hr	Hour
HBEC	Human bronchial airway epithelial cells
HEp-2	Human epithelial type 2
HLA	Human leukocyte antigen
HNAEC	Human nasal airway epithelial cells
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory factors
KCl	Potassium chloride
L	RNA-dependent RNA polymerase
LPS	Lipopolysaccharide
M	Matrix protein
MCP	Monocyte chemoattractant protein
MFI	Mean fluorescence intensity
MIP	Macrophage Inflammatory Proteins

MHC	Major Histocompatibility Complex
ml	Millilitre
mg	Milligram
MOI	Multiplicity of infection
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response 88
N	Nucleoprotein
NaCl	Sodium chloride
ng	Nanogram
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NPA	Nasopharyngeal aspirate
NS	Non-structural protein
P	Phosphoprotein
PAMP	Pathogen-associated molecular patterns
PE	Phycoerythrin
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque-forming units
pg	Picogram
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern Recognition Receptors
qPCR	Quantitative polymerase chain reaction
RANTES	Chemokine (C-C motif) ligand 5 (CCL5)
RIG-I	Retinoic acid inducible gene
RNA	Ribonucleic acid
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
T cell	Lymphocyte
TGF	Transforming growth factor
Th1	T cell helper 1
Th2	T cell helper 2
TLR	Toll like receptor
TNF	Tumor necrosis factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
µg	Microgram
µl	Microliter

Chapter 1. Introduction

1.1. Human respiratory syncytial virus

1.1.1. Disease burden and epidemiology

RSV is the most common cause of lower respiratory tract illnesses, such as bronchiolitis and pneumonia. Disease caused by RSV represents a global burden with an estimated 64 million individuals worldwide having RSV-related disease each year (WHO 2009). RSV was first isolated from chimpanzees in 1956 and then one year later infants (1,2). RSV gained its name following the observation of syncytia formation by infected ciliated AECs during viral culture. RSV infection follows a seasonal pattern with hospital admissions increasing during the colder months in temperate climate regions or rainy periods in tropical regions (Figure 1.1) (3,4). Figure 1.1 details this seasonality in England and Wales, with RSV infection having remained dominant over other respiratory viruses. This seasonality is not seen with other respiratory viruses, such as rhinovirus (RV) which is seen throughout the year. RSV infects almost everyone by 2 years of age, but can infect anyone at any time in life. This is owing to the inability to induce long-term immunity towards RSV and a skewed and unfavourable Th2-response seen in infants (5,6). RSV usually causes mild symptoms such as cough, wheeze and fever, however in some infants, the elderly, immunocompromised and those with pre-existing conditions, RSV causes can cause severe disease requiring hospitalisation and in some cases requiring transfer to an intensive care unit (7–9).

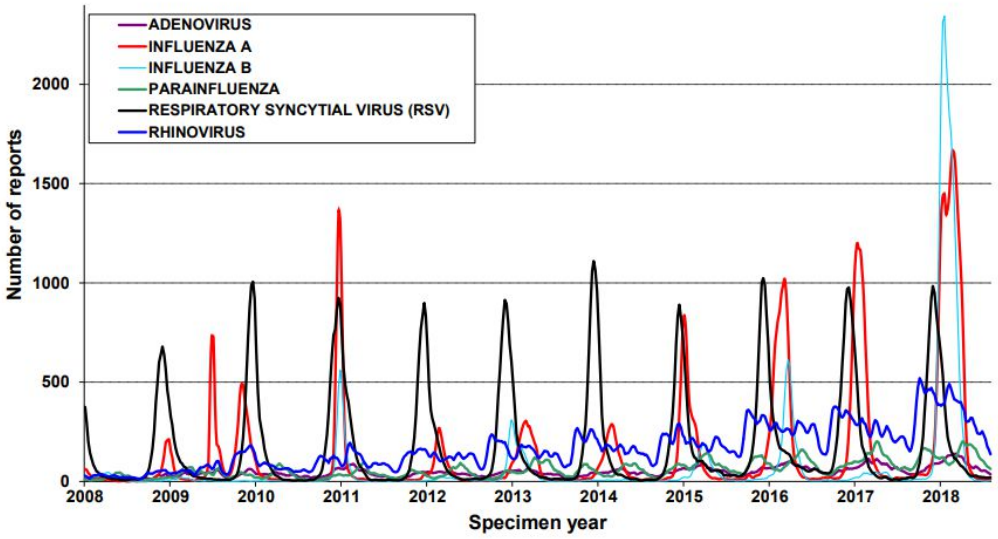


Figure 1.1. Weekly distribution of major viruses in England and Wales. Data is collected from Public Health England and NHS laboratories surveillance from 2004 to 2018 and shows a 3-week moving average. Lines represent adenovirus (purple), influenza A (red), influenza B (aqua), parainfluenza (green), RSV (black) and RV (blue). Reproduced from Public Health England (accessed October 2018).

In the USA between 1997-2009, an estimated average of 11,300 RSV-related deaths occurred annually (10). The burden of disease is greater in resource poor countries. RSV was reported to be responsible for 5.6% of deaths in a study of 8 African countries (11). Figure 1.2 details that infants who are born during the winter months had more RSV-attributable admission to hospital, which was more prevalent for those aged under 6 months as well (12–14). Predisposing factors resulting in an increased risk of severe RSV disease include preterm birth, low birth weight, not being breast fed, having a mother who smokes during pregnancy, genetic differences in innate immune responses, being from a low income household, and having other medical conditions such heart or respiratory problems (15–18). Overall, disease caused by this virus and its seasonal nature results in a large economic burden. Paediatric intensive care unit stays of up to 52 days are not uncommon and medical care for the elderly is more costly for those infected with the additional complications caused by RSV infection (19,20).

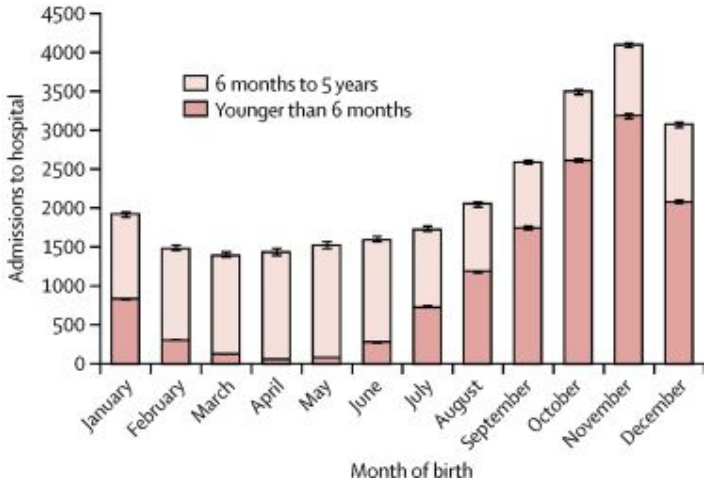


Figure 1.2. RSV-specific GP consultations and hospital admissions by month of birth. Graph represents the number of admissions to hospital associated with RSV disease for those born in each month. Light pink bars represent ages 6 months to 5 years and salmon bars represent infants under 6 months old. Reproduced from Cromer *et al.* 2018 (12).

1.1.2. Classification and structure

RSV is a single-stranded, negative-sense, enveloped, RNA virus belonging to the *Orthopneumovirus* genus of the *Pneumoviridae* family (21). The viral genome is 15.2kb nucleotides long and has 10 genes that encode 11 proteins (Figure 1.3A). RSV has two major antigenic subtypes, A and B which are concurrently in circulation, with subtype dominance during RSV seasons lasting around 1-2 years (22,23). Antigenic subtypes A and B can furthermore be sub-grouped into genotypes, or clades, based on G gene variability (24). Worldwide, the most prevalent clades for RSV A are GA2 and GA5 and for RSV B the BA (25,26).

Structurally each RSV particle has three transmembrane glycoproteins contained with a lipid bilayer; the glycoprotein (G), the fusion protein (F) and a small hydrophobic protein (SH) (Figure 1.3B). G protein structure includes variable glycosylation of 'mucin-like' domains which determine antigenicity and give rise to the A or B serotypes according to antibody reactivity (24,27,28). The G protein interacts with cell surface proteins including the CX3CR1 receptor found on ciliated AECs via a CX3C motif and enhances cell binding (29–32). CX3CR1

deficient mice do not display complete inhibition of viral replication and anti-CX3CR1 treatment of the cell line Hela did not affect viral replication (30). This is possibly due to the multiple receptors expressed by AECs that RSV can utilise for enhanced cell entry such as ICAM-1, TLR4, nucleolin and HSPG (33–36). The F protein glycoprotein is essential for cell entry (37,38) and has both pre and post fusion states which form a trimeric structure at the membrane surface (39). Due to its essential requirement for cell entry, the F protein has a highly conserved region seen across RSV A and B subtypes, with overall 89% amino acid sequence similarity compared with 50% for the G protein (24,40). The F-protein binds to ICAM-1 and upon binding, virus particles are then endocytosed into the cell (41,42). The role of SH protein has still not been fully determined. The SH protein can form ion channels in human embryonic kidney cell line, HEK 293, however the role of this is still undetermined (43–45). RSV lacking both G and SH proteins is still able to infect cells and recombinant RSV lacking SH protein has a higher replicative ability than wild-type virus *in vitro* (46). Replication of RSV lacking SH protein has also shown to be different between upper and lower airways and also between difference species. Mice showed a 10-fold reduction in replication of RSV lacking SH protein in the upper airways, not seen in the lower airways compared to wild-type RSV, whilst in chimpanzees reduced replication was observed in the lower airways compared to the upper airways (47,48). This suggests that the SH protein may aid in RSV replication *in vivo*.

Below the lipid bilayer is an inner layer consisting of two matrix proteins M and M2-1. M matrix protein is essential for the production of infectious viral particles and can associate with the nucleocapsid (49–51). The M2 protein aids in antitermination (the ability to prevent premature termination of RNA synthesis) which is especially important for longer RNA viruses (52,53). Figure 1.3C illustrates the RSV life cycle and details the stages of viral genomic replication, viral protein synthesis and virion budding. Within the virus particle is the nucleocapsid which is released into the cytoplasm of the host cell after fusion. The

nucleocapsid, consisting of N, P and L proteins, forms a decametric ribonucleoprotein ring which provides protection of RSV genomic RNA and aids in viral replication (54). Concurrently occurring is the translation of the viral mRNA using the host cell's ribosomes to produce viral proteins (55). P protein associates with N protein to form 'nucleocapsid-like' structures which gives specificity for viral RNA-L complex RNA which is a cofactor in RNA synthesis and stabilises the L protein (56–58). The L protein is an RNA-dependent RNA polymerase involved in transcription of the viral RNA genome (59,60). M2-1 then combines to the nucleocapsid complex which allows M protein to bind. When M protein associates to the nucleocapsid complex the genomic RNA forms tighter coils and terminates transcriptase activity. Finally the nucleocapsid and envelope glycoproteins move to the cell surface where the M protein of the nucleocapsid interacts with the F and G protein and a new virion buds from the host cell membrane (58).

Non-structural proteins, NS1 and NS2, are expressed during transcription and play a role in inhibiting host cell innate responses, such as IFN-α and IFN-β expression (61–63). This is a mechanism that may aid in enhanced viral replication within the host by suppression of innate immune response and viral clearance mechanisms.

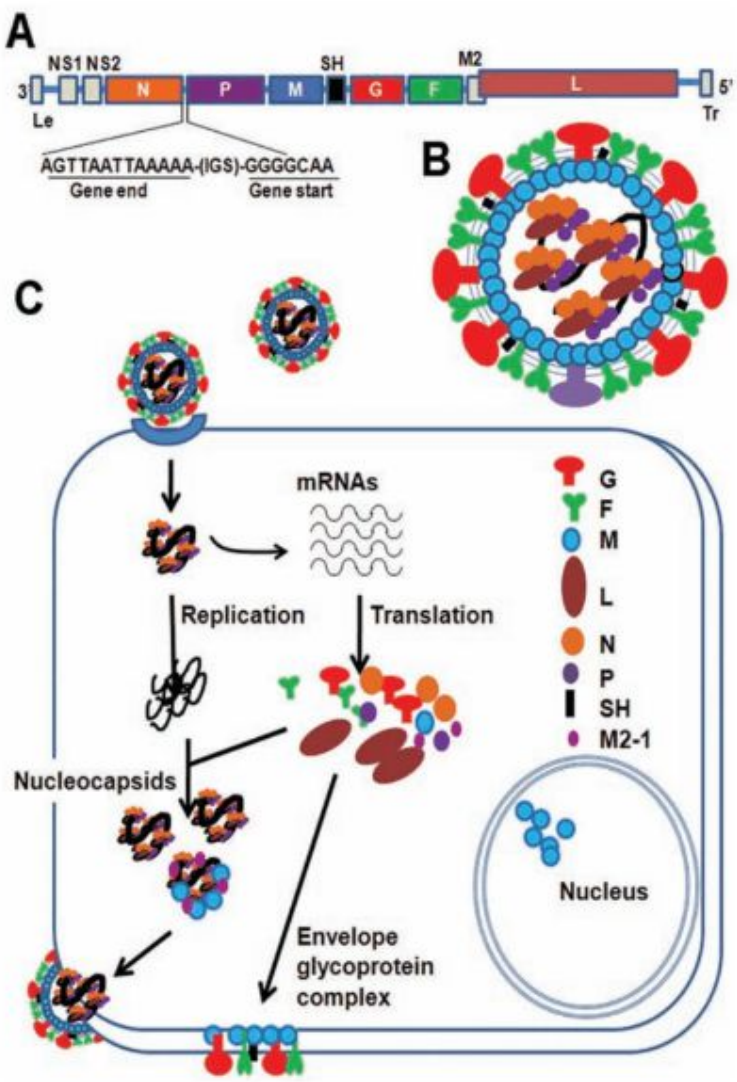


Figure 1.3. The genetic organisation, structure and infectious cycle of RSV. A) The genomic sequence of RSV which is 15.2 kb long, encoding 10 genes, encoding 11 proteins. NS – non-structural protein, N – nucleocapsid, P – phosphoprotein, M – matrix protein, SH – small hydrophobic protein, G – glycoprotein, F – fusion protein, L – large polymerase. B) Cartoon diagram of virion particle. The outer lipid bilayer layer consists of glycoproteins; G attachment protein, F fusion protein and SH small hydrophobic protein. The envelope lies below the outer layer consists of the M matrix and M2 protein. Within the envelope is the ribonucleoprotein complex, consisting of negative sense (-), single stranded (ss) RNA, genome and proteins N, P and L. C) RSV attaches to the host cell via G protein. Fusion of virus and cellular membranes occurs allowing release of viral genome into the cytoplasm. The genome is transcribed into mRNAs which are then transcribed into viral proteins by host cell ribosomes. New genomes associate with N, P, L, M and M2-1 proteins to form nucleocapsids. Envelope glycoprotein are processed through the host cells ER-Golgi secretory pathway. The glycoprotein complex and nucleocapsids associate to form a new budding virion which leaves the host cell. Reproduced from Ghildyal *et al.* (58).

1.1.3. Airway epithelium recognition of RSV

RSV transmission can occur through viral particles present in aerosol sprays and also by direct contact from person to person. RSV has an incubation period of around 3-6 days, which is delayed in comparison to influenza virus (64,65). Infection begins in the nasopharynx and may then spread to the lower respiratory tract (66,67). RSV predominantly infects ciliated cells entering through the apical surface but can also infect other non-ciliated, bronchial, bronchiolar and alveolar epithelial cells such as basal cells (68). In the lower airways, RSV has also been shown to infect type 1 pneumocytes (responsible for gas exchange) in the alveoli, indicating a potential for viral spread throughout the entire airways (69). The extent of resultant epithelial damage includes sloughing, apoptosis of ciliated epithelial cells, loss of cilia function and increased goblet cell numbers, which leads to mucus overproduction (70,71).

Infection of AECs initiates an innate immune response which progresses with the combined response of resident lung innate immune cells. AECs, alveolar macrophages, dendritic cells (DCs) and innate lymphoid cells (ILCs) express pattern recognition receptors (PRRs) which include toll-like receptors (TLRs), retinoic acid inducible gene (RIG-I) and nucleotide-binding oligomerisation domain (NOD)-like receptors. These bind pathogen-associated molecular patterns (PAMPs), such as viral RNA, and initiate signal cascades that induce localisation of two main transcription factors, nuclear factor-κB (NF-κB) and interferon regulatory factors (IRFs) (72,73). Activation of these signal cascades, as shown in Figure 1.4, leads to synthesis of cytokines and chemokines, including type I interferons (IFN), IFN-α and IFN-β, and type III interferons, IFN-λ, critical for virus elimination (Figure 1.4) (74,75).

Ten TLRs have been identified in humans and although all ten types of TLR mRNA are expressed throughout the airway epithelium, protein expression can vary depending on airway location (76). TLRs also show differential compartmentation within AECs. TLR3 is

expressed on both apical and basolateral sides in contrast to TLR1, TLR4, TLR5, TLR7, TLR9 and TLR10 which are predominantly expressed on the apical side, providing multiple sites for the initiation of an immune response when RSV binds and then enters AECs (77). TLRs TLR1, TLR2, TLR3, TLR5 and TLR6 have been shown to be the most highly expressed in AECs (77,78). In the AEC line, A549, RSV induces increases expression of TLR3, TLR4 and RIG-I which initiates activation of transcription such as NF-kB and IRF-3 (79–81). TLR3 recognises RSV dsRNA within the cell and also activates NF-κB leading to IL-8 synthesis (82). Knockdown of TLR3 in A549s reduced immune response-initiating gene expression of IFN-β, CXCL10 and CCL5 (83). RSV NS2 protein was also shown to inhibit the TLR3 pathway in A549s which again could lead to reduced interferon response (84). The role TLRs play in RSV-recognition provide a varied and crucial initiation of immune response cytokine expression in AECs. Polymorphisms in TLR structure could therefore reduce AEC recognition of RSV. For example TLR4 polymorphisms may correlate to a more severe RSV illness, but the role of TLR polymorphisms during RSV infection are not well defined (85–87). RSV F protein has been reported to bind TLR4 leading to NF-κB-led transcription of cytokines IL-6, IL-8, IL-10 and IFN-β and an increase in disease severity has been associated with lower TLR4 gene expression (88,89). RSV has been shown to also upregulate TLR4 in A549s cells, however the role of RSV F protein binding and interactions with TLR4 is still debated with other studies having suggested RSV-TLR4 interactions do not influence RSV pathogenesis at all (88–92). Overall, RSV activated pattern recognition receptors (PRRs) and induced cytokine and chemokine expression leading to an infiltration of immune cell populations and subsequently development of acquired immunity.

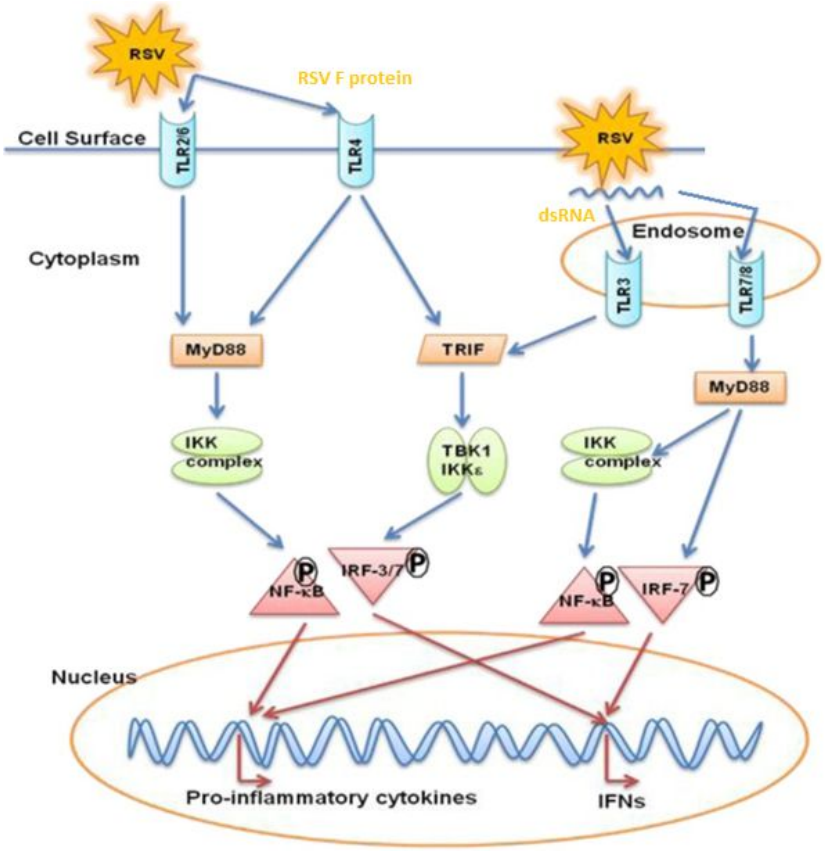


Figure 1.4. RSV-induced toll-like receptor (TLR) signalling pathway. RSV proteins and RNA are recognised by extracellular and intracellular TLRs which signal through MyD88 or TRIF adaptor molecule pathways to induce transcription factors NF-κB and IRFs 3 and 7. This leads to production of interferons (IFNs) and pro-inflammatory cytokines. Reproduced and adapted from Kolli *et al*. (74).

1.1.4. Therapies

Delivering therapeutic advances for the prevention and treatment of RSV infection has been challenging. To date, there is still no accepted RSV vaccine. In the 1960's, a formalin-inactivated RSV vaccine was trialled on a small group of infants but when they were exposed to natural RSV infection, this resulted in more severe disease and two fatalities. In 1998 Palivizumab (brand name Synagis), a monoclonal antibody that binds to the RSV F protein inhibiting viral entry into host cells, was approved for prophylactic use. This treatment, which takes the form of passive immunisation, is given to individuals at risk of RSV infection including infants born prematurely and those with other underlying diseases. High risk

children that do not receive RSV prophylaxis are more likely to be hospitalised and those who do not complete treatment have longer hospital stays (93,94). Palivizumab treatment has been shown to reduce overall risk of hospitalisations by ~50% in term infants and by 78% for infants born prematurely (95). However, prophylaxis treatment may still not fully inhibit symptoms such as wheezing (96).

Currently, F and G proteins are thought to be attractive therapeutic targets for vaccine design. However, these vaccines may not provide lifelong protection from RSV disease. For example, an RSV F nanoparticle vaccine only resulted in increased anti-F IgG levels for up to a year in young adults (97). Alternative therapeutic strategies showing promise include low-molecular-weight organic compounds which block viral entry to the cell by inhibiting fusion of RSV to host cell surface receptors (98).

1.2. The host immune response to RSV

1.2.1. Innate immunity

The innate immune response provides the first line of defence against pathogens and other environmental substances. It is composed of anatomical barriers such as the skin, epithelial cells that line the airways and gastrointestinal tract, innate immune cells and leukocytes which coordinate the initiation of inflammatory and immune responses often through recognition of common pathogen features through PRR sensing. Although the innate immunity can be considered to lack classical immunological memory, it is an essential initiator of the highly specific adaptive immune response and thus also a regulator of the type, strength and longevity of that response. Innate immunity helps shape the adaptive response including immunological memory and subsequent response to infections.

1.2.1.1. Airway epithelium

The airway epithelium is comprised of AECs that form a continuous layer in both large and small airways. The main cell types of the airway epithelium include ciliated columnar epithelial cells, secretory cells and basal cells (Figure 1.5). Over 50% of the airway epithelium are ciliated cells which transport mucus from the lower airways up to the throat (99). Goblet cells secrete mucin glycoproteins into the airway which consists of antimicrobial peptides, aiding in trapping of environmental substances (100). Serous cells form glands and secrete ions and airway liquid (101). Basal cells are progenitors of columnar cells and respond to airway cell loss, renewal or damage (102). Together, this mixed population of cells ensure the airway epithelium stays intact and acts quickly to initiate an immune response to pathogens. Pathogens are recognised by external and internal PRRs, TLRs, c-type lectin receptors, retinoic acid-inducible gene-I-like and NODs, described above in Section 1.1.3 (103).

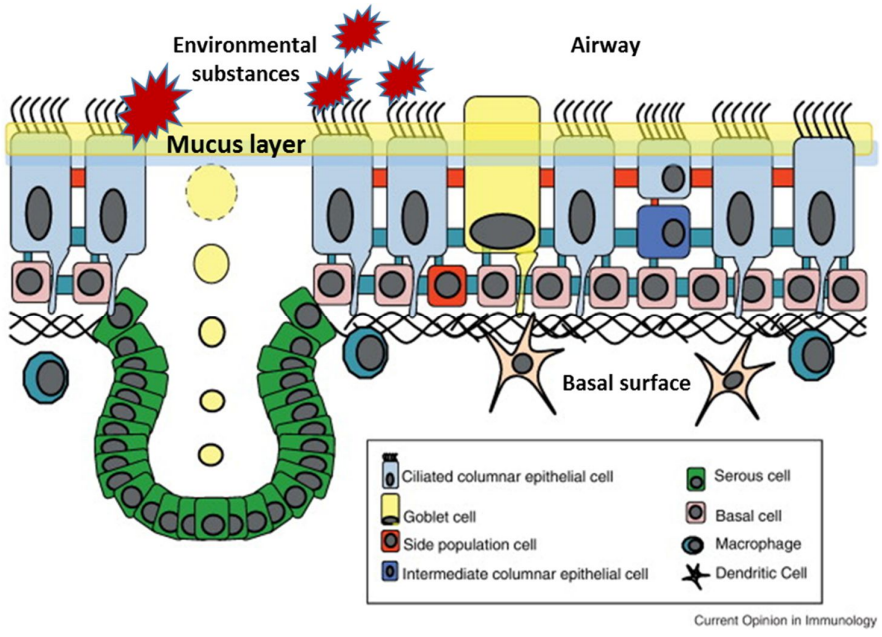


Figure 1.5. Anatomy of the airway epithelium. Serous and goblet cells provide the mucus layer at the airway interface and ciliated columnar epithelial cells aid in moving the mucus up to the throat. Progenitor basal cells differentiate into columnar cells to ensure the epithelium stays as one continuous layer. Innate immune cells such as macrophages and macrophages reside at the basal surface. Modified from Hirota *et al*. (104).

The apical side of the airway epithelium consists of a mucus layer of secreted molecules that provides an important barrier to inhaled pathogens and substances to limit contact with AECs. Secreted molecules include antimicrobial substances such as β-defensins, LL-37 (or cathelicidin) antimicrobial peptide, lysozyme, lactoferrin, IgA, nitric oxide, and mucins (Figure 1.6) (105–107). These molecules are multifunctional, acting on many types of microbes and microbial products. β-defensins are antimicrobial peptides that aid in pathogen clearance in which their expression can be induced by bacterial lipopolysaccharide (LPS), fungus, TNF-α and IL-1β (108,109). LL-37 expression stimulated by LPS induces infected AEC apoptosis and proliferation to maintain epithelium integrity (110–112). The iron binding protein lactoferrin has the ability to inhibit entry of various viruses, including RSV, into AECs and inhibit bacterial growth (113–115). IgA has potent virus neutralising properties, such as for influenza virus, however RSV specific IgA has shown little correlation to RSV neutralisation (116–118). Lysozyme cleaves bacteria cell wall peptidoglycans and has antimicrobial properties against common lung bacteria such as *E.coli*, *P.aeruginosa* and *S.aureus* (119,120). Nitric oxide has various roles within the lung such as ion channel regulation, airway secretions and ciliary motility which all aid in pathogen clearance (121). MUC5AC and MUC5B are the main secreted mucins and trap pathogens within the mucus layer, however overproduction of MUC5AC and MUC5B have been associated with increase in disease severity to RSV and chronic bronchitis possibly by reducing airflow (122–124).

Functions of the basolateral side of the epithelium include secretion of pro-inflammatory cytokines and chemokines. These have the ability to aid migration, differentiation and activation of both innate and adaptive immune cells (125,126). Figure 1.6 details some innate responses during RSV infection. RSV upregulates the production of many chemokines and cytokines from AECS via the NF-κB pathway of which IL-1, IL-6, IL-11, CXCL10, IL-8, CCL5, CCL2, CCL3, CCL4, TNF-α, interferons such as IFN-α/β, and GM-CSF are the most prominently upregulated. Table 1.1 details some key AEC-derived chemokines during RSV infection (127–

130). Differential expression of key AEC-derived chemokines and cytokines has been correlated to both RSV disease severity and also to protection from enhanced disease. In humans, increased serum CCL5 levels and a CCL5 (−403 G/A) polymorphism has been reported to be associated with increased risk of recurrent wheezing following RSV infection, potentially by leading to prolonged inflammatory cell recruitment in the lungs after infection (131). In mice, CCL3 expression was shown to protect from TNF-α induced weight loss, an indicator of disease severity, which was potentially through reducing the amount of TNF-α expressing $CD8^+$ T cells in the lungs (132). Differential expression of AEC-derived chemokines may therefore drive and protect from excessive inflammation.

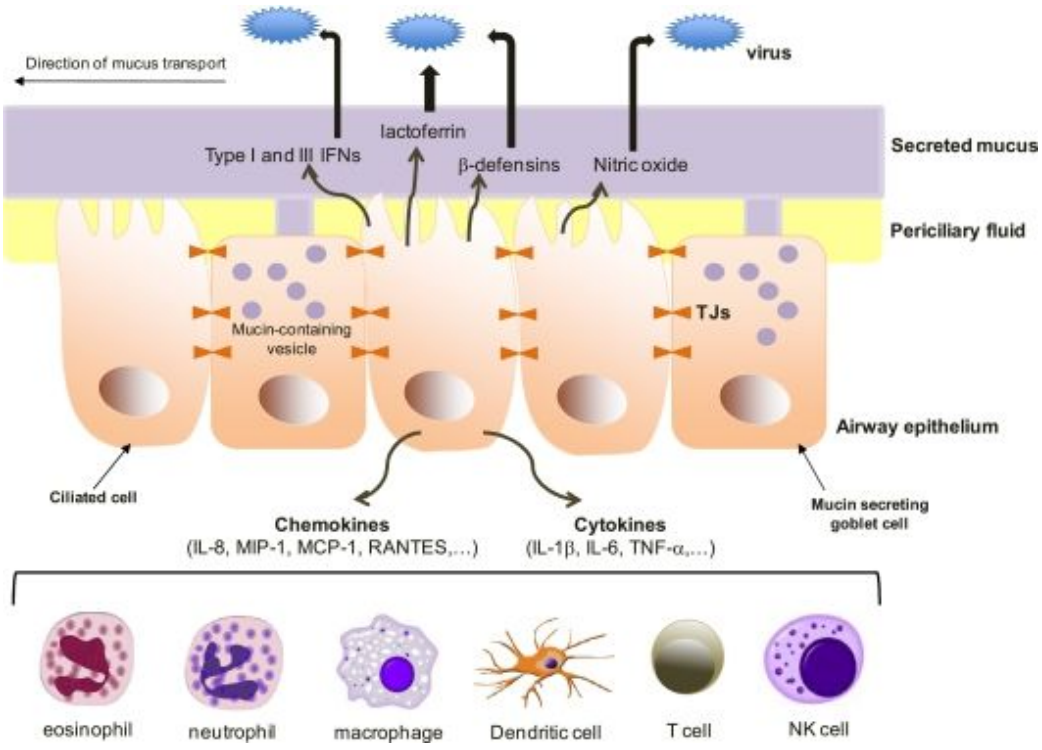


Figure 1.6. Innate immune responses of the airway epithelium to viral infections. The first line of defence against respiratory viruses is a mucus layer which aids in trapping pathogens and holding secreted molecules such as β-defensins, interferons, nitric oxide and lactorferrin. Upon infection, AECs then co-ordinate an innate and adaptive immune response with the secretion of various chemokines and cytokines which attract and activate immune cells. Taken from Vareille *et al.* (133).

Table 1.1. AEC-derived chemokines during RSV infection. Modified from Zhang *et al.* (129).

Chemokine (also 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

The anti-viral interferon response is especially important during RSV infection. AEC-derived interferons including type I (IFN-α, IFN-β) and type III (IFN-λ1/IL-29, IFN-λ2/IL-28A and IFN-λ3/IL-28B) expressed during viral infection are thought to limit viral replication (134–136). IFN-α and IFN-β inhibit viral replication and spread through the induction of IFN-inducible proteins such as RNase L, which inhibits viral RNA synthesis, and expression of apoptotic proteins, which reduces cell-to-cell viral spread (137–140). However, RSV NS1 and NS2 proteins restrict the expression of IFN-α, IFN-β, IFN-λ1 and IFN- λ2/3, which would otherwise aid in viral clearance (141,142). The interferon response during RSV infection is important in initiating adaptive responses. Mice lacking IFNAR1 showed reduced expression of memory $CD8^+$ T cell–derived granzyme B during secondary RSV infection, suggesting an impaired interferon response from AECs could reduce protective responses on secondary infection (143). Expression of AEC-derived BAFF, a crucial B cell activating cytokine, is IFN-β dependent. As RSV NS1 and NS2 proteins reduce IFN-β gene expression this could potentially

reduce IFN-β-induced AEC-derived BAFF expression and hence reduced B cell responses (144–146). Reduced B cell responses could increase the likelihood for secondary infection, with less RSV-neutralising antibodies raised and reduced adaptive immune responses. Overall, an AEC-derived interferon response is essential to provide effective RSV innate immune cell elimination and adaptive immunological memory.

During an inflammatory response, AECs can be further stimulated by cytokines in their environment including TNF-α and IFN-γ, to further increase or modify AEC cytokine and surface receptor expression. This could enhance the immune orchestration from AECs. TNF-α and IFN-γ AEC-stimulation includes increased expression of ICAM-1 receptor, CCL11 (eotaxin), CCL5, IL-6 and IL-15, which aid in eosinophil, neutrophil and T cell recruitment to infected airways and then immune cell adherence to infected AECs (147–152). RSV infection of AECs accompanied with IFN-γ treatment has been shown to act synergistically, that is the expression level by combined RSV infection and IFN-γ treatment produced an amount that was greater than the sum of levels observed with infection or treatment alone, to enhance CXCL10 expression which could increase recruitment of monocytes, T cells, natural killer (NK) cells and DCs to infected sites (153). Therefore upon recruitment of immune cell populations, a co-ordinated response between AECs and immune cell populations may occur.

1.2.1.2. Lung resident and recruited immune cells

Lung resident immune cells include cells such as mast cells, DCs, macrophages, NK cells and ILCs. Immune cells that are recruited during infection or injury include neutrophils, eosinophils, monocytes and other lymphocytes (154). These cells, along with AECs, can enhance an innate immune response and provide a bridge between the innate and adaptive immune response leading to activation of the adaptive immune response.

DCs can be classified as either myeloid (mDC) or plasmacytoid (pDC) based on expression of surface proteins, CD11c, CD13, CD33 and CD11b and CD123, CD303 and CD304, respectively (155). mDCs prime T cells whilst pDCs express type I interferons, especially IFN-α (156). DCs are an essential cell population for the presentation of pathogen antigens to T and B cells, however their role during RSV infection is not fully defined. Both mDCs and pDCs were found in nasal washes from children with RSV infection along with a decrease in peripheral blood DC numbers suggesting recruitment to infected airways (157). RSV has been shown to directly infect and replicate in monocyte derived DCs, with preferential infection of mDCs over pDCs (158,159). RSV NS1 and NS2 proteins have shown to reduce DC function and hence impaired DC-T cell interactions (160–162). Taking these results together suggest that upon recruitment to sites of infection, DC functions could be impaired such as by NS1 and NS2 modulation of DC IFN-α expression. This was shown in mice with depletion of pDCs leading to enhanced lung inflammation and mucus production during RSV infection, which could have been due at least in part to an observed reduction in IFN-α protein expression (160). Furthermore DCs from neonatal mice were shown to have reduced ability to process RSV antigens and showed reduced expression IFN-α, resulting in enhanced airway pathology in comparison to that observed in adult mice. (161,162). A reduction in both the expression of IFN-α and RSV-antigen presentation could lead to reduced T cell responses. Overall, reduced DC-derived IFN-α expression could lead to the reduction of other cytokines, such as IFN-γ, CXCL10, IL-6 IL-1β and TNF-α, which has been observed in IFNAR1 deficient mice (147,148) and thus reduced adaptive responses and increased RSV illness.

Alveolar macrophages present antigen or antigenic peptides from phagocytosed pathogens or infected-apoptotic cells to T or B cells (163) and enhance the cytokine response to RSV infection, including production of IFN-α, IFN-β, TNF-α, IL-6, CCL3, and CXCL10 (164,165). Macrophages seem to aid in immune cell recruitment and retention in the lungs of mice. Macrophage depleted mice showed reduced NK cell numbers and activation over an 8 day

period, compared to wild-type RSV infected mice that had NK cell recruitment lasting over 4 days (166). However not all mouse studies show supporting results. In one study, changes in bronchoalveolar lavage (BAL) cellularity during RSV infection of macrophage depleted mice resulted in increased numbers of DCs, neutrophils, $CD4^+$ and $CD8^+$ T cells (167). Using New Zealand black mice, which have inherent macrophage functional deficiencies, results showed reduced lung $CD4^+$ and $CD8^+$ T cells and increased NK cells and DCs numbers compared to BALB/c mice (168). Here impaired macrophage function was associated with RSV pathology and increased viral load which could suggest T cell responses aid in resolution whilst NK cells and DCs could be involved in pathology. Mouse macrophage studies show it is apparent that macrophages have a role in recruiting and activating different immune cell populations, however their role has not been well defined in humans.

Another group of emerging innate immune cells that are being extensively studied throughout many different inflammatory diseases are ILCs (169). There are three groups of ILCs which include ICL1 (including NK cells), ILC2 and ILC3 (including lymphoid tissue inducer cells) (170). ILCs derive from a common lymphoid progenitor which express ID2, a transcriptional repressor. Figure 1.7 briefly details how ILCs are classified based on phenotypes and functions. For instance, different ILCs groups express different cytokines, such as IFN-γ and TNF-α for ILC1s, IL-5, IL-6 and IL-13 for ILC2s and IL-22 and IL-17 for ILC3s, in response to different stimuli (Figure 1.7). All ILCs, except NK cells, express CD127 (IL-17RA) and all lack lineage markers for other hematopoietic-derived cells, such as CD3 (T cells), CD19 (B cells), CD11b (neutrophils and eosinophils) or CD11c (DCs and monocytes). In healthy human lungs, the main ILC group are ILC3 (~57%) then followed by ILC2s representing around ~33% and finally ILC1s ~10% (171). Expansion and activity of specific ILC groups have been reported in different lung disease including ILC3 in COPD, ILC1/NK cells and ILC2s during RSV infection and ILC2 in allergic asthma (171–173). NK cells are discussed in more detail in Section 1.3 and their role during RSV infection in Section 1.4.

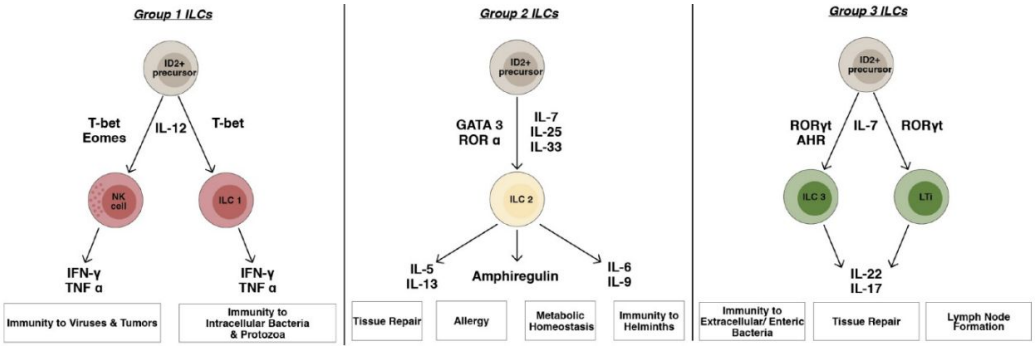


Figure 1.7. Innate lymphoid cell groups and roles during infection. ILCs derive from a common lymphoid progenitor which express ID2, a transcriptional repressor. ILCs are then classified into one of three groups based on transcription factor expression, expansion by different cytokines and cytokine expression. Taken and modified from Elemam *et al.* (174).

Neutrophils are the main inflammatory cell population recruited to the lungs during severe RSV infection where their functions include phagocytosis of pathogenic particles, neutrophil extracellular trap formation and degranulation of pro-inflammatory molecules. Neutrophil chemo-attractants present during RSV infection include IL-6, IL-8, CCL3 and CCL5 (175). Up to 75% of nasal lavage cells isolated from patients with bronchiolitis are neutrophils and airway neutrophilia has been suggested to correlate with disease severity (176,177). SNPs in the IL-8 gene associated with increased production of this potent neutrophil chemoattractant, have been associated with more severe RSV- disease (178). This may suggest a role in RSV pathogenesis, however the overall role of neutrophils is still unclear.

Eosinophils are another group of recruited cells that have also been implicated in RSV pathogenesis. Recruited to the lung by chemo-attractants such as CCL5, CCL11 and CCL3 (179,180), eosinophils produce cytokines and other inflammatory mediators, including nitric oxide, which aid viral clearance (181). Although the role of eosinophils during primary RSV has again not yet fully been characterised, their roles after RSV infection may be particularly relevant to the development of allergy or asthma. Eosinophil counts have been found to be higher in infants with persistent wheeze following RSV infection and reinfection after exposure to RSV early after birth in mice leads to an eosinophilic inflammation which is more

pronounced in IFN-γ deficient mice (182–185). Administration of IFN-γ was only able to protect from eosinophilia in IFN-γ deficient mice if given during primary infection indicating the importance of early IFN-γ expression in controlling eosinophil recruitment to the lungs both during first and subsequent infections (185). Mice first infected with RSV during the neonatal period and then later re-challenged with RSV show elevated Th2 responses which was also accompanied by a greater number of eosinophils in BAL, suggesting an imbalance Th1/2 responses (186). This data suggests eosinophils may not be involved in severe disease during primary RSV infection but may have a role in subsequent infections and promoting airway allergy.

1.2.2. Adaptive immunity

In contrast to the innate immune response, the adaptive immune response is highly specific and forms an immunological memory, providing faster and enhanced pathogen clearance on subsequent exposures. Two broad arms of adaptive immunity are the antibody or humoral response, initiated by B cells, and the cell-mediated response, initiated by T cells.

1.2.2.1. Cell-mediated immunity

Antigen presenting cells, such as DCs and macrophages described above, present antigen as peptide fragments associated with MHC class I or II which interact with $CD8^+$ and $CD4^+$ T cells, respectively (187). T cells can be classed into two main subsets $CD4^+$ or $CD8^+$. $CD4^+$ T cells can be further differentiated into Th1 (T helper), Th2, Th9, Th17, Th22, Treg (regulatory), and Tfh (follicular helper) (188). $CD8^+$ T cells include naïve, T_{CM} (central memory), T_{EM} (effector) and T_{EMRA} (effector memory) cells (189). Upon activation, depending on the subset, T cells can produce cytokines including IFN-γ, TNF-α, TGF-β, IL-4, IL-5, IL-9, IL-13, IL-17, IL-22 and cytotoxic proteins, granzymes and perforin. During RSV infection, $CD8^+$ T cells aid in viral

clearance by killing infected cells and are an important part of the memory response to RSV (23,190,191). $CD4^+$ T cells during RSV infection are the main producers of the pleotropic cytokine IL-10, which has been shown the both reduce and increase pathology by influencing Th1 and Th2 responses (192,193). Both T cell subsets are important for viral clearance through expression of cytokines.

In general the association between RSV severity with T cell numbers, T cell activation and T cell cytokine expression is unclear. RSV infection results in an expansion in the T cell population in infected children compared to non-infected children of which 1.9% of total BAL cells were T cells and of these ~28% were $CD4^+$ and 62% RSV-specific $CD8^+$ T cells (194). In adults, during more severe RSV disease there was an increase in proliferating and activated $CD8^+$ T cells, although this study did not examine if these were RSV-specific $CD8^+$ T cells (195). Brand *et al.* showed that peripheral blood from RSV infected children contained reduced absolute numbers of $CD4^+$ and $CD8^+$ T cells (196). Furthermore, this was most evident in severe disease which could suggest recruitment to sites of infection and an increase in lung T cell numbers may be associated with disease severity. Other studies have made similar observations with lower absolute numbers of peripheral blood $CD8^+$ and $CD4^+$ T cells during more severe disease and lower $CD4^+$ T cells counts in infants needing ventilation (197,198). Reduced peripheral blood T cell number suggests T cell recruitment to the lungs, however many of these studies are limited by a lack of functional characterisation of the T cell subsets. Transcriptome analysis of $CD4^+$ T cells from RSV infected infants showed increased SOCS2, SCOS3 and SOCS5 gene expression during severe illness compared to mild illness (199). SOCS proteins are involved in $CD4^+$ T cell polarisation, and could indicate a Th2 polarised environment during RSV infection which may induce more severe disease, although phenotypic $CD4^+$ T cell analysis was not conducted in the study above (200).

Animal models indicate T cells are necessary for effective viral clearance. Combined deficiency of both $CD4^+$ and $CD8^+$ T cells resulted in prolonged RSV viral titres up to day 14 post infection. This was not observed in wild-type mice or mice depleted of $CD8^+$ T cells only suggesting $CD4^+$ T cells have a greater role in limiting viral spread than $CD8^+$ T cells (201). Mice studies have been used to further determine T cells during secondary RSV infection. In one study mice immunised with formalin-inactivated RSV showed a strong memory $CD4^+$ T cell response that induced a severe response on re-infection with RSV (202). In a different study, on RSV re-challenge wild-type mice showed little weight loss 4 days however mice depleted of $CD4^+$ T cells showed the greatest weight loss and disease severity after re-infection (201). This was not seen with $CD8^+$ T cell depletion. This study indicates that $CD4^+$ T cells may also play a protective role in subsequent infections such as initiating a stronger immunological memory against RSV. RSV has also been reported to directly infect $CD4^+$ T cells which was suggested to result in reduced expression of the Th1 associated cytokines IL-2 and IFN-γ (197). Overall, impaired $CD4^+$ T cell responses in humans could result in prolonged RSV illness and the potential for subsequent repeated infections.

Depletion of $CD8^+$ T cells in mouse studies has also demonstrated their importance during RSV infection. $CD4^+$ T cells, from RSV infection of mice that had been previously vaccinated with an RSV-F protein expressing vaccine and depleted of $CD8^+$ T cells, expressed higher intracellular levels of Th2 cytokines IL-4 and IL-13 and reduced levels of the Th1 cytokine IFN-γ (203). In this study, $CD8^+$ T cell depletion was also associated with eosinophilia which may have been directed by $CD4^+$ T cells as the result of reduced IFN-γ expression and an enhanced Th2 response. $CD8^+$ T cells may therefore reduce $CD4^+$ T cell-derived Th2 responses. Other mouse studies also show similar results stressing the importance of RSV specific $CD8^+$ T cells in also controlling a Th2 response and reducing eosinophilia on subsequent re-infection (204,205). Furthermore, as shown in murine models, Tregs may supress early $CD8^+$ T cell functions during RSV infection and result in enhanced pathology (206,207). These studies

indicate a dynamic interplay between $CD4^+$ and $CD8^+$ T cell populations is needed to control RSV infection and highlights a further beneficial role for $CD8^+$ T cells. Overall both $CD4^+$ and $CD8^+$ T cell subsets appear to be mutually associated with overall RSV disease severity.

Another important T cell subset during RSV infection are the IL-17 producing, Th17 cells. Infants express higher levels of IL-17 in tracheal aspirates and serum during RSV bronchiolitis which may be associated with disease severity (198,208,209). The role of Th17 cells during RSV infection is not well established, although they are believed to play a role in regulating airway inflammation. AECs stimulated with IL-17 show increased expression of inflammatory cytokines, mucus proteins and chemokines (210). In mice, IL-17 levels in the lung peaked 8 days after infection suggesting that Th17 cells can contribute to RSV disease possibly through prolonged inflammation (208). This study also suggested enhanced IL-17 induced mucus production, inhibition of viral clearance and enhanced a Th2 cytokine profile. The role of Th17 cells needs further characterisation in humans and is relatively less characterised than other T cell subset responses.

1.2.2.2. Humoral immunity

Viral neutralising antibodies produced during infection has been shown to be important in preventing subsequent reinfection. In Infants protection can begin with passive immunity though transfer of maternal antibodies of IgG type to the foetus. Maternal anti-RSV IgG detected in mothers is also seen in their infants, but levels fall to around 6% of those at birth by 3-6 months of age, an age at which many infants are infected (211). Additionally whilst maternal RSV-specific IgG antibodies have been shown to have some protective role against RSV, including delaying infection, serum neutralising antibody levels do not fully correlate with protection against disease (212–214).

Natural RSV infection results in production of neutralising antibodies, particularly against the G and F proteins. However repeat infections are common and it appears RSV-specific antibody levels fall after infection and provide protection for only a limited time (5,215). In one study of participants after voluntary intranasal RSV infection, those who were negative for RSV after challenge had a significantly higher pre-inoculation nasal RSV-specific IgA titre, suggesting the level of IgA may provide protection, however nasal RSV IgA titres and RSV titres showed no correlation (216). Another study also observed that adults who were naturally infected with RSV had lower nasal RSV-specific IgA and serum IgG titres compared to non-infected individuals and an inverse relationship was seen between serum RSV neutralising antibodies and hospitalisation (217,218). This suggests RSV infection leads to an incomplete humoral response. A lack of correlation has also been observed between infant serum antibody levels and severity to RSV infection (219). Lower antibody levels and the short-term nature of their expression may also lead to reduced immune responses upon infection.

At a cellular level RSV infection induces AEC-derived BAFF and APRIL expression, which are required for B cell activation (145,151,220). RSV infected infants showed an increase in B cell numbers in peripheral blood compared to age matched controls and the B cell absolute count was shown to continue to increase one week after hospital admission (221). A histology based study showed B cells, BAFF and APRIL are all present in the human lung during RSV infection (222). BAFF protein is also greatly increased in the BAL fluid of RSV infected infants of which a key source of BAFF could be the airway epithelium (145). B cells grown in culture supernatant from RSV infected BEAS-2B cells, an AEC line, show enhanced B cell survival indicating that B cells could be active during *in vivo* infection through AEC-derived molecules (151). Overall, B cells can be activated during RSV infection however it appears subsequent antibody production may not be fully protective against RSV.

1.2.3. Imbalance in Th1/Th2 responses during RSV infection.

RSV disease severity has been strongly associated with an imbalance in the Th1 and Th2 response. This was first evidenced after the failed 1960's FI-RSV vaccine in which children given the vaccine experienced severe RSV illness with natural infection. Subsequent research lead to the characterisation of FI-RSV vaccine-induced Th2 responses (223). Th1 cells highly express CXCR3 and CCR5 whilst Th2 cells highly express CCR2, CCR3 and CCR4, and therefore differential chemokine expression during RSV infection could influence which T cells are recruited to the lungs and influence inflammatory outcome (224).

Early expression of IFN-γ was lower in nasal lavage fluid from infants with acute RSV bronchiolitis compared to upper respiratory tract infection, along with an increase in IL-4 at days 5-7 after hospitalisation (225). This was taken to indicate that a lack of early IFN-γ expression could lead to a Th2 response. Some studies show a Th2 profile is associated with more severe RSV illness, described as an increase in nasal fluid IL-4/IFN-γ ratio (225–227). However, not all clinical studies show that a reduced Th1 and increased Th2 response correlate to RSV disease severity (228,229). Others have observed an increase in nasal fluid IFN-γ/IL-4 ratio, indicating a Th1 response may also contribute to RSV illness (230–232). Vojvoda *et al.* found serum levels of Th2 cytokines TARC (CCL17) and CCL22 negatively correlated with RSV disease severity, whereas an increase in Th1 cytokine CXCL10 correlated with disease severity (233). Out of a selection of chemokines, CXCL10 had the highest expression at 33,000pg/ml in the BAL of RSV infected infants and although this study did not examine correlations with severity, these infants were hospitalised suggesting overexpression of Th1 associated chemokines, particularly CXCL10, early in infection contributes to more severe illness (234). Overall, clinical data indicates that an imbalance in either a Th1 or Th2 response can lead to enhanced RSV bronchiolitis in infants.

Murine models have provided further knowledge about the Th1/Th2 response to RSV infection. Neonatal mice re-infected as adults with RSV expressed more Th2 cytokine expressing $CD4^+$ T cells compared to adult mice (186). IFN-γ deficient mice, a Th1-assoctiated cytokine, had no difference in viral load or airway inflammation compared to wild type mice during first RSV infection (185). However on second RSV re-challenge IFN-γ deficient mice developed severe airway inflammation along with eosinophilia, which could not be restored by IFN-γ treatment. This indicates Th1 associated cytokine IFN-γ shapes the initial adaptive immune response to RSV infection. Neonatal mice with enhanced IFN-γ expression at the beginning of RSV infection had less IL-4 expressing $CD4^+$ T cells and overexpression of IL-4 resulted in enhanced a Th2 response (235). These mouse models suggests that expression of IFN-γ or a controlled Th1 cytokine environment, is essential for a reduced and/or controlled Th2 response. Most significantly, these mouse models highlight the importance for IFN-γ and the development of acquired memory to RSV to reduce Th2 pathology on subsequent infections.

However the overall balance of Th1/Th2 responses are also not well defined in mouse models. Decreased RSV load, reduced weight loss and reduced IFN-γ expression was observed in the lungs of mice which overexpressed of IL-13, a Th2 associated cytokine (236). Furthermore IL-13 deficient mice showed opposite results of higher RSV load, greater weight loss and more IFN-γ. Therefore a balance between Th1 and Th2 environments are likely to produce non-pathogenic responses to RSV but disease outcome is not exclusive of other T cell subsets. Tregs and Th17 cells have also been shown to regulate the Th1 and Th2 responses in mice. Depletion of Tregs led to increased IFN-γ expression by $CD4^+$ and $CD8^+$ T cells and IL-13 by $CD4^+$ T cells which was associated with Th2 pathology (237). IL-17 was shown to induce IL-13 expression during RSV infection, indicating Th17 cells may prolong a Th2 environment (208). Therefore, the interplay between Th1, Th2 and Th17 cells and

associated cytokines either before, during and/or after infection determine RSV disease outcome and is not well defined in humans.

1.3. Natural killer cells

NK cells were originally believed to have a 'natural' ability to kill infected or tumorous cells. It has since been demonstrated that NK cells are a complex cell population that interact with their local environment to not only provide innate immunity, but also act as a bridge between innate and adaptive immunity (238–240). NK cells are large granular lymphocytes and as mentioned in Section 1.2.1.1, NK cells have been defined as a distinct population within the ILC1 group that express IFN-γ, TNF-α and T-box transcriptional factors T-bet and eomes (241). Although NK cells respond in a wide variety of infectious diseases and show significant anti-tumour activity, this section mainly focuses on NK cell activation, receptor expression and cytokine and cytotoxic responses during RSV infection.

1.3.1. Maturation, phenotype, markers and lung specific NK cells

NK cells are of lymphocyte lineage and derived from $CD34^+$ hematopoietic progenitor cells (242). NK cell precursors express the IL-15 receptor β chain (CD122) which binds IL-15 and is essential for NK cell maturation (243). Human NK cells are classically defined as expressing CD56 (NCAM) and lacking expression of the pan T cell marker CD3 (244). NK cells can be further characterised by their differential expression of CD16 and divided into two phenotypically diverse sub-sets of $CD56^{dim}CD16^{bright}$ and $CD56^{bright}CD16^{dim}$ (245). $CD56^{bright}CD16^{dim}$ NK cells produce higher amounts of cytokines, including IFN-γ and TNF-α, compared to $CD56^{dim}CD16^{bright}$ NK cells which have a higher cytotoxic function and expression of perforin and granzyme B, as shown in Figure 1.8 (246–249). The $CD56^{dim}CD16^{bright}$ NK cell population may exhibit a more cytotoxic nature due to higher expression of cell surface

receptor CD16 (FcγRIIIa). CD16 binds to the Fc portion of antibodies, leading to antibody-dependent cellular cytotoxicity and subsequently target cell lysis (250,251).

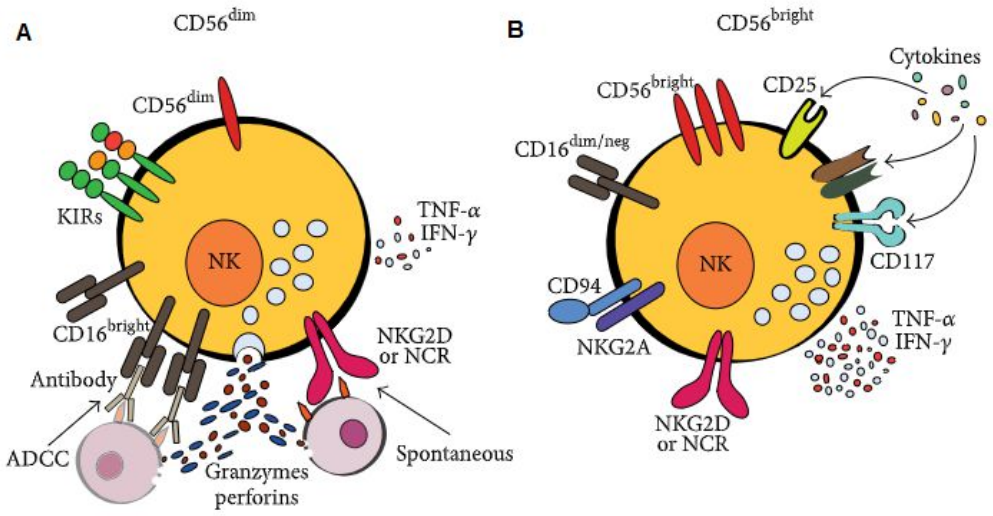


Figure 1.8. Functional differences between NK cell subsets. NK cells can be divided into two broad subsets, $CD56^{bright}CD16^{dim}$ and $CD56^{dim}CD16^{bright}$. Although the two subsets have overlapping functions, each subset has either greater cytokine producing or cytotoxicity activity. $CD56^{dim}CD16^{bright}$ NK cells (A) have greater cytotoxicity function compared to that of $CD56^{bright}CD16^{dim}$ NK cells (B) which express higher amounts of cytokines. Taken from Gabrielli *et al.* (252).

NK cells are resident in lymphoid and non-lymphoid tissues throughout the body and make up between ~5-25% of peripheral blood lymphocytes with ~90% of peripheral blood NK cells being $CD56^{dim}CD16^{bright}$ and 10% $CD56^{bright}CD16^{dim}$ (253–256). The percentage of NK cells within the lymphocyte population found in each tissue varies throughout the body. Human lung NK cells represent ~10-30% of the lymphocyte population present in this tissue compared to ~5-10% of the lymphocyte population in the liver (257–260). In mice, lung NK cells numbers are thought to be generally lower at ~10% of the lung lymphocyte population (261). In the human lung, ~80% of lung resident NK cells are $CD56^{dim}$ which correlates with a higher expression of perforin (260,262,263). This suggests a primarily cytotoxic NK cell activity in response to respiratory infections.

1.3.2. Activating and inhibitory receptors

Unlike B, T and NKT cells, NK cells are not activated by antigen-presentation via MHC class I or II molecules. Initially, it was thought that cells lacking HLA class I molecules were targeted by NK cells. This HLA class I inhibition of NK cell cytotoxicity ensures NK cell lysis does not occur against 'self' or host cells expressing HLA class I (264,265). It is now apparent that NK cell activation or inhibition of activation also depends on a combination of signals from multiple activating and inhibitory receptors (266). In brief as detailed in Figure 1.9, healthy cells express inhibitory and survival receptors which will keep NK cells in a resting state whilst infected or tumour cells can express a different array of receptors and NK cell stimulating cytokines which lead to NK cell activation. Both activating and inhibitory receptors fall into two classes, killer Immunoglobulin-like receptors (KIR) and c-lectin receptors which include CD94-NKG2C/E/H heterodimeric receptors and NKG2D (267). The precise interactions between activating and inhibitory receptor signals resulting in NK cell activation or inhibition are complex and not yet fully defined (268). Even between different diseases and tissues, differences in receptor expression has been examined which may lead to different NK cell functional outcomes (269).

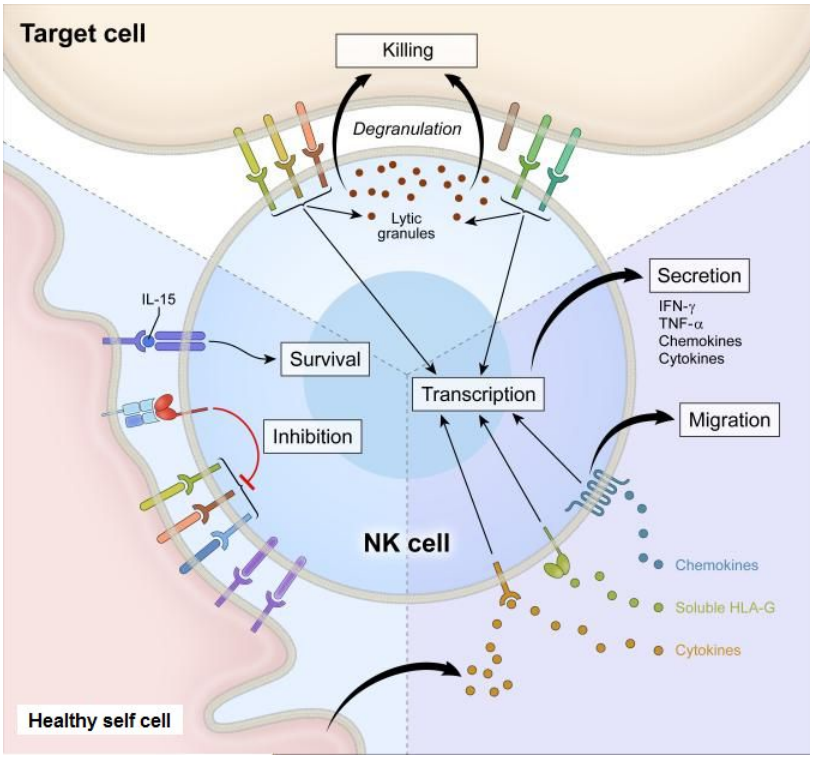


Figure 1.9. Inhibitory and activating signals determine NK cell response. NK cells express a range of inhibitory and activating receptors. Ligand interactions with healthy or 'self-cells' inhibit NK cell-mediated cell lysis and expression of cytokines and chemokines whilst ligand interactions with infected, non-self or tumour cells induces NK cell activation. Modified from Long *et al.* (270).

Activating receptors signal through immunoreceptor tyrosine-based activating motifs (ITAMs) and include natural cytotoxicity receptors such as NKp46, NKp44 and NKp30 (271). NKp44 and NKp46 have been reported to bind viral proteins presented on virally infected cells and initiates NK cell cytotoxicity (272–274). Nkp30 receptor activation can initiate tumour cell lysis and binds tumour associated proteins and transcriptional factors including B7-H6, BAT3 and heparan sulfate proteoglycans (275–277). Cytokine and chemokine receptors are also part of the activating receptor repertoire. Activating cytokines include IL-12, IL-15, IL-18, IL-21 and IFN-α which bind to IL-2R, IL-12R, IL-15R, IL-18R, IL-21R and IFNAR respectively (278,279). Human peripheral blood $CD56^{bright}$ NK cells preferentially express chemokine receptors CXCR3 and CCR5 (which bind CXCL9, CXCL10, CXCL11, CCL3, CCL4, CCL5

and CC8) compared to $CD56^{dim}$ NK cells which preferentially express CXCR1 and CXCR2 (which bind IL-8) (280). Other chemokine receptors expressed on human NK cells include CCR1, CCR4, CCR7, CCR9, CXCR5 and CXCR6 (281). Differential cytokine and chemokine receptor expression by NK cell subsets indicates an ability for functionally different NK cell subsets to migrate towards different stimuli.

Inhibitory signals for NK cell activation act through immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Inhibitory receptors include KIRs, which interact with HLA-A, HLA-B and HLA-C molecules and CD94/NKG2A which interacts with HLA-C, HLA-E and HLA-G molecules (282–284). HLA molecules expressed by healthy 'self-cells' inhibit NK cell-mediated cell lysis and are also important for NK cell development and then activation of mature NK cells against self-cells during infection (285,286). This further illustrates the complex nature of differential receptor expression which determines NK cell functions. Inhibitory receptor expression can also be influenced by viruses which have been shown to inhibit NK cell activity and the immune system through manipulation of inhibitory receptors (287).

1.3.3. Cytokine activation of NK cells

IL-12, IL-15, IL-18 are classically associated with NK cell development, homeostasis, cytokine production and cytotoxicity. Other cytokines such as IL-4, IL-7, IL-10, TGF-β and type I interferons also influence NK cell activity. Furthermore the cytokine environment NK cells are in can greatly influences their function and receptor expression (269).

1.3.3.1. IL-2

IL-2, part of the four alpha helix bundle cytokine family, is a pleiotropic cytokine which has greatest affects towards NK cells and T cells. IL-2 and IL-15 share two receptor subunits, IL-

2Rβ and IL-2Rγ/common γ (γC) with IL-2 binding with a higher affinity to IL-2Rα and IL-15 with ~1000-fold higher affinity to IL-15Rα than IL-2 (288,289). IL-2 aids in NK cell survival and proliferation although IL-2 is most commonly known to be a potent inducer of NK cells cell lysis and cytotoxicity against tumour cells (290–292). IL-2 also induces the expression of IFN-γ from NK cells, particularly from $CD56^{bright}$ NK cells with IL-12 co-stimulation (293,294). IL-2 stimulated NK cells can also express IL-10 which aids in dampening inflammatory responses (295).

1.3.3.2. IL-12

IL-12 is a heterodimer protein consisting of a p35 alpha chain and a p40 beta chain which form an active p70 protein. IL-12p70 binds to a dimerised receptor of IL-12Rβ1 and IL-12Rβ2 subunits which initiates JAK (JAK2 and TYK2) and STAT (STAT3, 4 and 5) signalling pathways (296–298). The p40 chain can also form monomers which bind to IL-12 receptor and act antagonistically against signalling by preventing IL-12p70 binding (299). p40 also binds another subunit, p19 to form IL-23 (300). In the perspective of NK cells, IL-12 was first called NKSF (NK cell stimulatory factor) and alone was shown to induce cytotoxicity in both $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets (296). Resting human NK cells express higher levels of IL-12Rβ1 and IL-12Rβ2 subunits than T cells and their expression may be negatively regulated by IL-12 itself indicating a possible protective role by reducing over activation (297). IL-12 has potent activity on IFN-γ expression from NK cells and alone is able to induce IFN-γ production (301). IL-12 also regulates CD16 expression on human NK cells which could enhance NK cell cytotoxicity (302). IL-2Rβ and IL-12Rβ2 knockout mice show reduced NK cell cytolytic ability (303,304).

Major producers of IL-12 are antigen presenting cells such as DCs and macrophages (305–307). The DC-NK synapse and particularly DC-derived IL-12 has recently been highlighted to

be important for NK cell activation. DC-specific siRNA knockdown of IL-12p40 or use of neutralising IL-12p20 antibody inhibited IFN-γ secretion by NK cells (308).

1.3.3.3. IL-18

IL-18 is part of the IL-1 family, a group of 11 pro-inflammatory cytokines (309). Pro-IL-18 requires cleavage into an mature protein which is instigated through activation of the NLRP3 inflammasome and then cleavage of pro-caspase-1 into of active caspase-1 (310,311). IL-18 binds to IL-18Rα which recruits IL-18Rβ and finally forms a heterodimer which leads to MyD88 signalling and NFKB activation (312,313). The ability of IL-18 to act on target cells is also influenced by the presence of a soluble receptor IL-18BP which is seen in the plasma and serum of healthy individuals (74,314,315). NK cells constitutively express IL-18Rα and expression is not changed by negative or positive feedback loops to IL-18 (316,317). IL-18 alone can directly induce NK cell proliferation and target-cell lysis possibly through an increase in degranulation markers such as CD107a (317–319). IL-18 can also induce NK cell apoptosis at higher concentrations indicating a modulatory role for IL-18 on mature NK cells (320). Mouse NK cells taken from IL-18BP deficient mice display an immature phenotype yet enhanced TNF-α production suggesting that IL-18 is important during NK cell maturation process to form balanced mature NK cell responses (321). However *in vitro* human NK cell stimulation with IL-18 alone may not be enough to induce IFN-γ expression (322). As discussed in Section 1.3.3.4, IL-18 plays an important role in enhancing NK cell responses by synergistically enhancing responses in combination with other cytokines.

1.3.3.4. IL-15 and IL-15Rα receptor

IL-15 is a pleotropic cytokine that shares a 4-alpha-helix bundle along with other cytokines such as IL-2, IL-3, IL-4 and IL-6 (289). As described above in section 1.3.3.1, IL-15 shares the two subunits two receptor subunits, IL-2Rβ and IL-2Rγ/common γ (γC) with IL-2, but shows

greatest affinity to IL-15Rα. IL-15 signalling in human NK cells has been shown to involve STAT1, STAT3, STAT4 and STAT5, which initiate maturation and proliferation (322,323). IL-15 is also important in the later stages of NK cell responses for both survival and sustained and enhanced cytokine production through synergistic effects with IL-12 (324–326).

IL-15 mRNA is expressed by many tissues, including placenta, skeletal muscle, lungs and kidney (327). The IL-15 pre-mRNA undergoes alternative splicing resulting in production of two protein isoforms a shorter 21-amino acid peptide and a longer 48-amino acid peptide which both bind to IL-15Rα and have been shown to be biologically active (328). Mature IL-15 protein is coded by exons 5, 6, 7 and 8 and with of 114 amino acids (329). The shorter peptide has been detected within the cytoplasm and nucleus and the longer peptide is seen within the endoplasmic reticulum and Golgi apparatus (330,331). In regards to the work here, the longer IL-15 peptide is thus likely to be studied in Chapters 3, 4 and 5.

An increase in IL-15 mRNA expression does not always reflect increased protein secretion. For instance, when stimulated monocytes showed induced expression of IL-15 mRNA, IL-15 protein was not detected in the culture supernatants indicating tight regulation of IL-15 at both the transcriptional and translational levels (332). Furthermore, serum IL-15 in healthy adults is rarely detected or at low levels even though many tissues and cell types constitutively express IL-15 mRNA (333–335). One potential reason for the lack of secreted IL-15 during infection could be due to the formation of an IL-15/IL-15Rα complex. Dubois *et al.* first described IL-15/IL-15Rα complex presentation at the cell surface in the mouse T cell line, CTLL-2. CTTL-2 cells transfected with IL-15Rα showed surface expression of IL-15Rα mirroring expression of surface IL-15 (336). This study also showed that IL-15 bound to IL-15Rα, and when expressed on the cell surface, allowed IL-15 to stimulate neighbouring cells through the IL-12Rβ/γC receptor. This unique presentation of IL-15 to a responding cell was then termed ‘trans-presentation’. Three dimensional structures of the complex show that

negatively charged IL-15 binds to the positively charged binding domain of IL-15Rα providing many charge/charge interactions leading to a stable and high affinity complex (337). Presenting IL-15 in this manner, at the cell surface as part of an IL-15/IL-15Rα complex, may be important for additional control of NK cells by presenting cells

IL-15Rα is expressed by both hematopoietic cells, such as T cells B cells and non-hematopoietic cells, such as mouse tubular epithelial cells, lung epithelial cells and found within various tissues in particular the liver, skeletal muscle, kidney, heart and lungs (288,338–341). This pattern of expression highlights a potentially important role for non-hematopoietic cells in modulating the immune response through IL-15. Figure 1.10 details how the IL-15/IL-15Rα complex is thought to be formed inside the cell. IL-15 and IL-15Rα proteins independently translocate to the endoplasmic reticulum and then form a complex with IL-15Rα essential for translocation of IL-15, in complex form, to the Golgi apparatus (342). IL-15Rα processing is also known to be dependent upon removal of certain exons which once removed allow for the binding of IL-15 to be possible and for cell surface expression (338,343). Furthermore release of the complex may also rely on additional IL-15Rα isoform modifications and its release may also only be observed by specific isoforms (343). Secretory vesicles then transport the IL-15/IL-15Rα complex to the cell surface. Trans-presentation then occurs when responding cells express receptor chains IL-2Rβ and IL-2Rγ/common γ (γC) at their cell surface and interact with the IL-15, which is part of the IL-15/IL-15Rα complex on presenting cells, shown in Figure 1.10B.

Co-ordinated expression of both IL-15 and IL-15Rα is essential for IL-15/IL-15Rα complex formation before transport to the cell surface (344–347). IL-15Rα also enhances the half-life of IL-15 activity (348,349). In human embryonic kidney cell line 293, co-expression of both IL-15 and IL-15Rα enhances surface expression IL-15 and in studies using the African green monkey kidney fibroblast-like cell line, COS-7, surface IL-15 was only detected when cells

were transfected with both IL-15 and IL-15Rα (342,347). Therefore, detection of IL-15 at the cell surface can indicate IL-15/IL-15Rα trans-presentation in which IL-15 is still biologically active. To date, cells shown to express IL-15 at the cell surface include CTTL-2 cells, human activated monocytes, mouse bone marrow-derived DCs and the human monocyte-like cell line THP-1 (336,350–353). Furthermore complex secretion or release by proteolytic cleavage from the cell surface may be another method of signalling with mice showing IL-15/IL-15Rα complexes in the serum and thus the potential for non-local IL-15 signalling (347,354).

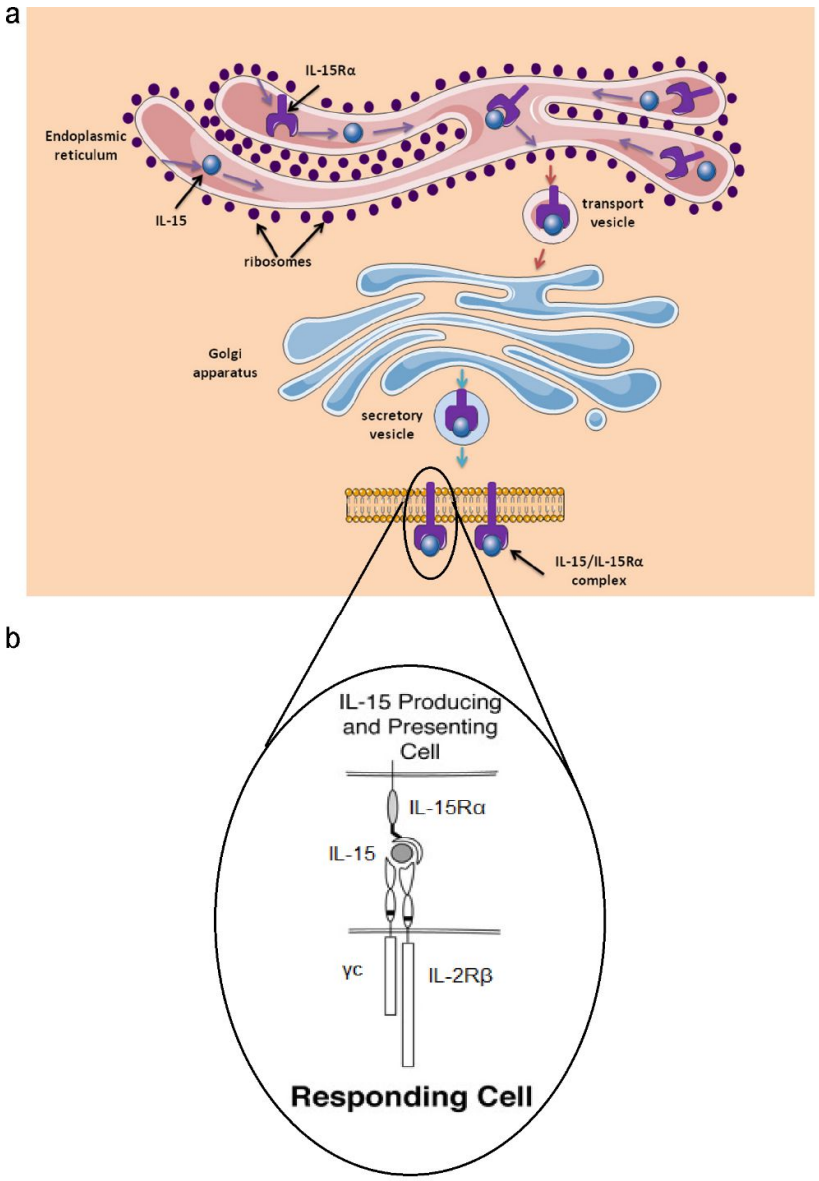


Figure 1.10. IL-15/IL-15Rα intracellular complex formation and trans-presentation. IL-15 and IL-15Rα proteins are formed within the endoplasmic reticulum and form a complex before being transported to the Golgi apparatus. The IL-15/IL-15Rα complex is then transported to the cell surface by a secretory vesicle. Modified from Burkett *et al.* and Jakobisiak *et al.* (344,355).

Human NK cells have also been shown to express IL-15 at the cell surface which is dependent on IL-15Rα expression. This could suggest a method of cis-presentation and self-activation (350,356). Cis-presentation occurs where expression of both the IL-15/IL-15Rα complex and

IL-2Rβ and γC receptors on the same cell leads to same cell activation. Human NK cells overexpressing IL-15 at the cell surface, and hence cis-presentation, showed a potential for increased survival, expansion and cytotoxicity compared to wild-type NK cells co-cultured with K562 cells expressing IL-15 in trans (357). However these NK cells were transformed to overexpress IL-15 at the cell surface and the relevance of cis-presentation *in vivo* in humans has still not been determined, if it exists at all. Overall the cis- model has not be characterised in as much detail as trans-presentation.

The importance of both IL-15 and IL-15Rα has been demonstrated through use of knock-out mice. IL-15 has shown to be essential for NK development and mice deficient in IL-15 are almost devoid of NK cells (325,358). Mice injected with plasmid DNA encoding both IL-15 and IL-15Rα showed increased NK cell numbers in the lung, liver and spleen with additional increase in serum IL-15 when compared to those that received plasmid DNA encoding IL-15 only or wild-type mice (347). This study suggests that co-expression of IL-15 and IL-15Rα enhances NK cell proliferation compared to IL-15 alone. Bone marrow derived DCs taken from mice deficient in either IL-15 or IL-15Rα and co-cultured with NK cells could not induce expression of NK cell-derived IFN-γ and NK cell activation was only seen with co-ordinated expression of IL-15 and IL-15Rα by wild-type DCs (345). Other studies demonstrate the importance of co-ordinated IL-15 and IL-15Rα expression to form IL-15/IL-15Rα complexes (344,359). Furthermore, the IL-15/IL-15Rα complex has shown to have enhanced activity on both NK cells and T cells such as increased proliferation and cytokine secretion (347–349). Overall, data indicates a key role for both IL-15, IL-15Rα and an enhanced effect of the IL-15/IL-15Rα complex for NK cell development and activation.

1.3.3.5. Synergistic effects of multiple cytokines on NK cells

In vitro NK cell stimulation with different combinations of cytokines can either stimulate or inhibit NK cell maturation, proliferation, cytokine expression, cytolytic function or receptor expression. Human NK cells show increased proliferation with IL-12 and IL-18 (360). IL-2 plus IL-12 enhances NK cell cytotoxicity (297,360). IL-12 in combination with IL-15, IL-18 or TNF-α synergistically to increase IFN-γ positive NK cells and IFN-γ secretion (247,301,361). IL-15 in combination with IL-18 and IL-21 have also been shown to enhance IFN-γ expression by NK cells (322). NK cells cultured in the presence of IgG and IL-18 expressed higher levels of IFN-γ (362). When human NK cells were cultured in the presence of IgG and IL-2 or IL-12, a 2-fold increase in IL-8 and CCL3 expression was observed (363). Receptor expression such as CD25 may also be increased through cytokine synergy (364,365).

Priming NK cells with certain cytokines may also be important for NK cell responses. Priming of mouse NK cells with IL-18 subsequently enhanced IL-12-induced NK cell-specific IFN-γ expression (366). Priming of human NK cells also provides enhanced cytokine expression when co-cultured with DCs. NK cells primed with a combination of IL-12 and IL-18 or IL-12 and IL-2 40hrs before co-culture with DCs displayed increased IFN-γ and TNF-α expression in co-culture supernatants compared to NK cells primed with individual cytokines (367). This could suggest the environment NK cells reside in before activation can lead to not only different NK cell function but also influence other immune cell functions through NK cell-induced effects.

However combinations of cytokines do not always enhance NK cell cytokine expression. NK cells cultured with both IL-4 and IL-12 resulted in IL-4 mediated inhibition of IL-12-induced CD69 surface expression (367). Addition of TGF-β reduced the synergistic effect of IL-15 with IL-12 on CCL3 expression and TGF-β may reduce IL-15 enhanced NK cell metabolism (368,369). A combination of IL-12 and IL-10 reduces the number of IFN-γ positive NK cells

compared to IL-12. This may be an intrinsic mechanism of NK cell regulation as some NK cells express IL-10 (301,370). For mouse NK cells IL-21 stimulation inhibits the proliferative effect of IL-15 but does not compromise NK cell cytokine and cytolytic functions (371).

Overall, these studies show that the cytokine environment NK cells are exposed to can greatly modulate NK cell proliferation and function. This also includes the environment before NK cell activation or interaction with other immune cells, such as DCs. Therefore, an imbalance in these key NK cell activating cytokines either before and/or during infection could lead to an abnormal NK cell responses.

1.3.4. NK cell cytotoxicity and cytokine expression

NK cell effector functions fall into two broad classes, cytotoxicity and cytokine expression. As described in Section 1.3.1, NK cells can be classed as $CD56^{dim}$ NK cells which have higher cytotoxic activity than $CD56^{bright}$ NK cells which have greater cytokine expression. First described here is NK cell cytotoxicity.

Healthy self-cells express MHC class I molecules which inhibit NK cell activation. NK cell cytotoxicity can be induced by lack of MHC class I molecules on infected or non-self-cells. NK cell-mediated cell death is achieved through two mechanisms, granule exocytosis and death receptor pathways (372). For the granule exocytosis pathway, NK cells contain lytic granules which contain molecules that initiate cell-death pathways including granzymes, granulysin, FasL and perforin (373). These lytic granules are pre-formed in resting NK cells with NK cells expressing more perforin than cytotoxic $CD8^+$ T cells (374). Furthermore, NK cell triggered cell apoptosis is much faster than that induced by cytotoxic $CD8^+$ T cells indicating a pivotal role for NK cells controlling the spread of infection (375). This process is tightly regulated via the formation of lytic synapses ensuring specificity to infected cells and preventing damage to surrounding healthy cells (376). Granzymes are serine proteases that activate caspases

and non-caspase molecules that induces intracellular apoptotic pathways in the target cell (377,378). The glycoprotein perforin can form pores in target cell membranes under *in vitro* conditions (379). However the current model for perforin and granzyme B entry is via endocytosis of both molecules into the target cell which work together to induce increased apoptosis (380,381). The death receptor pathway is initiated by ligands expressed on NK cells such as FasL, TNF-α and TRAIL which bind to TNF receptor family death receptors on target cells (382).

IFN-γ and TNF-α are classical NK cell-derived cytokines that act synergistically to reduce viral replication (383). IFN-γ and TNF-α secretion is not restricted to immunological synapses like perforin and granzymes and their secretion is separate from that of lytic granules (381). This is believed to optimise NK cell action allowing induction of apoptosis of infected cells whilst simultaneously initiating an anti-viral response in surrounding cells. Membrane bound TNF-α has also been shown to be constitutively expressed on human peripheral blood NK cells and the NK92 cell line which could lead to faster NK cell-induced cytotoxicity through direct cell-to cell contact (384,385). NK cells may also contribute to immune cell recruitment to sites of infection by the expression of chemokines, including CCL3, CCL4 and CCL5 as observed from NK cells during co-culture with K562 cells. Other chemokines expressed by NK cells include IL-8, macrophage-derived chemokine (MDC), and CCL2 suggesting NK cells can display diverse and differing roles during infection (363,368,386–388).

1.4. Immune response of NK cells in RSV infection

NK cells are an initial source of defence against viruses in the lungs. One study showed that NK cells represented ~0.4% of BAL with an absolute count of 1-6x10^4 cells/ml in RSV infected infants (194). Although NK cells represent a small fraction of total BAL lymphocytes during RSV infection, they are the first immune cell population to expand in number following RSV

infection. Figure 1.11 displays viral titre and immune cell population cell numbers profiles over time with RSV infection. The NK cell population expansion rises and falls along with the viral titre. This is followed by an increase in T cells, B cells and the expression of RSV-specific antibodies (Figure 1.11). Analysis of BAL from RSV infected mice show NK cell numbers in the lung peak around 3 to 4 days and then fall at day 6 (166,389–391). This same pattern was also observed for IFN-γ positive mouse NK cells and NK cell cytotoxicity with greatest activity observed at day 3 and absent at day 8 (391,392). NK cell derived IFN-γ reduces RSV lung titres and depletion of either NK cells or IFN-γ results in sustained viral titres (391). As early as days 1 and 2 after infection an increase in mouse $CD69^{+}$NK cells, CD69 being an early marker of activation, can occur which is dependent on the presence alveolar macrophages (166). TNF-α protein is also expressed in the lungs of RSV infected mice within the first 2 days after challenge (393). NK cells could be a source of this early expressed cytokine. Depletion of TNF-α throughout RSV infection in mice resulted in increased viral titres. This indicates that NK cell-derived TNF-α may also aid in restricting viral spread like IFN-γ (132). These data indicate there is rapid expansion and activation of NK cells that is limited to the early stages of infection and not seen in later stages of infection.

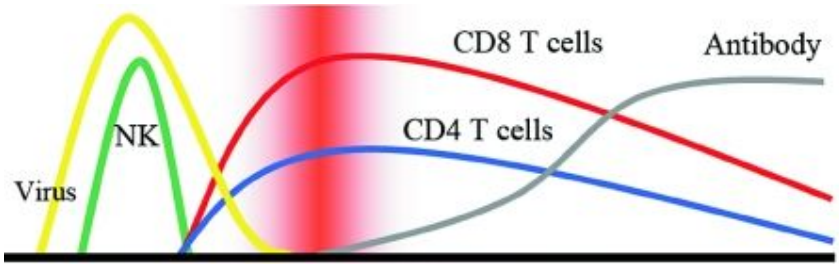


Figure 1.11. Time-scale of innate and acquired immunity during RSV infection. NK cell expansion follows RSV titres which are both seen to peak and reduce before arrival of adaptive immune cell populations such as $CD4^{+}$ and $CD8^{+}$ T cells. Taken from Openshaw & Tregoning (394).

The NK cell population in the BAL of infants with RSV bronchiolitis was 8-fold higher than non-infected infants indicating recruitment of NK cells from peripheral blood (395). Therefore, lung resident and recruited NK cells may both be present and activated during RSV infection. In infants, the absolute number of NK cells in peripheral blood were shown to be reduced during severe RSV disease compared to mild disease suggesting recruited NK cells to the lungs and/or prolonged NK cell activation may contribute to pathology (196,198). There is little knowledge about the role of resident NK cells in humans which would the earliest source of NK cell activity compared to those from the periphery. Other studies have given differing results regarding NK cell activity or the results were inadequate to support firm conclusions. For instance, immunohistochemical staining of post-mortem lung tissue from RSV infected infants showed little to no $CD56^{+}$ cells (67). However it cannot be concluded that there is a lack of NK cells as the timing of tissue sampling may not be at the peak of NK cell expansion. Another study compared NK cell responses *in vitro* during various respiratory virus infections and concluded the NK cell cytotoxic response was much lower during RSV infection compared to other respiratory viruses (396).

It is also important to take into consideration the function and level of activity of NK cells, which can in part be indicated by their specific pattern of NK cell receptor expression. This has rarely been examined in humans. One study of infants requiring hospitalisation reported a higher percentage of $CD16^{+}$ and $CD56^{bright}$ activated NK cells present in peripheral blood compared to those who were not hospitalised, which could suggest a role of NK cells in RSV pathogenesis (397). Another factor to consider in regards to NK cell numbers and clinical results is the age of patients and the mean age of patients within these groups. Absolute numbers of NK cell in healthy neonates gradually increase over time from 25 weeks gestation to 42 weeks gestation (398). In particular, babies born at 25-30 weeks gestation, characterised as very pre-term, had the lowest NK cell absolute counts and this was not observed between preterm and full term neonates for B cells or T cells. This raises the

possibility of a numerical or functional deficiency in NK cells in preterm or younger infants which could influence the initial NK cell response to RSV.

The exact role of NK cells during RSV has not yet been fully defined and their role has been debated. Some studies suggesting a beneficial activity for NK cells by inducing a Th1 response whilst others suggest that NK cells enhance a harmful Th2 response. IL-12 activated NK cells were shown to protect against eosinophilia through expression of IFN-γ in RSV G protein primed mice (390). In another study, RSV infected IL-12 deficient mice had reduced numbers of BAL NK cells and also reduced NK cell cytotoxicity *in vitro* compared to wild-type mice (384). Depletion of NK cells in adult mice resulted in an increase in viral load at day 4 post RSV infection and decreased lung histological inflammation scores suggesting NK cells may contribute to the earliest stages of lung inflammation (391,399). However viral load and inflammation were no different in later stages of infection indicating that NK cells are important at the initial or acute stages of infection and their depletion is not detrimental at the later stages of a first infection. NK cell depleted mice, at day 4 after RSV infection, normally when peak of NK cell numbers are seen, have reduced IFN-γ gene expression but increased IL-4, IL-13 and eotaxin expression indicative of a more Th2 environment which could develop as a result of deficient or impaired NK cells (389). This was later followed by increase in eosinophils and mucus positive AECs at day 9. Even when NK cell numbers had been restored, on secondary infection this Th2 environment was maintained. These data indicate that the early NK cell response can influence and shape responses at later stages of infection, possibly through IFN-γ expression.

The NK cell response in neonatal mice has also been investigated and results remain inconclusive on whether NK cells are beneficial or not during RSV infection. Neonatally RSV primed mice re-infected as adults show increased lung RSV titres compared to adult mice on both first and second infection (393). Tregoning *et al.* showed that during adult reinfection

of neonatally RSV primed mice, those which had previously been depleted of NK cells as neonates had increased anti-RSV IgG titres (400). This suggested NK cells supress antibody responses which was subsequently shown to be through expression of IFN-γ. This response was also observed on depletion of $CD8^+$ T cells. Tregoning *et al.* next examined the role of IFN-γ on antibody responses. Neonatal mice were shown to produce a strong IFN-γ response with ~2-fold increase in lung IFN-γ compared to non-infected mice at 5 days post infection. This was presumably from NK cells as this matched a ~2-fold increase in IFN-γ positive lung NK cells at day 5. There was also another significant increase in lung IFN-γ at day 11, determined to be from IFN-γ positive $CD4^+$ and $CD8^+$ at days 11 post infection. However, individual depletion of NK cell or T cells subsets did not decrease lung IFN-γ protein suggesting compensatory mechanisms operate between cell populations. This study then confirmed IFN-γ was inhibiting the antibody response using neutralising anti-IFN-γ antibodies which significantly increased anti-RSV IgG titres (400). Use of an IFN-γ expressing recombinant RSV also decreased antibody titres.

Another study reported that adult reinfection of neonatally RSV primed mice had more lung $CD69^+$ NK cells at 4 days post infection than mice primed and re-challenged (393). In this study Harker *et al.* show that more IFN-γ positive NK cells were observed after adult reinfection of neonatally RSV primed mice, although significance was not reached in this study (400). Harker *et al.* also demonstrate that there was a significant increase in $CD11b^+/CD27^+$ NK cells on adult reinfection of neonatally RSV primed mice. An increase in expression of CD69, CD27 and CD11b by NK cells could lead to enhanced cytokine expression and cytolytic responses which could have contributed to the increased weight loss and pathology observed by Harker *et al.* (401,402). Overall the results from studies of neonatal NK cell responses during RSV infection indicate early IFN-γ derived from NK cells, and later derived from T cells, reduces the antibody response in RSV infected neonatal mice, this persists into adulthood influences responses on reinfection.

The response of NK cells to RSV may also depend on expression of NK cell and AEC-derived inhibitory and activating receptors, which RSV could modulate to its advantage. Failure of RSV to induce AEC-derived NK cell activating receptors may reduce efficient RSV elimination. In RSV infected mice, lung NK cells express higher levels of activating receptors NKG2D and CD27 which mirrored an increase in IFN-γ expression in BAL (391). This study suggests that NK cell activation and NK cell-derived IFN-γ is involved in acute lung injury possibly through expression of these receptors. NKG2D has been shown to enhance NK cell induced injury whilst stimulation of CD27 by infected cells lead to NK cell cytotoxicity and cytokine expression (403,404). Examples of NK cell-activating receptors that are upregulated during RSV infection include the MHC class I-chain related protein MICA at the surface of human AECs and ULBP1 on human DCs, which bind NKG2D on NK cells (152,405,406). MICA polymorphisms have been associated with RSV infection with the MICA*002:01/A9 allele frequency significantly lower in RSV patients (407). In NKG2D deficient mice there was an increase in lung DC numbers during the early stages of RSV infection, however RSV-infected NKG2D deficient mice displayed no change in viral load, weight loss, NK cell recruitment to the lung or number of IFN-γ positive NK cells (408,409). This is possibly due to the wide range of inhibitory and activating receptors NK cells possess and the increase in DC numbers as a compensatory mechanism. For example NKG2D and LFA-1 act synergistically to enhance NK cell cytotoxicity yet activation only of individual receptors is not enough to induce cytotoxicity (410,411). This indicates a deficiency in only one activating receptor may be insufficient to alter the NK cell, although receptor deficiencies cannot be excluded as a possibility to describe NK cell responses during RSV infection.

Overall these data indicate that NK cells play a significant role in the early stages of RSV infection, influencing viral load and acute inflammation through expression of IFN-γ. However NK cell responses and NK cell-derived IFN-γ during neonatal infections may also influence subsequent infection as adults and attenuate adaptive immune responses. NK cells

may also aid in compensatory mechanisms seen in T cell deficient mice and maybe beneficial in those who are immune compromised or in neonates with immature adaptive immune cell populations (390,412). Furthermore the environment, or type of NK cell priming could, additionally influence the NK cell response. This complexity may have given rise to the debated roles NK cells have during RSV infection in both human and mice studies.

1.5. Aims of thesis

The early antiviral immune mechanisms and importance of AEC-NK cell communication in the immune response to RSV has not been fully characterised. The main aim of the work described in this thesis was to investigate the ability of RSV infected AECs to support immune responses through NK cell activation *in vitro*.

BEAS-2B cells, a cultured airway epithelial cell line, and human adult nasal airway epithelial cells (HNAECs) were first used to characterise the expression of NK cell activating cytokines IL-12, IL-15 and IL-18 and the IL-15 receptor, IL-15Rα, following RSV infection. The formation of IL-15/IL-15Rα complexes was also examined. These experiments are described in Chapter 3 for BEAS-2B cells and Chapter 4 for HNAECs.

To then determine if the infected airway epithelium can alone support NK cell activation, AEC-NK cell co-cultures were established for both BEAS-2B cells and HNAECs. NK cell activation was characterised by expression of IFN-γ and TNF-α. These experiments are described in Chapter 3 for BEAS-2B cells and Chapter 4 for HNAECs.

To determine if there was a relationship between NK cell activating cytokine expression and disease severity in infants, NK cell activating cytokines, IL-15, IL-18 and the IL-15/IL-15Rα complex were measured in NPAs from infants under 1 years of age PCR positive only for RSV or RV. Results were characterised by disease severity, age and oxygen requirement during

hospital admission. A comparison between RSV and RV expression was then used to determine if individual respiratory viruses gave different NK cell activating cytokine profiles. Additionally for the RSV NPAs, to determine if there was a correlation with NK cell activating cytokine expression TNF-α and IFN-γ levels were examined. This work is described in Chapter 4.

NK cell-derived cytokines could lead to enhanced airway epithelium responses during RSV infection. To further understand this unique aspect of AEC-NK cell communication expression of the Th1 associated chemokines, CXCL9, CXCL0 and CXCL11, Th2 cytokine TARC and B cell activating cytokine BAFF by AECs during NK cell co-culture as examined. This work is described in Chapter 5. This work was extended to determine if Th1 or Th2 cytokine environments influence BEAS-2B cell expression of IL-15, IL-15Rα, ICAM-1 and BAFF during RSV infection and then the NK cell response during BEAS-2B cell-NK cell co-culture.

An additional aim was to characterise the influence of RSV strain in AEC and NK cell responses. RSV lab strain A2 and an A strain clinical isolate, RSV X were used to determine if RSV strain influences NK cell responses during infection of BEAS-2B cells and subsequently if RSV X further resulted in an enhanced Th1 associated chemokine response compared to RSV A2. These experiments are described throughout Chapters 3, 4 and 5.

Chapter 2. Materials and methods

2.1. BEAS-2B cell culture

BEAS-2B cells, an adenovirus 12-SV40 virus transformed human bronchial epithelial cell line, were grown in complete Dulbecco's modified Eagle's medium (cDMEM) (Table 2.1, page 82) and cultured in T25 flasks (Nunc, Thermo Fisher Scientific) at 37°C with 5% CO_2. Cells were subcultured twice weekly when 80-90% confluent in T25 flasks (Corning Costar) with media changed every 2-3 days. For experiments cells seeded at 5000 cell/cm2. Flasks and plates were coated with 3µg/ml collagen diluted in 20mM acetic acid (Table 2.1, page 82) for 1hr then washed 3 times with sterile PBS before addition of cells.

For subculture, media was removed and cells washed with 5ml PBS. 1ml 0.25% trypsin (Table 2.1, page 82) was added for 3-5 minutes at 37°C. Flasks were visualised under the microscope until 90% dissociation was achieved. 6ml cDMEM was added to neutralise the trypsin and then all 7ml transferred into a 10ml sterile tube and centrifuged at 2200 rpm for 5 minutes to pellet cells. Supernatant was discarded and cells resuspended in 1ml media for counting using a Bright-Line™ haemocytometer (Reichert, USA). Cells were only used up to passage 15 before a new vial of cells were then used.

Cells were only used up to passage 4 for long-term storage. After centrifugation, cells were resuspended in 1ml of cDMEM containing 10% DMSO (Table 2.2, page 83). Further cDMEM + 10% DMSO was added so that cells were at a density of $1.5x10^6$cells/ml and 1ml added to cyrovials (Fisher, UK). Cyrovials were placed in a CoolCell® freezing container (BioCision) and incubated at -80°C for 24hr before being transferred to -180°C freezer for long-term storage. For revival, all 1ml was added to 5ml warm cDMEM in a collagen coated T25 flask.

2.2. Processing and culture of human nasal airway epithelial cells

HNAECs were obtained from consented healthy adult volunteers and otherwise healthy adults with asthma (A-HNAEC). This study was approved by Liverpool central research ethics committee study number 17/NW/0044 IRAS number 212223.

Nasal passages were brushed using a ConMed® Cytology Brush Ring Handle 3.00mm x 120.00cm (ConMed; 149R) after which the brush was repeatedly stirred and banged against the sides of a 10ml tube containing 7ml RPMI media to detach cells from the brush. Cells were centrifuged at 1500rpm for 5 minutes to pellet cells. Media was discarded and 500µl trypsin/EDTA solution added (Table 2.1, page 82) and cells incubated for 5 minutes at 37°C to allow for digestion of epithelial sheets. 500µl trypsin neutralising solution (Table 2.1, page 82) was then added to neutralise the trypsin along with 2ml of bronchial epithelium growth medium (BEGM) (Table 2.1, page 82). Cells were centrifuged at 1500rpm for 5 minutes to pellet cells, media discarded and cells resuspended in 1ml BEGM. A further 2ml of media was added and 1ml of cell suspension added to each well of a 12-well plate pre-coated with 10µg/ml collagen (Table 2.1, page 82).

Cells were incubated typically for 6-7 days until 100% confluent before subculture. Media was removed and cells washed with PBS and 200µl/well of 0.25% trypsin/EDTA added (Table 2.1, page 82) for 3-5 minutes at 37°C. Plates were visualised under the microscope until 90% dissociation was achieved. 200µl/well of trypsin neutralising solution (TNS) added to each well and the cell suspension transferred into a 10ml sterile tube. 1ml BEGM was added and cells centrifuged at 1500 rpm for 5 minutes to pellet cells. The supernatant discarded and cells resuspended in 1ml media for counting. Cells were seeded at $1x10^4$ in 94-well plates and $1x10^5$ in 12-well plates. After subculture, cells were typically incubated for 4-5 days before 100% confluence was reached and subcultured again. Cells were only used on passages 2 and 3 for experimentation and subculture was stopped at passage 3.

Figure 2.1 displays the growth of one donor's nasal AECs 24hr after isolating and plating to 6 days culture to form a confluent monolayer. Images were taken on the EVOS XL cell imaging system (Thermo Fisher Scientific). Figure 2.2 displays the same donor's nasal AECs after the first subculture. To confirm cells were of epithelial origin, cells were stained for cytokeratin-18 at either passages 2 or 3 (see Section 2.10.2).

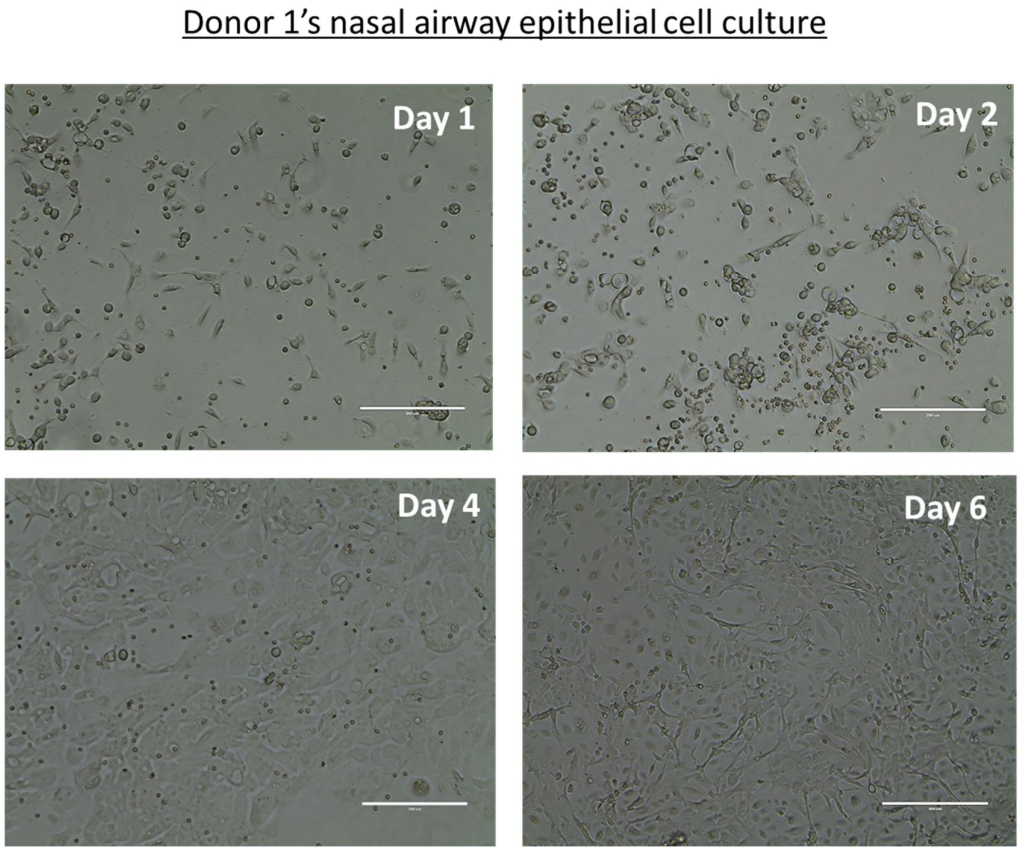


Figure 2.1. Culture of HNAECs to form a confluent monolayer. Images are representative of donor 1's nasal AECs and show images taken at days 1, 2, 4 and 6 after seeding. Images were taken using a light microscope. Scale bars represent 200µm for images days 1, 2 and 5, and 400µm for day 6.

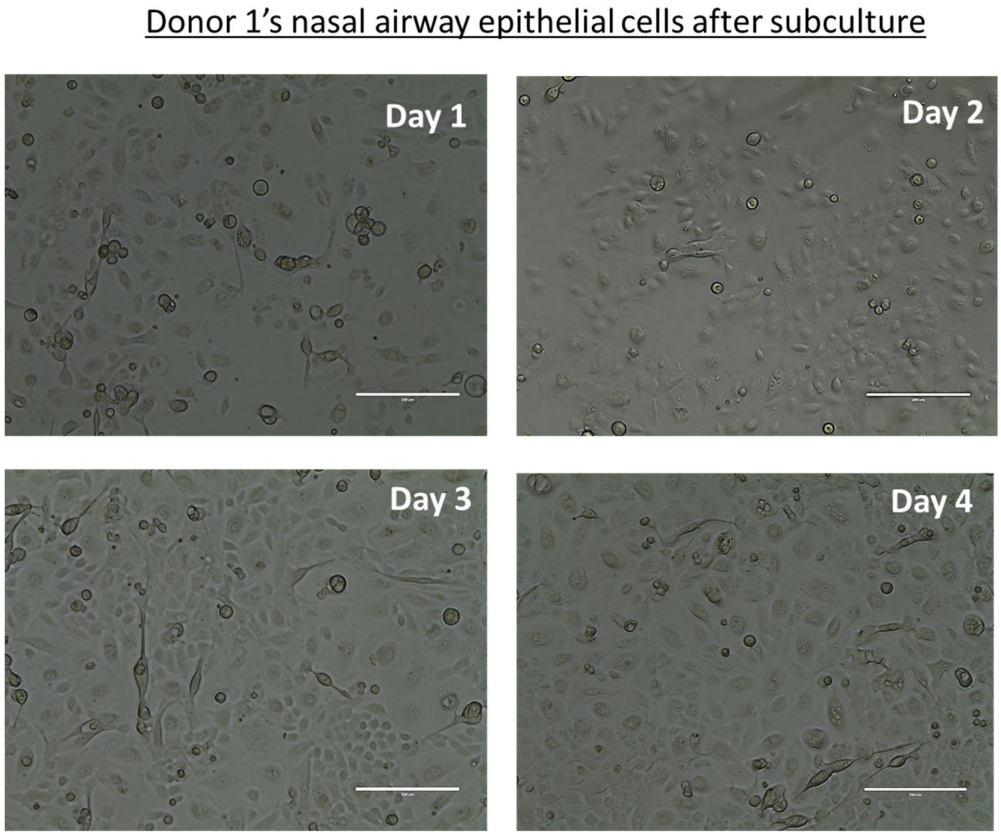


Figure 2.2. Culture of HNAECs after first subculture. After 100% confluency was reached, cells were subcultured into 12-well plates. Shown here are HNAECs days 1, 2, 3 and 4 after subculture. Images are representative of donor 1's nasal AECs. Images show photographs using a light microscope. Scale bars represent 200µm for all images.

2.3. RSV propagation

Hep2 cells were used for propagation of RSV A2 and RSV X. Hep2 cells were cultured similarly to BEAS-2B cells, with the difference of seeding at 30,000 cells/cm^2 in uncoated T175 flasks in a total volume of 15ml cDMEM. Cells were first grown at 37°C with 5% CO_2 for 24hr until 60-70%, confluent. Media was removed and cells washed with 15ml PBS. 5ml of serum free DMEM (Table 2.1, page 82) was added along with 1 vial of RSV stock. The flask was gently rocked back and forth and then placed on a gentle rocker to allow for adequate infection of cells for 2hr at 37°C with 5% CO_2 after which 11ml cDMEM was then added to give a final FBS concentration of 2%. Cells were assessed daily until a 50% cytopathic effect was visible,

typically at 48hr. 9ml of the media was removed and discarded. The monolayer was harvested using a cell scraper and cells collected in a 10ml sterile tube. Cells were lysed on ice with 10 passes through a 2ml syringe and 25-gauge needle. Lysate was then centrifuged at 1500 rpm in a pre-cooled centrifuge at 4°C for 5 minutes. The supernatant was aliquoted into pre-labelled cyrovials and snap frozen in liquid nitrogen. Cyrovials were quickly transferred to -80°C storage.

RSV X was a gift from the Netherlands National Institute for Public Health and the Environment (RIVM). RSV-X was isolated in 1998 from a patient in the Leiden University Medical Center (LUMC) (Leiden, NL) and passaged in Hep2. In 1999, RIVM received the virus and determined is to be A strain. RSV X has been propagating in Hep2 cells for an unknown number of times and was given purified in sucrose density gradient.

2.3.1. Determination of RSV titre by plaque assay

RSV viral titres were measured by titration on monolayers of the airway epithelial cell line A549. A549 cells were seeded into 96-well plates at 10,000 cells/cm^2 and incubated at 37°C with 5% CO_2 for 24hr. Cells were washed with PBS before serial dilutions of RSV in serum free DMEM were added in triplicate wells (50µl/well) for 2hr after which 150µl of complete media was added and cells incubated for 24hr. Cells were washed with PBS, fixed with methanol, stained with goat anti-RSV biotinylated antibody (1/200 dilution) (Bio Rad; 7950-0104) and labelled using an extravidin peroxidise colour development substrate (Table 2.2, page 83). Plaques were visualised under light microscope and wells with 100-200 plaques present used to determine the mean plaques, shown in Figure 2.3. Plaque forming units (PFU) were calculated as: number of plaques/dilution factor x volume added (µl).

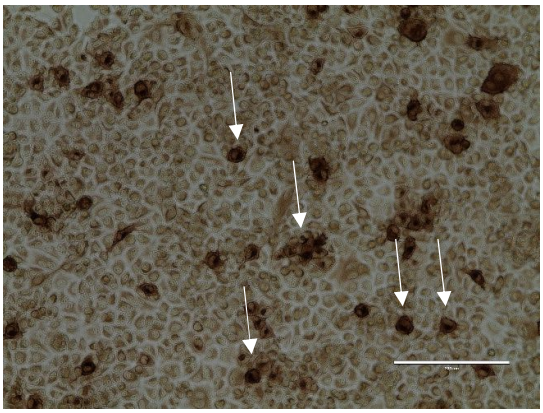


Figure 2.3. RSV plaque assay. RSV positive A549 cells, or plaques, are seen as dark brown spots. Selected plaques are indicated by arrows. Fixed cells were stained with goat anti-RSV biotinylated antibody and labelled using an extravidin peroxidise colour development substrate. Image was taken on the EVOS XL cell imaging system.

2.4. Natural Killer Cell isolation

2.4.1. Isolation of peripheral blood mononuclear cell (PBMCs)

Ethics from University of Liverpool Research Ethics Committee approved for the analysis of normal blood leukocyte function using cells from healthy volunteers (reference: RETH000773).

Venous blood was obtained from healthy adult donors and placed into 10ml tube containing lithium heparin coated beads (Sarstedt; 46.363.001). Peripheral blood mononuclear cells were isolated via density centrifugation using a histopaque-1077 gradient. Blood was diluted with equal amounts of PBS and layered onto histopaque (Table 2.2, page 83) at a volume equal to that of the original blood sample. Following centrifugation at 400g for 30 minutes, the layer above the histopaque interface containing PBMCs was transferred into a new tube. PBMCs were washed with PBS and pellet resuspended in 1ml RPMI. 9ml of red blood cell lysis buffer was added for 3 minutes, with gentle rocking for the first minute. Cells were centrifuged and NK cells were then isolated as described below (Section 2.4.2).

2.4.2. Natural killer cell negative isolation

White cells were washed twice in EasySep™ Buffer (Table 2.2, page 83) before NK cell isolations using EasySep™ Human NK Cell Enrichment Kit following the manufacturer's instructions (Table 2.2, page 83). After NK cell isolation, cells were washed twice in cRPMI before being counted and added to wells in 100µl cRPMI. Remaining cells where then washed twice in flow/IF buffer (Table 2.2, page 83) for preparation of flow cytometric purity analysis (Section 2.9.3). NK cells used for assays were above 95% purity.

2.5. RSV infection of airway epithelial cells

Cells were infected when 100% confluent. Media was removed and cells washed once with PBS before addition of RSV in serum free DMEM. Cells were incubated for 2hr at 37°C with 5% CO_2 before the inoculum was removed and complete media added. Biological replicates were considered as cells used from different passages. AECs were grown in 96-well plates for the majority of experiments in (200µl/well). 12-well plates (600 µl/well) was used for Figure 3.3B. 24-well plates (400 µl/well) were used for flow cytometry experiments. For further details for specific experiments and the multiplicity of infection (MOI) used can be found within each results chapter. Palivizumab, a monoclonal antibody that binds to the F-protein of RSV, was used as an anti-RSV control during infection and used at 100µg/ml (Abbvie, UK).

2.5.1 Airway epithelial cell and NK cell co-culture

Confluent BEAS-2B cells and HNAECs were infected for 24hr at MOI 1 or MOI 2.5 respectively, in 100ul cRPMI before addition of isolated NK cells (Section 2.4) in 100ul cRPMI for a further 24hr at AEC:NK cell ratios of 1:1, 1:2 and 1:3. For HNAECs, donor matched NK cells were used.

2.5.2. Transwell separation of BEAS-2B cells and NK cells

Figure 2.4 depicts the conditions used for this experiment. Corning® Transwell® polyester membrane cell culture inserts at 6.5mm diameter and pore size of 0.4µm were used (Corning; 3470). BEAS-2B cells were grown on the bottom of a 24-well plate or on in the

insert until confluent before infection with RSV A2 at MOI 1 for 24hr in 400ul cRPMI. Isolated NK cells (Section 2.4) were added for a further 24hr at a BEAS-2B cell: NK cell ratio of 1:3 into the lower chamber in 100ul cRPMI (A), into the insert in 100ul fresh cRPMI (B), into the insert in 100ul infected BEAS-2B cell culture supernatant (C) and into the lower chamber in 100ul cRPMI (D). Culture supernatants were taken from both lower compartment and insert and stored at -70°C.

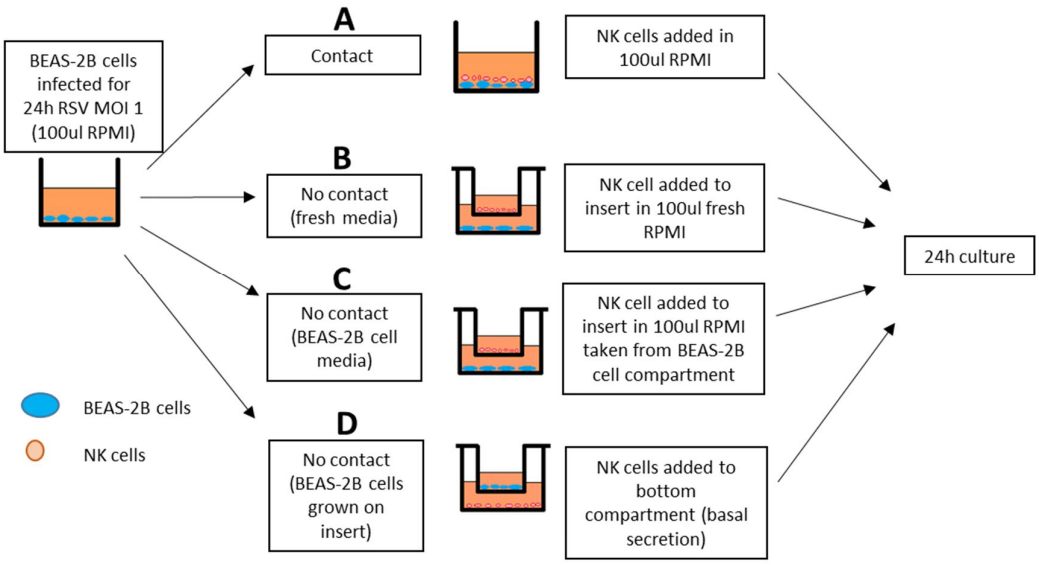


Figure 2.4. Diagram of transwell experiment conditions. BEAS-2B cells were infected with RSV A2 at MOI 1 for 24hr. Isolated NK cells (Section 2.4) were added for a further 24hr at a ratio of 1:3 into the lower chamber in 100ul cRPMI (A), into the insert in 100ul fresh cRPMI (B), into the insert in 100ul infected BEAS-2B cell culture supernatant (C) and into the lower chamber in 100ul cRPMI (D).

2.6. Polymerase Chain Reaction (PCR)

2.6.1. RNA isolation

RNA was isolated using TRI reagent (Sigma) as described by the manufacturer. For RNA extracted from experiments using 96-well plates, media was first removed and cells lysed by adding 80µl of TRI reagent (Table 2.2, page 83) to each well. Three replicate wells were collected as one sample with a total volume of 240µl and the lysate transferred to an RNase-

, DNase and pyrogen- free tube (Molecular BioProducts; 3412). At this point, samples were either frozen at -70°C in TRI reagent for future RNA isolation or RNA isolated immediately. Briefly 50µl of chloroform was added to each tube before it was vortexed at high speed for 10 seconds and then centrifuged for 15 minutes, 12,000g and at 4°C. The top clear fraction was transferred to a new nuclease-free tube and the bottom layer discarded. 200µl isopropanol was added to the eluent, vortexed and allowed to sit at room temperature for 10 minutes. Samples were then centrifuged for 15 minutes, 12,000g at 4°C to pellet the RNA. The eluent was discarded carefully so as to not disrupt the RNA pellet and the pellet washed with 300µl of 70% nuclease-free ethanol, after which samples were vortexed, centrifuged and eluent again discarded. The RNA pellet was allowed to dry at room temperature for 5 minutes before being dissolved in 20µl nuclease-free water. Eluted RNA was either stored at -70°C for future use or placed on ice for RNA quantity and quality assessment using a Nanodrop ND-1000 Spectrophotometer. 1µl per sample was used to measure the absorbance of the RNA samples at 260nm and A260/A280 ratio. RNA samples were then stored at -70°C.

2.6.2. Reverse transcription (RT)

A High Capacity cDNA Reverse Transcription Kit (Table 2.2, page 83) was used for synthesis of cDNA. 10µl of each RNA sample containing around 1 µg of total RNA was combined with 10µl of freshly prepared reverse transcription mastermix in an RNase-, DNase and pyrogen-free sterile tube (Thermo Fisher Scientific; 3451). The mastermix consisted of 2µl 10X RT buffer 10X RT Buffer, 2µl 10X RT Random Primers, 1µl 25X dNTP Mix, 1µl MultiScribe® Reverse Transcriptase and 4µl nuclease-free water for each 10µl RNA. Samples were incubated in a TC-512 thermal cycler (Techne, USA) for 1 hour at 37°C to complete cDNA synthesis. cDNA samples were then diluted 1:10 by addition of 180µl nuclease-free water. Samples were then either stored at -20°C or carried forward to qPCR analysis (Section 2.6.3).

2.6.3. Quantitative Polymerase Chain Reaction (qPCR)

cDNA was analysed using TaqMan® gene expression assays, either TaqMan™ Universal PCR Master Mix (Table 2.2, page 83) or PrecisionPLUS MasterMix with ROX buffer was used (Table 2.2, page 83). All probes spanned and exon boundary. For TaqMan™ Universal PCR Master Mix, 11.25µl cDNA was mixed with 12.5µl TaqMan™ Universal PCR Master Mix and 1.25µl TaqMan® primer/probe to give a final volume of 25µl. For PrecisionPLUS MasterMix, 9µl cDNA was mixed with 10µl PrecisionPLUS MasterMix and 1µl TaqMan® primer/probe to give a final volume of 20µl.

Each cDNA sample was probed for the control housekeeping gene, ribosomal L32 (L32) and gene/s of interest. Details of specific probes used can be found in Table 2.3 (Table 2.3, page 84).

Samples were run in duplicate in 96-well PCR plate (Starlabs, UK) on a 7300 Real-Time PCR System (Applied Biosystems, UK) using Sequence Detection Software V1.4 (Applied Biosystems, UK). Cycling conditions were 50°C for 2 minutes, followed by hold at 95°C for 10 minutes and then 40-50 cycles of 95°C for 15 seconds and 60°C for 1 minute. At the end of each run amplification plots were viewed and baseline and threshold values set individually for each gene.

Analysis of each gene was done using the comparative C_T method ($2^{-\Delta\Delta CT}$) using the equations below, with housekeeping gene L32 used as the internal reference gene.

1) Duplicates of Ct values were averaged
2) Ct values were corrected to housekeeping gene L32 to give Ct value relative to L32.

   $\Delta CT = CT\ (target\ mean) - CT\ (mean\ reference)$

3) ΔCT were then corrected to relative control.

   $\Delta\Delta CT = mean\ \Delta CT\ (treatment\ group) - mean\ \Delta CT\ (untreated\ control)$

4) The fold change in target gene expression relative to the internal reference gene, L32.

$$\text{Relative gene expression} = 2^{-\Delta\Delta CT}$$

Relative gene expression values were then used for graphical presentation and expressed as the percentage of L32 housekeeping gene expression by multiplying the value given for the relative gene expression by 100.

2.7. Enzyme Linked Immunosorbant Assay (ELISA)

For all plates, absorbance were read at 540nm using a BioTek ELx800 absorbance microplate reader. OD values were collected using KC junior software (version 1.4.1.8) (BioTek).

2.7.1. Chemokine and cytokines in cell supernatants

All ELISAs were carried out according to manufacturer's instructions. Table 2.4 provides details of the specific ELISAs and sample dilution factors used. Reconstituted capture antibodies, standards and detection antibodies were aliquoted stored at -70°C (Table 2.4, page 84). With the exceptions detailed below the specified reagent diluent was used for sample dilution. All assays supplied by R&D systems were carried out on MaxiSorp® flat bottom 96-well plates (Nunc, Thermo Fisher Scientific). Blank graphs represent that protein was examined however no protein was detected above the detection limit.

2.7.2. Preparation of NPA for ELISAs

NPAs were vortexed for 10 seconds at 1600rpm and centrifuged for 5 minutes at 2000rpm to pellet cells debris and mucus to before diluting 1:2 with RIPA buffer.

2.8. Luminex

Luminex Assay used for the detection of human IL-15 and BAFF protein in BEAS-2B cell and HNAEC supernatants were completed according to manufacturer's instructions (R&D; LXSAHM-02). The standard curve range for IL-15 was 3 - 1,540pg/ml and for BAFF 17.4 -

4,240pg/ml. Assays were performed on MAGPIX® System (Merck) with xPONENT (version 4.2.1324) (Luminex) software to acquire the data.

2.9. Flow cytometry

2.9.1. General staining procedure

Cells were washed twice in flow/IF buffer (Table 2.2, page 84) before addition of antibodies or isotype controls (Table 2.9, page 86). Cells were incubated on ice for 30 minutes with shaking at 100rpm in the dark with either un-conjugated or conjugated primary antibodies (Table 2.5, page 85). Cells were washed twice before either continuing to flow cytometric analysis if conjugated antibodies were used, or incubated for a further 30 minutes on ice in the dark with secondary antibody. Cells were then washed twice before resuspension in 200µl of flow/IF buffer and flow cytometric analysis.

2.9.2. Surface staining of airway epithelial cells

Media was removed and cells washed with PBS before addition of 0.48mM EDTA in PBS for 3-5 minutes at 37°C. Cells were further disrupted by pipette and transferred to eppendorf tubes for centrifugation for 5 minutes at 4000rpm and preparation continued as described in Section 2.10.

2.9.3. NK cell purity analysis

Following NK cell isolation (Section 2.4.2) purity was determined by flow cytometry using CD56 and CD3 antibodies. PBMCs were first used to set gates for these antibodies and a representation of the gating strategy used for NK cell purity of isolated cells is shown in Figure 2.5. This gating strategy was then slightly altered to lower isotype control as shown in Figure 2.6.

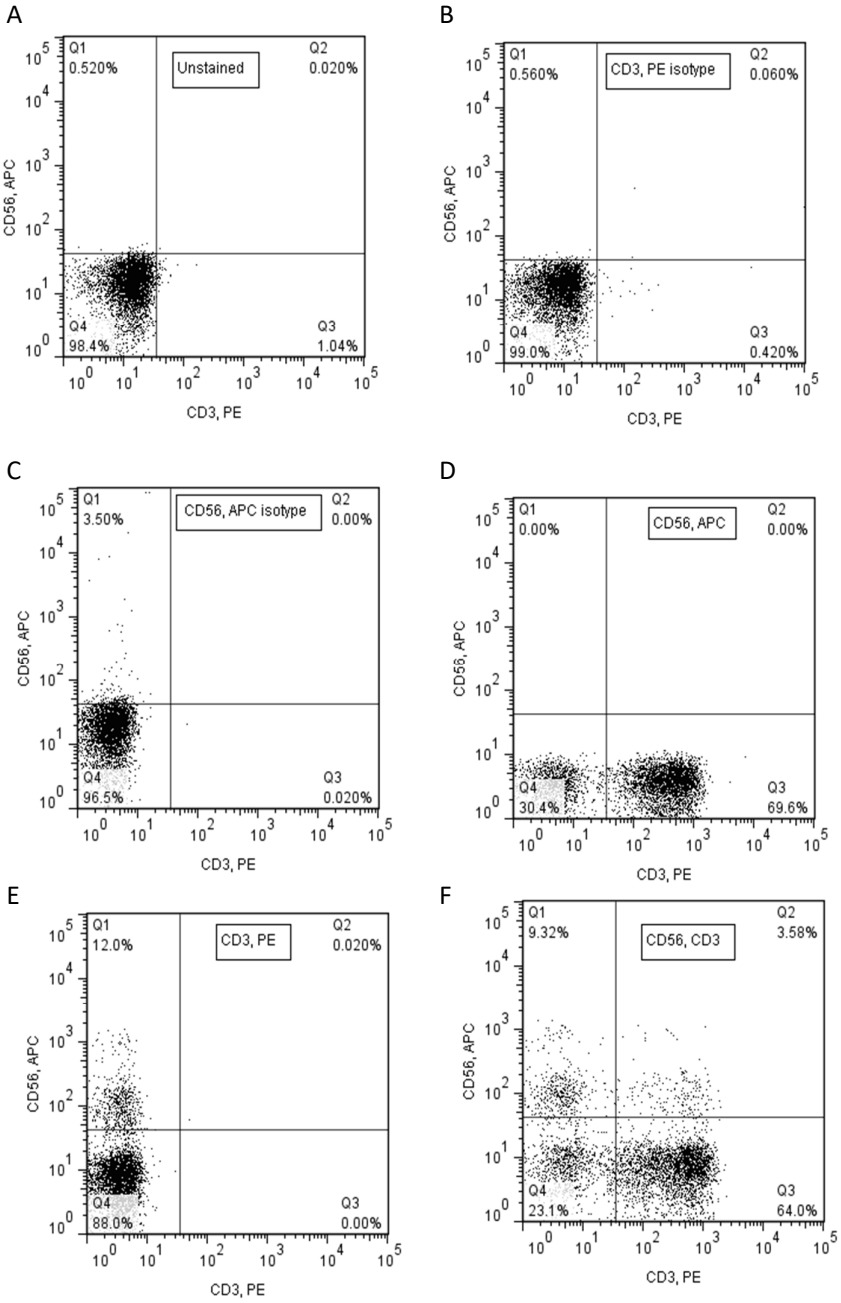


Figure 2.5. Representative flow cytometry gating set-up using PBMCs for NK cell purity analysis. Isolated PBMCs were left unstained A) or stained with B) CD3 PE isotype, C) CD56 APC isotype, D) CD3 PE, E) CD56 APC, F) dual stain CD56 APC and CD3 PE and analysed by flow cytometry to determine the NK cell population ($CD56^+$ $CD3^-$).

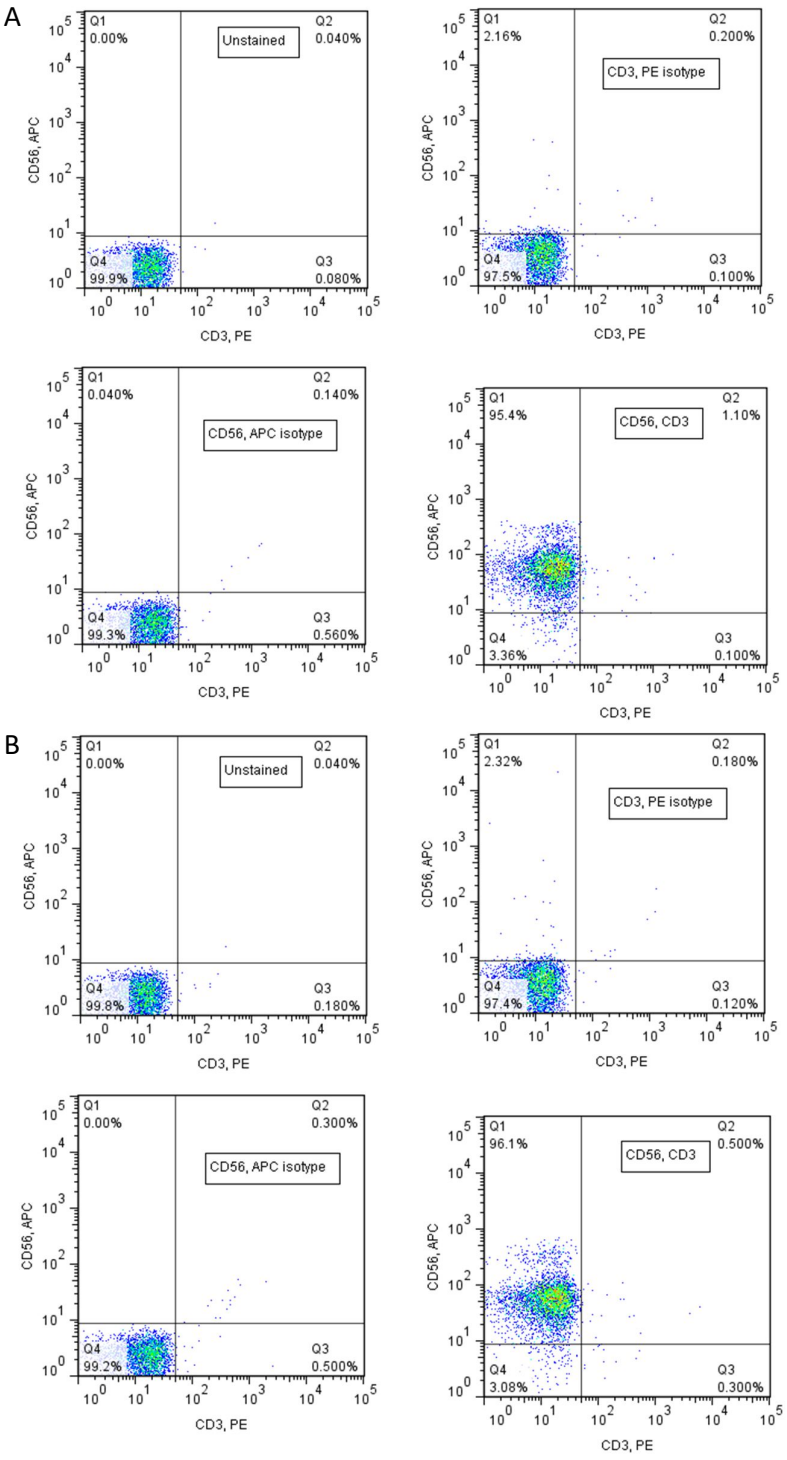


Figure 2.6. Representative flow cytometry of NK cell purity assessment after NK cell isolation. Isolated NK cells were left unstained or single stained with CD3 PE isotype, CD56 APC isotype or dual stained with CD56 APC and CD3 PE. Shown are two donor's NK cells, labelled as A and B. NK cells are the $CD56^{+}$ $CD3^{-}$ population, shown in Q1 region with dual CD56 and CD3 staining.

2.9.4. Intracellular IFN-γ staining of NK cells

2.9.4.1. Direct BEAS-2B cell:NK cell co-culture

Cells first incubated with brefeldin A (Table 2.2, page 84) for 4hr before preparation as in Section 2.9.2, without continuation to Section 2.11.1. Cells were permeabilised with Fixation/Permeabilization Solution Kit (Table 2.2, page 84) following the manufacturer's instructions before staining as described above (Section 2.9.1). Figure 2.7 shows gating strategy to distinguish BEAS-2B cells from NK cells during co-culture assays.

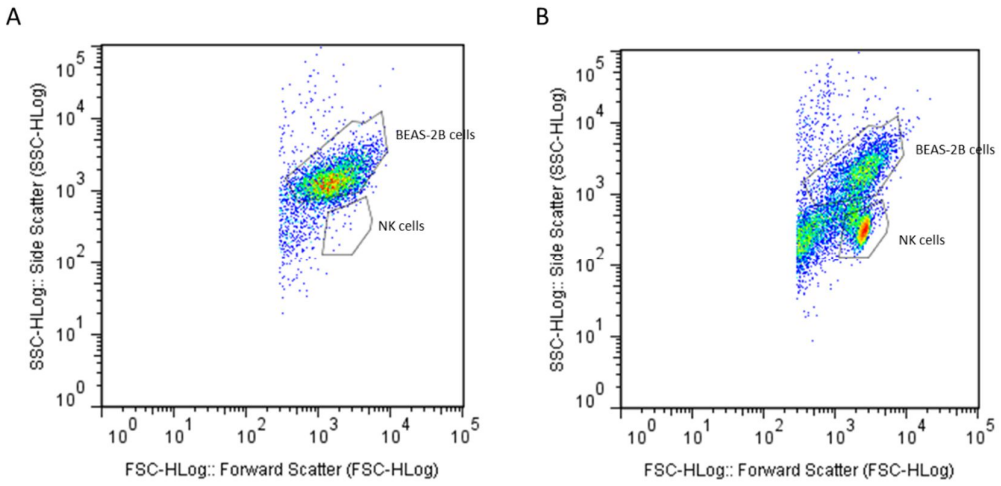


Figure 2.7. Representative flow cytometry for gating strategy to distinguish BEAS-2B cells from NK cells during co-culture. Forward scatter and side scatter was displayed in log to clearly distinguish between BEAS-2B cells and NK cell populations. A) BEAS-2B cell culture only and B) BEAS-2B cells co-cultured with NK cells.

2.9.4.2. NK cell stimulation with BEAS-2B cell culture supernatants

These methods describes those of a collaborative effort with Liz Van Erp for the detection of IFN-γ and perforin intracellular expression in healthy adult NK cells cultured with BEAS-2B cell culture supernatants.

Figure 2.8 details the procedure of production of culture supernatants and NK cell culture. BEAS-2B cells were infected with RSV A2 at MOIs 1 and 2.5 for 24 and 48hr and culture supernatant (200ul/well) stored at -70^{o}C. Three biological replicates were collected and supernatants shipped on dry ice to RIVM. Two separate donors were used and NK cells cultured in each three biological replicates of BEAS-2B cell culture supernatants and each replicate done in duplicate.

Briefly, NK cells were isolated from healthy adult controls by negative selection (MACS, Miltenyi Biotec). NK cells were cultured at a ratio of 1:1 with cell line K562, at 1x10^{5} cells/well in BEAS-2B cell culture supernatants with addition of Brefeldin A and anti-CD107a antibody (H4A3, Biolegend) for 4hr. Cells were fixed and permeabilised (eBioscience) before staining for CD56, (HCD56, Biolegend), CD3 (SK7, BD Biosciences) IFN-γ (B27, Biolegend) and perforin (B-D48, Biolegend) before flow cytometric analysis. Data was acquired using a BD LSR Fortessa X20 flow cytometer.

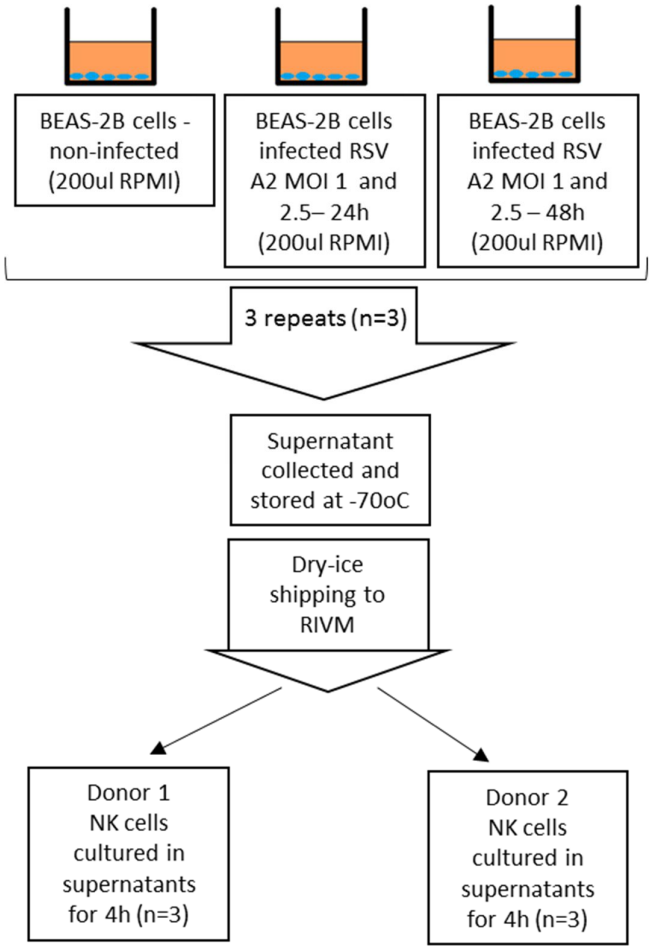


Figure 2.8. Experimental procedure for NK cells cultured in BEAS-2B cell supernatants. BEAS-2B cells were infected with RSV A2 at MOI 1 and 2.5 for 24hr and 48hr. NK cells were isolated from two healthy adult PBMCs and cultured in supernatants for 4hr before flow cytometric analysis. Non-infected cells contained cRPMI only as a control.

2.9.5. Acquisition and analysis of flow cytometry data

Flow cytometry was performed using Guava® easyCyte flow cytometer using guavaSoft (version 3.1.1) software. Data was analysed using FlowJo analysis software (version 7.6.5) (FlowJo, LLC).

2.10. Immunofluorescence staining

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

2.10.1. BEAS-2B cells

2.10.1.1. Surface staining

Primary antibody and secondary antibody staining of BEAS-2B cells was carried out as described in Section 2.9.1. Cells were either incubated with IL-15 and IL-15Rα alone or with combination of both (Table 2.6 and Table 2.8, pages 85 and 86). BEAS-2B cells were then immobilised onto glass slides using a cytospin centrifuge (ROTOαFIX 32A, Hettich). Cells were fixed with 4% paraformaldehyde (Table 2.2, page 84) for 15 minutes at room temperature and washed before being counter stained with DAPI (Sigma; D9542) for 10 minutes at room temperature, washed again and allowed to dry before addition of VECTASHIELD Antifade Mounting Medium (Vector laboratories; H-1000).

2.10.1.2. Intracellular staining

BEAS-2B cells were grown on sterile 13mm glass coverslips in 12-well plates and infected when at 50% confluency. Media was removed and cells washed with PBS before addition of 4% methanol for 15 minutes at -20^{o}C. Coverslips were incubated in permeabilising buffer PBST (Table 2.2, page 84) at room temperature for 15 mines before blocking in flow/IF buffer for 1hr at room temperature. Coverslips were then incubated with primary antibodies overnight at 4^{o}C in PBST washed with PBST and then incubated for 2hr at room temperature with secondary antibodies diluted in PBST. After further washing slides were counter stained with DAPI for 10 minutes at room temperature, washed again and allowed to dry before mounting in VECTASHIELD Antifade Medium.

2.10.2. HNAEC cytokeratin-18 staining

Approximately, $5x10^4$ HNAECs were immobilised on glass slides using a cytospin centrifuge and fixed using 4% paraformaldehyde (Table 2.2, page 84) at room temperature for 15 minutes. Slides were washed with PBS before incubation with anti- Cytokeratin 18 antibody (Table 2.6, page 85) in flow/IF buffer overnight at 4°C the washed again and incubated with secondary antibody (Table 2.6, page 85) for 1hr at room temperature. Slides were visualised using EVOS FLoid fluorescence microscopy (Thermo Fisher Scientific) (Figure 2.9).

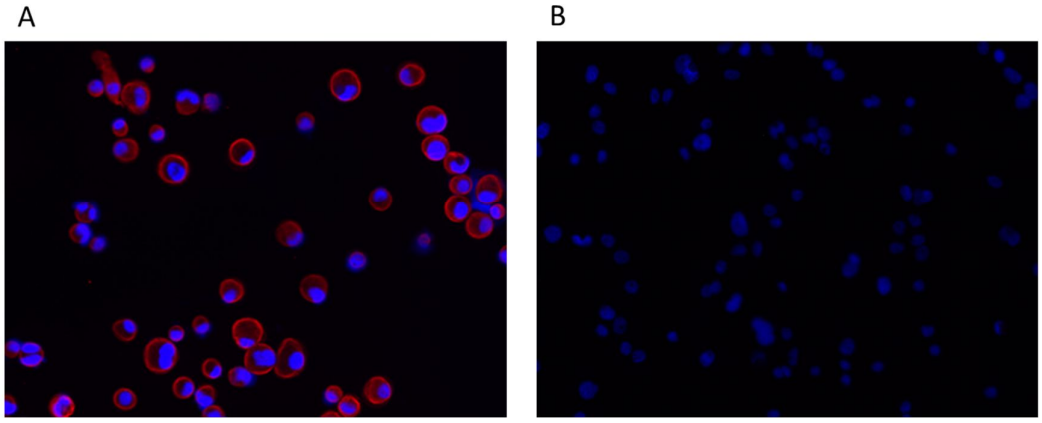


Figure 2.9. HNAECs express cytokeratin-18. After passage HNAECs were stained for cytokeratin-18 to determine if cells were of airway epithelial origin. Image is representation of one HNAEC donor. A) cytokeratin-18, B) isotype control. Cytokeratin staining is shown in red. Nuclei are stained in blue (DAPI).

2.11. Nasopharyngeal aspirates

Discarded nasopharyngeal aspirates (NPAs) collected between 1.11.2016 and 6.4.2017 RSV winter season were obtained from the microbiology department Alder Hey Children's Hospital. This study was approved by Liverpool central research ethics committee study number 17/NW/0044 IRAS number 212223.

PCR analysis of respiratory virus present in the samples had previously been carried out within the microbiology laboratory in Alder Hey Children's Hospital by the staff. Samples for this analysis were randomly selected from a list as follows;

- Infants aged 7 days to 1 years old.
- NPA requested by general paediatrics services only.
    - To exclude infants who were already in different departments at Alder Hey for issues potentially not related to respiratory illness.
    - Children were only included if they were admitted to hospital.
- NPA taken within 2 days of admission.
- Positive for RSV or RV only.
- No comorbidities.

Severity was determined as;

- Mild, no oxygen at all during admission
- Moderate, oxygen needed
- Severe, admission to paediatric critical care unit

2.12. Statistical Analysis

AEC experiments that required comparison between two groups used the paired, non-parametric Wilcoxon signed rank test. AEC experiments that required comparison between three or more groups used the paired, non-parametric Friedman test followed with Conover post-hoc test to calculate the statistical significance. For NPA protein analysis in Sections 4.3.8 and 4.3.9, data was first assessed for normal distribution using Shapiro normality test. All NPA protein values did not follow a normal distribution so non-parametric tests were used. Analysis between two groups were analysed by Mann-Whitney U test and between more than three groups analysed by Kruskal-Wallis test followed with Conover-Inman post-hoc test. Correlations between NPA cytokine levels were derived by Spearman's rank correlation coefficient analysis. All statistical analysis was completed using StatsDirect

3.0.171. A p value of $p < 0.05$ was considered to be significant. Figure legends detail statistical analysis used for each specific experiment.

Table 2.1. Media and media supplements.

Name	Composition	Application	Supplier; cat no.
Dulbecco's modified Eagle's medium (DMEM)	Refer to manufacturer's media composition	BEAS-2B, A549 and Hep2 culture	Sigma; D5796
Bronchial epithelium basal medium (BEBM)	Refer to manufacturer's media composition	Human nasal airway epithelial cell culture	Lonza; CC-3171
BEGM BulletKit	Refer to manufacturer's composition	Human nasal airway epithelial cell culture	Lonza; CC-3170
Bronchial epithelium growth medium (BEGM)	BEGM BulletKit in BEBM	Human nasal airway epithelial cell culture	As stated above
RPMI 1640 Medium, GlutaMAX™ Supplement	Refer to manufacturer's media composition	NK cell co-culture	Thermo Fisher Scientific; 61870-010
Fetal bovine serum (FBS)	Heat inactivated (60°C for 30 minutes in water bath)	BEAS-2B, A549 and Hep2 culture	Thermo Fisher Scientific; 16000-044
Gentamicin	Refer to manufacturer's composition	BEAS-2B, A549 and Hep2 and NK cell culture	Sigma; G1397
Ultroser-G	Refer to manufacturer's composition	Human nasal airway epithelial cell	Pall Life Sciences; 15950-017
Collagen I Rat Protein, Tail	Worked concentration at 50µg/ml in 20mM acetic acid (sterile filtered)	BEAS-2B (3µg/ml) and human nasal airway epithelial cell (10µg/ml) seeding	Thermo Fisher scientific; A1048301
Trypsin 10x solution	2.5% trypsin diluted in PBS to 0.025%	BEAS-2B, A549 and Hep2 sub-culture	Sigma; 59427C
0.25% trypsin/EDTA solution	Refer to manufacturer's composition	Human nasal airway epithelial cell sub-culture	Lonza; CC-5012
Trypsin neutralising solution (TNS)	Refer to manufacturer's composition	Human nasal airway epithelial cell sub-culture	Lonza; CC-5002
Complete DMEM (cDMEM)	10% FBS, 1% gentamicin in DMEM	BEAS-2B, A549 and Hep2 culture	As stated above
Serum free DMEM	1% gentamicin in DMEM	BEAS-2B, A549 and Hep2 infection media	As stated above
Complete RPMI (cRPMI)	10% FBS, 1% gentamicin in RPMI	NK cell co-culture	As stated above
Complete BEGM (cBEGM)	2% Ultroser-G	Human nasal airway epithelial cell culture	As stated above

Table 2.2. Regents, buffers and kits.

Name	Composition	Application	Supplier; cat no.
PBS (1x)	0.014M KH2PO4, 0.008M Na2HPO4-7H20, 0.0026M KCl, 0.137M NaCl	Cell culture	
Dimethyl sulfoxide (DMSO)	Refer to manufacturer's composition	Cell culture and general use	Sigma; 2650
ExtrAvidin®–Peroxidase	Refer to manufacturer's composition	RSV titration	Sigma; E2886
3-Amino-9-ethylcarbazole	Refer to manufacturer's composition	RSV titration	Sigma; 03005
Histopaque-1077	Polysucrose, 57 g/L and sodium diatrizoate, 90 g/L	Isolation of human peripheral PBMCs	Sigma; 10771
EasySep™ Buffer	PBS, FBS (2%), EDTA 1mM	Isolation of NK cells	Stem Cell; 20144
EasySep™ Human NK Cell Isolation Kit	Refer to manufacturer's composition	Isolation of NK cells	Stem Cell; 19055
Flow/IF buffer	PBS, 1% BSA	Flow cytometry	
TRI Reagent™ Solution	Refer to manufacturer's composition	RNA isolation	Invitrogen; AM9738
High capacity cDNA RT kit	10X RT Buffer, 10X RT Random Primers, 25X dNTP Mix (100 mM), MultiScribe® Reverse Transcriptase (50 U/µL)	Reverse transcription	Applied Biosystems; 4368814
4% paraformaldehyde	Refer to manufacturer's composition	Immunofluorescence	Electron Microscopy Sciences; 157-4-100
PBST	1x PBS, 0.3% Triton x-100	Immunofluorescence	
Fixation/Permeabilization Solution Kit	Refer to manufacturer's composition	Flow cytometry	BD Biosciences; 554714
TaqMan™ Universal PCR Master Mix	Refer to manufacturer's composition	qPCR	Life technologies; 4369016
PrecisionPLUS MasterMix with ROX	Refer to manufacturer's composition	qPCR	Primer Design; PPLUS-R-XXML

Table 2.3. Taqman® primer/probe assays.

Primer/probe	Gene	Assay ID	Amplicon length
L32	MRPL32	Hs00388301_m1	81
Interleukin 8	CXCL8	Hs00174103_m1	101
Interleukin 12β	IL12B	Hs01011518_m1	72
Interleukin 15	IL15	Hs01003716_m1	117
Interleukin 18	IL18	Hs01038788_m1	115
IL-15Rα	IL15RA	Hs00542604_m1	82
TNF-α	TNF	Hs01113624_g1	143
IFN-γ	IFN	Hs00989291_m1	73
CXCL10	CXCL10	Hs00171042_m1	98
BAFF	TNFSF13B	Hs00198106_m1	84
ICAM-1	ICAM1	Hs00164932_m1	87
TARC	CCL17	Hs00171074_m1	51

Table 2.4. Commercially available ELISA kits.

ELISA (human)	Standard curve range (pg/ml)	Supernatant dilution factor	Nasopharyngeal aspirate dilution factor	Supplier; cat no.
CXCL8/IL-8	31.3-2000	60	-	R&D; DY208
IL-12 p70	7.81-500	1	-	Invitrogen; 88-1726-22
IL-15	7.81-500	-	2	Biolegend; 435104
IL-18	11.7-750	1	2	R&D; DY318
IL-15/IL-15Rα complex	62.5-4000	2	2	R&D; DY6924
CXCL9/MIG	62.5-4000	30	-	R&D; DY392
CXCL10/IP-10	31.3-2000	30	-	R&D; DY266
CXCL11/I-TAC	7.81-500	30	-	R&D; DY671
IFN-γ	4-500	1	2	Invitrogen; 88-7316-88
TNF-α	4-500	1	2	Invitrogen; 88-7346-88
BAFF	39.1-2500	-	-	R&D; DY124
TARC	3.9-250	-	-	Biolegend; 441104

Table 2.5. Antibodies used in flow cytometry.

Antibody raised against	Host/Isotype	Conjugate	Working conc.	Supplier; cat no.
CD3	Mouse IgG2a κ	PE	2.5µg/ml	Invitrogen; 12-0039-41
CD56	Mouse IgG1 κ	APC	2.5µg/ml	Invitrogen; 12-0039
ICAM-1	Mouse IgG1 κ	APC	10µg/ml	Invitrogen; 17-0549-41
IL-15	Goat IgG	-	15µg/ml	R&D; AF315
IL-15Rα	Mouse IgG2b κ	PE	10µg/ml	Biolegend; 330207
IL-15Rα	Goat IgG	-	10µg/ml	R&D; AF247
IFN-γ	Mouse IgG1 κ	FITC	10µg/ml	Invitrogen; 11-7319-81

Table 2.6. Antibodies used in immunofluorescence.

Antibody raised against	Host/Isotype	Working conc.	Supplier; cat no.
IL-15	Goat IgG	5µg/ml	R&D; AF315
IL-15Rα	Rabbit IgG	1/400 dilution	Abcam; ab140618
GM130	Sheep IgG	5µg/ml	R&D AF8199
Cytokeratin-18	Sheep IgG		R&D; AF7619

Table 2.7. Antibodies used for neutralisation assays.

Antibody raised against	Host/Isotype	Working conc.	Supplier; cat no.
IFN-γ	Mouse IgG_{2A}	10ng/ml, 100ng, 1µg/ml	R&D; MAB285
TNF-γ	Goat IgG	10ng/ml, 100ng, 1µg/ml	R&D; AF-210-NA

Table 2.8. Secondary antibodies.

Antibody raised against	Conjugate	Working conc.	Used for primary antibody	Supplier; cat no.
Donkey anti-sheep	NL637	1/150 dilution	GM130	R&D; NL011
Donkey anti-goat	Alexa Fluor 488	10µg/ml	IL-15	Invitrogen; A11055
Donkey anti-rabbit	Alexa Fluor 488	10µg/ml	IL-15Rα (single stain)	Abcam; ab21206
Donkey anti-rabbit	DyLight™ 649	5µg/ml	IL-15Rα (dual stain)	Biolegend; 406406

Table 2.9. Isotype controls.

Name	Conjugate	Isotype control for	Supplier; cat no.
Goat IgG	-	IL-15	R&D; AB-108-C
Sheep IgG	-	GM130	R&D; 5-001-A
Rabbit IgG	-	IL-15Rα	SCBT; sc-13057
Mouse IgG2a	PE	CD3, IL-15Rα (Biolegend)	Invitrogen; 12-4724-41
Mouse IgG1 κ	APC	CD56, ICAM-1	Biolegend; 400119
Mouse IgG1 κ	PE	IFNGR1	Biolegend; 400111
Mouse IgG1 κ	FITC	IFN-γ	Biolegend; 400107

Table 2.10. Human recombinant proteins.

Name	Working conc.	Supplier; cat no.
TNF-γ	10ng/ml	Peprotech; 300-01A
IFN-γ	100ng/ml	Peprotech; 300-02
IL-15	10ng/ml	Peprotech; 200-15
IL-4	20ng/ml	Peprotech; 200-04
IL-13	20ng/ml	Peprotech; 200-13

Chapter 3. *In vitro* NK cell activating-cytokine profile of RSV A2 infected BEAS-2B cells and BEAS-2B cell-NK cell co-culture

3.1. Introduction

The epithelial response at the site of infection could have a crucial role in orchestrating inflammatory and immune responses through release of cytokines and chemokines. Airway epithelial-derived cytokines and chemokines act to activate and attract cells of the immune system. The work described in this chapter focusses on how epithelial cells, through cytokine release, may activate NK cells. Innate immune cells, such as NK cells, are present within the resting lung, and are believed to be one of the first immune cell populations to respond to infection (Section 1.4). As described in Chapter 1, the principle cytokines that activate NK cells are IL-12, IL-15 and IL-18 (Section 1.3.3). IL-15 may also form IL-15/IL-15Rα complexes which could act as a potent inducer of NK cell activation and cytokine expression (Section 1.3.3). Activated, NK cells secrete IFN-γ and TNF-α which can act to further induce both immune and inflammatory responses (Section 1.3.4) and induce further expression of AEC-derived cytokines (Section 5.1).

IL-15 expression by the airway epithelium following RSV infection has already been demonstrated (Section 1.3.3.3), but expression of IL-12, IL-15Rα, the IL-15/IL-15Rα complex and IL-18 by AECs during RSV infection has not been examined. IL-15Rα expression has been measured in different epithelial cells from other organs under various different stimuli and shown to be expressed by a number of epithelial cell types (Section 1.3.3.3). IL-15Rα mRNA has also been shown to be expressed by human bronchial epithelial cells (HBEC) and BEAS-2B cells under resting conditions, however expression and activity during RSV infection has not been characterised (341). In the work described in this chapter, I extend these findings to further define the pattern of NK cell activating cytokine expression including IL-12, IL-15,

IL-18, and the IL-15/IL-15Rα complex by airway epithelial cell line, BEAS-2B. To then further understand how the airway epithelium may activate NK cells during RSV infection I examined the hypothesis that alone AECs, after infection, can activate NK cells by expression of NK cell-activating cytokines and IL-15Rα receptor. This was achieved by using a novel BEAS-2B cell-NK cell co-culture model and by use of transwell cell separation to determine if direct cell contact, results in enhanced NK cell responses.

3.2. Aims

- To characterise the kinetics of cytokines IL-12, IL-15 and IL-18 and the IL-15Rα receptor expression by BEAS-2B cells in response to RSV A2 infection (Sections 3.31, 3.3.2 and 3.3.3).
- To determine if IL-15/IL-15Rα complexes are formed following RSV A2 infection of BEAS-2B cells (Sections 3.3.4, 3.3.5, 3.3.6 and 3.3.7).
- To establish a BEAS-2B cell-NK cell co-culture model, measuring IFN-γ and TNF-α as indicators of NK cell activation (Section 3.3.8 and 3.3.10).
- To determine if direct contact of BEAS-2B cells and NK cell are required for optimum NK cell activation (Section 3.3.8 and 3.3.9).
- To determine if infected BEAS-2B cell culture supernatants can induce NK cell cytotoxicity (Section 3.3.11).
- To compare the expression of IL-15 and IL-15Rα by BEAS-2B cells in response to infection with two different RSV A strains, A2 lab strain and clinical isolate RSV X. Then to determine if differences in AEC cytokine expression result in changes in IFN-γ and TNF-α levels in BEAS-2B cell-NK cell co-cultures (Section 3.3.12).

3.3. Results

In these experiments the bronchial epithelial cell line, BEAS-2B, were infected with RSV A2 strain at an MOI of 1 for 4, 8, 24 and 48hr, after which culture supernatants and RNA were collected (Section 2.5). Cells without infection were cultured in media only as a non-infected control and described as 'non-infected' in Figures. To confirm the observed effects were due to replicating virus and exclude any possible influence of contaminants in viral lysate or culture media, control cultures were treated with Palivizumab, a neutralising monoclonal antibody, which binds the RSV F-protein and inhibits RSV entry into the cell. This is detailed as 'anti-RSV control'.

3.3.1. RSV replication and IL-8 expression in BEAS-2B cells

After infection RSV N gene expression was measured by qPCR (Section 2.6.3) to confirm viral replication and IL-8 gene expression measured to ensure the BEAS-2B cells were responding to challenge. The pattern of expression observed is illustrated in Figure 3.1.

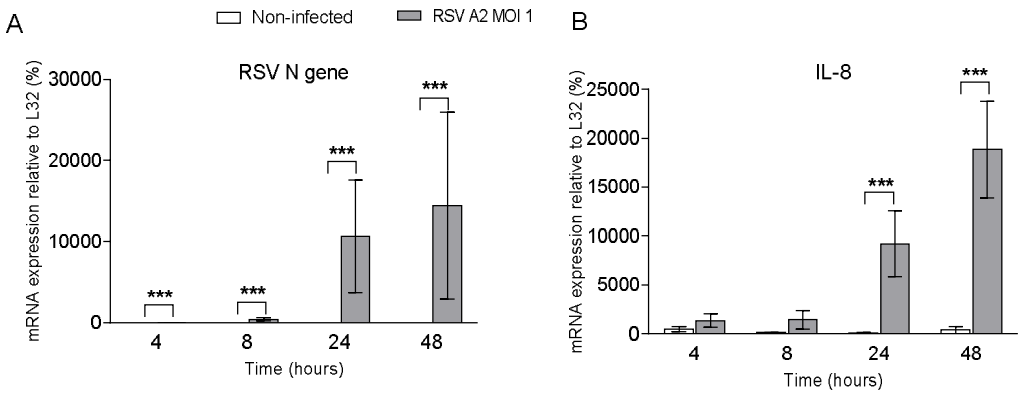


Figure 3.1. Expression of RSV N gene and IL-8 mRNA in RSV A2 infected BEAS-2B cells. BEAS-2B cells were infected with RSV A2 at an MOI of 1 for 4, 8, 24 and 48hr. Control non-infected cultures were cultured in the absence of RSV. A) RSV N gene (n=4) and B) IL-8 (n=4). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, ***p<0.001).

RSV N RNA was not detected in the absence of infection and was significantly increased at 4hr ($p<0.001$), 8hr ($p<0.001$), 24hr ($p<0.001$) and 48hr ($p<0.001$) compared to non-infected cells (Figure 3.1A). IL-8 mRNA was significantly upregulated at 24hr ($p<0.001$) and 48hr

p<0.001) compared to control non-infected cells (Figure 3.1B). These results indicate both RSV replication and response to infection by the BEAS-2B cells. Similar measurements were made during all later experiments to ensure infection had taken place.

3.3.2. Expression of IL-12 and IL-18 during RSV A2 infection of BEAS-2B cells

IL-12 and IL-18 have been shown to act synergistically to enhance NK cell activation (Section 1.3.3.4). Here IL-12β and IL-18 mRNA was first measured by qPCR as an indicator of IL-12 and IL-18 gene expression following RSV infection and subsequently IL-12p70 and IL-18 protein expression in culture supernatants measured by ELISA (Section 2.6.3 and 2.7).

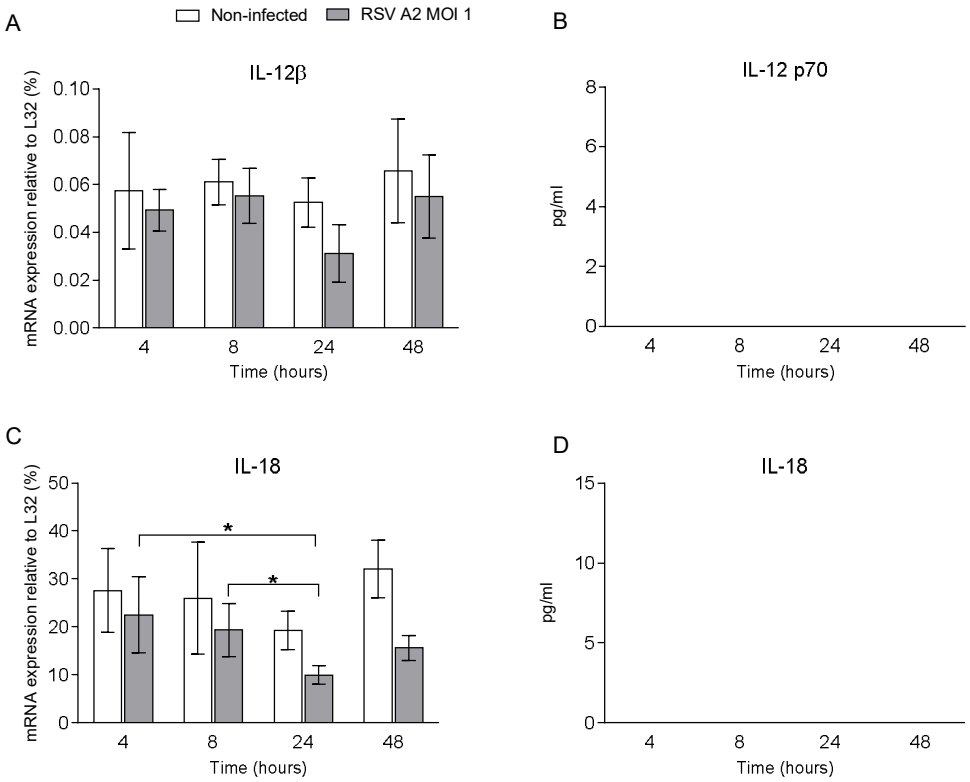


Figure 3.2. Expression of IL-12 and IL-18 mRNA and protein in RSV A2 infected BEAS-2B cells. BEAS-2B cells were infected with RSV A2 at an MOI of 1 for 4, 8, 24 and 48hr. Control non-infected cultures were cultured in the absence of RSV. A) IL-12β mRNA (n=4), B) IL-12 protein (n=3), C) IL-18 mRNA (n=4), D) IL-18 protein (n=3). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05).

There was no significant difference in IL-12β mRNA expression between control non-infected and RSV infected BEAS-2B cells (Figure 3.2A). IL-12β mRNA expression was less than 0.1% of housekeeping gene L32, suggesting little if any protein would be expressed by BEAS-2B cells with or without RSV infection. This was confirmed by ELISA with no IL-12p70 protein detected in undiluted culture supernatants, above the detection limit of 7.8pg/ml (Table 2.4) (Figure 3.2B).

IL-18 mRNA was expressed in both control and infected BEAS-2B cells at ~10-30% relative to L32 housekeeping gene (Figure 3.2C). No significant differences were observed in IL-18 mRNA expression between non-infected and infected cells at any time point. For the infected BEAS-2B cells, IL-18 mRNA expression was significantly lower at 24hr compared to 4hr ($p<0.05$) or 8hr ($p<0.05$). No IL-18 protein was detected using undiluted supernatants above the detection limit of 11.7pg/ml (Table 2.4) (Figure 3.2D).

These results indicate that BEAS-2B cells are most likely not a source of IL-12 protein during RSV infection. IL-18 mRNA expression was reduced at 24hr after RSV infection compared to early time points of infection, however no significant difference was seen between control and RSV infected BEAS-2B cells at 24hr. IL-18 protein was not detected above the detection limit, although IL-18 mRNA levels indicate protein may be present.

3.3.3. Expression of IL-15 during RSV A2 infection of BEAS-2B cells

IL-15 is another key NK cell activating cytokine and IL-15 expression has already been characterised in AECs during RSV infection (Section 1.3.3.3). Here the influence of RSV infection on BEAS-2B IL-15 mRNA and protein expression was measured by qPCR and Luminex, respectively (Section 2.6.3 and 2.7).

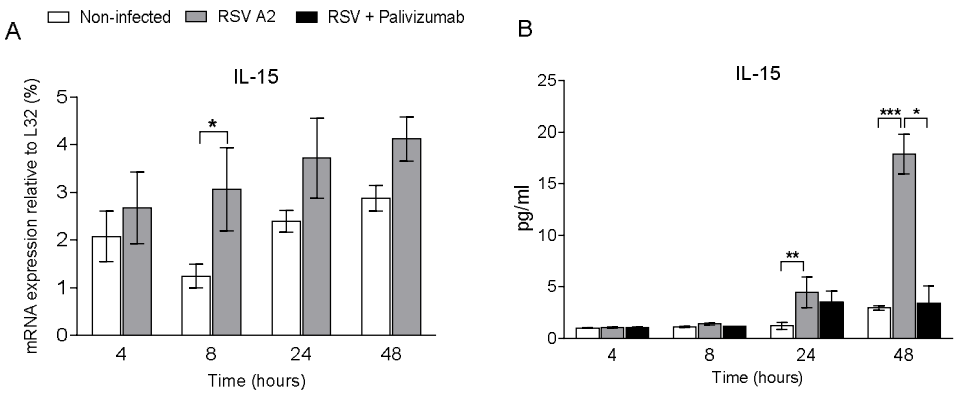


Figure 3.3. Expression of IL-15 mRNA and protein in RSV A2 infected BEAS-2B cells. BEAS-2B cells were infected with RSV A2 at an MOI of 1 for 4, 8, 24 and 48hr (A,B) or for 48hr at MOIs 0.5, 1 and 2.5 (C,D). Control non-infected cultures were cultured in the absence of RSV and Palivizumab used as an anti-RSV control. A) IL-15 mRNA (n=4), B) IL-15 protein (n=3). Dashed line shows lower detection limit. Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01, ***p<0.001).

IL-15 mRNA was significantly increased by ~2-fold at 8hr with infection compared to non-infected cells ($p<0.05$) (Figure 3.3A). For detection of IL-15 protein in culture supernatants protein expression measured using a Luminex assay (Section 2.8). IL-15 protein was significantly increased at 24hr ($p<0.01$) and 48hr at ~17.9 pg/ml $p<0.001$) in comparison to non-infected cultures (Figure 3.3B). At 24hrs, Palivizumab treated cells showed comparable levels of IL-15 with that of infected culture supernatants. At 48hrs, IL-15 expression was significantly higher in infected cultures compared to those treated with anti-RSV control Palivizumab ($p<0.05$). IL-15 protein was not found above the detection limit in non-infected cultures at 4 and 8hrs culture with levels being below the limit of detection.

These results show that RSV induces secretion of IL-15 from BEAS-2B cells at 48hr after infection. The Palivizumab treated control cultures were negative for IL-15 protein indicating expression was specifically induced by replicating RSV and not contaminants in the viral lysate used.

3.3.4. Expression of IL-15Rα during RSV A2 infection of BEAS-2B cells

The IL-15/IL-15Rα complex is a potent inducer of NK cell activation (Section 1.3.3.3). IL-15Rα expression by AECs has not previously been examined during RSV infection. It has been suggested that co-ordinated expression of both IL-15 and IL-15Rα is essential for complex formation (Section 1.3.3.3). Therefore IL-15Rα mRNA and cell surface expression was characterised by qPCR and flow cytometry, respectively (Section 2.6.3 and 2.9). For flow cytometric analysis, BEAS-2B cells were infected at RSV A2 MOIs of 0.5, 1 and 2.5 for 48hrs.

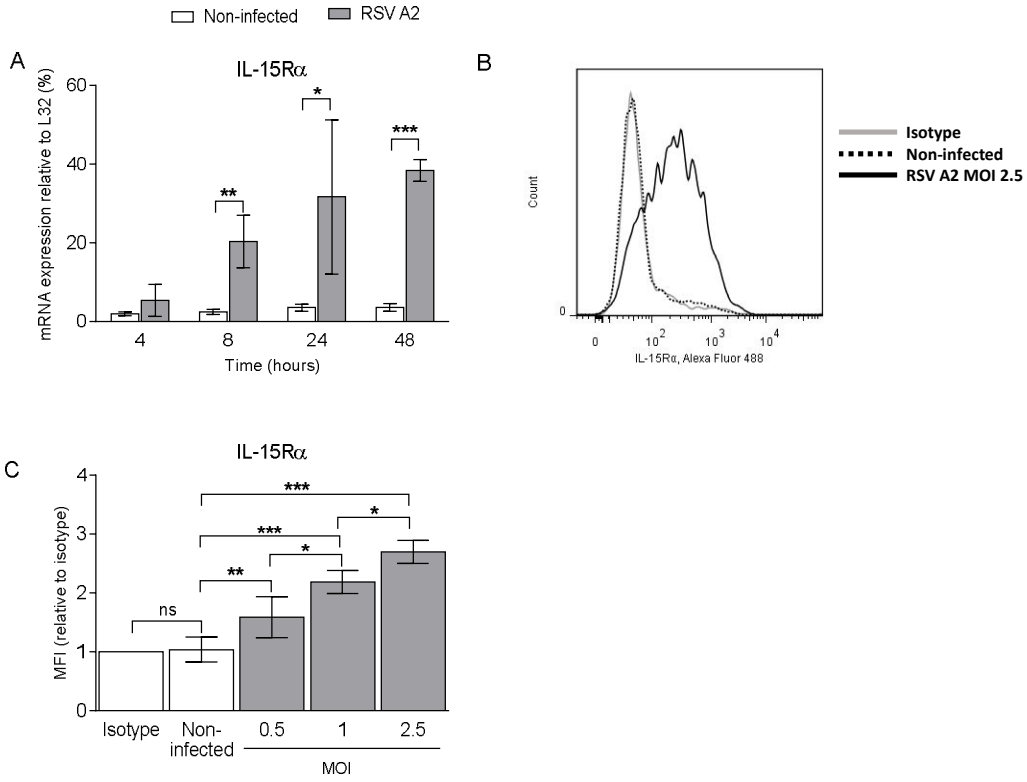


Figure 3.4. Expression of IL-15Rα mRNA and protein in RSV A2 infected BEAS-2B cells. BEAS-2B cells were infected with RSV A2 at an MOI of 1 for 4, 8, 24 and 48hr (A) or for 48hr at MOIs 0.5, 1 and 2.5 (B,C). Control non-infected cultures were cultured in the absence of RSV. A) IL-15Rα mRNA (n=4), B) representative flow histogram of surface IL-15Rα, C) flow cytometric analysis of surface IL-15Rα (n=3). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01, ***p<0.001).

Basal expression of IL-15Rα mRNA in control BEAS-2B cells, was ~1-5% of the L32 mRNA level (Figure 3.4A). RSV infection resulted in a significant increase in IL-15Rα mRNA expression at

8hr ($p<0.01$), 24hr ($p<0.05$) and 48hr ($p<0.001$) compared to control non-infected cells. At 4, 8 and 48hr, IL-15Rα mRNA was ~10-fold higher in infected when compared to non-infected BEAS-2B cells.

Flow cytometric analysis was used to determine if IL-15Rα was expressed at the surface of BEAS-2B cells (Figure3.4B). This data is presented as relative change compared to isotype control. No difference was observed between isotype control and control non-infected cells indicating an absence of surface expression on non-infected cells (Figure 3.4B). A single peak was observed during RSV infection indicative of one homogenous positive population (Figure 3.4B). Significant increases in mean fluorescence intensity (MFI) were seen between MOIs 0.5 ($p<0.01$), 1 ($p<0.001$) and 2.5 ($p<0.001$) compared to non-infected control. At an MOI of 2.5, the MFI was ~3-fold higher than that of control cells. Significant increases in MFI were also observed with increasing level of infection at MOI 0.5 ($p<0.05$) and MOI 2.5 ($p<0.05$) compared to MOI 1 (Figure 3.4C). Experiments described in Section 5.3.5 show Palivizumab pre-treatment effectively inhibits RSV-induced IL-15Rα surface expression.

These results indicate that RSV infection increases the expression of IL-15Rα mRNA in BEAS-2B cells and that IL-15Rα protein was expressed at the cell surface only on infection. Surface expression of IL-15Rα also followed an RSV MOI dose-dependent increase in expression.

3.3.5. Surface expression of IL-15 during RSV infection of BEAS-2B cells

Having established that RSV infected BEAS-2B cells express cell surface IL-15Rα (Section 3.3.4), IL-15 could be bound to IL-15Rα and also detected at the cell surface. Surface IL-15 has been explored in monocytes and the murine cytotoxic T cell line CTTL-2 (Section 1.3.3.3). Here, BEAS-2B cell surface IL-15 expression during RSV infection was measured using flow cytometry (Section 2.9). BEAS-2B cells were infected at RSV MOIs of 0.5, 1 and 2.5 for 48hrs.

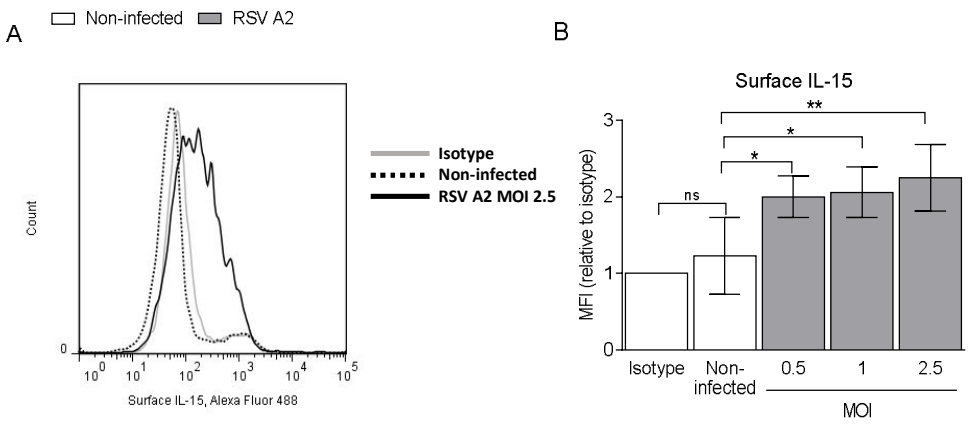


Figure 3.5. Expression of surface IL-15 of RSV A2 infected BEAS-2B cells. BEAS-2B cells were infected with RSV A2 for 48hr at MOIs of 0.5, 1 and 2.5. Control non-infected cultures were cultured in the absence of RSV. A) representative flow histogram of surface IL-15, B) flow cytometric analysis of surface IL-15 (n=3). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01).

A single peak was observed during RSV infection indicative of one homogenous positive population (Figure 3.5A). Data is shown as relative change compared to isotype control. No difference was observed between isotype control and non-infected cells indicating an absence of surface expression on control non-infected cells. There was a significant increase in MFI between non-infected control and MOIs 0.5 ($p<0.05$), 1 ($p<0.05$) and 2.5 ($p<0.05$) (Figure 3.5B). At an MOI of 2.5, the MFI was ~3-fold higher than non-infected cells. Experiments described in Section 5.3.5 show Palivizumab pre-treatment effectively inhibits RSV-induced IL-15 surface expression.

These results indicate that IL-15 was not only secreted as a soluble protein, as shown in Section 3.3.3, but also present on the cell surface after RSV infection.

3.3.6. Intracellular and extracellular expression of IL-15 and IL-15Rα during RSV A2 infection of BEAS-2B cells

Intracellular expression of IL-15 and IL-15Rα has previously been reported within the Golgi apparatus and endoplasmic reticulum, indicating that IL-15/IL-15Rα complex formation occurs within the Golgi apparatus before presentation at the cell surface, detailed in Section 1.3.3.3. To further evaluate intracellular and extracellular expression of IL-15 and IL-15Rα during RSV infection of BEAS-2B cells, immunofluorescence staining was used to define their cellular location. BEAS-2B cells were either permeabilised for intracellular staining or not permeabilised for surface staining as detailed in Section 2.10.1. An antibody to GM130, a cis-Golgi matrix protein, was used as a marker for intracellular staining within the Golgi apparatus (413).

Surface expression was first determined by use of non-permeabilising method (Section 2.10.1.1). Surface expression of both IL-15 (green) and IL-15Rα (red) were minimally detected in control non-infected BEAS-2B cells (Figure 3.6 upper). Stronger positive surface staining for both IL-15 and IL-15Rα was seen in RSV infected cells (Figure 3.6 lower). No staining was observed with the isotype control.

Intracellular expression was then determined using a permeabilising method (Section 2.10.1.2). Single staining for IL-15 (Figure 3.7) and IL-15Rα (Figure 3.8) showed intracellular expression of both proteins in both control non-infected and infected BEAS-2B cells. Both IL-15 and IL-15Rα were localised within the Golgi apparatus, shown here stained with GM130 (red). No staining was observed with the isotype control.

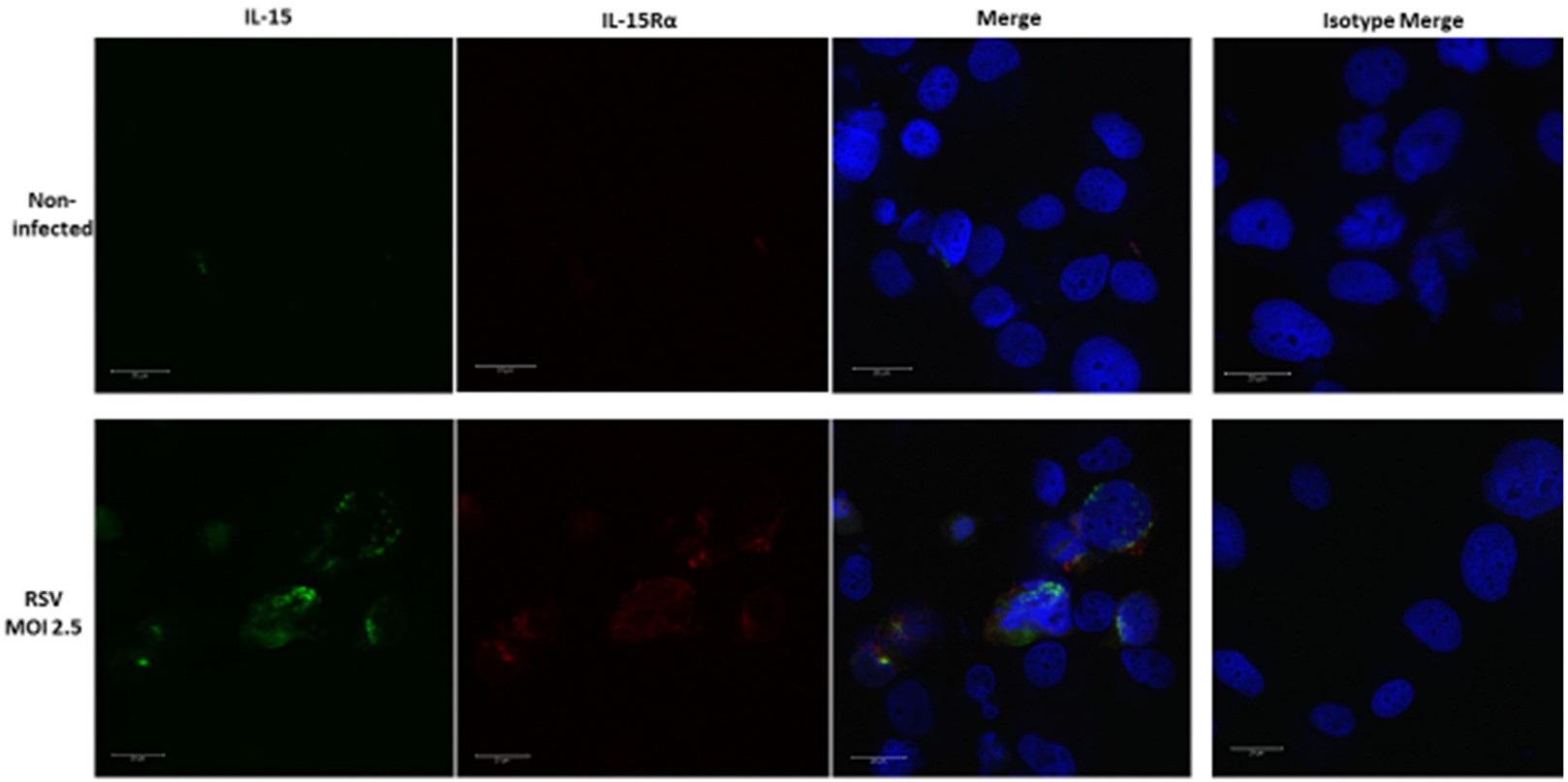


Figure 3.6. Immunofluorescence detection of surface IL-15 and IL-15Rα in RSV A2 infected BEAS-2B cells. BEAS-2B cells were infected with RSV A2 at MOI 1 for 24hr. Control non-infected cultures were cultured in the absence of RSV. Cells were stained as a single cell suspension with IL-15 antibody (green) and IL-15Rα antibody (red) or 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 2.10.1.1). Better images are supplied on the enclosed CD.

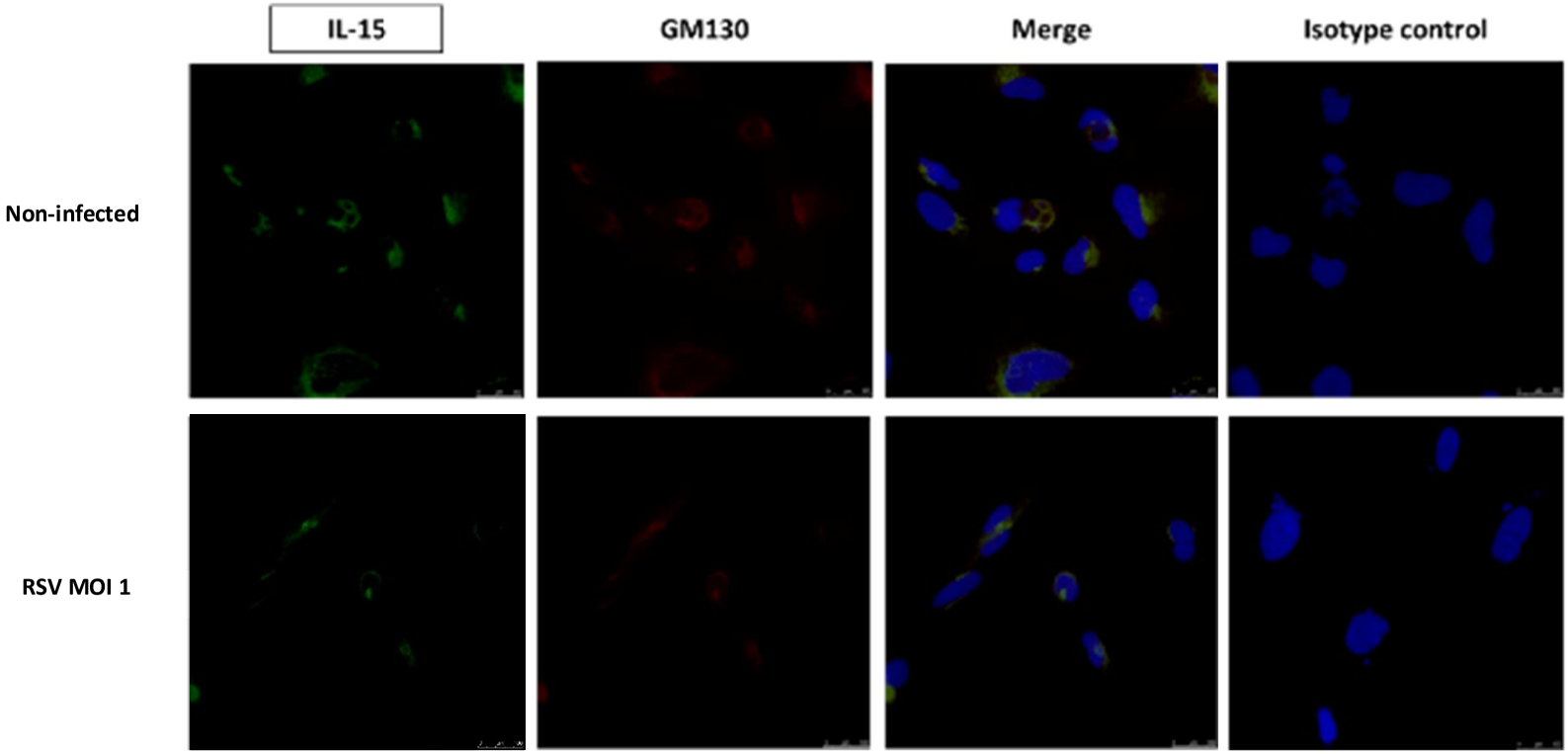


Figure 3.7. Localisation of intracellular IL-15 in RSV A2 infected BEAS-2B cells. BEAS-2B cells were grown until 50% confluence on coverslips in a 12-well plate and infected with RSV A2 MOI 1 for 24hr. Control non-infected cultures were cultured in the absence of RSV. Cells were permeabilised before incubation with IL-15 antibody (green) or with matched isotype controls. Cells were then incubated with secondary antibodies, Alexa Fluor 488 (green) and NL637 (red). Nuclei are represented as blue (DAPI). Images were obtained using confocal microscopy at 40x magnification (section 2.10.1.2). Better images are supplied on the enclosed CD.

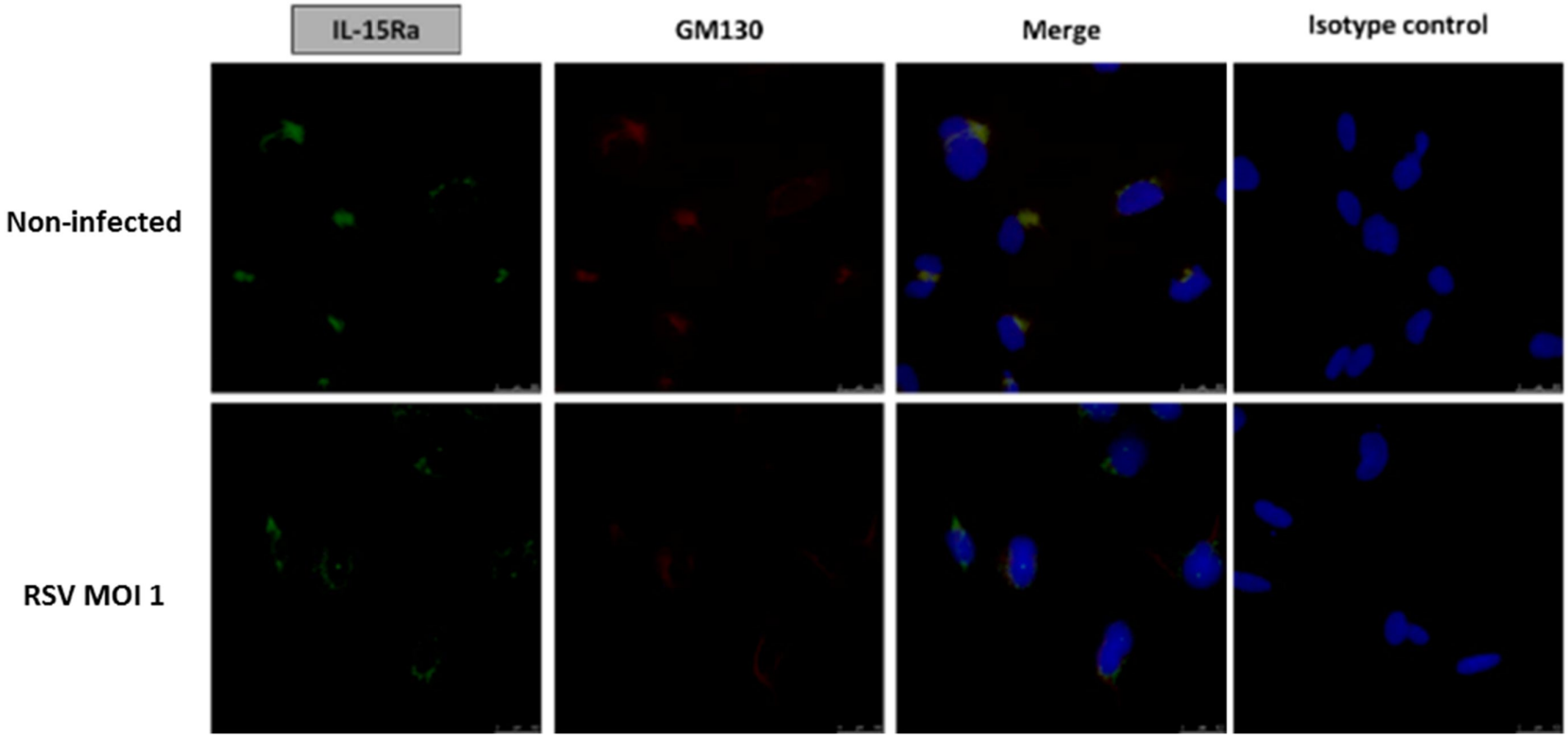


Figure 3.8. Localisation of intracellular IL-15Rα in RSV A2 infected BEAS-2B cells. BEAS-2B cells were grown until 50% confluence on coverslips in a 12-well plate and infected with RSV A2 MOI 1 for 24hr. Control non-infected cultures were cultured in the absence of RSV. Cells were permeabilised before incubation with IL-15Rα antibody (green) or with matched isotype controls. Cells were then incubated with secondary antibodies, Alexa Fluor 488 (green) and NL637 (red). Nuclei are represented as blue (DAPI). Images were obtained using confocal microscopy at 40x magnification (section 2.10.1.2). Better images are supplied on the enclosed CD.

These confocal images compliment the cytometry analysis results in Section 3.3.4 and 3.3.5, with expression of IL-15 and IL-15Rα seen at the cell surface in RSV infected BEAS-2B cells. Single staining for IL-15 and IL-15Rα show that intracellular expression was localised within the Golgi apparatus of both non-infected and RSV infected BEAS-2B cells.

3.3.7. Expression of IL-15/IL-15Rα complex during RSV A2 infection in BEAS-2B cells

The IL-15/IL-15Rα complex has been detected in serum of mice (Section 1.3.3.3). Here above it has been shown that infected BEAS-2B cells express both IL-15 and IL-15Rα at the cell surface. Therefore IL-15/IL-15Rα complex levels were also measured in culture supernatants following RSV infection of BEAS-2B cells.

The IL-15/IL-15Rα complex was detected in 2/5 RSV infected BEAS-2B cell supernatants, above the detection limit of 62.5pg/ml (Table 2.4) detailed in an experiment shown in Section 5.3.5.2, Figure 5.15.

This indicates that the IL-15/IL-15Rα complex was secreted or released from RSV infected BEAS-2B cells. The comparatively high minimum detection limit of this assay may have prevented detection in one or more of the 3 negative samples and requires further investigation.

3.3.8. Co-culture of RSV A2 infected BEAS-2B cells and NK cells

In order to be able to further characterise the ability of AECs to initiate NK cell activation during RSV infection, an *in vitro* co-culture model was established. Here, BEAS-2B cells were co-cultured with freshly isolated human peripheral blood NK cells from the blood of normal healthy adult volunteers (Section 2.4). *In vivo* infection of mice with RSV results in maximal airway NK cell number around 2-4 days after infection (Section 1.4). To reproduce an *in vivo* AEC-NK cell response to RSV, BEAS-2B cells were infected with RSV at MOI 1 for 24hr before addition of NK cells for a further 24hr (Section 2.5.1). This was to ensure that infected BEAS-2B cells expressed IL-15 and IL-15Rα protein and to mimic any potential *in vivo* 24hr delay in

NK cell population expansion. An MOI of 1 was chosen as the above results show that this induces expression of both IL-15 and IL-15Rα protein. Also, an MOI of 1 could be considered moderate so as to minimise RSV-induced cell lysis before NK cells are added (66,144,414). NK cells were added at increasing ratios of 1:1, 1:2 and 1:3 compared to BEAS-2B cells (BEAS-2B cell:NK cell), described in text as 'ratio of…'.

To confirm that BEAS-2B cells without RSV infection do not stimulate NK cells, control non-infected BEAS-2B cells were co-cultured at the highest NK cell ratio of 1:3 used with infected BEAS-2B cells. The control BEAS-2B cell:NK cell are shown in result figures as 'Non-infected 1:3'. To confirm that NK cells alone were not stimulated by culture media, NK cells were cultured alone. As the results described above show that it is unlikely that IL-12 or IL-18 protein was expressed by BEAS-2B cells, NK cells were stimulated with 10ng/ml human recombinant IL-15 as a stimulated NK cell positive control. These NK cell controls also provide additional information on the expression of mRNA and protein specifically from NK cells. Finally the neutralising anti-RSV antibody Palivizumab was used with the highest NK cell ratio of 1:3 to ensure observed results where produced by viral infection and not by other agents potentially present in the viral preparation. Production of TNF-α and IFN-γ were used as measures of NK cell activation.

3.3.8.1. Visualisation of BEAS-2B cell-NK cell co-culture

Light microscopy was used to determine if NK cells were in contact 4hr after addition to BEAS-2B cells BEAS-2B cell-NK cell co-cultures. NK cells were in contact with BEAS-2B cells after 4hr co-culture, as shown in Figure 3.9.

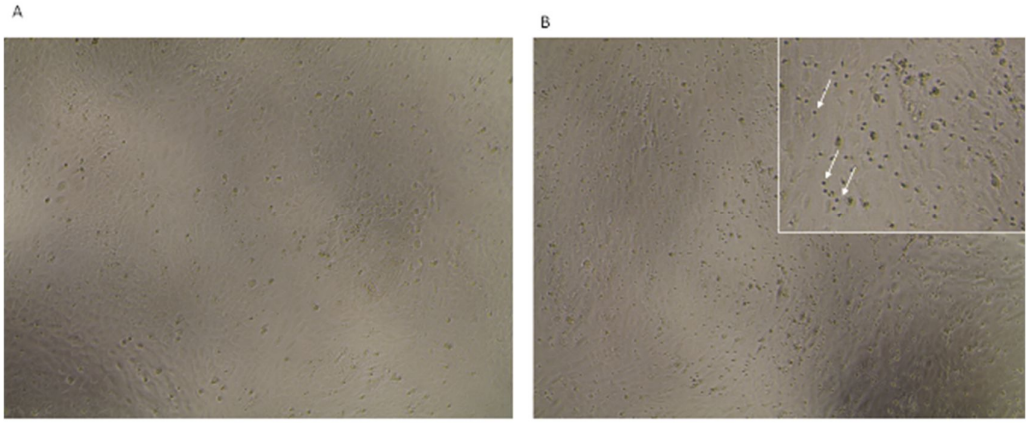


Figure 3.9. BEAS-2B cell-NK cell co-culture. BEAS-2B cells were infected with RSV A2 at MOI 1 for 24hr. NK cells were isolated from healthy adult PBMCs and added at a ratio of 1:3 to BEAS-2B cells for a further 24hr. Images were taken 4hr after NK cell addition to culture. NK cells are seen as small darker cells above the BEAS-2B monolayer. Non-infected cell cultures contained cRPMI only as a control. A) BEAS-2B cell without NK cells, B) infected BEAS-2B cells co-cultured with NK cells. Arrows in the zoomed image indicated NK cells.

3.3.8.2. Expression of RSV N gene and IL-8 protein in BEAS-2B-NK cell co-cultures.

To confirm and assess the level of infection in each culture RSV N gene expression was measured by qPCR. As previously described IL-8 protein expression in culture supernatants, measured by ELISA, was used as a control to show cells were responding to RSV infection (Section 2.6.3 and 2.7). Both BEAS-2B cells and NK cells would contribute to the L32 housekeeping gene expression.

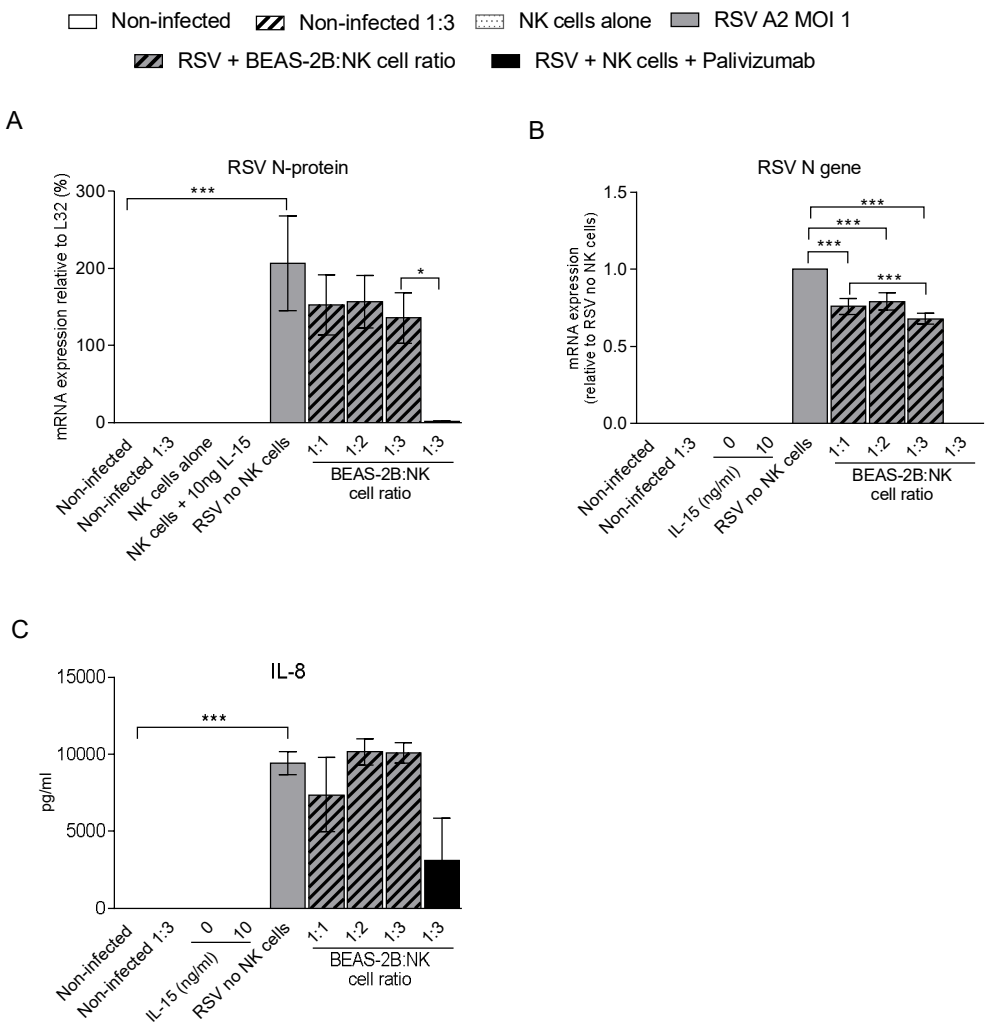


Figure 3.10. Expression of RSV N gene and IL-8 protein during RSV A2 infection of BEAS-2B cells co-cultured with NK cells. BEAS-2B cells were infected with RSV A2 at MOI 1 for 24hr. NK cells were isolated from healthy adult PBMCs and added at increasing ratios to BEAS-2B cells for a further 24hr. Control non-infected cultures were cultured in the absence of RSV and Palivizumab used as an anti-RSV control. A) RSV N RNA (n=3), B) RSV N gene relative to 'RSV no NK cells' (n=3), C) IL-8 protein (n=4). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, ***p<0.001).

RSV N RNA was not detected in control non-infected BEAS-2B cells, control BEAS-2B cells co-cultured with NK cells or NK cells cultured alone (Figure 3.10A). A significant increase in RSV N RNA was observed in infected BEAS-2B cells compared to control BEAS-2B cells ($p<0.001$). Palivizumab inhibited RSV replication with significantly reduced RSV N RNA in these anti-RSV control cultures ($p<0.05$). No significant difference in RSV N gene level was observed

between infected BEAS-2B cells cultured alone and those co-cultured with NK cells (Figure 3.10A). However it was noticed that infected BEAS-2B cells co-cultured with NK cells expressed lower RSV N RNA. Therefore data was expressed relative to infected BEAS-2B cells without NK cells, as shown in Figure 3.11B (Figure 3.10B). A significant decrease in RSV N RNA was seen with increasing NK cell ratios of 1:1 ($p<0.001$), 1:2 ($p<0.001$) and 1:3 ($p<0.001$) in comparison to infected BEAS-2B cells without NK cells. RSV N gene expression in cultures at a ratio of 1:3 was significantly lower than that observed in cultures at a ratio of 1:1 ($p<0.001$).

IL-8 protein was not detected in control non-infected BEAS-2B cell cultures, when non-infected BEAS-2B cells were co-cultured with NK cells or when NK cells were cultured alone (Figure 3.10C). There was a significant increase in the amount of IL-8 protein in culture supernatants from infected BEAS-2B cells compared to non-infected cells ($p<0.001$). No significant difference in IL-8 expression was observed when NK cells were added to infected BEAS-2B cells.

These results confirm, as expected, that RSV and IL-8 protein are found only in cultures following RSV infection. Relative expression of RSV N gene was decreased by addition of NK cells to infected BEAS-2B cells whereas IL-8 protein was not changed by addition of NK cells.

3.3.8.3. Expression of IFN-γ and TNF-α in BEAS-2B-NK cell co-cultures.

To measure activation of NK cells during co-culture with BEAS-2B cells, expression IFN-γ and TNF-α mRNA and protein were evaluated using qPCR and ELISA, respectively (Section 2.6.3 and 2.7). As previously described, BEAS-2B cells were infected for 24 hours before addition of NK cells then cells were co-cultured for 24hr before sample collection. Hence the results here show effect of NK cell activation over a 24hr period.

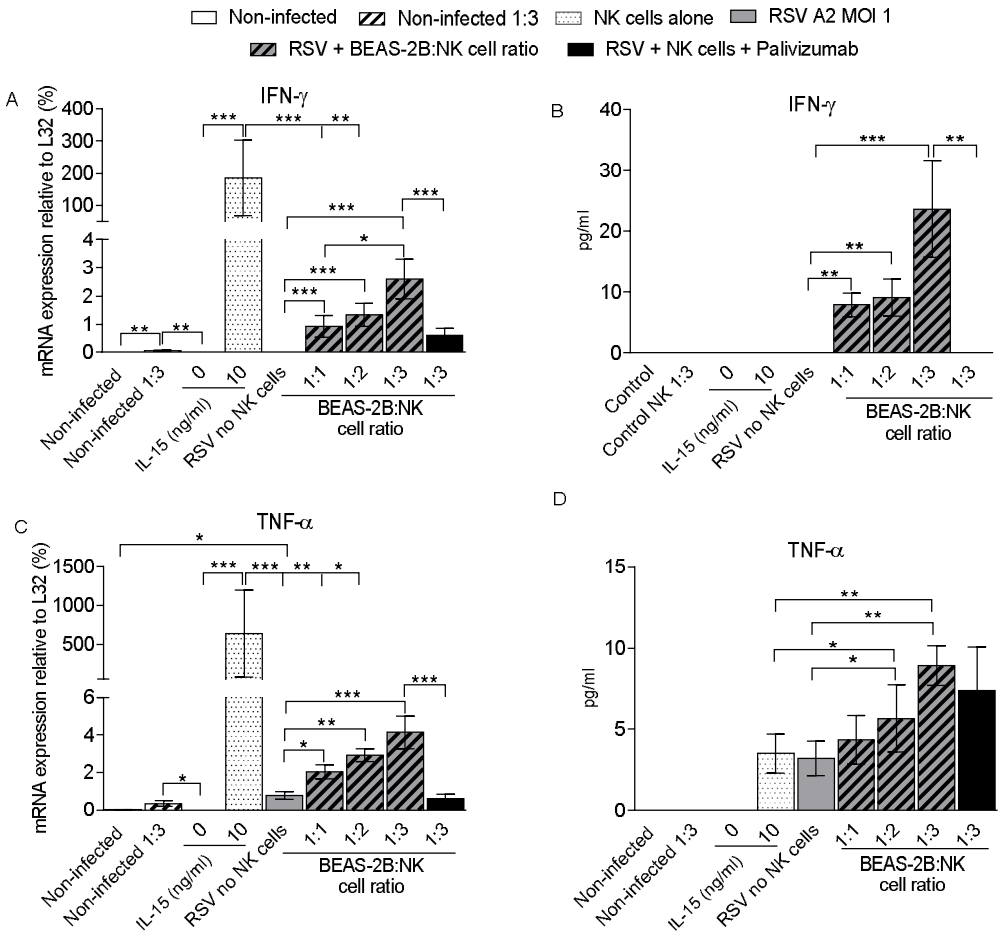


Figure 3.11. Expression of IFN-γ and TNF-α mRNA and protein during RSV A2 infection of BEAS-2B cells co-cultured with NK cells. BEAS-2B cells were infected with RSV A2 at MOI 1 for 24hr. NK cells were isolated from healthy adult PBMCs and added at increasing ratios to BEAS-2B cells for a further 24hr. Control non-infected cultures were cultured in the absence of RSV and Palivizumab used as an anti-RSV control. NK cells were cultured in cRPMI without or with 10ng/ml recombinant human recombinant IL-15. A) IFN-γ mRNA (n=4), B) IFN-γ mRNA (n=4), C) TNF-α mRNA (n=4), D) TNF-α protein (n=4). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01, ***p<0.001).

Neither control BEAS-2B cells, infected BEAS-2B cells, shown as 'RSV no NK cells', or NK cells cultured in media only expressed detectable levels of IFN-γ mRNA (Figure 3.11A). IFN-γ mRNA was present at levels below 0.1% of the housekeeping gene L32 in samples from non-infected BEAS-2B cells co-cultured with NK cells at a ratio of 1:3. This was significantly higher than control BEAS-2B cells without NK cells ($p<0.01$) and NK cells cultured in media alone ($p<0.01$). NK cells alone responded to recombinant IL-15 at 10ng/ml with expression of IFN-

γ mRNA at levels between 17-412% of the housekeeping gene L32 (Figure 3.11A). This was significantly increased compared to NK cells cultured in media alone. NK cells co-cultured with infected BEAS-2B cells showed a significant increased expression of IFN-γ mRNA at NK cell ratios of 1:1 ($p<0.001$), 1:2 ($p<0.001$) and 1:3 ($p<0.001$) compared to infected BEAS-2B cells cultured without NK cells (Figure 3.11A). Increasing the NK cell number present in culture with the BEAS-2B cells from a ratio of 1:1 to 1:3 resulted in a significant increase in IFN-γ mRNA ($p<0.05$). Addition of Palivizumab to cultures at a 1:3 significantly reduced IFN-γ mRNA expression ($p<0.001$), confirming expression was dependant on virus infection.

IFN-γ protein was only detected in culture supernatants from RSV infected BEAS-2B cells co-cultured with NK cells (Figure 3.11B). A significant increase in IFN-γ protein expression was observed between infected BEAS-2B cells without NK cells and those with NK cell ratios of 1:1 ($p<0.01$), 1:2 ($p<0.01$) and 1:3 ($p<0.001$). IFN-γ protein expression was ~2-fold higher at ~23pg/ml when a ratio of 1:3 was used compared to ratios of 1:1 and 1:2 at ~8-9pg/ml. IFN-γ protein was not present in co-cultures where Palivizumab had been used to neutralise RSV. IFN-γ protein expression was significantly higher at a ratio of 1:3 compared to Palivizumab treated co-culture ($p<0.01$).

Only comparatively low levels of TNF-α mRNA were present when BEAS-2B cells were cultured in the absence of infection (Figure 3.11C). Cultures of BEAS-2B cells alone contained TNF-α mRNA equivalent to ~0.02% of housekeeping gene L32. Expression in co-cultures containing control non-infected BEAS-2B cells with NK cells was ~0.4% of housekeeping gene L32 and this difference was significant ($p<0.05$) (Figure 3.11C). IL-15 stimulation, when compared to that of NK cells cultured alone, resulted in increased NK cell-derived TNF-α mRNA levels of between ~28-1700% in comparison to the housekeeping gene L32 ($p<0.001$). RSV infection significantly induced TNF-α mRNA expression by infected BEAS-2B cells in the absence of NK cells to ~0.8% of housekeeping gene L32 ($p<0.05$) (Figure 3.11C). Addition of

NK cells to infected BEAS-2B cells resulted in increased TNF-α mRNA expression. This was significantly increased in cultures containing NK cells at ratios of 1:1 ($p<0.05$), 1:2 ($p<0.01$) and 1:3 ($p<0.001$) when compared to infected BEAS-2B cells without NK cells (Figure 3.11C). Here, TNF-α mRNA expression was ~2%, ~2.9% and ~4% of housekeeping gene L32 at ratios of 1:1, 1:2 and 1:3, respectively. Treatment with Palivizumab resulted in significantly reduced TNF-α mRNA expression in cultures of infected BEAS-2B cells with NK cells ($p<0.001$), confirming expression was dependant on virus infection.

TNF-α protein was detected in 3/4 culture supernatants from NK cells stimulated with 10ng/ml of IL-15 and 3/4 from RSV infected BEAS-2B cell cultures without NK cells at ~4pg/ml each (Figure 3.11D). There was a significant increase in TNF-α protein from infected BEAS-2B cells co-cultured with NK cells at a ratio of 1:2 at ~5.6pg/ml compared to NK cells stimulated with 10ng/ml IL-15 ($p<0.05$) and infected BEAS-2B cells without NK cells ($p<0.05$). TNF-α protein was significantly higher in co-cultures of infected BEAS-2B cells and NK cells at a ratio of 1:3 at ~9pg/ml compared to NK cells stimulated with 10ng/ml IL-15 ($p<0.01$) or infected BEAS-2B cells without NK cells ($p<0.01$). There was no significant difference in TNF-α protein expression between co-cultures of infected BEAS-2B cell NK cell ratio 1:3 and infected BEAS-2B cell plus Palivizumab at a ratio of 1:3.

Within this assay, results indicate that NK cells do not express IFN-γ and TNF-α protein when cultured alone. The positive NK cell control cells stimulated with IL-15 expressed IFN-γ and TNF-α mRNA but only TNF-α protein. BEAS-2B cells also expressed TNF-α mRNA at low levels after virus treatment, whereas IFN-γ expression was specific to cultures with NK cells present. During co-culture, NK cells expressed more IFN-γ protein than TNF-α protein, with IFN-γ being ~2-fold higher. In general, prior exposure of BEAS-2B cells to RSV followed by co-culture with NK cells resulted in production of IFN-γ and TNF-α. A dose-response relationship was also observed for both IFN-γ and TNF-α mRNA and protein expression in cultures with

NK cells and infected BEAS-2B cells. The anti-RSV control Palivizumab prevented RSV cell entry and replication, as shown by RSV N RNA, however only expression of IFN-γ and not TNF-α protein was not significantly reduced. This could suggest either that another factor in the RSV lysate (or Palivizumab solution) stimulates TNF-α protein secretion from NK cells without inducing TNF-α mRNA expression.

3.3.9. Cell contact between NK cells and RSV A2 infected BEAS-2B cells was required to induce TNF-α and IFN-γ protein expression

In the experiments described in Section 3.3.2, cultures of BEAS-2B cells infected with RSV at an MOI 1 for 48hr contained around ~17pg/ml IL-15. In the above Section 3.3.7.3 NK cells were stimulated with 10ng/ml IL-15 which was ~580 fold more than present in infected BEAS-2B cell culture supernatants. The results illustrated in Figure 3.11 (Section 3.3.8.3) show that IFN-γ protein was not present in culture supernatants from NK cells stimulated with IL-15 and TNF-α detected at ~4pg/ml. However, co-cultures of NK cells with infected BEAS-2B cells contained significantly increased IFN-γ and TNF-α protein. This raises the possibility that direct cell-to-cell contact is necessary for expression of IFN-γ and TNF-α protein. Expression of IL-15Rα and IL-15 at the surface of RSV infected cells has been demonstrated in Sections 3.3.3, 3.3.4, 3.3.5 and 3.3.6, which could suggest IL-15/IL-15Rα complex presentation at the cell surface of BEAS-2B cells which may lead to enhanced NK cell activation compared to stimulation with IL-15 alone. Furthermore NK cells express an array of activating and inhibitory receptors which, during direct BEAS-2B cell contact, may or may not initiate further NK cell activation (Section 1.3.2). The fourth aim of this chapter questioned if direct contact of BEAS-2B cells and NK cells is required for optimum NK cell activation. To answer the fourth aim of this chapter, a transwell assay was used to separate BEAS-2B cells from NK cells in co-culture to determine if BEAS-2B cell-NK cell contact is required for optimum NK cell activation (Section 2.5.2).

Figure 2.4 in Section 2.5.2 depicts the conditions used for this experiment, shown here in Figure 3.12 as conditions A, B, C and D and discussed below. Another difference between this experiment and the co-culture experiment in Section 3.3.8 is that a 24-well plate was used instead of a 96-well, therefore a higher total number of NK cells were used. Inserts consisted of 0.4µm pores which allow cytokines but not cells to pass through (Section 2.5.2). Cultures with direct contact between BEAS-2B cells and NK cells were used as a positive control (A). NK cells were either incubated in the transwell insert with fresh RPMI media (B) or culture supernatant taken from the lower BEAS-2B cell compartment (C). BEAS-2B cells were also grown on the transwell insert and NK cells placed in the lower compartment to allow for IL-15 secretion from the basal surface of the epithelial cells (D). IFN-γ and TNF-α protein expression in supernatants from both upper and lower compartments was determined by ELISA.

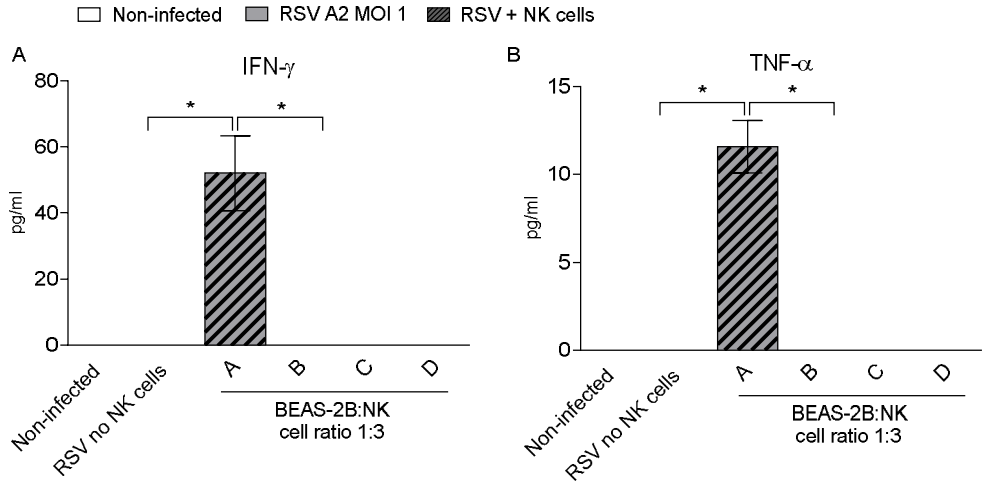


Figure 3.12. Expression of IFN-γ and TNF-α protein during RSV A2 infection of BEAS-2B cells co-cultured with NK cells or separated by a transwell insert. BEAS-2B cells were infected with RSV A2 at MOI 1 for 24hr. Control non-infected cultures were cultured in the absence of RSV. Isolated NK cells (Section 2.4) were added for a further 24hr at a ratio of 1:3 into the lower chamber in 100ul cRPMI (co-culture) **A**, into the insert in 100ul fresh cRPMI **B**, into the insert in 100ul infected BEAS-2B cell culture supernatant **C** and into the lower chamber in 100ul cRPMI **D**. A) IFN-γ protein (n=3), B) TNF-α protein (n=3). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05).

Expression of TNF-α and IFN-γ protein was only detected in culture supernatants from NK cells in direct contact with BEAS-2B cells at a ratio of 1:3 (Figure 3.12A, B). TNF-α protein ($p<0.05$) and IFN-γ protein ($p<0.05$) was significantly increased in comparison to cultures without NK cells. Cytokine protein was not found when NK cells were cultured in the inserts separated and above the BEAS-2B cell monolayer or co-cultured in the lower compartment with BEAS-2B cells in the upper insert.

These results indicate that NK cells do not express IFN-γ or TNF-α protein when cultured in supernatant from infected BEAS-2B cells alone and require direct contact with infected BEAS-2B cells to stimulate secretion.

3.3.10. Expression of intracellular IFN-γ protein in NK cells during BEAS-2B cell-NK cell co-culture

To further validate the hypothesis that NK cells are activated by RSV infected BEAS-2B cells, flow cytometric analysis was used to examine intracellular expression of IFN-γ protein in NK cells following direct BEAS-2B cell contact (Section 2.9.4). NK cells were cultured in media alone or with 10ng/ml IL-15 to determine negative and positive intracellular IFN-γ protein levels of expression. Direct contact stimulation of the NK cells followed the same methodology as used in Section 3.3.7, but with a 24-well plate instead and a BEAS-2B cell:NK cell ratio of 1:1.

Figure 2.7 in Section 2.9.4.1 shows the gates used to distinguish between BEAS-2B cells and NK cells. Figure 2.7A shows BEAS-2B cells cultured alone and Figure 2.7B shows BEAS-2B cell-NK cell co-culture. A Log scale was chosen to be able to distinguish between cell populations which was not clear in linear scale. The NK cell population was then confirmed by staining of co-cultured BEAS-2B cells and NK cells with CD56 and CD3. As shown in Figure 3.13 the selected NK cell population was $CD56^{+}$ $CD3^{-}$ and the BEAS-2B cell population $CD56^{-}$ $CD3^{-}$,

confirming correct gated for the NK cell population. Single staining for intracellular IFN-γ protein was then completed by gating the NK cell population.

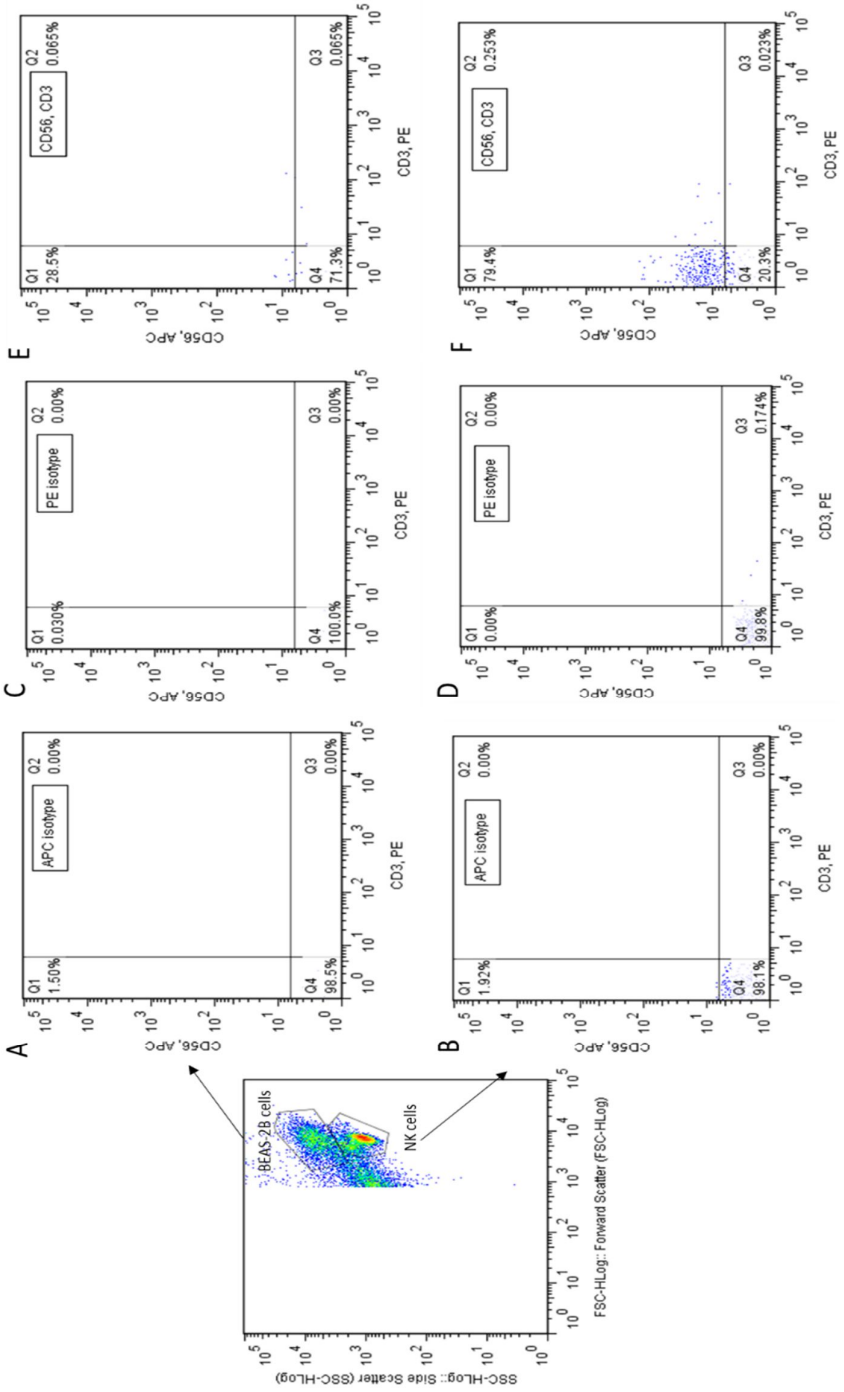


Figure 3.13. Representative flow cytometry gating to distinguish BEAS-2B cells ($CD56^-$, $CD3^-$) from NK cells ($CD56^+$, $CD3^-$) during co-culture. BEAS-2B cells were infected with RSV A2 at MOI 1 for 24hr. NK cells were isolated from healthy adult PBMCs and added at a ratio of 1:1 (BEAS-2B cells:NK cells) for a further 24hr. Cells were stained with A,B) CD56 APC isotype, C,D) CD3 PE isotype and E,F) dual stain CD56 APC and CD3 PE.

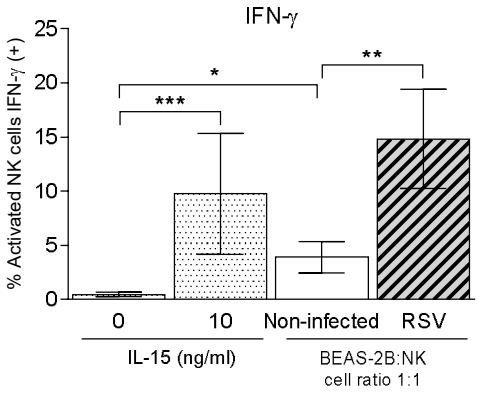


Figure 3.14. Expression of intracellular IFN-γ in NK cells co-cultured with RSV infected BEAS-2B. BEAS-2B cells were infected with RSV A2 at MOI 1 for 24hr. NK cells were isolated from healthy adult PBMCs and added at ratios of 1:1 (BEAS-2B cell:NK cells) for a further 24hr. Control non-infected cultures were cultured in the absence of RSV. Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01, ***p<0.001) (n=3).

As expected the percentage of IFN-γ positive NK cells was significantly higher when NK cells were cultured with 10ng/ml IL-15 compared to media only ($p<0.001$) increasing to ~10% IFN-γ positive (Figure 3.14). NK cells co-cultured with control non-infected BEAS-2B cells had a significantly higher percentage IFN-γ positive NK cells at ~5 %, ~3-fold higher, compared to NK cells cultured in media only ($p<0.05$). NK cells co-cultured with infected BEAS-2B cells had a significantly higher percentage of IFN-γ positive NK cells, at ~15% and ~3-fold higher, compared to NK cell co-cultured with non-infected BEAS-2B cells ($p<0.001$).

These results further indicate that direct contact with RSV infected BEAS-2B cells induces NK cell intracellular expression of IFN-γ protein. The percentage of IFN-γ positive NK cells was not significantly different between cultures in which NK cells were stimulated with 10ng/ml IL-15 alone and NK cells co-cultured with infected BEAS-2B cells, at ~10% and 15% respectively. Control non-infected BEAS-2B cells did induce intracellular expression of IFN-γ protein, but this was ~3-fold lower than observed in NK cells co-cultured with infected cells. This data also suggests that co-culture with non-infected BEAS-2B cells may induce a small percentage of NK cells to express intracellular IFN-γ under resting conditions.

3.3.11. NK cell markers of cytotoxicity

3.3.11.1. Expression of intracellular perforin in NK cells during culture with BEAS-2B cell supernatants

NK cell secretory granules contain granzymes and perforin which are secreted through an immunological synapse when NK cells are in contact with target cells (Section 1.3.4). Intracellular perforin expression can be examined as an indicator of the NK cell cytotoxic response. To determine if BEAS-2B cell culture supernatants could induce a NK cell cytotoxic response, intracellular expression of perforin was determined in NK cells cultured with effector cell line, K562. These experiments where produced in collaboration with Liz Van Erp at the National Institute for Public Health and the Environment (RIVM, Netherlands). Section 2.9.4.2 details the experimental procedure here also illustrated in Figure 3.15. Briefly, BEAS-2B cell culture supernatants from non-infected and infected were prepared in Liverpool and sent on dry ice to Liz Van Erp who carried out the NK cell cytotoxicity assay. Isolated NK cells from two healthy adult donors were incubated for 4hr with K562 cells in BEAS-2B cell culture supernatants. NK cells were then assessed by flow cytometry for intracellular perforin expression.

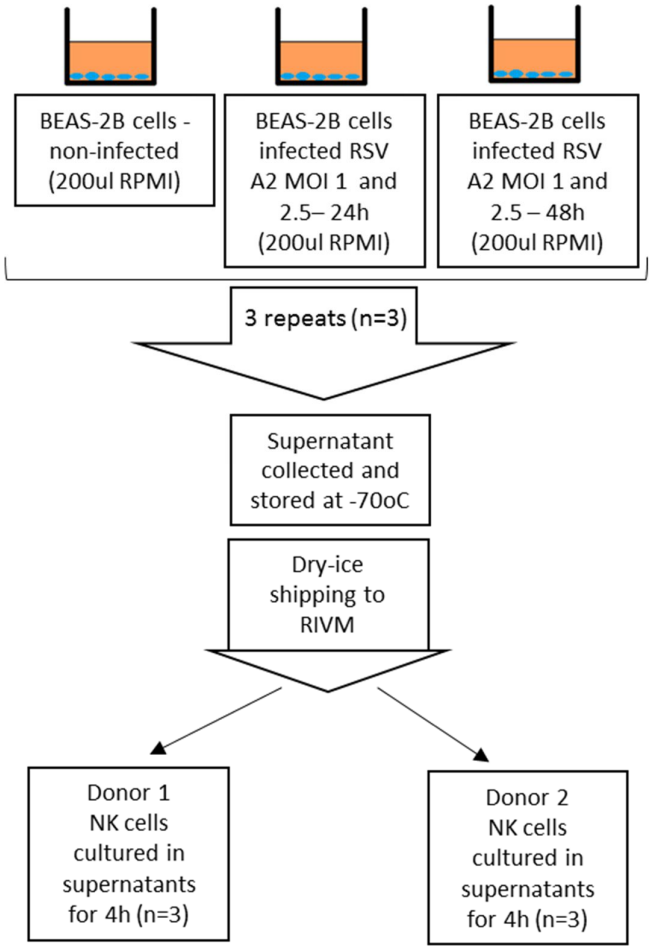


Figure 3.15. Experimental procedure for NK cells cultured in supernatants. BEAS-2B cells were infected with RSV A2 at MOI 1 and 2.5 for 24hr and 48hr. NK cells were isolated from two healthy adult PBMCs and cultured in supernatants for 4hr before flow cytometric analysis. Non-infected cells contained cRPMI only as a control.

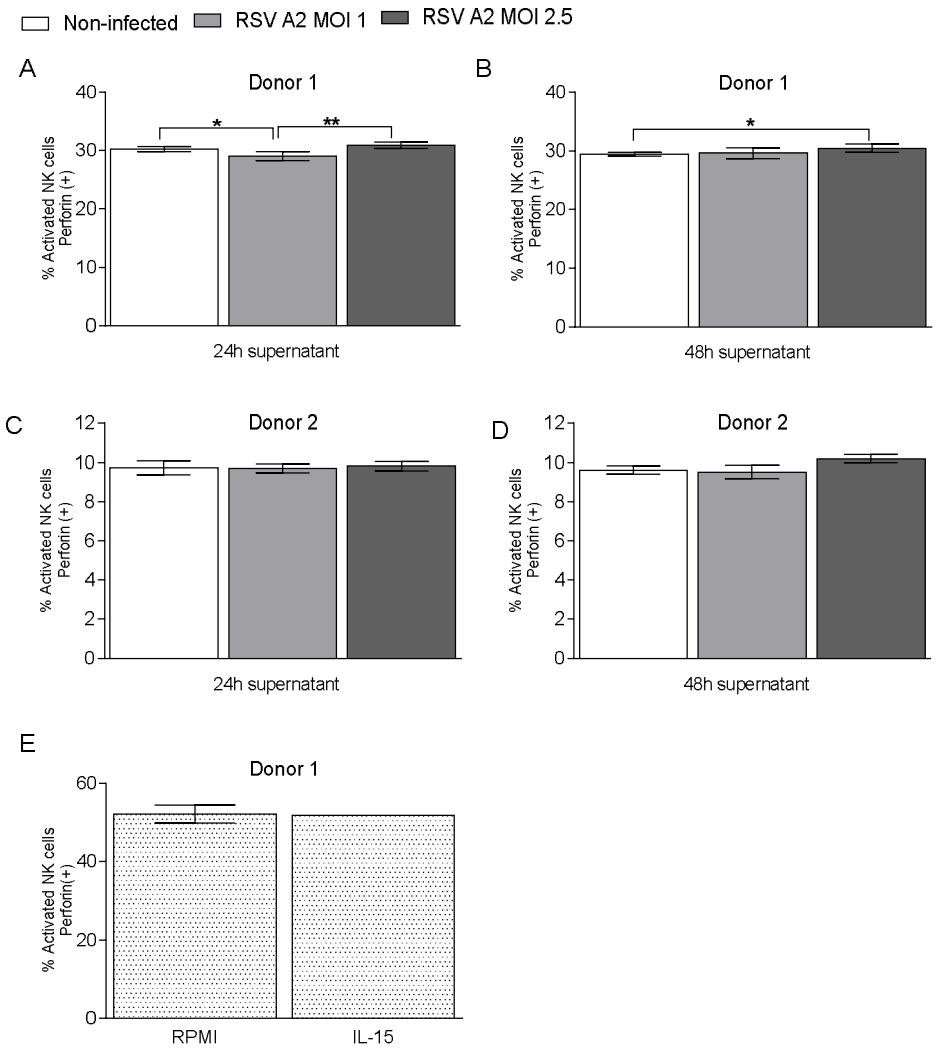


Figure 3.16. Expression of intracellular perforin in NK cells cultured in RSV infected BEAS-2B culture supernatant. BEAS-2B cells were infected with RSV at MOIs 1 and 2.5 for 24 and 48hr (n=3). Control non-infected cultures were cultured in the absence of RSV. NK cells from two separate donors (donor 1 A, B, E and donor 2 C, D) were cultured in 24hr (A, C) and 48hr BEAS-2B cell culture supernatant for 4hr before flow cytometric analysis. Each donor's NK cells were cultured in three separate supernatants and done in duplicate. E (RPMI n=1, IL-15 n=1). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01).

Culture of NK cells from donor 1 in BEAS-2B cell culture supernatants taken after 24hrs infection showed a minor but significant decrease in the percentage of perforin positive NK cells at a MOI 1 compared to NK cells cultured in supernatants from non-infected BEAS-2B cells ($p<0.05$) (Figure 3.16A). Culture of NK cells in MOI 2.5 supernatants showed a minor but

significant increase in the percentage of perforin positive NK cells compared to MOI 1 ($p<0.01$). For culture of NK cells from donor 1 in BEAS-2B cell culture supernatants taken after 48hr infection, a minor but significant increase in the percentage perforin positive NK cells cultured was observed at MOI 2.5 compared to NK cells cultured in supernatants from non-infected BEAS-2B cells ($p<0.05$) (Figure 3.18B). There were no changes in perforin expression from donor 2's NK cells (Figure 3.16C, D).

Donor 1 NK cells were used to determine intracellular expression of perforin protein when cultured in media only or 10ng/ml IL-15 (Figure 3.16E). No difference in perforin expression was observed which could suggest IL-15 did not stimulate perforin expression in this assay.

Overall, the changes in the percentage of intracellular perforin positive NK cells between non-infected and infected culture supernatants was not much and it cannot be concluded that perforin expression was influenced by BEAS-2B cell culture supernatants. IL-15 alone may not be enough to induce perforin expression by NK cells.

3.3.11.2. Expression of intracellular IFN-γ in NK cells during culture in BEAS-2B cell supernatants

As part of the cytotoxicity assay performed by Liz Van Erp above, intracellular IFN-γ expression was also measured.

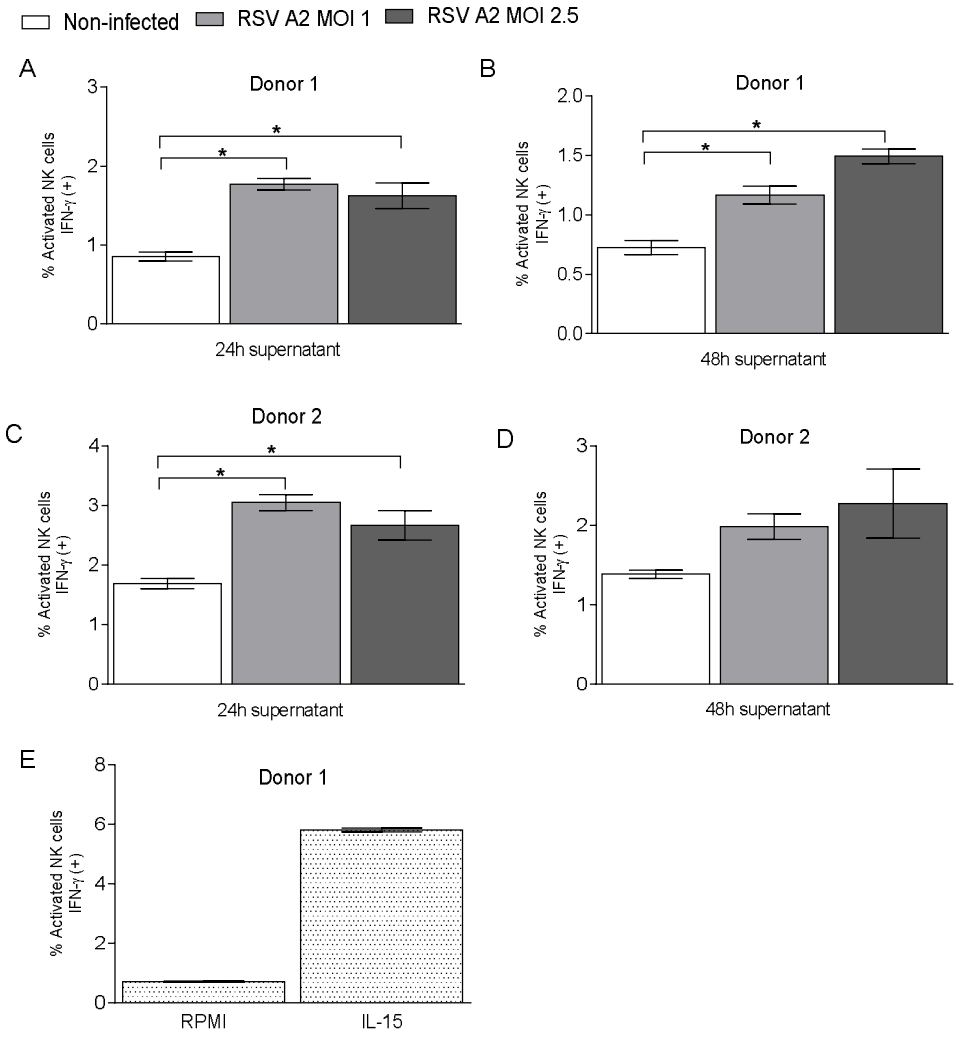


Figure 3.17. Expression of intracellular IFN-γ in NK cells cultured in RSV infected BEAS-2B culture supernatant. BEAS-2B cells were infected with RSV at MOIs 1 and 2.5 for 24 and 48hr (n=3). Control non-infected cultures were cultured in the absence of RSV. NK cells from two separate donors (donor 1 A, B, E and donor 2 C, D) were cultured in 24hr (A, C) and 48hr BEAS-2B cell culture supernatant for 4hr before flow cytometric analysis. Each donor's NK cells were cultured in three separate supernatants and done in duplicate. E (n=2). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05).

Donor 1's NK cells showed a significant increase in the percentage of IFN-γ positive cells when cultured with supernatants from BEAS-2B cells challenged with RSV for 24hrs at MOI 1 ($p<0.05$) and 2.5 ($p<0.05$) when compared to NK cells cultures with control non-infected supernatants (Figure 3.17A). A significant increase in the percentage of IFN-γ positive NK cells present was also observed with 48hr culture supernatants at MOI 1 ($p<0.05$) and 2.5 ($p<0.05$)

(Figure 3.17B). Donor 1 NK cells were also used to determine intracellular expression of IFN-γ protein when cultured in media only or 10ng/ml IL-15 (Figure 3.17E).

For donor 2 there was a significant increase in the percentage of IFN-γ positive NK cells when cultured with 24hr RSV infected BEAS-2B culture supernatants (MOI 1 ($p<0.05$) and 2.5 ($p<0.05$)) compared to non-infected culture (Figure 3.18C). No significant differences were seen for donor 2 using 48hr culture supernatants (Figure 3.17D).

These results indicate that BEAS-2B cell supernatants from infected cells could induce intracellular expression of IFN-γ in NK cells, which was ~2-fold more than NK cells cultured in non-infected BEAS-2B cell supernatant.

3.3.12. Differential activation of BEAS-2B cells by specific RSV strains

A better understanding of how different RSV isolates or strains modulate or induce specific immune responses by AECs could improve our knowledge of RSV pathogenesis. Characterising the airway epithelial responses *in vitro* with use of clinical RSV isolates may provide a more accurate representation of *in vivo* responses and aid in better vaccine and treatment development. The last aim of the work in this chapter was compare the expression of IL-15 and IL-15Rα by BEAS-2B cells in response to infection with two different RSV A strains, A2 lab strain and clinical isolate RSV X (Section 2.3.1). This was followed by comparing the expression of IFN-γ and TNF-α between RSV A2 and RSV X infected BEAS-2B cell-NK cell co-cultures. In these experiments BEAS-2B cells were infected with RSV A2 or RSV X at an MOI of 2.5 for 48hrs, after which culture supernatants and RNA were collected.

3.3.12.1. Differences between RSV A2 and RSV X replication and infectivity

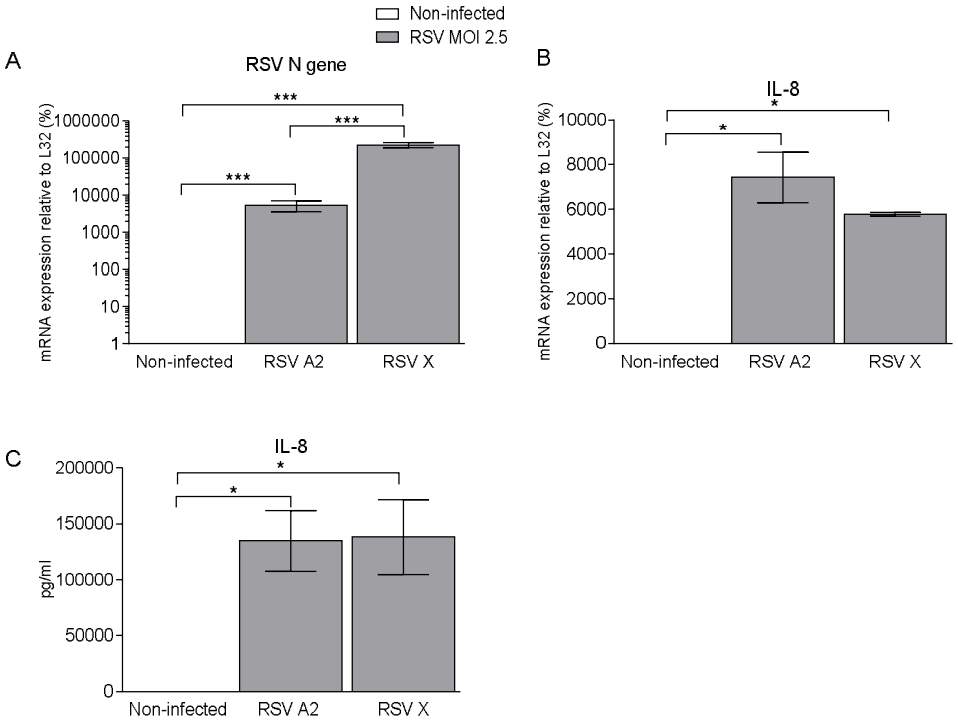


Figure 3.18. Comparison of RSV N gene and IL-8 mRNA expression induced by RSV A2 and RSV X. BEAS-2B cells were infected with RSV A2 and RSV X at an MOI of 2.5 for 48hr. Control non-infected cultures were cultured in the absence of RSV. A) RSV N gene (n=3), B) IL-8 mRNA (n=3), IL-8 protein (n=3). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, ***p<0.001).

The pattern of RSV N gene and IL-8 mRNA and protein expression observed for RSV A2 and RSV X strains is illustrated in Figure 3.19. As expected RSV N RNA was not detected in the absence of infection and was significantly increased with both RSV A2 ($p<0.001$) and RSV X ($p<0.001$) infection (Figure 3.18A). RSV N gene expression was ~56-fold greater in BEAS-2B cells infected with RSV X compared to RSV A2 ($p<0.001$). IL-8 mRNA was not detected in the absence of infection and was significantly increased with both RSV A2 ($p<0.05$) and RSV X ($p<0.05$) infection (Figure 3.18B). IL-8 protein was not detected in the absence of infection and was significantly increased with both RSV A2 ($p<0.05$) and RSV X ($p<0.05$) infection

(Figure 3.18C). For IL-8 mRNA and protein, no significant difference was observed between strains (Figure 3.18B, C).

The results indicate that although RSV infectivity was greater for RSV X than RSV A2 in BEAS-2B cells although no difference was observed in the RSV-induced expression of IL-8.

3.3.12.2. Differences between RSV A2 and RSV X in expression of IL-15 and IL-15Rα

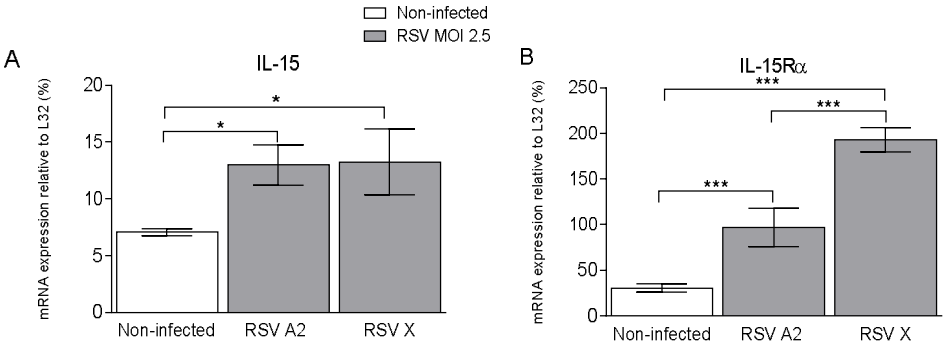


Figure 3.19. Comparison between the expression of IL-15 and IL-15Rα mRNA in RSV A2 and RSV X infected BEAS-2B cells. BEAS-2B cells were infected with RSV A2 and RSV X at an MOI of 2.5 for 48hr. Control non-infected cultures were cultured in the absence of RSV. A) IL-15 mRNA (n=3), B) IL-15Rα mRNA (n=3). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, ***p<0.001).

IL-15 mRNA was significantly increased by ~2-fold by 48hr infection at an MOI of 2.5 for both RSV A2 ($p<0.05$) and RSV X ($p<0.05$) compared to non-infected cells (Figure 3.19A). IL-15Rα mRNA was significantly increased by ~2-fold with RSV A2 ($p<0.001$) and increased by ~5-fold by RSV X ($p<0.001$) compared to non-infected cells (Figure 3.19B). Between RSV A2 and RSV X, RSV X infected cultures showed significantly more IL-15Rα mRNA expression, ~2-fold more than RSV A2 ($p<0.001$).

The results here indicate that both strains of RSV, A2 and X, induce comparable amounts of IL-15 mRNA. However RSV X was able to induce more IL-15Rα mRNA than RSV A2.

3.3.12.3. NK cell activation during BEAS-2B co-culture with different RSV A2 and RSV X

The results illustrated in Figure 3.19B indicate that RSV X induces the expression of more IL-15Rα mRNA by BEAS-2B cells when compared to RSV A2. IL-15Rα has been shown to be play an essential role in trafficking of IL-15 to the cell surface and in trans-presentation which induces NK cell activation (Section 1.3.3.3). Although cell surface IL-15Rα was not determined here, an increase in IL-15Rα mRNA by RSV X over RSV A2 could increase expression of activated NK cell cytokines during infected BEAS-2B cell-NK cell co-culture. Here, BEAS-2B cells were infected with RSV A2 or RSV X at an MOI of 1 for 24hr before addition of NK cells at a ratio of 1:3.

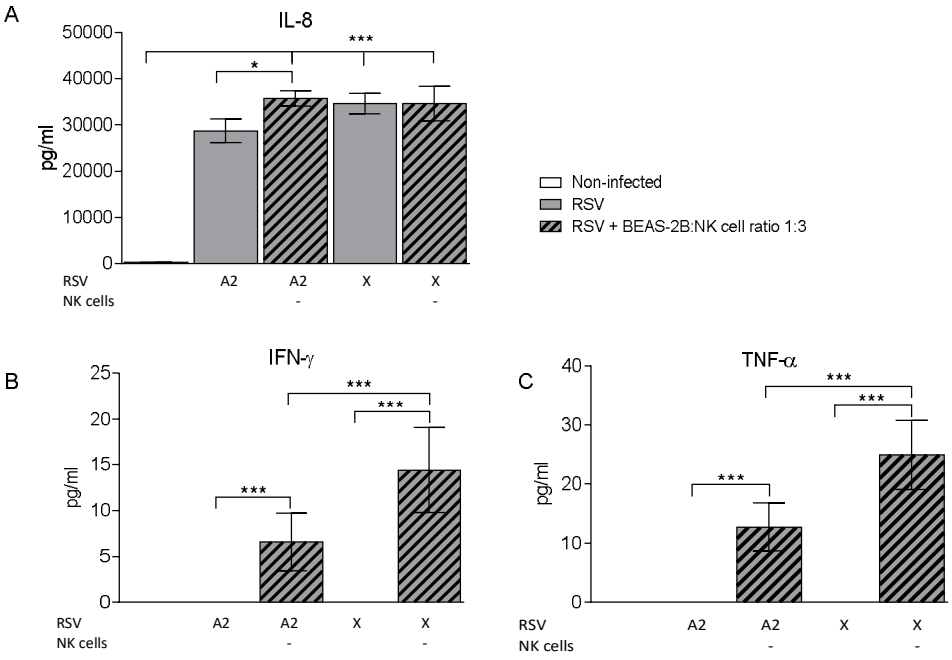


Figure 3.20. Comparison between the expression of IL-8, IFN-γ and TNF-α protein during RSV A2 and RSV X infection of BEAS-2B cells co-cultured with NK cells. BEAS-2B cells were infected with RSV A2 or clinical isolate, RSV X, at MOI 1 for 24hr. NK cells were isolated from healthy adult PBMCs and added at a ratio of 1:3 to BEAS-2B cells for a further 24hr. Control non-infected cultures were cultured in the absence of RSV. A) IL-8 protein (n=3), B) IFN-γ protein (n=3), C) TNF-α protein (n=3). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, ***p<0.001).

Expression of IL-8 protein was measured to ensure BEAS-2B cells were responding to RSV challenge. IL-8 protein was not detected in culture supernatants in the absence of infection (Figure 3.20A). IL-8 protein was significantly increased in all infected cultures, apart from RSV A2 without NK cells ($p<0.001$) compared to non-infected BEAS-2B cells. Addition of NK cells to RSV A2 infected BEAS-2B cells significantly increased expression of IL-8 protein by ~7000pg/ml ($p<0.05$) compared to infected culture without NK cells.

IFN-γ protein was only found in supernatants where NK cells had been present in the cultures. A significant increase in IFN-γ protein expression was observed between infected BEAS-2B cells without NK cells and those with NK cells (RSV A2, $p<0.001$ and RSV X, $p<0.001$) (Figure 3.20B). IFN-γ protein expression was significantly higher with RSV X infection compared to RSV A2 ($p<0.001$). IFN-γ protein was ~2-fold higher at ~15pg/ml with RSV X infection compared to RSV A2 infection at ~6.5pg/ml.

TNF-α protein was only detected in supernatants were NK cells had been present in the culture. A significant increase in TNF-α protein expression was observed between infected BEAS-2B cells without NK cells and those with NK cells (RSV A2, $p<0.001$ and RSV X, $p<0.001$) (Figure 3.20C). TNF-α protein expression was significantly higher following RSV X infection compared to RSV A2 ($p<0.001$). TNF-α protein was ~2-fold higher at ~25pg/ml with RSV X infection compared to RSV A2 infection at ~13pg/ml.

These results indicate that IFN-γ and TNF-α proteins are only expressed during NK cell co-culture with infected BEAS-2B cells and that RSV X, like RSV A2, was also able to induce their expression. Importantly, IFN-γ and TNF-α protein was significantly higher in culture supernatants during RSV X infection compared to RSV A2 infection.

3.4. Discussion

One of the first innate immune cell populations present in the lung to expand during RSV infection and be activated are NK cells. This suggests AECs, the site of RSV replication are important NK cell activators at least during the early stages of RSV infection. The main hypothesis tested in the experiments described in this chapter is that RSV infected AECs can alone activate NK cells through the expression of NK cell-activating cytokines and the IL-15Rα receptor. To test this hypothesis, the expression of NK cell activating cytokines was first measured in infected BEAS-2B cells. A main focus was on IL-15Rα receptor expression, which could indicate IL-15/IL-15Rα complex formation and thus an enhanced NK cell response. Next, a BEAS-2B cell-NK cell co-culture model was established to determine if infected BEAS-2B cells could alone initiate a cytokine response from NK cells. The nature of this interaction between NK cells and AECs was further examined using cell-to-cell contact inhibition to determine the importance of direct cell contact in initiating NK cell cytokine expression. The BEAS-2B cell response and then response of NK cells during co-culture with a clinical RSV isolate was also characterised and compared to the response obtained with RSV A2. Experiments here include the first use of an AEC-NK cell co-culture model to further understand RSV infection.

3.4.1. Expression of NK cell activating cytokines IL-12, IL-15 and IL-18 and the IL-15Rα receptor expression by BEAS-2B cells in response to RSV A2 infection

3.4.1.1. BEAS-2B cells do not express soluble IL-12p70 protein during RSV infection

Here, evidence of only a low expression of IL-12β mRNA was seen over a time period of 4-48hrs (Figure 3.2A). IL-12β mRNA expression was less than 0.1% of housekeeping gene L32 and unchanged by RSV infection. Further results showed no evidence of soluble IL-12p70 protein expression following RSV infection of BEAS-2B cells (Figure 3.2B). Other studies have also shown that BEAS-2B cells express very little, if at all any IL-12 subunits p35 or p40 in

response to other stimuli such as air-borne particulate matter (415,416). In conclusion, IL-12 expression by BEAS-2B cells is not influenced by RSV infection and BEAS-2B cells may not express IL-12 protein at all.

3.4.1.2. BEAS-2B cells express IL-18mRNA but not protein during RSV infection

Both non-infected and RSV infected BEAS-2B cells expressed IL-18 mRNA at levels ~10-30% of the L32 housekeeping gene (Figure 3.2C). RSV did not change IL-18 mRNA expression at any specific time point over a 4 to 48hr period. However, analysis of RSV infected cells showed a significant decrease in IL-18 mRNA expression at 24hr when compared to 4hr and 8hrs by ~2-fold (Figure 3.2C). Interestingly, this pattern of reduced IL-18 mRNA expression with RSV infection at a 48hr time point was also observed for infected HNAECS, specifically at lower MOIs of 0.1 and 1, but not for a higher MOI at 2.5 (Figure 4.4, Section 4.3.2). This is described and discussed in more detail in Chapter 4. Similarly to IL-12, IL-18 protein was not detected in culture supernatants either with or without infection (Figure 3.2D). This could be due to the detection limit of this assay, which was 11.7pg/ml (Table 2.4). RSV may fail to induce IL-18 protein expression from BEAS-2B cells above 11.7pg/ml and may reduce IL-18 mRNA expression. This cannot be concluded as mature IL-18 protein requires caspase-1 cleavage from a pro-IL-18 form (Section 1.3.3.2). BEAS-2B cells have been shown to lack expression of caspase-1 and this could be a possibly explain why IL-18 secretion was not observed here (417). However caspase-1 presence was not been examined here for BEAS-2B cells but could have offered further explanation of these results.

3.4.1.3. BEAS-2B cells express low levels of soluble IL-15 during RSV infection

IL-15 mRNA level was only shown to be significantly increased at 8hr with RSV infection by ~1-2% of the housekeeping gene level. This suggests that RSV infection does not extensively alter IL-15 mRNA expression in BEAS-2B cells at the MOI of 1 used here. Experiments using different RSV MOIs at 24 and 48hrs could have provided further insight on the ability of RSV

to modulate IL-15 mRNA expression in BEAS-2B cells. IL-15 protein was not present in culture supernatants from control BEAS-2B cells and only found above the lower limit of detection following RSV infection for 48hrs (Figure 3.3B). Furthermore, Palivizumab inhibited expression indicating replicating RSV induced IL-15 secretion and not other factors in the viral lysate.

IL-15 transcription and translation has been described as tightly regulated and could account for the relatively low soluble protein detected. For instance, the 5' noncoding region of IL-15 mRNA can prevent its translation and co-ordinated expression of IL-15Rα is essential for IL-15 protein to leave the Golgi apparatus (342,418). Another possibility is RSV NS1 and NS2 proteins potentially inhibiting IL-15 secretion. As described in the next section, the low levels of secreted IL-15 observed could also be due to complex formation, which provides a method of controlled and local use of IL-15 activity. This tight control may be especially beneficial for the prevention of over stimulation of IL-15 responsive immune cells. Low levels of secreted IL-15 could be beneficial so as to only act locally. For instance, mice overexpressing IL-15 have elevated NK cell and memory T cell throughout the body and was later accompanied by weight loss, respiratory distress, leukaemia and premature death (419).

The data described here show some similarities and some minor differences to that of other *in vitro* RSV-AEC studies. IL-15 expression in BEAS-2B cells was first examined by Ge *et al.* with reported resting BEAS-2B cells IL-15 protein expression being less than 1pg/ml (341). BEAS-2B cells have similarly shown expression of IL-15 mRNA under resting conditions, as well as A549 cells (420). IL-15 expression during *in vitro* RSV infection in airway cell lines A549s, BEAS-2B cells and commercially bought HBECs was first described by Zdrenghea *et al*. (152). Data here shows that IL-15 mRNA expression in BEAS-2B cells remained the same over a 48hr period and that IL-15 protein was not detected from control BEAS-2B cells and that RSV induced ~18pg/ml protein expression at 48hr and MOI of 1. Zdrenghea *et al.* show that

1) for A549s IL-15 mRNA decreased over a 72hr period, 2) non-infected BEAS-2B cells expressed ~1pg/ml IL-15 protein and 3) for infected BEAS-2B cells, only ~7pg/ml was detected at a 48hr and at an MOI 1. The ELISA used by Zdrenghea *et al.* had a lower limit of detection than the Luminex used here which could explain why no protein was detected in control supernatants. This study also showed that following RSV infection A549s expressed more IL-15 protein than BEAS-2B cells. This could suggest that different types of AEC lines or primary AECs, even from different airway locations, could have different IL-15 responses to RSV and thus differentially influence immune cell responses.

3.4.1.4. BEAS-2B cells express IL-15Rα during RSV infection

The next aim was to determine if IL-15/IL-15Rα complexes are formed following RSV A2 infection of BEAS-2B cell. BEAS-2B cell IL-15Rα expression has not been characterised during RSV infection. Here, BEAS-2B cells expressed IL-15Rα mRNA which was ~1-5% of housekeeping gene and did not increase over time suggesting an unchanging basal level of IL-15Rα mRNA expression in the absence of infection (Figure 3.4A). RSV greatly increased IL-15Rα mRNA expression. This was observed early in infection at 8hr and maintained up to 48hrs at around ~4-10-fold higher. IL-15Rα surface expression was then examined at 48hrs infection using increasing RSV MOIs to determine if viral titre effected IL-15Rα expression. Control BEAS-2B cells did not express cell surface IL-15Rα (Figure 3.4B, C). RSV, at an MOI of 0.1 to 2.5, induced an increase in cell surface expression of IL-15Rα interestingly showing a dose-dependent increase in expression with increase in MOI (Figure 3.4C). This is not the first account of BEAS-2B cells expressing IL-15Rα mRNA under resting conditions and RSV has previously been shown to increase IL-15Rα mRNA expression in A549s (341,420,421). However in these studies surface expression of IL-15Rα protein was not examined during RSV infection. The data presented here is the first report of RSV to inducing an increase in IL-15Rα both at gene and protein level in BEAS-2B cells. RV has also been shown to induce IL-

15Rα mRNA expression in the lungs of mice and may be a common airway epithelial response to viral infection (422).

In conclusion RSV induces IL-15Rα mRNA expression, seen early after 8hrs infection, and induces IL-15Rα protein cell surface expression which may also be dependent on viral titre. Described in the section below are reasons why a dose-dependent increase in IL-15Rα expression may be beneficial.

3.4.2. Do BEAS-2B cells express IL-15/IL-15Rα complex during RSV infection?

IL-15Rα and IL-15 protein were separately shown to be expressed on the cell surface of RSV infected BEAS-2B cells (Figure 3.4 and 3.5). Therefore, there is a possibility that IL-15 is complexed to IL-15Rα and present at the cell surface. To investigate this, immunofluorescence staining was done to examine both intracellular and cell surface expression of IL-15 and IL-15Rα. Dual immunofluorescence staining of IL-15 and IL-15Rα showed positive staining on infected BEAS-2B cells at the cell surface (Figure 3.6). A very small amount of surface IL-15 and IL-15Rα were observed on non-infected BEAS-2B cells. Intracellular individual staining for IL-15 and IL-15Rα was then performed. Both non-infected and RSV infected BEAS-2B cells expressed IL-15 (Figure 3.7) and IL-15Rα (Figure 3.8) which were both located in the Golgi apparatus as evidenced by co-localisation with GM130. Expression of IL-15 by non-infected BEAS-2B cells was expected as IL-15 translation is complex and tightly regulated with detection and expression of IL-15 mRNA not correlating with IL-15 protein secretion (330,332). Finally, soluble IL-15/IL-15Rα complex was only observed in culture supernatants from RSV infected BEAS-2B cells (Figure 5.15, Section 5.3.5.2). However, this needs further study as not all supernatants across experiments expressed IL-15/IL-15Rα complex.

Being able to increase IL-15/IL-15Rα complex at the cell surface on AECs based on viral titre could be of a great benefit during RSV infection. As described above, IL-15 expression is under tight regulation which could be of great benefit to restrict its action locally and reduce excessive immune responses. Therefore with greater viral titres, this could require a greater and or faster immune response. One way to achieve this could be through a rheostat-like expression of immune cell modulators. For instance during the end stages of disease when the virus titre is lower, reducing the expression of IL-15Rα and thus IL-15 signalling, could reduce excessive NK cell and T cell responses. This also allows for NK cells only present locally to be stimulated.

3.4.3. Following RSV infection, can BEAS-2B cells activate NK cells?

The next aim was to establish a BEAS-2B cell-NK cell co-culture model measuring IFN-γ and TNF-α as indicators of NK cell activation. So far, it has been determined that RSV infected BEAS-2B cells do not express NK cell-activating cytokines IL-12 and IL-18 but do express soluble and cell surface IL-15 with the assumption that this is through an IL-15/IL-15Rα complex, a potent inducer of NK cells. The absence of IL-12 and IL-18 in this model could mean activation is dependent on the ability of IL-15 and the IL-15/IL-15Rα complex to alone induce NK cell cytokine responses. These assays may therefore also provide information of the activity of these molecules. However it should be noted that other NK cell activating cytokines, such as IL-2 and IL-21, or NK cell inhibitory cytokines, such as IL-18BP, IL-10 and TGF-β, have not been measured here and could also be active within these experiments. Nevertheless, it is still a valid question to ask if BEAS-2B cells alone can activate NK cells during RSV infection.

3.4.3.1. Does co-culture of NK cells with RSV infected BEAS-2B cells influence viral replication and IL-8 response?

Figure 3.9 shows that NK cells after addition to infected BEAS-2B cells settle above the cell monolayer by 4hr after addition to culture media. This would allow for cell-to-cell contact and potential stimulation of NK cells via cell surface expression of IL-15 on BEAS-2B cells. Settling of NK cells is likely to be due to the expression of RSV-induced chemokines expressed by infected BEAS-2B cells, as detailed in Section 5.3.1.

Within this co-culture model it was observed that expression of RSV N gene within individual experiments decreased. When data was expressed as a ratio of infected BEAS-2B cells without NK cells, BEAS-2B cell-NK cell co-culture resulted in a significant decrease in RSV N gene compared to levels in infected BEAS-2B cells without NK cells (Figure 3.11B). A further, but minor decrease in RSV N gene expression was also seen between NK cells co-cultured at a higher ratio of 1:3 compared to 1:1. This is similar to results observed in Section 4.4.4.1 and is discussed further in that chapter's discussion. Here briefly, this could be due to added NK cell derived L32 reducing the apparent RSV N gene expression or through NK cell-mediated lysis of infected cells.

3.4.3.2. IFN-γ and TNF-α are expressed during co-culture of BEAS-2B cells and NK cells.

Controls were set up to 1) determine if IFN-γ and/or TNF-α are expressed by BEAS-2B cells and if RSV influence their expression, 2) to characterise expression of IFN-γ and TNF-α from NK cells cultured alone or in the presence of IL-15 and 3) to determine if non-infected BEAS-2B cells induced NK cell derived IFN-γ and/or TNF-α expression during co-culture.

First to be answered was the question, are IFN-γ and TNF-α expressed by BEAS-2B cells? Only TNF-α mRNA was observed in non-infected BEAS-2B cells without NK cells at less than 0.02% of the housekeeping gene expression (Figure 3.11). TNF-α mRNA was increased by RSV infection to a level equal to ~0.8% of the housekeeping gene. IFN-γ mRNA was not expressed

by BEAS-2B cells and this indicates that within this co-culture, IFN-γ protein was unlikely to be expressed by BEAS-2B cells. Indeed, IFN-γ protein was not found in any BEAS-2B cell culture supernatants with or without RSV infection whereas TNF-α protein was from RSV infected BEAS-2B cells (Figure 3.11). Other studies have reported BEAS-2B cell-specific TNF-α expression under resting conditions and increased by different stimulus, such as lipoteichoic acid, LPS via TLR4 signalling, dust extract, RSV and dsRNA (423–427). Therefore within this co-culture model, TNF-α cannot be described as NK cell specific whereas IFN-γ protein was likely to be NK cell specific.

The second control used was NK cells cultured alone. This was included to determine basal IFN-γ and TNF-α expression specific to NK cells and their expression with IL-15 stimulation. BEAS-2B cell-derived L32 mRNA will be included in the calculations for NK-cell derived IFN-γ mRNA expression and will not represent NK cell-specific values. NK cells cultured in media only did not express detectable IFN-γ or TNF-α mRNA and following IL-15 stimulation where expressed at ~400% and ~1700%, respectively (Figure 3.11). Interestingly, even though IL-15 induced IFN-γ and TNF-α mRNA expression by NK cells, TNF-α protein was only detected at ~4pg/ml in supernatants from NK cells cultured alone (Figure 3.11B, D). IL-15 treatment may preferentially induce TNF-α protein secretion and not IFN-γ, although this amount is relatively low.

The final control used was expression of IFN-γ and TNF-α during NK cell co-culture with non-infected BEAS-2B cells. Here IFN-γ and TNF-α mRNA were detected at less than 0.1% and 0.4% housekeeping gene, respectively (Figure 3.12A, C). No IFN-γ or TNF-α protein was found in culture supernatants when NK cells were co-cultured with non-infected BEAS-2B cells (Figure 3.12B, D). Overall, the controls mentioned above suggest that IFN-γ protein was specific to NK cells whereas TNF-α mRNA and protein could be made by both BEAS-2B cells

and NK cells. This should be considered when evaluating any apparent increases in TNF-α expression with co-culture.

The most important outcome of this assay was the ability of RSV infected BEAS-2B cells to induce NK cell cytokines, IFN-γ and TNF-α. This is best considered in the context of protein expression, rather than gene expression, as these could exert a biological effect during RSV infection. Here, a dose-dependent increase in IFN-γ mRNA followed an increase in the number of NK cell co-cultured with infected BEAS-2B cells (Figure 3.11A). This dose-dependent increase was also observed for IFN-γ protein, with the amount reaching ~23pg/ml at the highest co-culture ratio (Figure 3.11B). Furthermore IFN-γ protein was only detected from cultures with both RSV infected BEAS-2B cells and NK cells (Figure 3.11B). IFN-γ protein was also ~2.5 fold greater than TNF-α protein at the highest co-culture ratio. No IFN-γ protein was detected in culture supernatants from NK cells stimulated with IL-15, only IFN-γ mRNA. This suggests that infected BEAS-2B cell-NK cell cultures result in increased expression of actual IFN-γ protein production whereas the IL-15 stimulation does not induce its secretion to the same level. Possibly a post transcriptional regulatory mechanism is active during co-culture. For TNF-α protein, as mentioned above, ~4pg/ml was seen in supernatants from NK cells stimulated with IL-15 and infected BEAS-2B cells (Figure 3.11D). TNF-α protein also followed a dose-dependent increase reaching ~9pg/ml at the highest co-culture ratio.

Here the anti-RSV controls showed interesting results. For the anti-RSV control, addition of Palivizumab, NK cells were co-cultured at the highest ratio of 1:3 and these results compared to RSV infected BEAS-2B cells at a ratio of 1:3. As shown in Figure 3.11A Palivizumab successfully inhibited RSV N gene expression. Both IFN-γ and TNF-α mRNA were present in these anti-RSV control cultures. Both were significantly reduced compared to RSV infected BEAS-2B cells at a ratio of 1:3 ratio (Figure 3.11A, C). This suggests that the responses are specific to RSV infection. For IFN-γ, this was confirmed by lack of protein in anti-RSV control

supernatants (Figure 3.11B). However TNF-α protein was also present in the anti-RSV control supernatants and at the same concentration as RSV infected BEAS-2B cells at a ratio of 1:3 ratio (Figure 3.11D). This suggest that something in the viral lysate or Palivizumab solution stimulates TNF-α protein but does not induce expression of soluble IFN-γ.

Intracellular NK cell expression of IFN-γ was also examined. Figure 3.14 complements the data obtained in Figure 3.11A. For culture of NK cells only, stimulation with IL-15 enhanced IFN-γ expression resulting in ~10% of cells being IFN-γ positive, compared to ~1% for NK cells cultured in media only (Figure 3.14). NK cells co-cultured with non-infected BEAS-2B cells showed significantly more IFN-γ positive NK cells at around ~5% than NK cells cultured in media alone. This suggests some basal stimulation of NK cells by BEAS-2B cells under a non-infectious environment which does not lead to IFN-γ protein secretion (Figure 3.14). This could possibly be through expression of more inhibitory rather than activating NK cell receptors by BEAS-2B cells. Furthermore, this could be beneficial for rapid NK cell activation and cytokine expression during RSV infection if NK cells already express some intracellular IFN-γ protein. Following RSV infection of BEAS-2B cells, as illustrated in Figure 3.14, the percentage of IFN-γ positive NK cells was significantly increased to ~15% compared to NK cells co-cultured with non-infected BEAS-2B cells. This was comparable to and not significantly different from the number of IFN-γ positive NK cells present when NK cells were cultured alone and stimulated with 10ng/ml IL-15. This indicates that IL-15 alone was able to induce approximately the same amount of intracellular IFN-γ protein in NK cells as when co-cultured with infected BEAS-2B cells.

As shown in Figure 3.11B, IFN-γ was not detected in culture supernatants from NK cells stimulated with IL-15, only during co-culture with infected BEAS-2B cells. BEAS-2B cells in these co-cultures may express ~17.9 pg/ml IL-15, as shown in Figure 3.3, during RSV infection. This is ~550-fold less than the 10ng/ml IL-15 added to NK cells alone, yet no soluble

IFN-γ was detected. Therefore, NK cells require direct cell-to-cell contact with BEAS-2B cells, or other stimulus within the co-culture, for IFN-γ secretion. This is an important observation as it details how NK cell responses *in vivo* during RSV infection could be greatly determined by the airway epithelium and close proximity.

This co-culture model was the first of its kind and highlights how RSV infected BEAS-2B cells can activate NK cells. More specifically, this co-culture model shows that NK cells express IFN-γ protein only when co-cultured with RSV infected BEAS-2B cells and this followed a dose-dependent increase with increase in NK cells. As TNF-α production by RSV infected BEAS-2B cells was observed, it cannot be said that TNF-α protein was NK cell specific, but could show an additive value from both from BEAS-2B cells and NK cells during co-culture. This co-culture model also highlights a possibility that NK cells may require direct cell-to-cell contact during RSV infection for full activation.

3.4.3.3. Direct cell contact is essential for NK cell cytokine expression during RSV infection of BEAS-2B cells.

Results obtained using the BEAS-2B cell-NK cell co-culture model shown in Figure 3.11 and discussed above led to the next aim which was to determine if BEAS-2B cell-NK cell contact is required for optimum NK cell activation. This followed from the key observation that although IL-15 induced IFN-γ and TNF-α mRNA expression by NK cells, no protein was detected in culture supernatants and also that NK cells may require stimulation of their activating receptors for optimum responses.

To test this, NK cells were separated from BEAS-2B cells by use of transwell inserts. As expected, IFN-γ and TNF-α protein was only observed in culture supernatants when NK cell were in direct contact with RSV infected BEAS-2B cells (Figure 3.12). Results also demonstrate the observation that NK cells do not express IFN-γ and TNF-α protein when cultured in supernatant from infected BEAS-2B cells. The transwell results show ~2-fold

higher expression of IFN-γ protein than the direct co-culture results shown in Figure 3.11B. This could be due to a higher total number of NK cells used in the 24-well plate transwell compared to 96-well plates. However this was not observed for TNF-α protein with similar expression at ~9-12pg/ml. This could indicate that the TNF-α response was restricted in some way, such as by the ratio of BEAS-2B cells-NK cells or that the TNF-α response was not as prominent as IFN-γ during RSV infection. IFN-γ expression could be associated with total number of NK cells. Therefore the more NK cells present during RSV infection, the more IFN-γ protein may be expressed *in vivo*.

During the NK cell cytotoxicity assay, performed by Liz Van Erp, intracellular NK cell IFN-γ expression was also measured (Figure 3.17). Both of the two donor's NK cells cultured in supernatants taken from infected BEAS-2B cells showed a significant increase in the percentage of IFN-γ positive NK cells (Figure 3.17A-C). Results for donor 1 using 48 hour supernatant, showed a dose-dependent and significant increase in the percentage of IFN-γ positive NK cells with increase in RSV MOI (Figure 3.17B). This was also observed for donor 2 but significance was not reached (Figure 3.17D). The 24hr culture supernatant induced an increase in IFN-γ positive NK cells by ~2-fold for both donors and an increase in the MOI did cause a dose-dependent increase, as shown for 48hr supernatant (Figure 3.17A, C). Overall, the percentage of IFN-γ positive NK cells reached no more than ~4%, which was lower than ~15% positive observed during direct infected BEAS-2B cell-NK cell contact (Figure 3.14). The data shown in Figure 3.17 may therefore also reflect the requirement of NK cells to be in direct contact with infected BEAS-2B cells for optimal activation.

In conclusion, data shown in Figures 3.11, 3.12, 3.14 and 3.17 indicate that NK cells require direct cell contact with infected BEAS-2B cells for NK cell-derived IFN-γ protein expression. TNF-α expression within these assays may be both from BEAS-2B cells and NK cells. The IFN-

γ response from NK cells may be stronger than the TNF-α response during RSV infection *in vivo*.

3.4.4. BEAS-2B culture supernatants do not induce NK cell cytotoxicity

Finally, the potential for BEAS-2B cell culture supernatants to induce NK cell cytotoxicity during NK cell-K562 effector cell co-culture was also examined by Liz Van Erp. From the previous data, it was apparent that NK cells require direct contact with AECs for optimum IFN-γ protein expression. However, this may not be true for the cytotoxicity assay in which NK cells are cultured with effector cells providing a form of direct cell contact.

Figure 3.16 shows the intracellular expression of perforin in NK cells from two separate donors. In conclusion, the percentage of perforin positive NK cells was not increased when NK cells were cultured in BEAS-2B cell supernatants from non-infected or infected cells (Figure 3.16A-D). The lack of perforin positive NK cells cultured in infected BEAS-2B supernatants could be because the NK cells were not in direct contact with BEAS-2B cells. Additionally, IL-15 alone may not be able to induce perforin expression by NK cells as shown from the IL-15 stimulated NK cells (Figure 3.16E) whereas IL-15 was able to induce IFN-γ expression (Figure 3.17E). The addition of IL-15 to NK cell-K562 cell co-culture was not able to induce intracellular perforin expression which showed the same percentage of perforin positive NK cells as those cultured in media only (Figure 3.16E). In other studies, isolated NK cells cultured with 10ng/ml IL-15 were shown to induce targeted cell-lysis by ~20%, however these results suggest targeted cell-lysis may not occur here (361,428). In conclusion, BEAS-2B cell supernatants were not able to induce NK cell intracellular perforin expression, however it cannot conclude that no NK cell lysis was done by NK cells during BEAS-2B cell co-culture. This could have been examined by granzyme B expression in culture supernatants by ELISA.

3.4.5. RSV X infects and replicates in BEAS-2B cells and induces a similar expression of IL-8 as RSV A2

An MOI of 2.5 was used for both RSV A2 and RSV X, notably RSV RNA expression was ~56-fold higher during RSV X infection compared to RSV A2 (Figure 3.18A). RSV X clinical strain may have higher immune evasion strategies compared to RSV A2, such as reducing RNase L expression, detailed in Section 1.2.1. However IL-8 mRNA and protein expression were similar and not significantly different between the two strains (Figure 3.18B, C). This suggests that although the RSV X RNA expression was greater than that in RSV A2 cultures, expression of IL-8 was not enhanced by the higher viral titre in RSV X cultures. IL-8 expression may have reached saturation for RSV X and possibly this is why there was no observed difference between strains. Viral growth kinetics may be different between strains. Use of different MOIs and expression over time would have provided more information this. Overall, this was a very limited experiment design with only one MOI and one time point used but as discussed below allows an interesting comparison between these two strains.

3.4.6. Comparison between the expression of IL-15 and IL-15Rα by BEAS-2B cells in response to infection with two different RSV A strains, A2 lab strain and clinical isolate RSV X

Expression of IL-15 mRNA was not significantly different between RSV A2 and RSV X (Figure 3.19A) and IL-15Rα mRNA was significantly more with RSV X infection compared to RSV A2 (Figure 3.19B). As no protein analysis was done, such as cell surface expression by flow cytometry, it is hard to make a final comparison between RSV A2 and RSV X as protein and not mRNA influence immune responses.

Figure 3.3A shows that at an MOI of 1 for 48hr infection, RSV A2 did not induce a significant difference in BEAS-2B cell IL-15 mRNA expression, with IL-15 mRNA for both being ~3-5% of the housekeeping gene. In comparison, the results in Figure 3.21A show that RSV A2 at an

MOI of 2.5 for 48hr infection significantly induced IL-15 mRNA expression to ~13% housekeeping gene compared to non-infected control. Therefore, the expression of IL-15 mRNA may be dependent on viral RSV A2 titre, with more virus induced expression in comparison to changes in mRNA over time at the same MOI. An RSV dose-response at 48hr would need to be completed to confirm this.

There was also a difference in expression of IL-15Rα mRNA between MOI 1 and 2.5 with 48hr RSV A2 infection. In Figure 3.4A 48hr RSV A2 infection at an MOI of 1 induced expression of IL-15Rα mRNA to ~40% housekeeping gene compared to Figure 3.21B in which IL-15Rα mRNA expression was induced to ~100% housekeeping gene, L32. This suggests that increase in MOI may induce an increase in IL-15Rα mRNA expression. A RSV A2 and RSV X dose-response experiment would verify this.

Overall, an increase in IL-15Rα mRNA by RSV X could have influenced the expression cell surface IL-15Rα and possibly also IL-15/IL-15Rα complex formation and trans-presentation. If this is so, RSV X clinical isolate infection of BEAS-2B cells may be able to further activate NK cells during co-culture.

3.4.6.1. Is the expression of IFN-γ and TNF-α protein during NK cell co-culture of RSV infected BEAS-2B cells different between laboratory strain A2 and clinical isolate, X?

As discussed above, with RSV A2 and RSV X infection during BEAS-2B cell-NK cell co-culture, IFN-γ was only detected with infection and NK cell co-culture. Therefore, providing further confirmation that IFN-γ detected was NK cell derived. RSV X induced ~2-fold more IFN-γ and TNF-α protein than RSV A2 infection of BEAS-2B cells from NK cells during co-culture. This may be reflect the increase in IL-15Rα mRNA expression by BEAS-2B cells infected with RSV-X illustrated in Figure 3.19B. Potentially, the more IL-15Rα mRNA expressed, the increased likelihood of further IL-15 trans-presentation to NK cells.

3.4.7. Limitations of an *in vitro* BEAS-2B cell-NK cell co-culture model compared to *in vivo* AEC-NK cell responses during RSV infection

The exact role of NK cells has not been fully characterised during human RSV infection. A protective role of NK cells can be seen in IL-15 deficient mice that lack NK cell population expansion and on challenged with virus, show more fatalities then wild type mice (Section 1.3.3.3). However NK cells have also been implicated in enhancing RSV disease (Section 1.4). Here, the expression of IFN-γ and TNF-α was characterised using a RSV infected BEAS-2B cell-NK cell co-culture, the first co-culture of its kind. This model allowed the interactions between infected AECs and NK cells alone, to be studied without other immune cell influences. Furthermore, this model details that expression of IL-15 and IL-15Rα during RSV infection can induce NK cell activation. Although as mentioned previously, the whole NK cell activating cytokine repertoire has not been characterised here. The dependence of NK cells on BEAS-2B cell-derived IL-15 and IL-15Rα during RSV infection could have been examined by siRNA knock-down of BEAS-2B cell IL-15 and/or IL-15Rα mRNA. This was tested, but it proved difficult using IL-15 and IL-15Rα siRNA which inherently have a complex regulation of translation. Treatment of BEAS-2B cells with these siRNAs resulted in more IL-15 and IL-15Rα mRNA expression by BEAS-2B cells (data not shown). Addition of anti-IL-15 and anti-IL-15Rα to infected BEAS-2B cell-NK cell co-cultures would not be a valid option either as these antibodies may stimulate NK cells.

This model also has some limiting factors including the lack of IL-12 and IL-18 protein which have been observed in lung during RSV infection. This would otherwise change the NK cell response. Additionally it is not a whole lung model. It includes the use of an AEC line, BEAS-2B, a model lacking other immune cells or other cellular sources of inflammatory mediators. A whole lung model would ultimately determine the NK cell response, however under

experimental conditions, this can only been studied in mice and equally is not representative of a full human response. The model used here is less complex but allows a clear dissection of the response and BEAS-2B cell-NK cell interactions which is not possible *in vivo*.

A limitation that can be considered for all NK cell activating cytokines examined here is that only apical secretion was determined. These proteins may undergo basolateral secretion from infected BEAS-2B cells. A more thorough evaluation of apical and basolateral secretion by BEAS-2B cells would have been achieved by infecting BEAS-2B cells grown on transwell inserts. This would allow for detection of basolateral secretion, supernatants used from the bottom chamber and apical secretion, supernatants used from the upper chamber, concurrently. This may reflect why IL-12 and/or IL-18 and requires further investigation.

Further limitations of this model are discussed in Chapter 7. A possibility to improve this model is use by using primary AECs. To improve this model, Chapter 4 extends these results with use of HNAECs and donor matched NK cells.

3.5. Summary

RSV infection of BEAS-2B cells induces expression of both IL-15 and IL-15Rα mRNA and protein, but not IL-12 and IL-18 protein. IL-15 protein was detected at relatively low levels in culture supernatants and only at 48hrs infection. IL-15 and IL-15Rα were expressed intracellularly by both non-infected and infected BEAS-2B cells. However, co-expression of cell surface expression of IL-15 and IL-15Rα was observed only following infection which suggests IL-15/IL-15Rα complex trans-presentation. Cell surface expression of IL-15Rα also followed a dose-dependent increase with increase in RSV MOI, suggesting the possibility for a fine regulation of IL-15-responsive immune cells. It was then shown that RSV infected BEAS-2B cells could stimulate IFN-γ expression from NK cells over a 48hr period. TNF-α was also expressed within this co-culture model but it was not specific to NK cells. This BEAS-2B cell-NK cell co-culture model lacked IL-12 and IL-18 protein and therefore IFN-γ expression by NK

cells could possibly be through stimulation by the more potent IL-15/IL-15Rα complex. This could be explained by the inhibition of IFN-γ protein expression observed when direct BEAS-2B cell-NK cell contact was prevented. Figure 3.21 details a summary of the results observed within this chapter. RSV induces the expression of cell surface IL-15/IL-15Rα complex and may be presented in trans to NK cells. This could then aid in NK cell activation and expression of IFN-γ and possibly TNF-α protein. Soluble IL-15 may also activate NK cells via cis-presentation. Overall, these results demonstrate that the RSV infected airway epithelium can alone initiate an IFN-γ, and possibly a TNF-α, response from NK cells which is dependent on direct cell-to-cell contact.

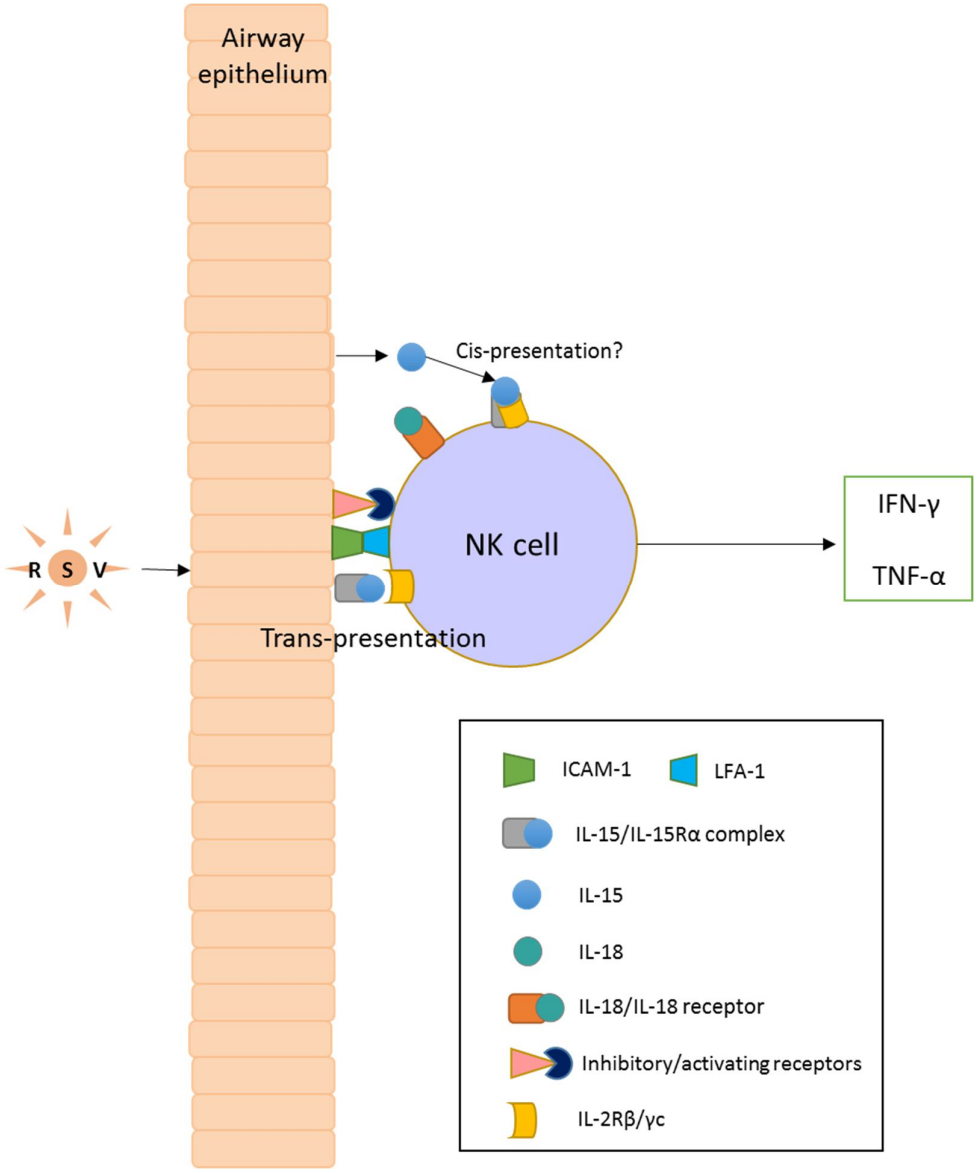


Figure 3.21. Direct contact between RSV infected BEAS-2B cells and NK cells is essential for NK cell activation. RSV infected BEAS-2B cells express cell surface IL-15/IL-15Rα complex which through trans-presentation, and through other cellular interactions and secreted molecules, stimulate NK cells to express IFN-γ and TNF-α

Chapter 4. *In vitro* NK cell activating-cytokine profile of RSV A2 infected human nasal airway cells, human nasal airway cell-NK cell co-culture and infant nasopharyngeal aspirate analysis

4.1. Introduction

The experiments described in Chapter 3 demonstrate that after RSV infection the airway epithelial cell line BEAS-2B express the NK cell activating cytokine IL-15, the IL-15Rα protein and additionally the more potent NK cell activating IL-15/IL-15Rα complex. A BEAS-2B cell-NK cell co-culture model was established in which NK cell-derived IFN-γ protein was expressed only during direct NK cell contact with RSV infected BEAS-2B cells and TNF-α was produced by both BEAS-2B cells and NK cells. Limitations of the BEAS-2B cell model include a potential tissue miss match between donor NK cells and the BEAS-2B cells, which could greatly influence the NK cell response (Section 1.3.2). Use of primary AECs would add further *in vivo* relevance to these specific studies of AEC-NK cell interactions. Therefore to extend the studies described in Chapter 3 indicating AECs, after infection, can activate NK cells by expression of NK cell activating cytokines and IL-15Rα receptor, HNAECs were cultured and the AEC-NK cell model modified to use HNAEC and NK cells from the same donor.

In these experiments the kinetics of NK cell activating cytokines expression, including IL-12, IL-15 and IL-18, and IL-15Rα was first characterised during RSV infection of HNAECs. Expression of IL-15 mRNA and protein by HBECs and human airway basal cells has already been reported (152), but IL-12p70, IL-18, IL-15Rα and IL-15/IL-15Rα complex expression following RSV infection has not been investigated. The work described in Chapter 3 shows the clinical isolate RSV X can induced significantly more IL-15Rα mRNA expression in BEAS-2B cells compared to RSV A2. Therefore a comparison between the expression of IL-15 and IL-15Rα by HNAECs during infection with RSV A2 and clinical isolate RSV X was also made,

especially considering a clinical isolate may more closely match *in vivo* infection compared to RSV A2.

Second, HNAECs and donor matched NK cells were co-cultured and IFN-γ and TNF-α expression measured as potential indicators of NK cell activation. Characterising the NK cell response during a co-culture model with donor matched NK cells will provide a more accurate representation of the *in vivo* response than BEAS-2B cells.

Finally, NPAs from infants under the age of 1 year naturally infected with RSV were analysed and expression of NK cell activating cytokines IL-15, IL-15/IL-15Rα complex and IL-18 and the NK cell-derived cytokines IFN-γ and TNF-α measured. Here, cytokine expression was compared to patient oxygen requirement as a disease severity score and age group. Furthermore, the levels of IL-15, IL-15/IL-15Rα complex and IL-18 in NPAs from infants infected with another common respiratory virus, RV, allow for comparison to the results obtained for RSV.

4.2. Aims

- To determine the kinetics of IL-12, IL-15 and IL-18 NK cell activating cytokines and cytokine receptor IL-15Rα expression, including the IL-15/IL-15Rα complex, by RSV A2 infected HNAECs (Sections 4.3.1, 4.3.2, 4.3.3, 4.3.4 and 4.3.5).
- To compare the expression of IL-15 and IL-15Rα by HNAECs in response to infection with two different RSV A strains, A2 lab strain and clinical isolate X (Section 4.3.6).
- To establish if HNAECs can activate NK cells in a HNAEC-NK cell co-culture model, measuring IFN-γ and TNF-α as indicators of NK cell activation (Section 4.3.7).

- To characterise expression of IL-15, IL-15/IL-15Rα complex, IL-18, IFN-γ and TNF-α in NPAs from RSV infected infants and IL-15, IL-15/IL-15Rα complex and IL-18 in NPAs from RV infected children (Sections 4.3.8 and 4.3.9).
- To determine if the IL-15, IL-15/IL-15Rα and IL-18 protein levels in NPAs from infants infected with RSV and RV differ (Section 4.3.10).

4.3. Results

To assess the NK cell activating cytokine profile of human primary AECs, HNAECs were isolated and grown from healthy adult donors (Section 2.2). HNAECs were infected with RSV A2 at MOIs of 0.1, 1 and 2.5 for 48hr. Palivizumab was used with RSV at an MOI 2.5 as an anti-RSV control. To reduce variation that could occur between different viral lysate preparations the same batch of RSV was used for all experiments. Here, unless stated all data is presented as the fold change to compensate for inter-individual differences in base-line gene expression and individual donor responses to RSV infection.

4.3.1. RSV replicates in HNAECs leading to IL-8 expression

To confirm infection, RSV N gene expression and IL-8 gene expression was measured by qPCR and IL-8 protein by ELISA (Section 2.6.3 and 2.7).

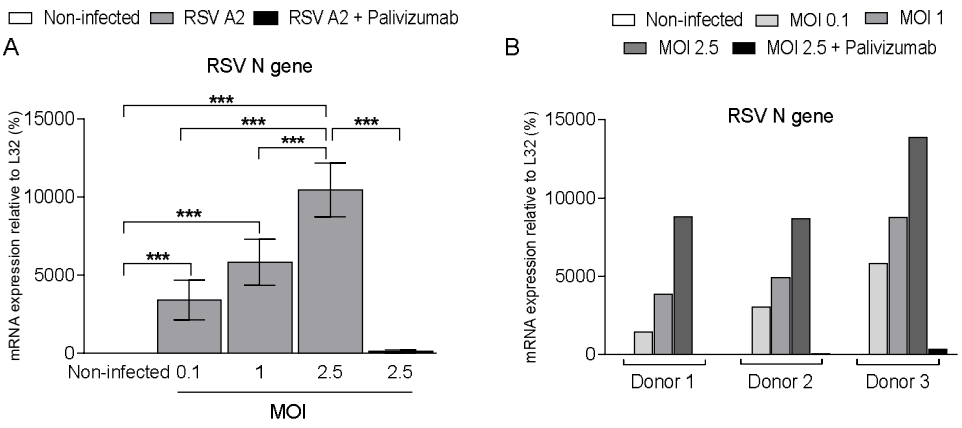


Figure 4.1. Expression of RSV N gene in RSV A2 infected HNAECs. HNAECs were infected with RSV A2 at MOIs of 0.1, 1 and 2.5 for 48hr. Control non-infected cultures were cultured in the absence of RSV and Palivizumab used as an anti-RSV control. A) RSV N gene (n=3), B) individual donor expression of RSV N gene (n=1 per donor). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, ***p<0.001).

RSV N RNA was not detected in the absence of RSV infection and was significantly increased at MOIs 0.1 ($p<0.001$), 1 ($p<0.001$) and 2.5 ($p<0.001$) (Figure 4.1A) compared to non-infected HNAECs. A significant increase in RSV N RNA was seen between MOI 2.5 compared to MOIs 0.5 ($p<0.001$) and 1 ($p<0.001$). Palivizumab anti-RSV control significantly decreased expression of RSV N RNA between MOI 2.5 and MOI 2.5 plus Palivizumab ($p<0.001$). RSV N gene expression in anti-RSV control was less than 0.01% L32.

Individual donor expression of RSV N RNA is shown in Figure 4.1B, with a dose-dependent increase in mRNA expression that reflected the increasing MOIs. Donor 3 had ~0.3-fold higher expression than donors 1 and 2 (Figure 4.1B).

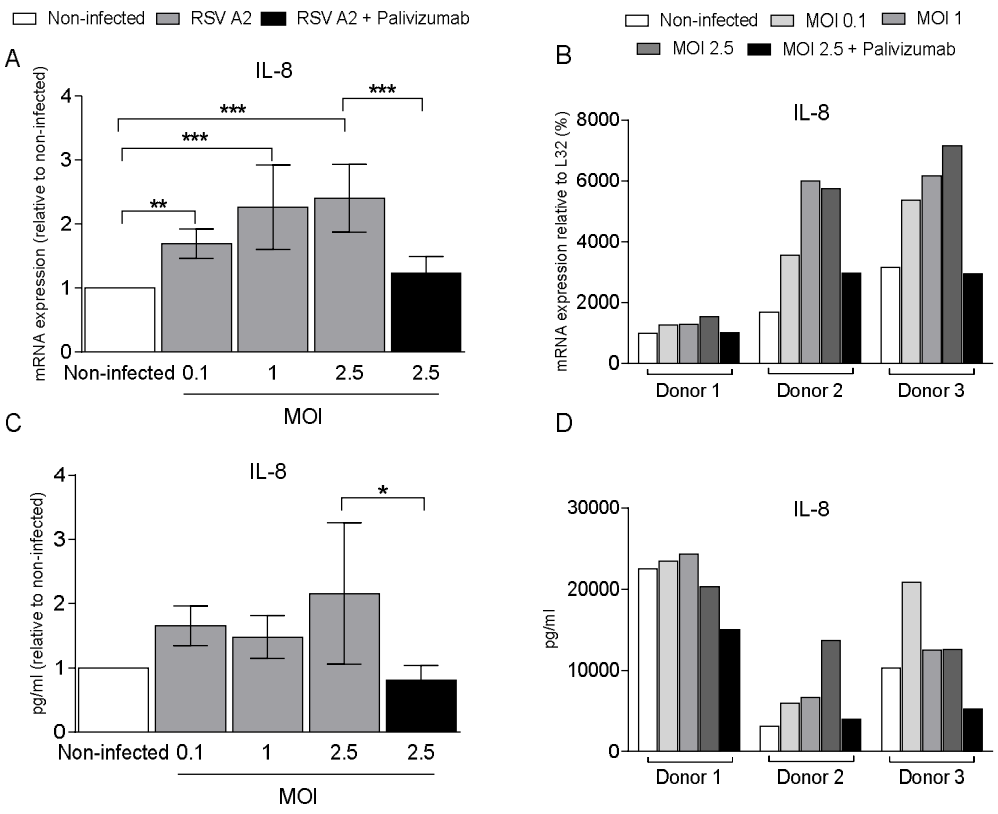


Figure 4.2. Expression of IL-8 mRNA and protein in RSV A2 infected HNAECs. HNAECs were infected with RSV A2 at MOIs of 0.1, 1 and 2.5 for 48hr. Control non-infected cells were cultured in the absence of RSV and Palivizumab used as an anti-RSV control. A) IL-8 mRNA (n=3), B) individual donor expression of IL-8 mRNA, C) IL-8 protein (n=3), D) individual donor expression of IL-8 protein. Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01, ***p<0.001).

IL-8 mRNA expression was significantly increased at MOIs 0.1 ($p<0.01$), 1 ($p<0.001$) and 2.5 ($p<0.001$) in comparison to non-infected HNAECs (Figure 4.2A). Palivizumab anti-RSV control significantly decreased expression of IL-8 mRNA between MOI 2.5 and MOI 2.5 plus Palivizumab ($p<0.001$). Individual donor IL-8 mRNA expression is shown in Figure 4.2B with donor 1 showing ~3-fold lower expression than donors 2 and 3 and also not following a clear dose-response (Figure 4.2B).

IL-8 protein was not significantly different between non-infected and infected HNAECs culture supernatants (Figure 4.2C). IL-8 protein was significantly reduced between MOI 2.5

and MOI 2.5 plus Palivizumab ($p<0.05$) (Figure 4.2C). Individual donor expression of IL-8 is shown in Figure 4.2D with donor 1 showing ~2-fold higher expression than donors 2 and 3, both with and without RSV infection (Figure 4.2D). Donor 2 cultures showed an expected dose-response with increase in RSV MOI and donors 1 and 3 did not show an increase in IL-8 protein with RSV infection.

These results indicate successful *in vitro* RSV infection and replication in HNAECs, a shown by RSV N RNA and IL-8 mRNA, however differences were not seen at the protein level with IL-8. Individual donor plots show that mRNA does not necessarily correlate to protein expression with donor 1 expressing the lowest level of IL-8 mRNA yet the highest level of IL-8 protein.

4.3.2. Expression of IL-12 and IL-18 during RSV A2 infection of HNAECs

BEAS-2B cells do not express IL-12β mRNA, IL-12p70 protein and IL-18 protein (Section 3.3.2). IL-18 mRNA was present and a reduction in IL-18 mRNA was observed with RSV infection at 24hr and an MOI of 1 from BEAS-2B cells. The lack of expression could be specific to BEAS-2B cells, therefore IL-12β mRNA, IL-12p70 protein and IL-18 protein expression by HNAECs was also examined and the kinetics of IL-18 mRNA during RSV infection determined.

IL-12β and IL-18 mRNA was first measured by qPCR as an indicator of IL-12 and IL-18 gene expression following RSV infection and subsequently IL-12p70 and IL-18 protein expression in culture supernatants measured by ELISA (Section, 2.6.3 and 2.7).

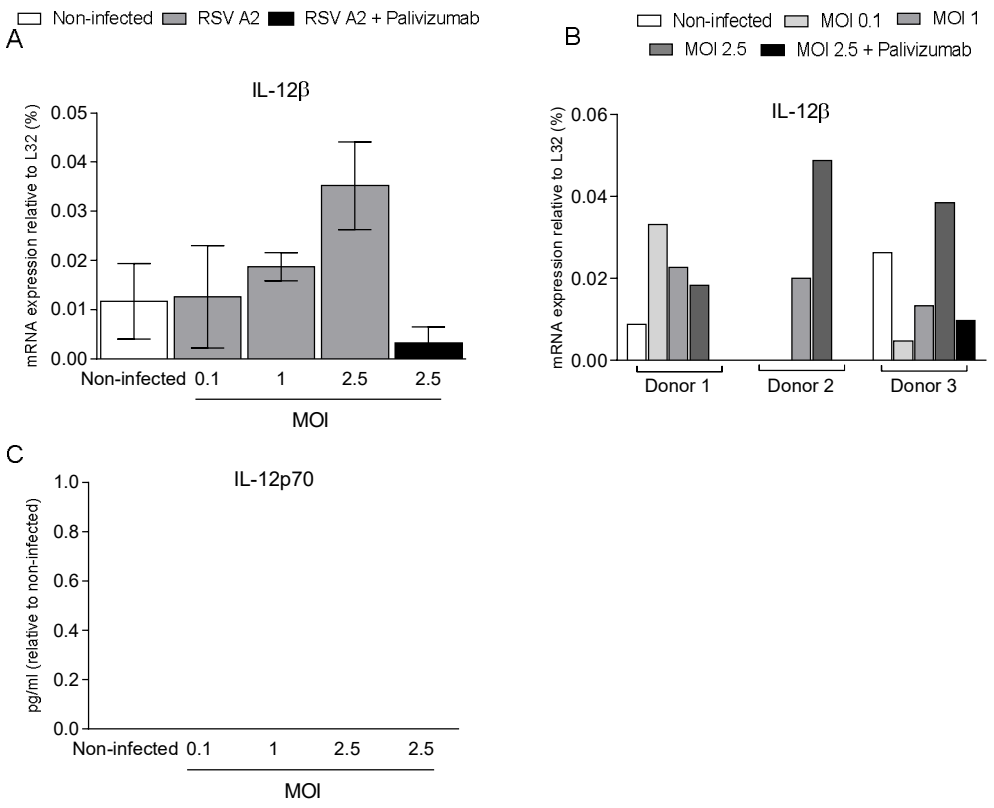


Figure 4.3. Expression of IL-12β mRNA and IL-12p70 protein in RSV A2 infected HNAECs. HNAECs were infected with RSV A2 at MOIs of 0.1, 1 and 2.5 for 48hr. Control non-infected cultures were cultured in the absence of RSV and Palivizumab used as an anti-RSV control. A) IL-12β mRNA (n=3), B) individual donor expression of IL-12β mRNA, C) IL-12p70 protein (n=3). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test).

Similarly to the results observed for BEAS-2B cells in Section 3.3.2 for IL-12β mRNA expression, no significant differences were observed between non-infected and infected HNAECs with IL-12β mRNA levels less than 0.05% of the housekeeping gene L32 (Figure 4.3A). Individual donor expression of IL-12β mRNA is shown in Figure 4.3B with donor 2 only expressing IL-12β mRNA at MOIs 1 and 2.5 (Figure 4.3B). No IL-12p70 protein was detected (Figure 4.3C).

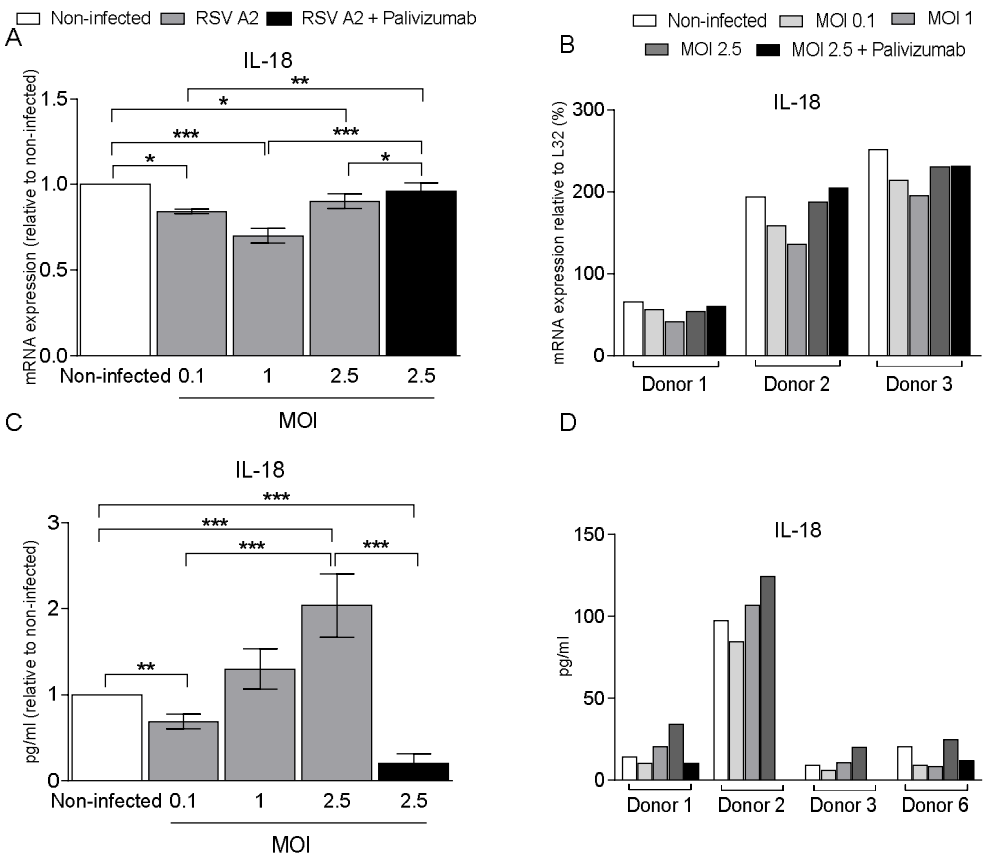


Figure 4.4. Expression of IL-18 mRNA and protein in RSV A2 infected HNAECs. HNAECs were infected with RSV A2 at MOIs of 0.1, 1 and 2.5 for 48hr. Control non-infected cultures were cultured in the absence of RSV and Palivizumab used as an anti-RSV control. A) IL-18 mRNA (n=3), B) individual donor expression of IL-18 mRNA, C) IL-18 protein (n=4), D) individual donor expression of IL-18 protein. Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01, ***p<0.001).

In comparison to non-infected HNAECs, IL-18 mRNA expression by infected HNAECs was significantly reduced at MOIs 0.1 ($p<0.05$), 1 ($p<0.001$) and 2.5 ($p<0.05$) with levels at ~80%, ~70% and ~90% of those in non-infected HNAECs, respectively (Figure 4.4A). IL-18 mRNA expression by infected HNAECs at MOIs 0.1 ($p<0.001$), 1 ($p<0.001$) and 2.5 ($p<0.05$) was significantly greater than observed in cultures treated with Palivizumab. Individual donor IL-18 mRNA expression is shown in Figure 4.4B, with donors 2 and 3 showing 2-3-fold increase in basal IL-18 mRNA expression compared to donor 1 (Figure 4.4B). IL-18 mRNA expression by non-infected HNAECs ranged from ~70-250% of the L32 house keeping gene level (Figure

4.4B). Each individual donor expression pattern also demonstrates the reduction at MOI 1 observed for each donor, regardless of the relative level of basal IL-18 mRNA expression.

IL-18 protein was present in culture supernatants from non-infected HNAECs at ~20-40pg/ml (Figure 4.4C). The apparent reduction in IL-18 mRNA expression following RSV infection was also reflected for protein expression with a significant decrease in IL-18 protein at MOI 0.1 compared to non-infected HNAECs ($p<0.01$). At a higher MOI of 2.5, there was a significant increase in IL-18 protein expression compared to non-infected HNAECs ($p<0.001$). IL-18 protein expression was significantly higher at MOI 2.5 compared to MOI 1 ($p<0.001$). For the Palivizumab anti-RSV control, only 2/4 culture supernatants were positive for IL-18 protein. Palivizumab treatment resulted in significantly decreased expression of IL-18 protein in comparison to infection at MOI 2.5 ($p<0.001$) and non-infected HNAECs ($p<0.001$). Individual donor IL-18 protein expression is shown in Figure 4.4D, with donor 2 having ~2-fold higher expression than donors 1, 3 and 6 at ~100-125pg/ml (Figure 4.4D). Donors 1, 3 and 6 had similar expression levels of IL-18 protein at ~20-40pg/ml.

These results indicate that similarly to BEAS-2B cells (Section 3.3.2), HNAECs do not express IL-12p70 protein neither with or without RSV infection. HNAECs express IL-18 protein both with and without RSV infection. At a lower MOI of 0.1 the reduction in IL-18 mRNA matched IL-18 protein. A higher MOI of 2.5 showed a significant increase in IL-18 protein, which was not reflected by individual donor mRNA levels at this time point. Palivizumab inhibited the reduction in IL-18 mRNA expression, however this was not seen at the protein level.

4.3.3. Expression of IL-15 during RSV A2 infection of HNAECs

IL-15 protein was detected in culture supernatants from RSV infected BEAS-2B cells (Section 3.3.3). IL-15 mRNA and protein expression by HBECs infected with RSV at an MOI of 1 for 24hr at ~2pg/ml has been reported (152). However, HBECs in the published study had been bought and recovered from frozen which may give a different response to the freshly isolated HNAECs used here. Here, IL-15 mRNA was measured by qPCR following RSV infection and IL-15 protein expression in culture supernatants measured by Luminex (Section 2.6.3 and 2.8).

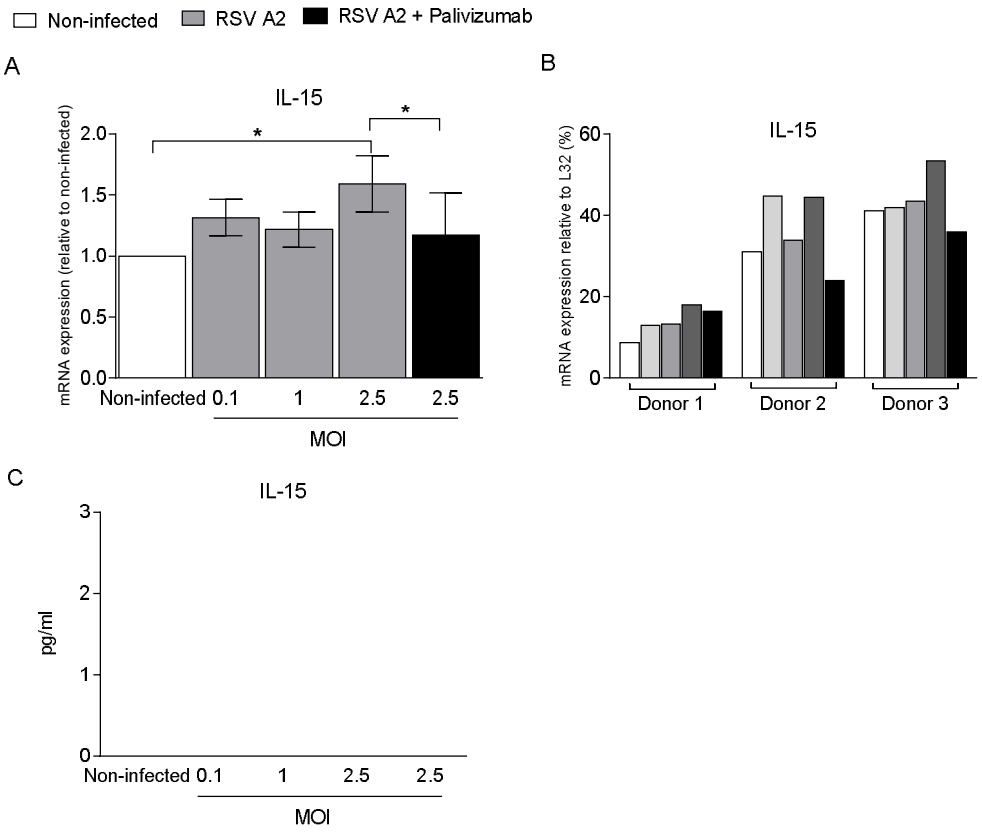


Figure 4.5. Expression of IL-15 mRNA and protein in RSV A2 infected HNAECs. HNAECs were infected with RSV A2 at MOIs of 0.1, 1 and 2.5 for 48hr. Control non-infected cultures were cultured in the absence of RSV and Palivizumab used as an anti-RSV control. A) IL-15 mRNA (n=3), B) individual donor expression of IL-15 mRNA, C) IL-15 protein (n=4). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, ***p<0.001).

IL-15 mRNA was significantly increased following infection at MOI 2.5 ($p<0.05$) as compared to non-infected HNAECs (Figure 4.5A). Palivizumab anti-RSV control significantly decreased

IL-15 mRNA expression compared to MOI 2.5 ($p<0.05$). Individual donor IL-15 mRNA expression is shown in Figure 4.5B with donors 2 and 3 expressing ~3-fold higher basal expression of IL-15 mRNA compared to donor 1 (Figure 4.5B). Basal IL-15 mRNA ranged from 10-40% of the housekeeping gene L32. Donor 1 shows the largest fold increase in IL-15 mRNA at ~2-fold compared to donors 2 and 3. The detection limit for IL-15 was 3pg/ml (Section 2.8), however no protein was detected in any sample (Figure 4.5C).

The results here indicate that RSV induces expression of IL-15 mRNA in HNAECs which was only seen at MOI 2.5 in comparison to non-infected HNAECs. IL-15 protein was not detected above the detection limit of 3pg/ml and matches the approximate level expressed by primary AECs from published data.

4.3.4. Expression of IL-15Rα during RSV A2 infection

RSV induced both IL-15Rα mRNA and cell surface expression in BEAS-2B cells (Section 3.3.3 and 3.3.4). Here expression of IL-15Rα by infected HNAECS was also evaluated. IL-15Rα mRNA was measured by qPCR following RSV infection and IL-15Rα cell surface expression measured by flow cytometry (Section 2.6.3 and 2.9).

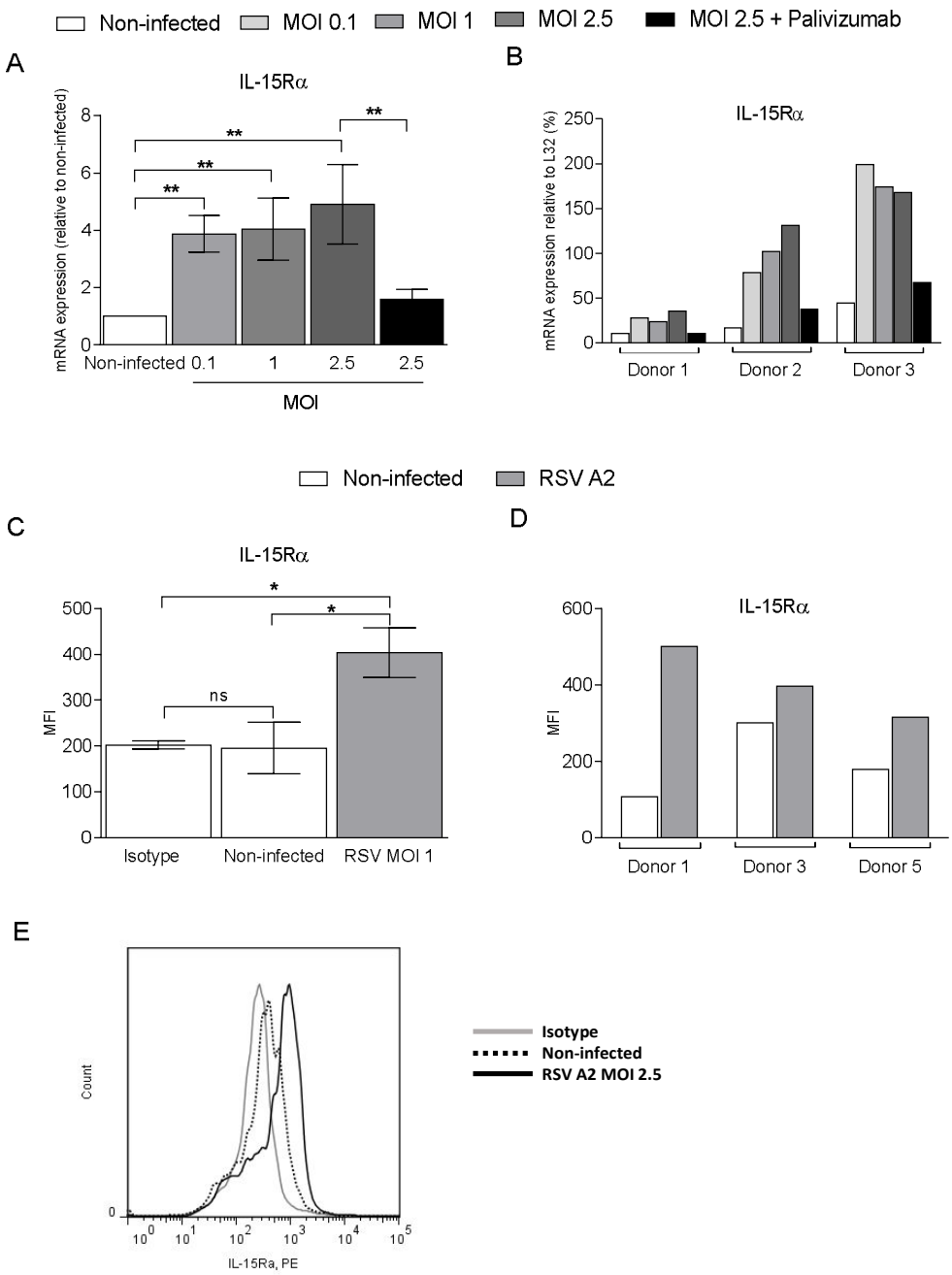


Figure 4.6. Expression of IL-15Rα mRNA and protein in RSV A2 infected HNAECs. HNAECs were infected with RSV A2 at MOIs of 0.1, 1 and 2.5 for 48hr (A, B) or MOI 1 for 48hr (C, D, E). Control non-infected cultures were cultured in the absence of RSV and Palivizumab used as an anti-RSV control. A) IL-15Rα mRNA (n=3), B) individual donor expression of IL-15Rα mRNA, C) IL-15Rα protein (n=3), D) individual donor expression of IL-15Rα protein, E) representative flow histogram of surface IL-15Rα. Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01).

RSV infection resulted in a significant increase in IL-15Rα mRNA expression at MOIs 0.1 ($p<0.01$), 1 ($p<0.01$) and 2.5 ($p<0.01$) compared to non-infected HNAECs (Figure 4.6A). RSV increased IL-15Rα mRNA expression by ~3-6 fold compared to non-infected HNAECs. Addition of Palivizumab as an anti-RSV control led to significantly reduced IL-15Rα mRNA expression ($p<0.001$). Individual plots show that the lowest MOI used 0.1, induced an ~3-fold increase in IL-15Rα mRNA expression in donors 1 and 2 and an ~5-fold increase in donor 3 compared to non-infected HNAECs (Figure 4.6B). IL-15Rα mRNA was between ~15-50% of the housekeeping gene L32 in non-infected HNAECs. Between MOI 0.1 and 2.5, donor 1 and 3 showed similar expression of IL-15Rα mRNA and donor 2 showed a dose-dependent increase with MOI.

Flow cytometric analysis was used to measure surface IL-15Rα expression. No difference was observed between isotype control and IL-15Rα staining on non-infected cells indicating an absence of surface expression (Figure 4.6C). A single peak was observed during RSV infection indicative of one homogenous positive population (Figure 4.6E). There was a significant increase in MFI at MOI 1 ($p<0.05$) compared to isotype control and non-infected HNAECs. Individual donor expression of surface IL-15Rα is shown in Figure 4.6D with donor 1 showing an ~5-fold increase in IL-15Rα surface expression with infection, whereas donor 3 showed very little increase in expression and donor 6 ~0.5-fold increase.

These results indicate that RSV induces IL-15Rα mRNA expression by HNAECs with no apparent increase in expression level with increase in RSV titre. IL-15Rα protein was also expressed at the cell surface during infection with variable levels of expression between donors.

4.3.5. Expression of soluble IL-15/IL-15Rα complex

The IL-15/IL-15Rα complex was detected in 2/5 infected BEAS-2B cell supernatants (Section 5.3.5.2, Figure 5.15). HNAECs culture supernatants were also examined for IL-15/IL-15Rα complex and no protein was detected below the detection limit of 62.5pg/ml.

4.3.6. Comparison between the response of HNAECs during RSV A2 and RSV X infection

As described in Section 3.3.12.2, BEAS-2B cells expressed ~10-fold more IL-15Rα during infection with a clinical isolate RSV X compared to RSV A2 (Figure 3.19). This was then complimented by significantly higher expression of IFN-γ and TNF-α during NK cell co-culture (Figure 3.20). It was also observed that gene expression of RSV N RNA with infection of RSV X was ~56-fold more than RSV A2 suggesting that RSV X may be able to better utilise receptors for cell entry and induce increased inhibition of anti-viral gene expression (Figure 3.18). However using HNAECs with a clinical isolate, like RSV X, may provide a more accurate response to *in vivo* infection compared to infection of HNAECs with RSV A2.

The aims set out in this section are to compare the gene expression of IL-15 and IL-15Rα by HNAECs infected with RSV A strains, laboratory A2 and a clinical isolate, RSV X (Section 2.3). In these experiments HNAECs were infected with RSV A2 and RSV X at an MOI of 2.5 for 48hrs, after which culture supernatants and RNA were collected.

4.3.6.1. Differences between RSV A2 and RSV X replication and infectivity

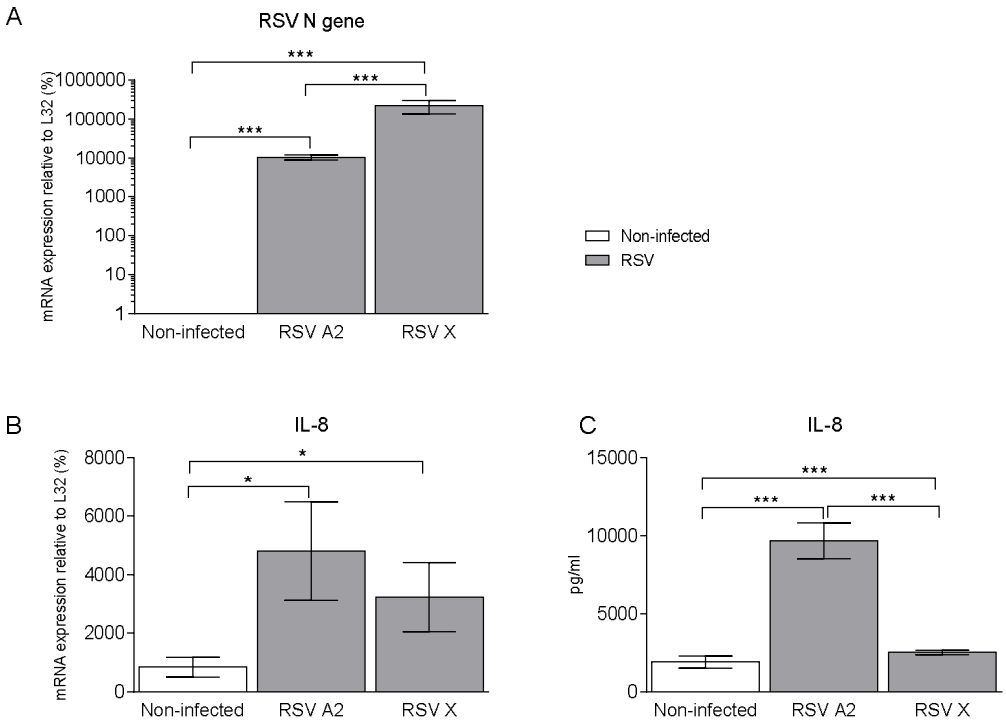


Figure 4.7. Comparison between the expression of RSV N gene and IL-8 protein in RSV A2 and RSV X infected HNAECs. HNAECs were infected with RSV A2 and RSV X at an MOI of 2.5 for 48hr. Control non-infected cultures were cultured in the absence of RSV. A) RSV N gene (n=3), B) IL-8 mRNA (n=3), IL-8 protein (n=3). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, ***p<0.001).

RSV N RNA was significantly increased by treatment with both RSV A2 (p<0.001) and RSV X (p<0.001) infection (Figure 4.7A). RSV N gene expression was ~20-fold greater in HNAECs infected with RSV X compared to RSV A2 (p<0.001). IL-8 mRNA was detected at ~842% of the housekeeping gene L32 level in the absence of infection and was significantly increased with both RSV A2 (p<0.05) and RSV X (p<0.05) infection by ~50-fold (Figure 4.7B). IL-8 protein was detected at ~1900pg/ml in the absence of infection and was significantly increased with both RSV A2 (p<0.001) and RSV X (p<0.001) infection to ~9670pg/ml and ~2530pg/ml, respectively (Figure 4.7C). IL-8 protein was significantly lower with RSV X (p<0.001) than RSV A2 by ~4-fold (Figure 4.7C).

The results indicate that although RSV infectivity was greater for RSV X than RSV A2 in HNAECs, RSV A2 infection induces the most IL-8 protein synthesis.

4.3.6.2. Differences between RSV A2 and RSV X and expression of IL-15 and IL-15Rα

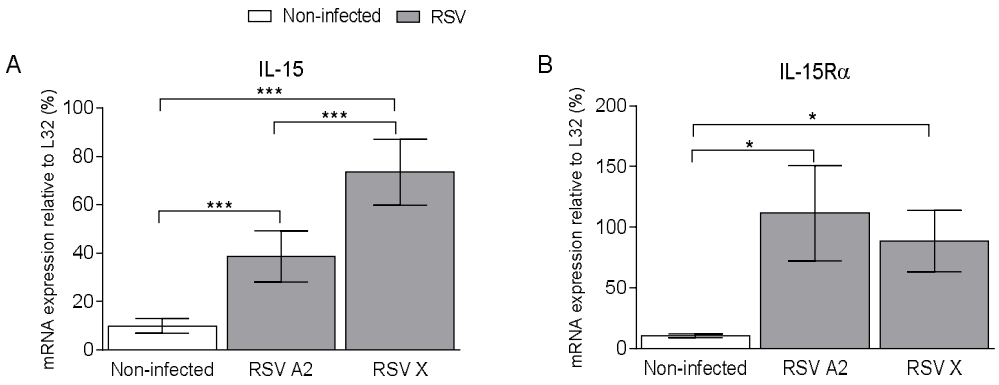


Figure 4.8. Comparison between the expression of IL-15 and IL-15Rα mRNA in RSV A2 and RSV X infected HNAECs. HNAECs were infected with RSV A2 and RSV X at an MOI of 2.5 for 48hr. Control non-infected cultures were cultured in the absence of RSV. A) IL-15 mRNA (n=3), B) IL-15Rα mRNA (n=3). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, ***p<0.001).

IL-15 mRNA was significantly increased after 48hr infection at an MOI of 2.5 by both RSV A2 ($p<0.001$) and RSV X ($p<0.001$), ~4-fold and ~~7-fold, respectively, compared to non-infected cells (Figure 4.8A). IL-15 mRNA expression was significantly greater, ~2-fold, after RSV X infection ($p<0.001$) when compared to RSV A2 infection. No IL-15 protein was observed in culture supernatants. IL-15Rα mRNA level was significantly increased ~10-fold by RSV A2 ($p<0.001$) and increased ~8-fold by RSV X ($p<0.001$) when compared to non-infected cells (Figure 4.7B).

The results here indicate that both strains of RSV, A2 and X, induce a similar amount of IL-15Rα mRNA level. However RSV X was able to induce more IL-15 mRNA expression than RSV A2.

4.3.7. Co-culture of RSV A2 infected HNAECs and donor matched NK cells

In Section 3.3.7 cultures using freshly isolated human adult peripheral blood NK cells were used to characterise BEAS-2B cell-induced NK cell activation. Co-culture of HNAECs with NK cells may be more characteristic of *in vivo* responses compared to experiments using an AEC line, BEAS-2B, although still not a full representation of lung infection. Co-culturing HNAEC and donor matched NK cells could give a more representative NK cell response. Secondly, HNAECs express IL-18 protein as shown in Section 4.3.2 which could enhance NK cell protein expression whereas BEAS-2B cells do not (Section 3.3.2). This suggests an enhanced NK cell cytokine response may be seen overall during HNAEC-NK cell co-cultures when compared to BEAS-2B cell-NK cell co-cultures. The next aim in this chapter was to establish if HNAECs can activate NK cells in an HNAEC-NK cell co-culture model again with IFN-γ and TNF-α as indicators of NK cell activation.

The method used here for HNAEC-NK cell co-culture is the same as that used with BEAS-2B cells, detailed in Section 3.3.8, with the difference of an MOI of 2.5 for HNAECs in comparison to MOI 1 used for BEAS-2B cells.

4.3.7.1. Expression of RSV N gene and IL-8 protein in HNAEC-NK cell co-cultures

To confirm infection, RSV N gene expression was measured by qPCR and to confirm viral replication, IL-8 protein expression in culture supernatants was measured by ELISA (Section 2.6.3 and 2.7).

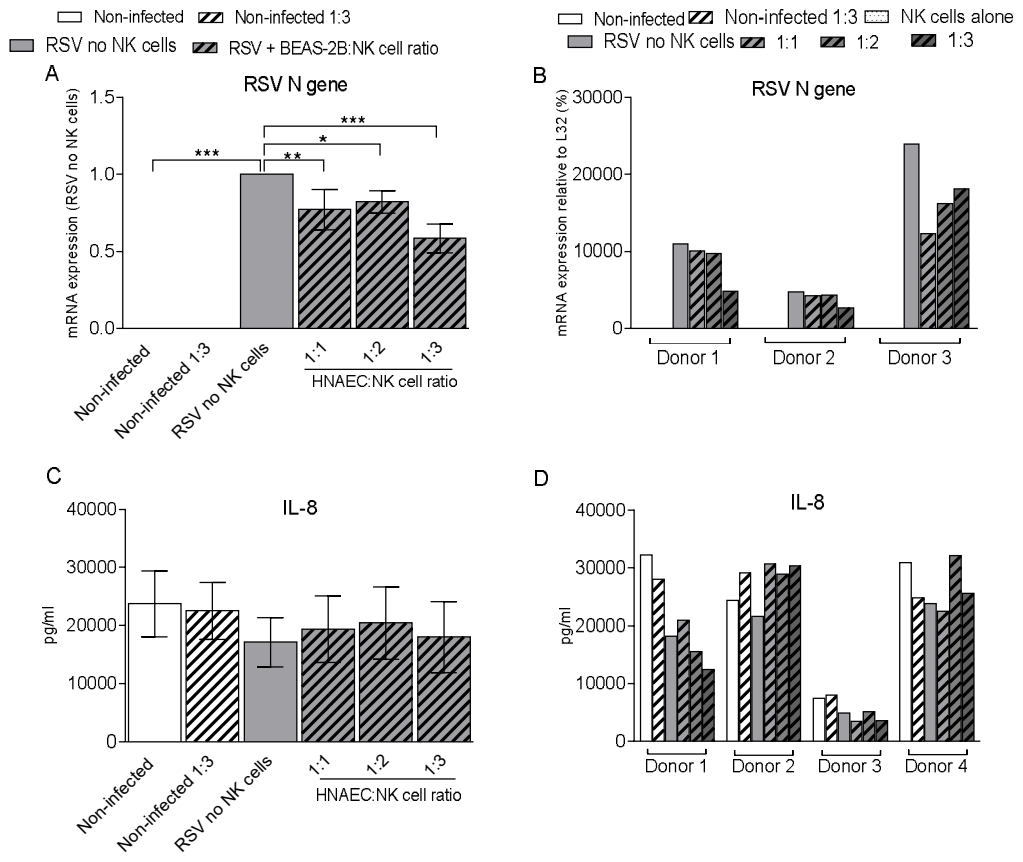


Figure 4.9. Expression of RSV N gene and IL-8 protein during RSV A2 infection of HNAECs cells co-cultured with NK cells. HNAECs were infected with RSV A2 at MOI 2.5 for 24hr. Donor matched NK cells were isolated from PBMCs and added at increasing ratios to HNAECs for a further 24hr. Control non-infected cultures were cultured in the absence of RSV. A) RSV N gene (n=3), B) individual donor expression of RSV N gene, C) IL-8 protein (n=4), D) individual donor expression of IL-8 protein. Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01, ***p<0.001).

RSV N RNA was not found in control HNAECs or non-infected HNAECs cultured with NK cells (Figure 4.9A). A significant increase in RSV N RNA was seen in infected HNAECs compared to non-infected HNAECs ($p<0.001$). A significant decrease in RSV N RNA was seen at ratios of 1:1 ($p<0.01$), 1:2 ($p<0.05$) and 1:3 ($p<0.001$) as compared to infected HNAECs without NK cells. This was ~0.5-fold lower in NK cell co-cultures at a ratio of 1:3. Individual donor expression of RSV N RNA is shown in Figure 4.16B with donor 3 showing ~2-fold higher expression than donors 1 and 2 (Figure 4.9B).

Similarly to the results in Section 4.3.1, IL-8 protein was present in supernatants from non-infected HNAECs cultures and no significant differences were seen between non-infected, infected or infected HNAECs/NK cell co-cultures (Figure 4.9C). Individual donor expression of IL-8 protein is shown in Figure 4.9D with donor 3 showing ~3-fold lower expression than donors 1, 2 and 4 (Figure 4.9D).

These results indicate infection only occurred in cultures where RSV was added and that RSV N gene expression decreased with addition of NK cells in infected HNAECs. Although qPCR shows RSV infection occurred, there was no increase in IL-8 protein in infected compared to non-infected HNAECs. This was seen with BEAS-2B cells (Section 3.3.8.2). IL-8 protein expression in HNAECs culture supernatants detailed in Section 4.3.1 also show that this was not significantly different, with or without RSV infection.

4.3.7.2. Expression of IFN-γ and TNF-α in HNAEC-NK cell co-cultures

To determine activation of NK cells during BEAS-2B cell co-culture, expression of NK cell-derived IFN-γ and TNF-α mRNA and protein were evaluated using qPCR and ELISA, respectively (Section 2.6.3 and 2.7).

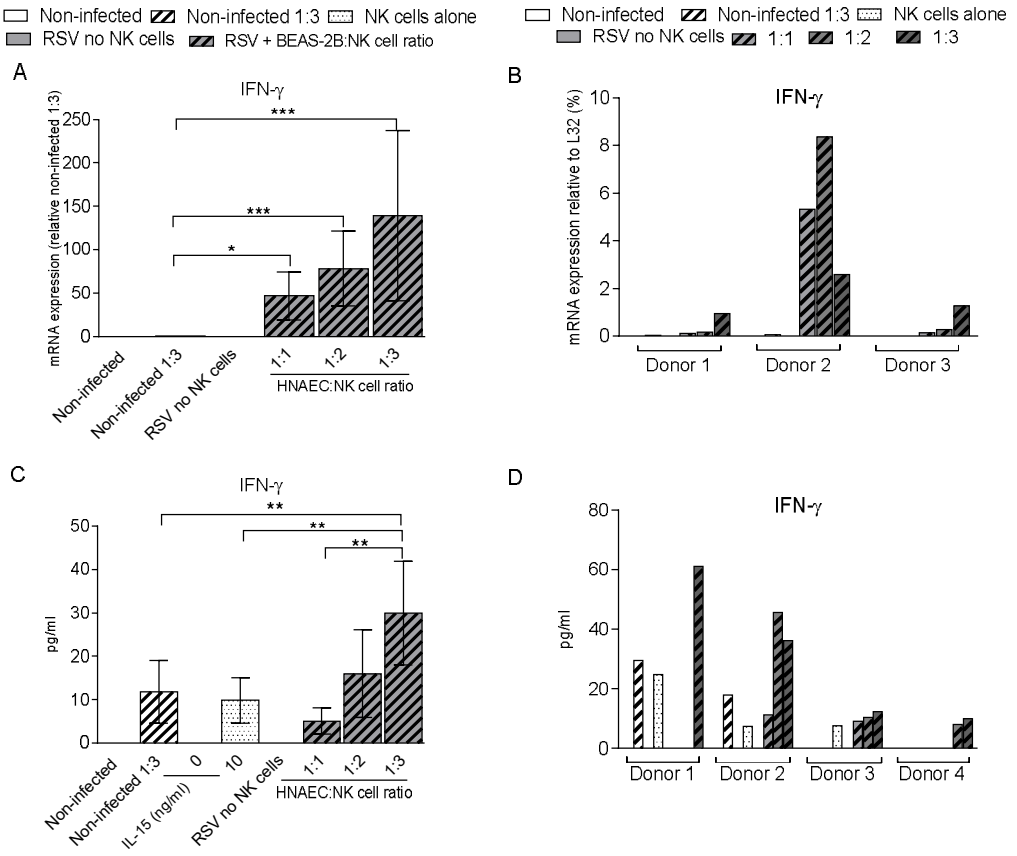


Figure 4.10. Expression of IFN-γ mRNA and protein during RSV A2 infection of HNAECs cells co-cultured with NK cells. HNAECs were infected with RSV A2 at MOI 2.5 for 24hr. Donor matched NK cells were isolated from PBMCs and added at increasing ratios to HNAECs for a further 24hr. Control non-infected cultures were cultured in the absence of RSV. A) IFN-γ mRNA (n=3), B) individual donor expression of IFN-γ mRNA, C) IFN-γ protein (n=4), D) individual donor expression of IFN-γ protein. Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01, ***p<0.001) (n=3).

IFN-γ mRNA was only detected in cultures with NK cells present and at less than 0.06% of the housekeeping gene L32 from non-infected HNAEC-NK cell co-cultures (Figure 4.10A). IFN-γ mRNA was significantly increased in HNAEC-NK cell co-cultures by infection with RSV at ratios of 1:1 ($p<0.05$), 1:2 ($p<0.01$) and 1:3 ($p<0.001$) compared to non-infected HNAECs co-cultured with NK cells at a ratio of 1:3. Individual donor expression of IFN-γ mRNA is shown in Figure 4.10B, with donor 2 showing ~8-fold higher expression than donors 1 and 3 (Figure

4.10B). Both donors 1 and 3 show a dose-dependent increase in IFN-γ mRNA with increase in NK cell ratio co-culture.

IFN-γ protein was only found in supernatants from cultures with NK cells present, however not all donors were positive at each NK cell co-culture ratio (Figure 4.10C, D). IFN-γ protein was present in 2/4 supernatants from non-infected HNAECs co-cultured with NK cells at ratio of 1:3 at ~20pg/ml. IFN-γ protein was found in 3/4 supernatants from NK cells cultured alone with 10ng IL-15 at ~10-20pg/ml. For HNAEC-NK cell co-cultures IFN-γ protein was detected in 2/4 supernatants from cultures at a ratio of 1:1, 3/3 supernatants at a ratio of 1:2 and 4/4 supernatants at a ratio of 1:3 (Figure 4.10C). IFN-γ protein was significantly increased at a ratio of 1:3 compared to non-infected HNAECs co-cultured NK cells at ratio of 1:3 ($p<0.01$), NK cells cultured alone with 10ng/ml IL-15 ($p<0.01$) and ratio of 1:1 ($p<0.01$). Individual donor expression of IFN-γ protein is shown in Figure 4.10D which details the culture supernatants that were negative for IFN-γ protein for each donor (Figure 4.10D). For donor 2, IFN-γ protein was found in all culture supernatants with NK cells present. At a co-culture ratio of 1:3 IFN-γ ranged from ~10-60pg/ml (Figure 4.10D).

These results indicate that infected HNAECs are able to express IFN-γ mRNA and protein during co-culture with donor matched NK cells. This appears to be NK cell specific and that the responses appear heterogeneous.

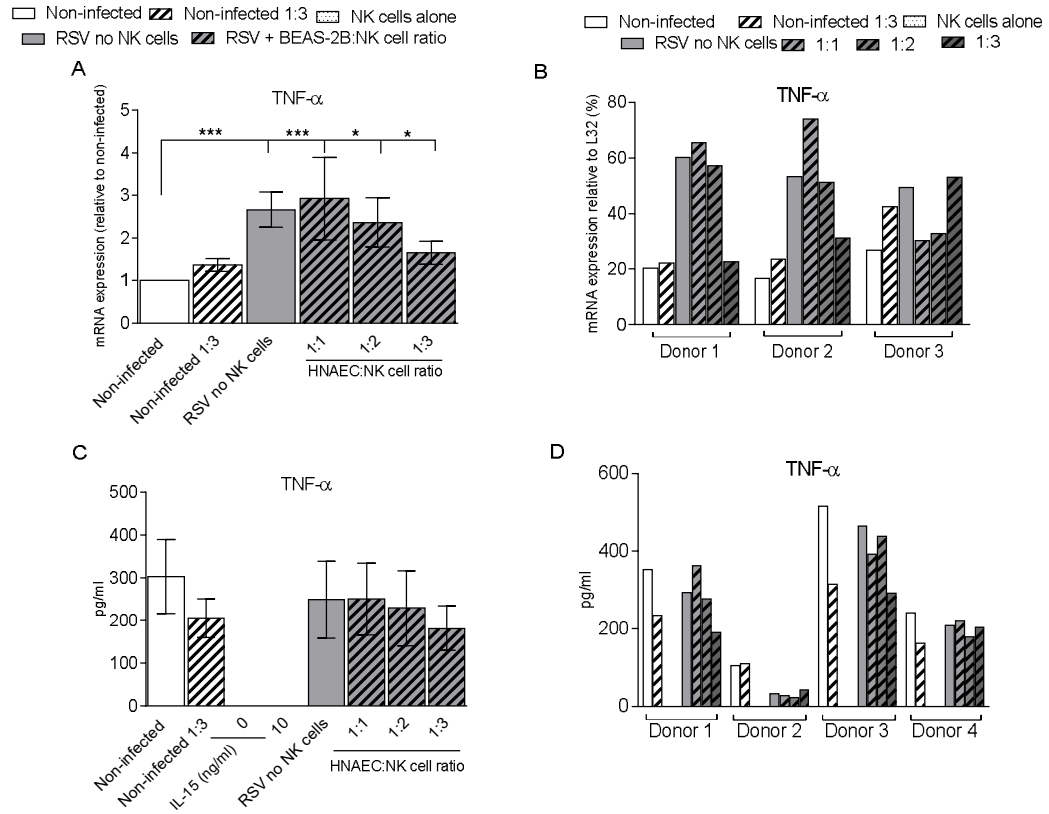


Figure 4.11. Expression of TNF-α mRNA and protein during RSV A2 infection of HNAECs cells co-cultured with NK cells. HNAECs were infected with RSV A2 at MOI 2.5 for 24hr. Donor matched NK cells were isolated from PBMCs and added at increasing ratios to HNAECs for a further 24hr. Control non-infected cultures were cultured in the absence of RSV. A) TNF-α mRNA (n=3), B) individual donor expression of TNF-α mRNA, C) TNF-α protein (n=4), D) individual donor expression of TNF-α protein. Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, ***p<0.001) (n=3).

TNF-α mRNA was detected in all cultures (Figure 4.11A). RSV significantly induced TNF-α mRNA expression in infected HNAECs culture without NK cells by ~2-3 fold ($p<0.001$). TNF-α mRNA was similarly significantly increased following addition of NK cells to infected HNAECs at ratios of 1:1 ($P<0.001$), 1:2 ($P<0.05$) and 1:3 ($p<0.05$). No other significant differences in TNF-α mRNA expression were seen. Individual donor expression of TNF-α mRNA is shown in Figure 4.11B with basal TNF-α mRNA expression of ~20% relative to the housekeeping gene L32, seen across all three donors (Figure 4.11B). RSV induced TNF-α expression in HNAECs to ~50-60% of the housekeeping gene L32 which was ~2-3-fold more than non-infected

HNAECs. Donors 1 and 2 showed a decrease in TNF-α mRNA expression with addition of NK cells at a ratio of 1:3 compared to non-infected HNAECs without NK cells (Figure 4.11B). Donor 1 and 2's TNF-α mRNA expression for was approximately the same as that in non-infected HNAECs. Donor 3 showed a decrease in TNF-α expression at ratios of 1:1 and 1:3 (Figure 4.11B).

TNF-α protein was present in culture supernatants from non-infected and infected HNAECs without NK cells present at ~200-400pg/ml (Figure 4.11C). RSV infection and co-culture with NK cells did not significantly increase TNF-α protein. Individual donor expression of TNF-α mRNA is shown in Figure 4.11D, with basal expression of TNF-α protein greatly differing between donors (Figure 4.11D). TNF-α protein expression was lowest from donor 2 and highest from donor 3. For donors 1 and 3, TNF-α protein was reduced when NK cells were added at a ratio of 1:3 compared to infected HNAECs without NK cells. For donors 2 and 4 TNF-α protein expression was similar between infected HNAECs with and without NK cell co-culture in.

These results also indicate that HNAECs express TNF-α protein which was not influenced by RSV infection. Addition of NK cells to infected HNAECs did not result in increased expression of TNF-α mRNA or protein.

4.3.8. Analysis of NK cell activating cytokine expression in nasopharyngeal aspirate samples from children with RSV infection

The final aim of the work described in this chapter were to measure expression of IL-15, IL-15/IL-15Rα complex, IL-18, IFN-γ and TNF-α in NPAs from RSV infected infants and from RV infected children.

A total of 56 NPAs from RSV infected infants were analysed by ELISA (Section 2.7.2). IL-15, IL-15/IL-15Rα complex and IL-18 were measured in all samples. Of these 56, 25 were also used

for IFN-γ analysis and 32 for TNF-α. Section 2.11 details the selection criteria for the NPAs used. Protein expression was compared to 1) whether patients required oxygen, 2) patient age by group, less than one month old (<1M), one to three months old (1-3M) and 3 months to 1 year old (3M-1Y), and 3) patient disease severity, mild (no oxygen required), moderate (oxygen required) and severe (oxygen and stay in paediatric critical care unit). No protein values followed a normal distribution.

4.3.8.1. IL-15 protein levels in NPAs from infants with RSV infection

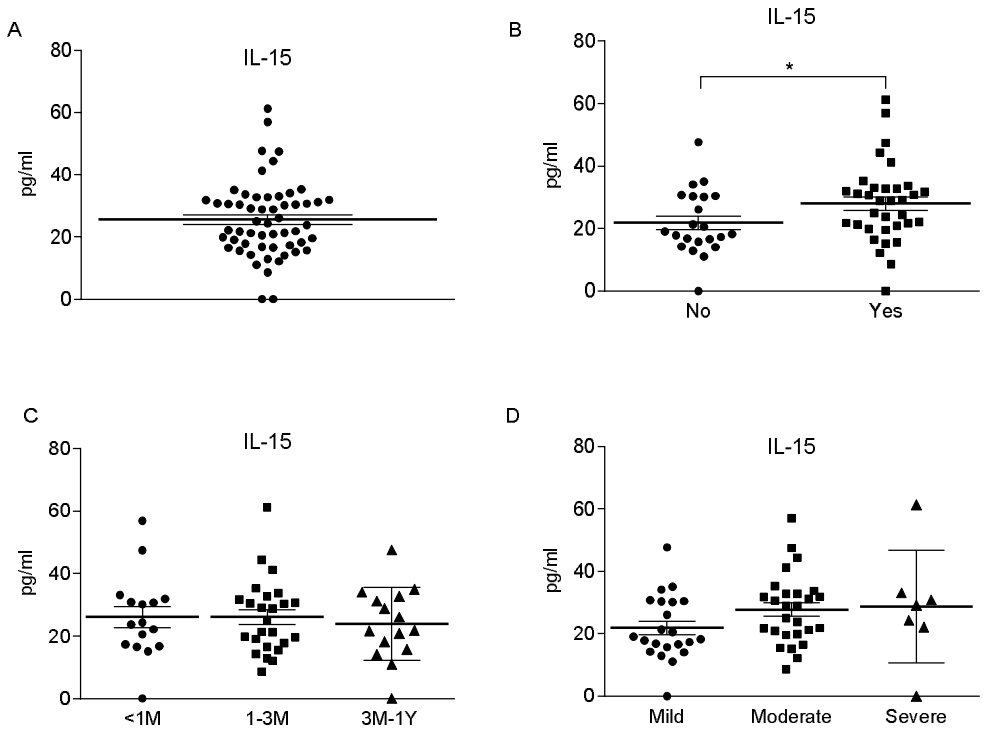


Figure 4.12. IL-15 concentrations in RSV-only positive NPAs from infants under 1 years old. Data is shown as protein expression in A) all NPAs, B) with oxygen administration, C) between age groups and D) disease severity. M, months and Y, year. Data is expressed as the mean ± SEM (n=56) (B, Mann-Whitney U-test and C, D Kruskal-Wallis with Conover-Inman post-hoc test), *p<0.05).

The mean NPA value for IL-15 was 25.5pg/ml with 2/56 NPAs negative in this assay. (Figure 4.12A). Samples from infants who were given oxygen contained significant more IL-15 ($p<0.05$) (Figure 4.12B). No significant differences in IL-15 protein were seen between age groups or with severity (Figure 4.12C, D).

These results indicate that an increase in IL-15 protein in NPAs was associated with the requirement for oxygen and level did not change between age groups or with different severities.

4.3.8.2. IL-15/IL-15Rα complex levels in NPAs from infants with RSV infection

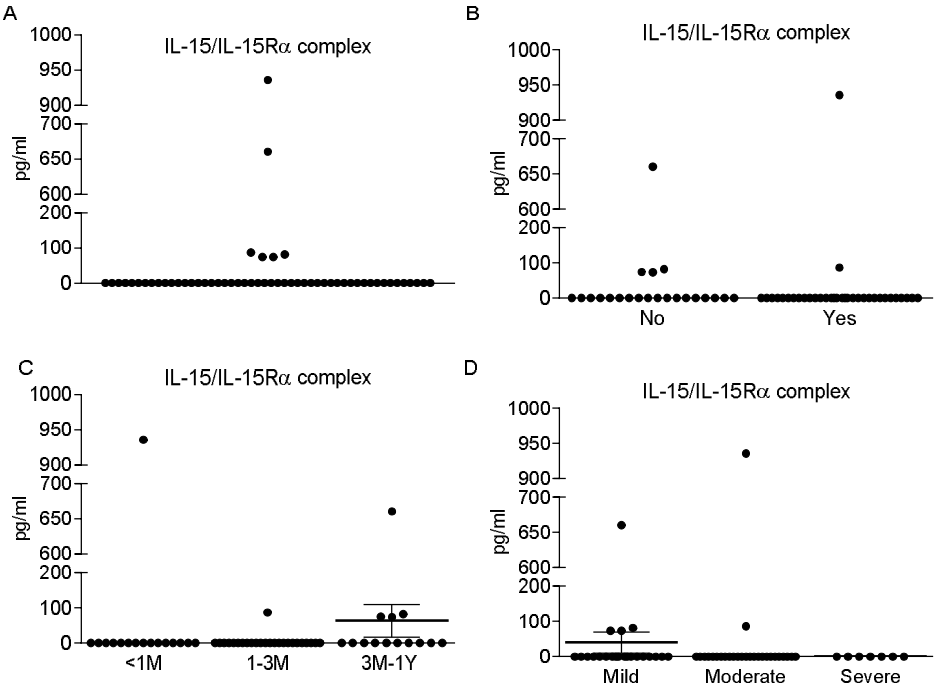


Figure 4.13. IL-15/IL-15Rα complex concentrations in RSV-only positive NPAs from infants under 1 years old. Data is shown as protein expression in A) all NPAs, B) with oxygen administration, C) between age groups and D) disease severity. M, months and Y, year. Data is expressed as the mean ± SEM (n=56).

Only 6/56 NPAs were positive for IL-15/IL-15Rα complex with values of 74, 74, 82, 86, 660, and 935 pg/ml (Figure 4.13A). Due to small number of positive values statistical analysis relating this data to oxygen requirement, age group or disease severity was not made. (Figure 7.13B, C, D).

These results indicate that in the majority of NPAs the IL-15/IL-15Rα complex was not present above the minimum detection limit of 62.5pg/ml.

4.3.8.3. IL-18 protein levels in NPAs from infants with RSV infection

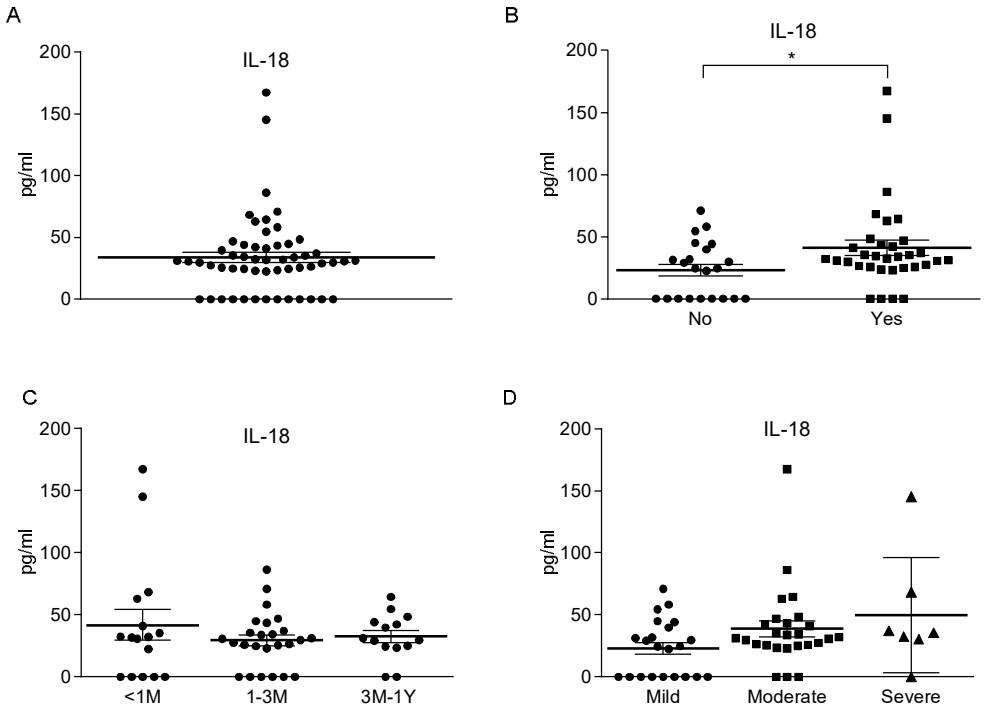


Figure 4.14. IL-18 concentrations in RSV-only positive NPAs from infants under 1 years old. Data is shown as protein expression in A) all NPAs, B) with oxygen administration, C) between age groups and D) disease severity. M, months and Y, year. Data is expressed as the mean ± SEM (n=56) (B, Mann-Whitney U-test and C, D Kruskal-Wallis with Conover-Inman post-hoc test).

The mean value for IL-18 was 33.8pg/ml with 13 NPAs below detection level of 11.7pg/ml (Figure 4.14A). There was a significant increase in IL-18 protein between those infants who

were given oxygen compared to those who did not ($p<0.05$) (Figure 4.14B). No significant differences in IL-18 protein were seen between age groups or with severity (Figure 4.14C, D).

These results indicate that an increase in IL-18 protein in NPAs was associated with the requirement for oxygen and its level did not change between age groups or with different severities.

4.3.8.4. IFN-γ protein levels in NPAs from infants with RSV infection

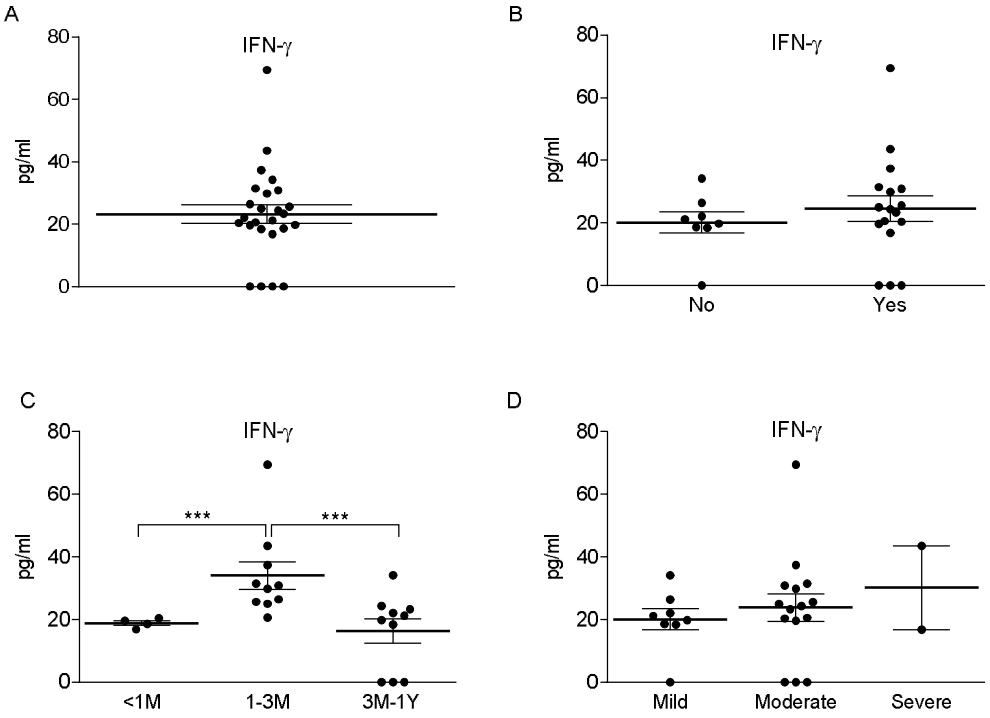


Figure 4.15. IFN-γ concentrations in RSV-only positive NPAs from infants under 1 years old. Data is shown as protein expression in A) all NPAs, B) with oxygen administration, C) between age groups and D) disease severity. M, months and Y, year. Data is expressed as the mean ± SEM (n=25) (B, Mann-Whitney U-test and C, D Kruskal-Wallis with Conover-Inman post-hoc test, *$p<0.05$, ***$p<0.001$).

The mean value for IFN-γ was ~23pg/ml with 4 NPAs below the minimum level of detection level (Figure 4.15A IFN-γ). There was a significant increase in IFN-γ protein between infants

aged 1-3M compared to those aged <1M ($p<0.001$) and 3M-1Y ($p<0.001$) (Figure 4.15C). No significant differences in IFN-γ expression was observed in relation to oxygen requirement or with severity were observed (Figure 4.15B, D).

These results indicate that an increase in IFN-γ protein in NPAs was associated with the age group 1-3M. Protein level did not change with oxygen requirement or with different severities.

4.3.8.5. TNF-α protein levels in NPAs from infants with RSV infection

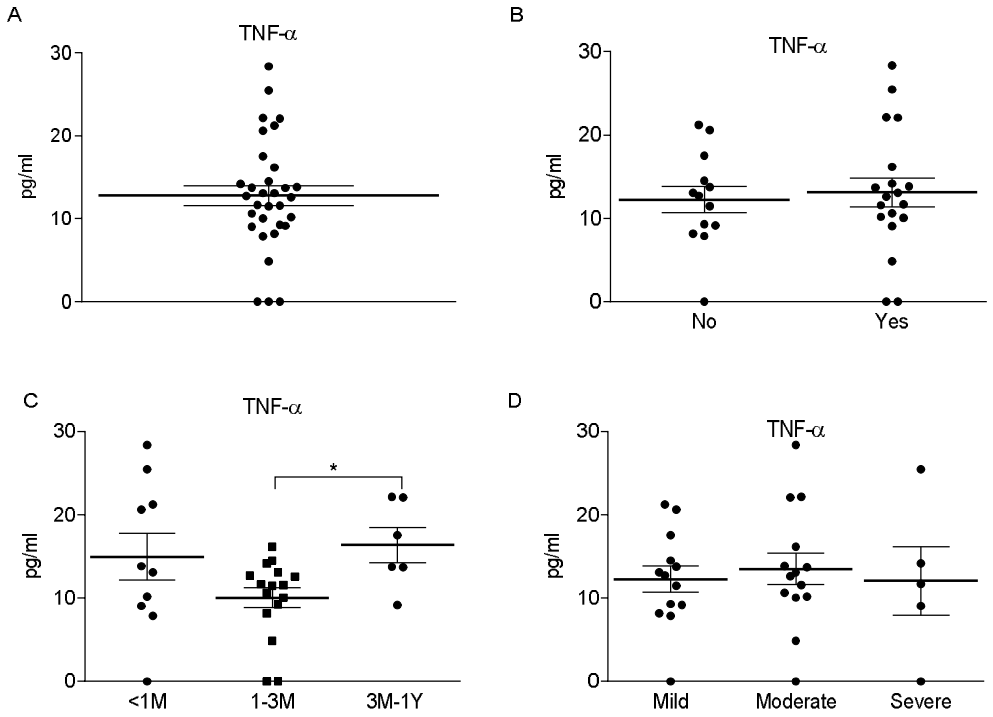


Figure 4.16. TNF-α concentrations in RSV-only positive NPAs from infants under 1 years old. Data is shown as protein expression in A) all NPAs, B) with oxygen administration, C) between age groups and D) disease severity. M, months and Y, year. Data is expressed as the mean ± SEM (n=32) (B, Mann-Whitney U-test and C, D Kruskal-Wallis with Conover-Inman post-hoc test, *$p<0.05$).

The mean value for TNF-α was ~12.8pg/ml with 3 NPAs below detection level (Figure 4.16A). There was a significant decrease in TNF-α protein between infants aged 1-3M ($p<0.05$)

compared to those aged 3M-1Y (Figure 4.16C). No significant differences in TNF-α protein were seen between oxygen requirement or with severity (Figure 4.16B, D).

These results indicate that an increase in IFN-γ protein in NPAs was associated with the age group 1-3M and level detected did not change with oxygen requirement or with different severities.

4.3.8.6. Correlations between IL-15 and other proteins in NPAs from infants with RSV infection

Next was to determine if protein levels observed above for IL-15/IL-15Rα complex, IL-18, IFN-γ and TNF-α positively or negatively correlated to IL-15. Figure 4.17 details these correlations between.

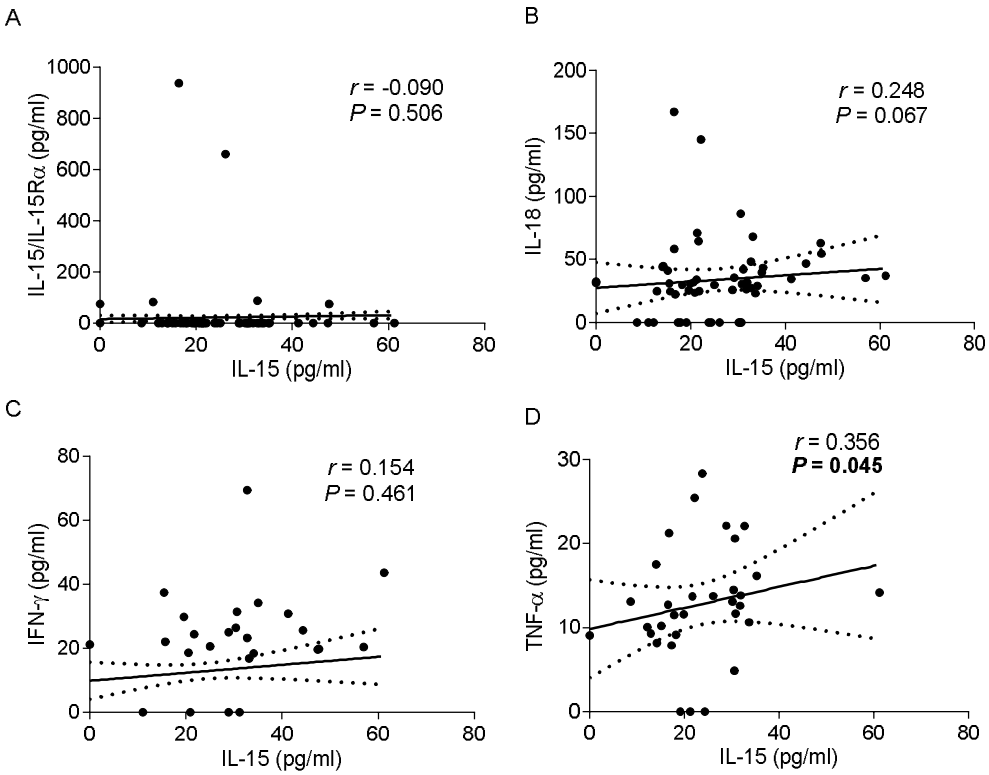


Figure 4.17. Correlations between protein levels of IL-15 against IL-18, IFN-γ and TNF-α in RSV-only positive NPAs from infants under 1 years old. A) IL-15/IL-15Rα complex (n=56), B) IL-18 (n=55), C) IFN-γ (n=25), D) TNF-α (n=32). Correlations were derived by Spearman's rank

order analysis, P two-tailed, n = number of XY pairs. Statistical significant correlation ($p<0.05$) is given in bold.

The correlation coefficients for IL-15 paired with IL-15/IL-15Rα complex, IL-18 and IFN-γ were -0.09, 0.248 and 0.154, respectively, with no significance observed (Figure 4.17A, B, C). A positive but weak correlation coefficient of 0.356, was observed between IL-15 and TNF-α ($p>0.05$). However the degrees of freedom, here being 30, based on a critical value table for Pearson's correlation shows that the r value of 0.356 is larger than 0.349 and therefore this result is not significant.

4.3.9. Analysis of NK cell activating cytokine expression in nasopharyngeal aspirate samples from children with RV infection

A total of 60 NPAs from RV infected infants were used for analysis. IL-15 and IL-18 were measured in all samples and 19 samples for IL-15/IL-15Rα complex.

4.3.9.1. IL-15 protein levels in NPAs from infants with RV infection

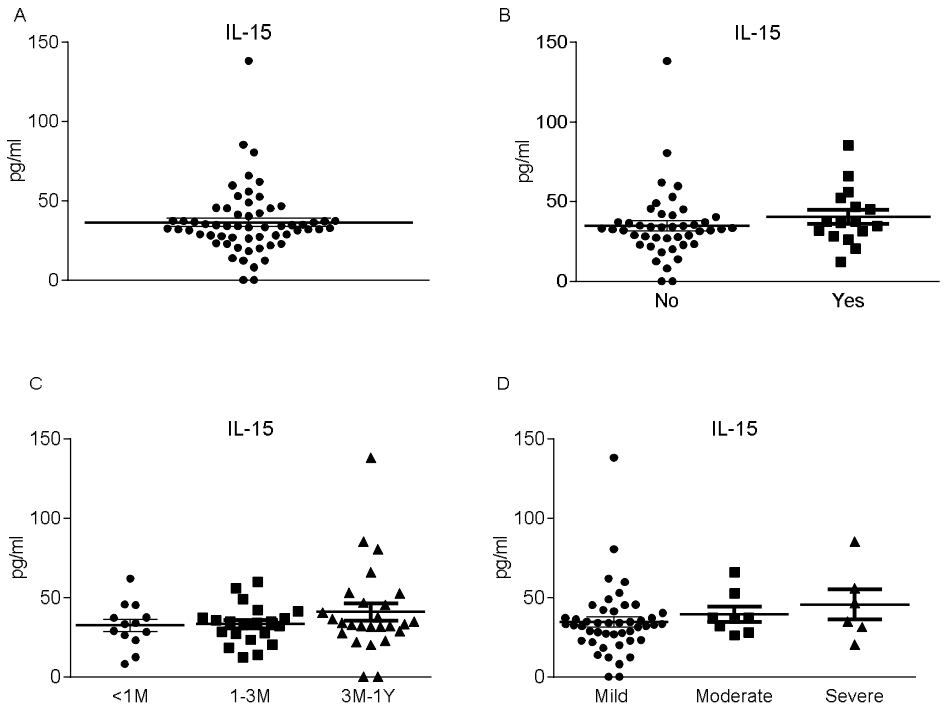


Figure 4.18. IL-15 concentrations in RV-only positive NPAs from infants under 1 years old. Data is shown as protein expression in A) all NPAs, B) with oxygen administration, C) between age groups and D) disease severity. M, months and Y, year. Data is expressed as the mean ± SEM (n=60) (B, Mann-Whitney U-test and C, D Kruskal-Wallis with Conover-Inman post-hoc test, *p<0.05).

The mean value for IL-15 was 36.4pg/ml with 2/60 NPAs were negative in this assay (Figure 4.18A). No significant differences were observed for IL-15 protein between oxygen requirement, age groups and disease severity (Figure 4.18B, C, D).

These results indicate that IL-15 protein in NPAs from infants under 1 years old and infected with RV do not show differences over age or disease severity.

4.3.9.2. IL-15/IL-15Rα complex levels in NPAs from infants with RV infection

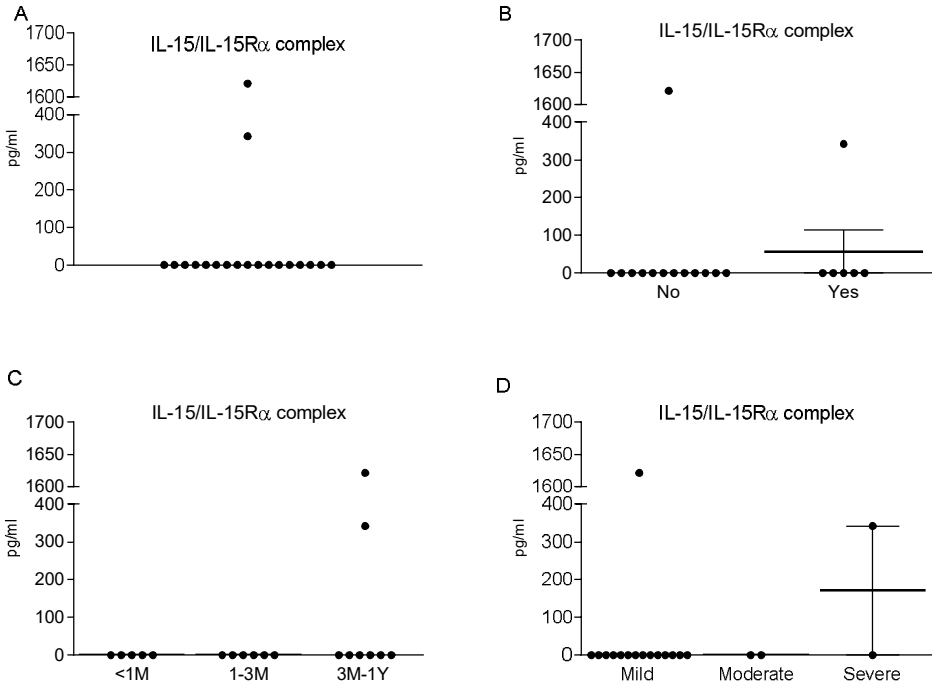


Figure 4.19. IL-15/IL-15Rα complex concentrations in RV-only positive NPAs from infants under 1 years old. Data is shown as protein expression in A) all NPAs, B) with oxygen administration, C) between age groups and D) disease severity. M, months and Y, year. Data is expressed as the mean ± SEM (n=19).

Only 2/19 NPAs were positive for IL-15/IL-15Rα complex with values of 342 and 1621 pg/ml (Figure 4.19A). Due to the small number of positive values (Figure 4.19B, C, D) statistical analysis and correlation with oxygen requirement, age groups and severity groups were not made.

These results indicate that with the exception of two samples, the IL-15/IL-15Rα complex was not present at levels above the minimum level of detection in this assay 62.5pg/ml.

4.3.9.3. IL-18 protein levels in NPAs from infants with RV infection

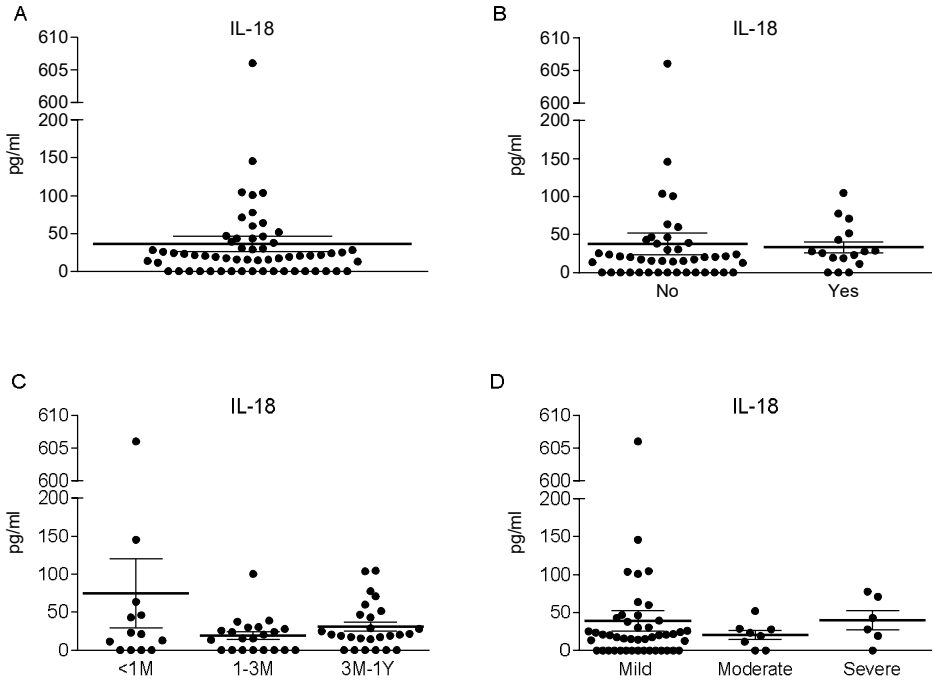


Figure 4.20. IL-18 concentrations in RV-only positive NPAs from infants under 1 years old. Data is shown as protein expression in A) all NPAs, B) with oxygen administration, C) between age groups and D) disease severity. M, months and Y, year. Data is expressed as the mean ± SEM (n=60) (B, Mann-Whitney U-test and C, D Kruskal-Wallis with Conover-Inman post-hoc test).

The mean value obtained for IL-18 expression was 36.35pg/ml with 18/60 NPAs negative in this assay (Figure 4.20A). One NPA had an IL-18 protein level of 600pg/ml. No significant differences in IL-18 expression dependant on between oxygen requirement, age groups or disease severity was observed (Figure 4.20B, C, D).

These results indicate that IL-18 expression in NPAs from infants under 1 years old and infected with RV do not differ with age or disease severity.

4.3.9.4. Correlations between IL-15 and other proteins in NPAs from infants with RV infection

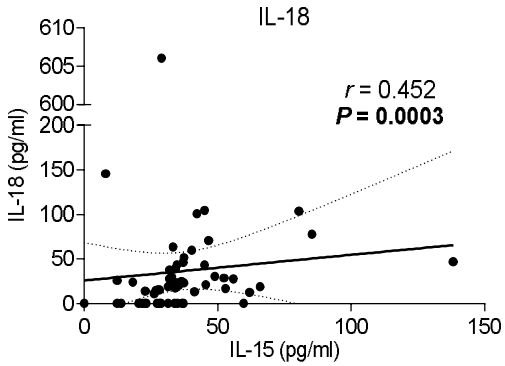


Figure 4.21. Correlations between protein levels of IL-15 against IL-18 in RV-only positive NPAs from infants under 1 years old. Correlations were derived by Spearman's rank order analysis, P two-tailed, n = number of XY pairs. Statistical significant correlation (p<0.001) is given in bold (n=60).

Also determined was if protein levels observed above for IL-18 show positive or negative correlation to IL-15. Figure 4.21 details the correlations between protein levels detected in NPAs. A positive but weak correlation coefficient of 0.45, was observed between IL-15 and IL-18, which was significant (p<0.001).

4.3.10. Comparison between IL-15 protein levels in paediatric nasopharyngeal aspirates during RSV and RV infection

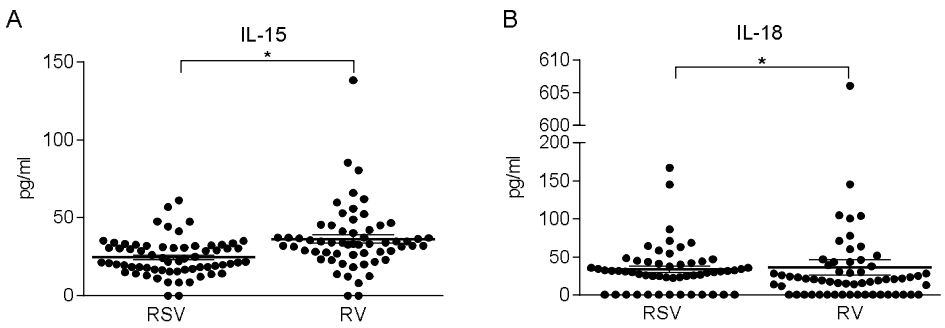


Figure 4.22. Comparison between IL-15 protein in NPAs from infants with RSV or RV infection. Data is shown as protein values for A) IL-15 (RSV n=56, RV n=60), B) IL-18 (RSV n=56, RV n=60) between RSV and RV. Data is expressed as the mean ± SEM (Mann-Whitney U-test, *p<0.05).

Comparison was made between the results obtained with samples from RSV patients and those with RV for both IL-15 and IL-18. NPAs from infants with RV infection contained significantly higher IL-15 protein ($p<0.05$) compared to those with RSV by ~11pg/ml (Figure 4.22A). NPAs from infants with RV infection had significantly higher IL-18 protein ($p<0.05$) than those with RV (Figure 4.22B).

The significant increase in IL-18 protein for those with RV than those with RSV was possibly due to the one sample containing ~600pg/ml protein. Therefore, only IL-15 was taken for further comparison to determine if those with RV infection had higher IL-15 protein levels with oxygen requirement, between age groups and with disease severity, compared to those with RSV infection.

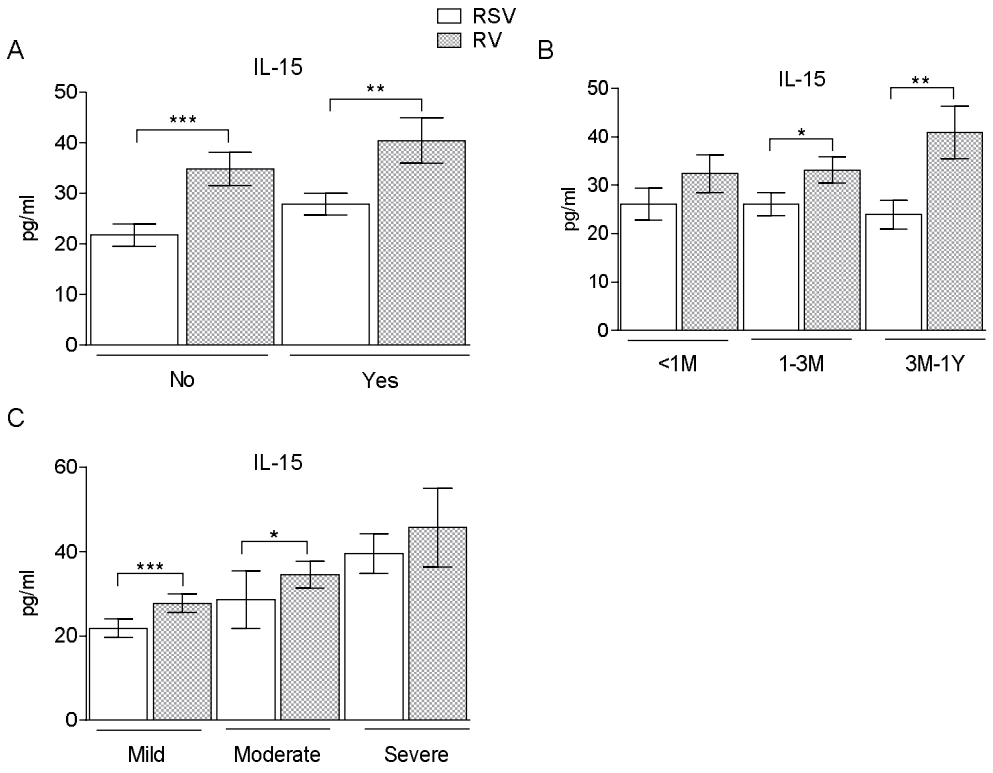


Figure 4.23. Comparison between oxygen requirement, age and disease severity for IL-15 protein levels in NPAs from infants with RSV or RV infection. Data is shown as protein expression in A) with oxygen administration (RSV no n=22, RSV yes n=34, RV no n=44, RV yes n=16), B) between age groups (RSV <1M n=16, RSV 1-3M n=24, RSV 3M-1Y n=15, RV <1M n=13, RV 1-3M n=21, RV 3M-1Y n=26) and C) disease severity (RSV mild n=22, RSV moderate n=27, RSV severe n=7, RV mild n=46, RV moderate n=8, RV severe n=6). M, months and Y, year. Data is expressed as the mean ± SEM and (Mann-Whitney U-test, *p<0.05, **p<0.01).

RV NPA samples from those who did (p<0.01) or did not (p<0.001) require oxygen treatment contained significantly higher IL-15 protein levels than those from RSV infected infants (Figure 4.23A). NPAs from RV infants who were aged 1-3M (p<0.05) and those aged 3M-1Y (p<0.01) had significantly higher IL-15 protein levels compared from those to RSV infected infants (Figure 4.23A). NPAs from RV infants from those with mild disease (p<0.001) and those with moderate disease (p<0.05) had significantly higher IL-15 protein levels compared to RSV infected infants (Figure 4.23C).

4.4. Discussion

This chapter aimed to extend the hypothesis detailed in Chapter 3 which was that AECs, after infection, can activate NK cells by expression of NK cell-activating cytokines and IL-15 bound to IL-15Rα receptor. Here the expression of NK cell activating cytokines IL-12, IL-15, IL-18 and IL-15Rα from infected HNAECs was first characterised. This included a comparison in expression of IL-15 and IL-15Rα mRNA during infection of HNAECs with RSV A2 and clinical isolate RSV X. Next, a HNAEC-NK cell co-culture model was established to determine if infected HNAECs could initiate a cytokine response from donor matched NK cells. Finally, NPAs from RSV and RV infected infants were analysed to show that these proteins are expressed 1) *in vivo* during RSV or RV infection and 2) a comparison was made between RSV and RV results to determine if these viruses induced significantly different patterns of NK cell cytokine expression.

4.4.1. Expression of NK cell activating cytokines IL-12, IL-15 and IL-18, the IL-15Rα receptor and IL-15/IL-15Rα complex expression by HNAECs in response to RSV A2 infection

4.4.1.1. HNAECs do not express IL-12 during RSV infection

Here, evidence of only a low expression of IL-12β mRNA was seen in HNAECs at 48 hours in the presence or absence of infection (Figure 4.3A). IL-12β mRNA expression was less than 0.05% of housekeeping gene L32 and significantly unchanged by RSV infection. For some donors, IL-12β mRNA was not observed in all samples. No evidence of IL-12 protein expression as determined by IL-12p70 subunit measurement, was obtained. Similar results were obtained with BEAS-2B cells (Figure 3.2C). In conclusion, IL-12 expression by HNAECs and BEAS-2B cells is not influenced by RSV infection and this may also be true for AECs *in vivo*.

4.4.1.2. HNAECs express IL-18 protein during RSV infection

Both non-infected and RSV infected HNAECs expressed IL-18 mRNA ranging between ~70-250% relative to the housekeeping gene L32 (Figure 4.4B). RSV infection reduced the expression of IL-18 mRNA at all MOI compared to non-infected HNAECs (Figure 4.4A). This observation was further confirmed by results obtained with the anti-RSV control which showed significantly higher IL-18 mRNA expression compare to infection. The reduction in IL-18 mRNA following RSV infection was only minor at ~0.25-fold less than control at an MOI of 1.

Unlike BEAS-2B cells, HNAECs expressed IL-18 protein. For each individual donor IL-18 protein was reduced following infection at an MOI of 0.1, which correlated with the observed decrease in IL-18 mRNA at MOI 0.1 (Figure 4.4C, D). At an MOI of 1, IL-18 protein expression was the same as non-infected HNAECs and at a higher MOI of 2.5, IL-18 protein was significantly greater. Protein expression was reduced in cultures with Palivizumab treatment indicating this expression is an RSV-specific response.

Interestingly, the results observed here using HNAECs and a reduction in IL-18 mRNA at an MOI 1 was also observed with BEAS-2B cells (Figure 3.2C). Furthermore, between infected BEAS-2B cells over time, IL-18 mRNA was significantly reduced (Figure 3.2C). One explanation for this could be a method of controlling IL-18 expression. Under resting conditions, IL-18 mRNA and pro-IL-18 is expressed however during infection, mRNA synthesis is inhibited to reduce excessive inflammatory responses. This was suggested as a mechanism for keratinocyte damage and wound healing with keratinocytes showing reduced IL-18 mRNA expression when stimulated with cytokines (TNF-α, IL-1β, IFN-γ) yet resulted in simultaneous and increasing IL-18 protein detected over time (429). Another possibility is that RSV infection leads to downregulation of IL-18 mRNA as a method to allow for increased viral spread at lower MOIs and thus early immune evasion. Inhibition in expression mediated by

NS1 and NS2 viral proteins has been shown for anti-viral IFNs (Section 1.2.1). Reduced early AEC-derived IL-18 expression could lead to reduced or altered NK cell activation, and could also affect other IL-18 responsive immune cells. Use of RSV KO strains without NS1 and NS1 proteins would allow for determine if RSV does modulate IL-18 mRNA expression in AECs.

4.4.1.3. HNAECs express IL-15 mRNA during RSV infection but soluble protein was not detected.

A significant increase in HNAECs IL-15 mRNA expression of ~1.5-fold, was observed at MOI 2.5 (Figure 4.5A). For all donors, RSV increased IL-15 mRNA levels, particularly at an MOI of 2.5. Although this indicates that the IL-15 response may be different between individuals, due to the complex nature and tight regulation of IL-15 translation and transcription, variation in IL-15 protein may not be observed. This may be true because IL-15 protein was not observed below 3pg/ml (Figure 4.5B, C). However, as described in Chapter 3, IL-15 is expressed at the cell surface on BEAS-2B cells during RSV infection. Therefore determining the cell surface expression of IL-15 on HNAECs would provide a better insight into the individual expression of IL-15, rather than soluble protein expression in culture supernatants, and therefore provide more information on the potential of IL-15 signalling by HNAECs.

In comparison to the results observed using RSV infected BEAS-2B cells, the overall expression of IL-15 mRNA by HNAECs was ~3-10-fold higher in non-infected HNAECs than non-infected BEAS-2B cells (Section 3.3.3). Similar to BEAS-2B cells at an MOI of 1 with 48hr infection, HNAECs also did not show a significant increase in IL-15 mRNA levels. Only with higher RSV MOI was there a significant increase in IL-15 mRNA levels by HNAECs. This could suggest that induction of IL-15 mRNA by AECs may only be seen during infection with higher viral titres. However, as HNAECs express a higher basal expression of IL-15m RNA, this could

suggest that primary epithelial cells may be primed for the initiation of NK cell stimulation and other IL-15 responsive immune cells during RSV infection.

The results here cannot be used to support the conclusion that IL-15 protein was not expressed by HNAECs as IL-15 has been shown to be ~2.5pg/ml with RSV infection in HBECs and ~1pg/ml during no infection, which is below the detection limit of the Luminex assay used here (152,341). In comparison to results observed by Zdrenghea *et al.*, IL-15 mRNA was increased by ~3-fold at an MOI of 1 with 24hr RSV infection, which is more than what was observed here in Figure 4.5A at ~1.5-fold (152). This may reflect the different cell types used, here being nasal epithelial cells and for Zdrenghea *et al*., bronchial epithelial cells. This could also suggest that lower AECs could be a better model to use for AEC-NK cell co-cultures and is discussed further in Chapter 6.

4.4.1.4. HNAECs express IL-15Rα during RSV infection

IL-15Rα mRNA levels were ~15-50% of the housekeeping gene L32 in non-infected HNAECs and increased by RSV by ~3-6 fold for all MOIs (Figure 4.6). IL-15Rα was also detected at the cell surface, similar to BEAS-2B cells (Figure 4.6D). Therefore, RSV can induce IL-15Rα expression in both BEAS-2B cells and HNAECs and therefore is likely to be a common response to RSV infection by AECs *in vivo*. HNAECs had a higher basal expression of IL-15Rα mRNA compared to BEAS-2B cells and with this in mind, HNAECs may potentially express more IL-15/IL-15Rα complex, either presented at the cell surface or as a secreted complex, compared to BEAS-2B cells. However, RSV produced a greater fold increase in IL-15Rα mRNA in BEAS-2B cells compared to HNAECs in relation to basal expression level. Culture supernatants were used for IL-15/IL-15Rα complex protein analysis but for HNAECs no protein was detected. This does not however, fully support the conclusion that IL-15/IL-15Rα complexes are not secreted by HNAECs and a method of protein detection below 62.5pg/ml

would clarify this. Overall, RSV induced expression of IL-15Rα by AECs could suggest a specific method of IL-15 signalling through cell surface complex expression in comparison to soluble IL-15 signalling. This could occur *in vivo* and is discussed further in Chapter 6.

4.4.2. Comparison between the expression of IL-15 and IL-15Rα by HNAECs in response to infection with two different RSV A strains, A2 lab strain and clinical isolate RSV X

Expression of IL-15 mRNA was significantly higher by ~4-fold with RSV X compared to RSV A2 infection (Figure 4.8A). IL-15Rα mRNA expression was similar for each RSV strain (Figure 4.8B). An increase in IL-15 mRNA level induced by RSV X compared to RSV A2 may not necessarily correlate to increased protein expression due to the complex nature of IL-15 post transcription controls. However, it is possible that there could be an increase in IL-15 protein synthesis and possibly IL-15/IL-15Rα complex formation with RSV X infection in HNAECs. This however would need to be demonstrated through cell surface flow cytometry and culture supernatant analysis.

4.4.3. Following RSV infection, can HNAECs activate NK cells?

4.4.3.1. Does co-culture of donor matched NK cells with RSV infected HNAECs influence viral replication and IL-8 response?

Similarly to infected BEAS-2B cells co-cultured with NK cells in Section 3.3.8.2, a lower expression of RSV N RNA was also observed when infected HNAECs were co-cultured with NK cells. Therefore RSV N RNA expression was expressed as relative to infected HNAECs without NK cell co-culture. RSV N gene expression was significantly reduced in all HNAEC-NK cell co-cultures (Figure 4.9A, B). At a ratio of 1:3, RSV N gene was ~0.5-fold lower than from infected HNAECs without NK cells. This could be reflective of NK cell-derived housekeeping gene L32 which would reduce true RSV N gene levels specific to infected BEAS-2B cells.

Another possibility is that the presence of NK cell derived anti-viral cytokines and/or targeted cell lysis of infected cells. A decrease in RSV L gene expression was observed during HBEC-$CD8^+$ T cell co-culture and these cells, similarly to NK cells, also express both anti-viral cytokines and display cytotoxic responses (430). Therefore, NK cells in this model may express a cytolytic phenotype and induce infected-AEC apoptosis. However as discussed in Section 3.4.3.1, this decrease in RSV RNA gene expression observed in this work and the published study could be due to additional housekeeping gene contribution from NK cells.

IL-8 mRNA and protein levels remained the same for all conditions, with and without RSV infection or NK cell co-culture (Figure 4.9C, D). This result was also similar for BEAS-2B cell-NK cell co-cultures (Figure 3.10C). Therefore, addition of NK cells to infected HNAECs does not alter IL-8 expression, regardless of the apparent decrease in RSV N gene expression. This may indicate that the decrease in RSV N gene during co-cultures is not due to NK cell cytotoxicity, which could have been displayed as a decrease in IL-8 expression. Again, this would need further evaluation.

4.4.3.2. IFN-γ is expressed during co-culture of HNAECs and NK cells

IFN-γ mRNA and protein were only expressed in co-cultures with NK cells present (Figure 4.10). IFN-γ protein was only present in all HNAEC-NK cell co-cultures at a ratio of 1:3 and only expressed when NK cells are present in cultures, again suggesting that the IFN-γ protein detected is NK cell specific (Figure 4.10C, D). IFN-γ protein was also expressed from 2/4 non-infected HNAEC-NK cell co-cultures which was not seen for BEAS-2B cells and could be due to donor matching (Figure 3.11B). In the lung, resting HNAECs will likely be in contact with resident NK cells and may provide survival of resident NK cells to maintain beneficial NK numbers for potential infection. Resident NK cells may therefore express a small level IFN-γ which could act to induce expression of certain IFN-γ inducible genes, like ICAM-1, which

could aid in continued AEC-NK cell interactions without infection. This possibility is examined further in Chapter 5.

4.4.3.3. TNF-α expression during co-culture is not NK cell specific

A basal level of TNF-α mRNA and protein was observed from HNAECs without NK cells which could be necessary for AECs, such as for survival and cell signalling. TNF-α mRNA, although not significant showed an apparent decrease in expression with addition of NK cells at a ratio of 1:3 compared to infected HNAECs without NK cells. Therefore these results demonstrate that soluble TNF-α may not be a suitable cytokine to measure NK cell activation within these co-cultures as it is not NK cell specific. However, detection of TNF-α protein by other means could have been used. Furthermore, membrane-bound TNF-α has also been reported on human peripheral blood NK cells, NK92 cells and IL-2 stimulated mouse NK cells (384,385,431), with IL-15 also being shown to upregulate membrane-bound TNF-α on human peripheral NK cells (384). Measurement by flow cytometry of intracellular or membrane-bound TNF-α, would have provided more information about NK cell-specific TNF-α expression during HNAEC co-cultures missed when using the ELISA for soluble protein analysis.

4.4.4. Heterogeneity in HNAEC responses to RSV

There is great heterogeneity between both level and patterns of gene and protein expression between HNAEC donors without and with RSV infection and also NK cell responses during co-culture. Although these experiments were done *in vitro*, differences in responses between individuals during *in vivo* infection may also occur. An individual's AEC-expression profile at any one time may reflect RSV disease severity and requires further characterisation. Overall, heterogeneity in individual donor responses has been observed for

expression of NK cell activating IL-15, IL-15Rα and IL-18 from HNAECs and this has also been reflected in expression of IFN-γ and TNF-α during HNAEC-NK cell co-cultures with some donors expressing IFN-γ and others not. For example during RSV infection, donor 2 showed the biggest induction of IL-15Rα mRNA, most IL-18 protein and was the only donor to show IFN-γ expression in all cultures with NK cells in comparison to other donors. However, this is only speculative and for conformation of this, repeated HNAEC isolations from the same donor would need to be done. The responses shown here also are only measured at one time point and individual donor kinetics of gene expression may result in different peaks in protein expression at different times.

4.4.5. Expression of IL-15, IL-15/IL-15Rα complex, IL-18, IFN-γ and TNF-α in NPAs from RSV and RV infected infants

To support the hypothesis here that alone AECs, after infection, can activate NK cells, *in vivo* expression of NK cell activating cytokines were examined in airway fluids from RSV and RV infected infants.

4.4.5.1. IL-15 and IL-18 are associated with oxygen administration with RSV infection only

For those who required oxygen, IL-15 protein was slightly but significantly increased by ~6pg/ml (Figure 4.12B). This is not much, however severity scores show that those with the highest IL-15 protein levels had moderate or severe disease (Figure 4.12D).

IL-15/IL-15Rα complex was only detected in 6/56 NPAs and this could be reflective of the detection limit of the assay as 4 out of these 6 positive were just about the detection limit (62.5pg/ml) at 74, 74, 82, 86pg/ml (Figure 4.13). Therefore it cannot be concluded that IL-15/IL-15Rα complex was not present in NPAs and requires further analysis.

IL-18 protein was slightly but significantly increased by ~18pg/ml in NPAs from those who required oxygen compared to those who didn't (Figure 4.14A, B). Furthermore, more NPAs

were negative for IL-18 protein from infants who did not require oxygen compared to those who did.

4.4.5.2. Those aged 1-3 months old and infected with RSV show different expression of IFN-γ and TNF-α protein

For those aged 1-3 months old, lower IFN-γ protein and higher TNF-α protein was observed compared to younger and older infants (Figures 5.23 and 5.24). However, this is a small sample size making it hard to conclude that this is a true response within this age group. Analysis of more samples might support the veracity of this observation. However, infants within this age group are likely to have waning maternal antibody titres compared to infants aged less than 1 month and less self-produced antibodies than those aged 3 months above. How this relates to the results shown here is difficult to explain without speculating.

Other studies observed no differences for IFN-γ protein between age during RSV infection (432,433). However, the overall levels of IFN-γ and TNF-α are similar to other studies. Nasal IFN-γ ranged from 0-~70pg/ml in children with RSV and was shown to be positively correlated to TNF-α levels (434). Tracheal aspirates from infants with RSV bronchiolitis observed IFN-γ ranged from ~10-54pg/ml and TNF-α ranged from ~11-18pg/ml (435). IFN-γ protein was higher in nasal washes from infants under 1 years old with influenza compared to those with RSV (433). Another study also showed significantly less IFN-γ in serum of RSV infected infants compared to influenza, parainfluenza and adenovirus, suggesting (436). Furthermore, non-ventilated infants with RSV bronchiolitis had higher levels of IFN-γ in NPAs than those who required mechanically ventilation or oxygen (432,437). RSV may fail to induce a robust IFN-γ response which lead to increased disease severity. Lower IFN-γ levels could limit IFN-γ-inducible gene expression, such as anti-viral proteins and IFN-γ-inducible chemokines, which

would otherwise reduce viral spread and recruit adaptive immune cell populations to sites of infection.

4.4.5.3. Correlations between NPA cytokines.

For infants with RV infection, a weak but positive correlation was observed between IL-15 and IL-18 levels (Figure 4.21). Increase in both these proteins at the same time could lead to synergistic enhancement of NK cell and T cell responses. However, analysis of proteins in this way does not conclude if increase in either IL-15 or IL-18 leads to increased expression of the other cytokine.

4.4.5.4. NPAs from infants with RV infection show higher expression of IL-15 and IL-18.

Infants with RV infection had higher NPA level of IL-15 and IL-18 protein compared to those with RSV (Figure 4.22). For IL-15, the difference was only small at ~11pg/ml and for IL-18 this significance could have been driven through one sample with ~600pg/ml IL-18. Comparisons between oxygen requirement show that those with RV had higher IL-15 levels than those with RSV infection whether they required oxygen or not (Figure 4.23A). This comparison details that overall RV infection may induce more IL-15 protein than RSV and only in those with RSV infection is a higher level of IL-15 protein potentially associated with increased disease severity.

Other clinical studies have also reported IL-15, IL-18, IFN-γ and TNF-α in mucosal and serum samples collected during RSV infection. This is the first account of IL-15/IL-15Rα complex analysis from infants infected with RSV and RV. Tracheal aspirates from infants with RSV bronchiolitis also showed similar IL-15 protein levels at ~30-37pg/ml suggesting low IL-15 protein levels throughout the lower and upper airways (435). In regards to the data shown here, low respiratory IL-15 levels could indicate that IL-15 secretion is highly regulated during

RSV infection and its expression may be seen as part of IL-15/IL-15Rα complexes presented at the AEC cell surface. Serum IL-15 has been shown to be increased with disease severity in RSV infected infants (438). Here, higher IL-15 levels in NPAs did not correlate to more severe RSV or RV disease, only with oxygen requirement for those with RSV infection, and could be reflective of the small sample size for the severe groups. The results of a comprehensive analysis of NPA from children with bronchiolitis show some similarities to observations in this work, with overall higher IL-15 levels in samples from infants with RV infection (439). Overall, these results could indicate that during RSV infection, IL-15 responses could be lower compared to RV infection which could alter immune response outcomes for each virus. However, airway fluid analysis does not regard IL-15 trans-presentation signalling though IL-15/IL-15Rα complex.

4.4.5.5. Limitations of analysing discarded NPAs

Use of discarded NPAs which were taken when infants displayed symptoms that led to guardians taking them to the hospital could suggest that the cytokines analysed here may be more reflective of later stages of RSV infection when NK cell population expansion is starting to decline, as shown in mice. It is impossible to collect NPAs from infants on the first day or second of RSV infection which would be more representative of the environment NK cells will be exposed to and IFN-γ and TNF-α potentially derived from NK cells. However, the NK cell population expansion and decline has not been studied in humans and may not represent that shown in mouse models. Regardless, IL-15 and IL-18 are still present in NPAs from infants with RSV infection during later stages of RSV infection and levels higher in those requiring oxygen. Higher expression of IL-15 and IL-18 many days after initial infection may lead to prolonged NK cell activation, population expansion and increased T cell responses which may reflect an increase in disease severity. Furthermore, hospital stays for infants with RSV infection are longer compared to those with RV bronchiolitis and additionally RV

symptoms present sooner than for RSV (440,441). Considering the published data together with results observed from the NPA analysis, RSV modulation of the immune response may lead to increased IL-15 and IL-18 expression which may reflect disease severity and longer hospital stays in comparison with RV.

Another limitation is use of NPA in comparison to analysis of BAL. BAL may have provided a more representative response within the lung and thus more accurately reflect RSV severity. To allow for further characterisation of NK cell activating cytokines and NK cell-derived cytokines during RSV infection, a prospective study could be developed in which NPAs and/or BAL are taken from infants each day after admission for up to 1 week. It may also be possible to collect NK cells from BAL which could allow for phenotyping. Comparison from infants with other respiratory disease may provide more detail into how RSV modulates the immune response and if one of these pathways affects IL-15 and IL-18 expressing and responsive cells.

Overall, the results for NPAs analysed here indicate that higher levels of IL-15 and IL-18 may be associated with RSV disease severity which is not observed for those with RV infection. Furthermore, although an increase in IL-15 and IL-18 levels was observed in NPAs from infants with RV in comparison to those with RSV, IL-15 and IL-18 levels were not associated with an increase in disease severity for those with RV infection. Therefore, IL-15 and IL-18 responsive cells during RSV infection may be the populations that are contributing to severity, such as NK cells and T cells, which is not seen for those with RV infection.

4.4.6. Limitations of an *in vitro* HNAEC-NK cell co-culture model compared to *in vivo* HNAEC-NK cell responses during RSV infection

Similar to the limitations mentioned with the BEAS-2B cell-NK cell co-culture model (Section 3.4.7), peripheral blood NK cells may not respond in the same way as resident lung NK cells. A comparison between human peripheral blood and nasal NK cells observed that gene

expression profiles differs between the two (442). Analysis indicated that nasal NK cells may express a less cytotoxic phenotype than peripheral blood NK cells. IL-12 was also not expressed by HNAECs and may therefore produce different NK cell response than that *in vivo.* This model is still an improvement on that with use of BEAS-2B cells to determine NK cell responses during co-culture. However, an even better model would be to use nasal NK cells with HNAECs as human nasal NK cells have a different phenotype compared to lung NK cells (262). Furthermore, ALI culture is conclusively a better model than that of submerged monolayers such as having similar gene profiles to *in vivo* cells, although is still not a perfect match (443,444). The best *in vitro* model that could be achieved would be the use of ALI cultured HBECs with donor matched lung NK cells. Regardless, the AEC-NK cell co-culture used here is still a better model than characterising responses to RSV with AECs or NK cells alone. A strength of this model has been its ability to demonstrate that recruited peripheral NK cells can be activated by RSV infected HNAECs to express IFN-γ. Furthermore, it has highlighted the heterogeneity of individual AEC responses to RSV and that impaired airway epithelium responses for some could ultimately lead to imbalances in immune cell responses.

4.5. Summary

RSV infection of HNAECs induces increased expression of both IL-15 and IL-15Rα mRNA, cell surface IL-15Rα protein and IL-18 protein. IL-15 protein was not detected in culture supernatants with 48hrs infection up to MOI 2.5. Clinical isolate RSV X induced more IL-15 mRNA than RSV A2. IL-18 mRNA protein has a reduced expression with RSV at an MOI of 0.5 compared to control non-infected HNAECs. It was then shown that RSV infected HNAECs could stimulate NK cell IFN-γ expression. This was observed for NK cells at a ratio of 1:3 for all donors. TNF-α protein was also expressed by non-infected and infected HNAECs within

this co-culture model with no apparent increase with co-culture with NK cells. These results show that different donors displayed different NK cell activating cytokine expression profiles which may reflect the different expression of IFN-γ during HNAEC-NK cell co-culture. NPA samples from infants infected with RSV and requiring oxygen during hospital admission show higher IL-15 and IL-18 protein levels than those who did not need oxygen treatment. Those aged 1-3 months displayed higher IFN-γ and lower TNF-α protein in NPAs compared to those younger and older which may reflect changes in the developing immune system. For infants infected with RV, IL-15 and IL-18 protein levels in NPAs were not different between disease severities. Infants infected with RV showed higher NPA IL-15 protein levels than those infected with RSV. This trend towards an increase with disease severity was not observed for IL-18. Overall, these results demonstrate that the RSV infected nasal airway epithelium can alone initiate an IFN-γ response from donor matched NK cells and that nasal IL-15 and IL-18 protein levels may correlate to disease severity in infants with RSV bronchiolitis.

Chapter 5. Enhanced immune responses during RSV infection of airway epithelial cells during co-culture with NK cells

5.1. Introduction

In Chapter 3, RSV infection of BEAS-2B cells was shown to induce the expression of IL-15 and IL-15Rα mRNA, cell surface IL-15 and IL-15Rα and soluble IL-15 protein. During co-culture of RSV infected BEAS-2B cells and NK cells, NK cells were shown to express IFN-γ indicating activation. In Chapter 4 these results were extended to primary AECs and co-culture with donor matched NK cells. RSV infection of HNAECs induced expression of IL-15 and IL-15Rα mRNA and cell surface IL-15Rα. During co-culture of RSV infected HNAECs, NK cells expressed IFN-γ. Overall these results indicate that during RSV infection, AECs are capable of activating NK cells. In turn, NK cell-derived cytokines within this co-culture model could influence the AEC response. IFN-γ and TNF-α were expressed in co-cultures and levels were higher when NK cells were present. NK cell derived cytokines, such as IFN-γ and TNF-α observed in Chapters 3 and 4 during co-culture, could lead to the expression of IFN-γ and TNF-α inducible gene expression by AECs. For example, TNF-α has been shown to induce gene expression of IL-15 by ~2-fold and IL-15Rα by ~8-fold, 10ng/ml and 20ng/ml, respectively, in A549 cells (445,446). This could then lead to enhanced inflammatory cell recruitment which is co-ordinated through both AECs and NK cells.

The work described in this chapter aimed to further characterise the interactions between AECs and NK cells using my established co-culture model. Emphasis was placed on measuring expression of IFN-γ and TNF-α induced AEC-derived immune mediators. I examined the hypothesis that NK cells, through expression of IFN-γ and TNF-α, influence the AEC immune and inflammatory responses and that the cytokine environment before infection also determines BEAS-2B cell activation of NK cells. A selected IFN-γ/TNF-α-inducible AEC-derived cytokine profile was measured. This profile was chosen because previous studies have shown

proteins within this group to be significantly upregulated during RSV infection including the Th1 response associated chemokines CXCL9, CXCL10, CXCL11 (234), Th2 response associated chemokine TARC (233,447), B cell activating cytokine BAFF (145,448) and immune cell adhesion molecule ICAM-1 (449–451).

First, expression of the selected IFN-γ-inducible AEC-derived cytokine profile was characterised within the AEC-NK cell co-culture models following infection of with either RSV A2 or RSV X. Neutralising antibodies were used to confirm the observed effects are dependent on IFN-γ and TNF-α. As described in Section 1.2.3, differential cytokine environments such (e.g.Th1 or Th2) may alter the resultant AEC inflammatory profile, both before and during RSV infection. Therefore the response of BEAS-2B cells primed in Th1 and Th2 cytokine environments was measured and responses further characterised during infected BEAS-2B cell-NK cell co-cultures.

5.2. Aims

- To characterise AEC-NK cell interactions through expression of AEC-derived CXCL9, CXCL10, CXCL11, TARC and BAFF during co-culture of RSV infected AECs and NK cells (Sections 5.3.1, 5.3.3 and 5.3.4).
- To compare the effect of NK cell-induced expression of BEAS-2B cell-derived Th1 associated chemokines in response to infection with two different RSV A strains, A2 lab strain and clinical isolate X (Section 5.3.2).
- To characterise how Th1 and Th2 cytokine environments influence the expression of BEAS-2B cell derived immune molecules which could then influence NK cell responses during BEAS-2B cell-NK cell co-culture (Section 5.3.5).

5.3. Results

5.3.1. Expression of chemokines, cytokines and adhesion molecules during BEAS-2B-NK cell co-culture

5.3.1.1. Th1 associated chemokines CXCL9, CXCL10 and CXCL11

To determine if NK cells influence the expression of BEAS-2B cell derived Th1 associated chemokines CXCL9, CXCL10 and CXCL11 proteins and CXCL10 mRNA expression were evaluated by ELISA and qPCR, respectively (Section 2.6.3 and 2.7).

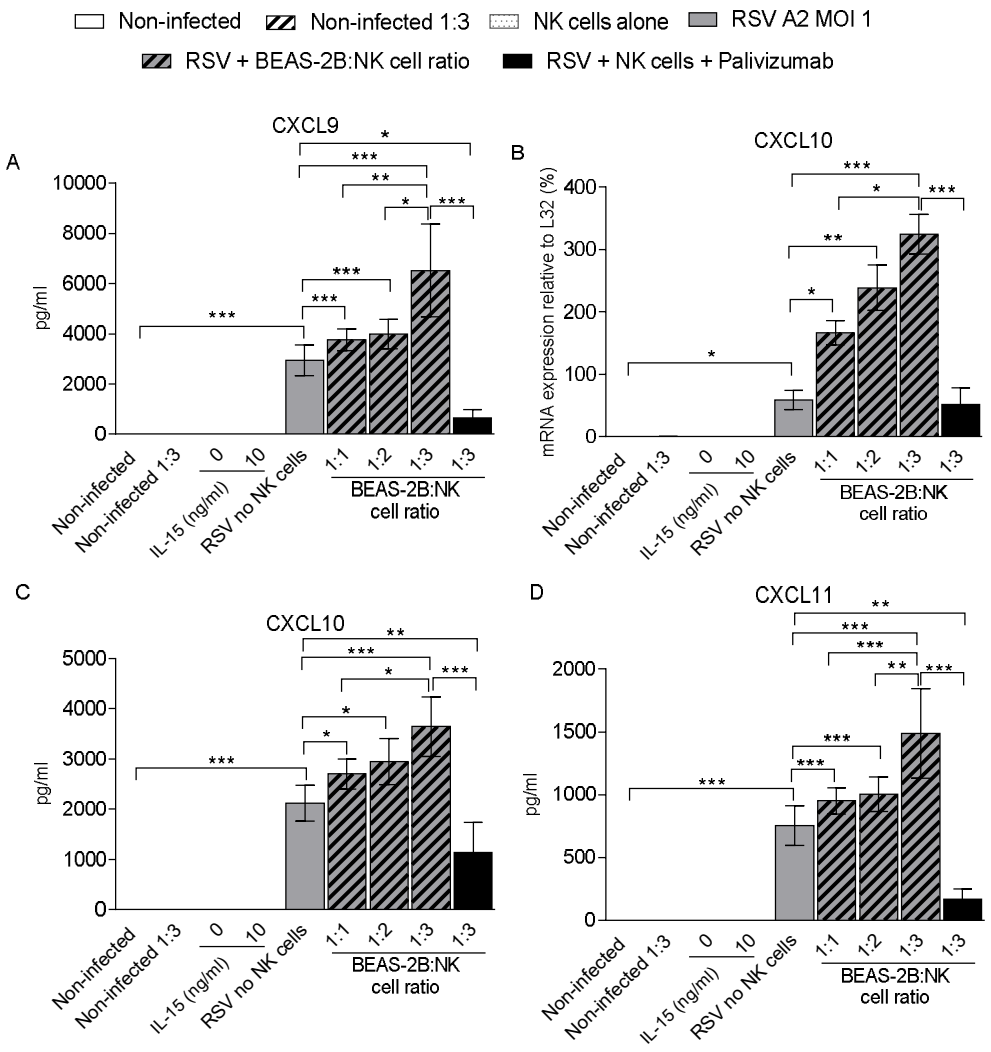


Figure 5.1. Expression of Th1 recruitment associated chemokines CXCL9, CXCL10 and CXCL11 in RSV A2 infected BEAS-2B cells co-cultured with NK cells. BEAS-2B cells were infected with RSV A2 at MOI 1 for 24hr. NK cells were isolated from healthy adult PBMCs and added at increasing ratios to BEAS-2B cells for a further 24hr. Control non-infected cultures were cultured in the absence of RSV and Palivizumab used as an anti-RSV control. A) CXCL9 protein (n=4), B) CXCL10 mRNA (n=3), C) CXCL10 protein (n=3), D) CXCL11 protein (n=4). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01, ***p<0.001).

CXCL9 protein was found in culture supernatants from infected BEAS-2B cells, cultured with or without NK cells (Figure 5.1A). NK cells cultured alone or with 10ng/ml IL-15 did not express CXCL9 protein. CXCL9 was not detected in culture supernatants from NK cells cultured with control non-infected BEAS-2B cells. There was a significant increase in CXCL9

protein detected from infected BEAS-2B cells alone without NK cells at ~2900pg/ml ($p<0.001$) compared to non-infected BEAS-2B cells. CXCL9 expression was significantly increased by addition of NK cells at ratios of 1:1 ($p<0.001$), 1:2 ($p<0.001$) and 1:3 ($p<0.001$) to ~3700pg/ml, ~4000pg/ml and ~6500pg/ml, respectively, compared to infected BEAS-2B cells alone. There was significantly higher expression of CXCL9 protein at an NK cell ratio of 1:3 compared to ratios of 1:1 ($p<0.01$) and 1:2 ($p<0.05$) (Figure 5.1A). At a ratio of 1:3, there was ~2-fold increase in CXCL9 protein expression compared to infected BEAS-2B cells without NK cells. Neutralisation of RSV with the control antibody Palivizumab significantly reduced expression of CXCL9 protein compared to infected BEAS-2B cells without NK cells ($p<0.05$) and infected BEAS-2B cells cultured with NK cells at a ratio of 1:3 ($p<0.001$).

CXCL10 mRNA was expressed at ~0.02% of the housekeeping gene L32 levels by non-infected BEAS-2B cells or NK cells cultured in media alone or with 10ng/ml IL-15 (Figure 5.1B). CXCL10 mRNA levels where similar ~0.23% of the housekeeping gene L32 level in cultures with non-infected BEAS-2B cells co-cultured with NK cells at a ratio of 1:3. There was a significant increase in CXCL10 mRNA expression by infected BEAS-2B cells to ~60% of the housekeeping gene L32 compared to non-infected BEAS-2B cells ($p<0.05$). CXCL10 mRNA expression was significantly increased in infected BEAS-2B cell-NK cell co-cultures at ratios of 1:1 ($p<0.05$), 1:2 ($p<0.01$) and 1:3 ($p<0.001$) being ~166%, ~239% and ~324% of the housekeeping gene L32 levels, respectively, compared to infected BEAS-2B cells alone. There was a significantly higher expression of CXCL10 mRNA in BEAS-2B cell-NK cell co-cultures at ratio of 1:3 ($p<0.05$) compared to ratio of 1:1. Compared to infected BEAS-2B cells without NK cells, CXCL10 mRNA expression was ~2-fold higher at ratios of 1:1 and 1:2 and ~3-fold higher for ratio 1:3 (Figure 5.1B). CXCL10 mRNA expression was significantly reduced in infected BEAS-2B cells cultured with NK cells at a ratio of 1:3 plus Palivizumab compared to infected BEAS-2B cells cultured with NK cells at a ratio of 1:3 ($p<0.001$).

CXCL10 protein was not expressed by control BEAS-2B cells, non-infected BEAS-2B cells co-cultured with NK cells, NK cells cultured in media alone or with 10ng/ml IL-15 (Figure 5.1C). RSV infection of BEAS-2B cells led to a significant increase in CXCL10 protein expression to ~2120pg/ml ($p<0.001$). CXCL10 protein expression was significantly increased further in co-cultures of infected BEAS-2B cells and NK cell at ratios of 1:1 ($p<0.05$), 1:2 ($p<0.05$) and 1:3 ($p<0.001$) to ~2700pg/ml, ~2950pg/ml and ~3650pg/ml, respectively, compared to infected BEAS-2B cells without NK cells. There was significantly higher expression of CXCL10 protein at an NK cell ratio of 1:3 compared to 1:1 ($p<0.05$). Compared to infected BEAS-2B cells without NK cells, CXCL10 protein was ~2-fold higher at a ratio of 1:3. Palivizumab anti-RSV control significantly reduced CXCL10 protein expression by infected BEAS-2B cells without NK cells ($p<0.01$) and infected BEAS-2B cells cultured with NK cells ($p<0.001$).

CXCL11 protein was not expressed by control BEAS-2B cells, non-infected BEAS-2B cells co-cultured with NK cells and NK cells cultured in media alone or with 10ng/ml IL-15 (Figure 5.1D). There was a significant increase in CXCL11 protein expression by infected BEAS-2B cells compared to non-infected BEAS-2B cells ($p<0.001$). CXCL11 protein expression was significantly increased in infected BEAS-2B cell-NK cell co-cultures at ratios of 1:1 ($p<0.001$), 1:2 ($p<0.001$) and 1:3 ($p<0.001$) to ~950pg/ml, ~1000pg/ml and ~1480pg/ml, respectively, when compared to infected BEAS-2B cells without NK cells which had expression at ~750% of the housekeeping gene L32. There was significantly higher expression of CXCL11 protein in co-cultures at a ratio of 1:3 compared to ratios of 1:1 ($p<0.001$) and 1:2 ($p<0.01$). Compared to infected BEAS-2B cells without NK cells, CXCL11 protein was ~0.5-fold higher at a ratio of 1:3. Cultures pre-treated with Palivizumab, an anti-RSV control, had significantly reduced CXCL11 protein expression when compared to infected BEAS-2B cells without NK cells ($p<0.01$) and infected BEAS-2B cells cultured with NK cells at a ratio of 1:3 ($p<0.001$).

These results firstly indicate that RSV induces the expression of CXCL9, CXCL10 and CXCL11 proteins and CXCL10 mRNA in BEAS-2B cells. Expression of CXCL11 protein was ~2-fold lower than CXCL10 during RSV infection. Secondly, NK cells do not express these proteins in culture media alone or with IL-15 stimulation. Thirdly, addition of NK cells to infected BEAS-2B cells increased the expression of these proteins and CXCL10 mRNA. The induced increase in CXCL9 protein expression during NK cell co-culture was greater than observed for CXCL10 and CXCL11.

5.3.1.2. Th2 associated chemokine TARC

TARC has been shown to be expressed by HBECs and AEC lines, A549 and BEAS-2B (452,453). TARC expression was furthermore shown to be induced by IFN-γ. Therefore, to determine if NK cells influence TARC expression by infected BEAS-2B cells, TARC mRNA and protein expression was measured by qPCR and ELISA, respectively (Section 2.6.3 and 2.7).

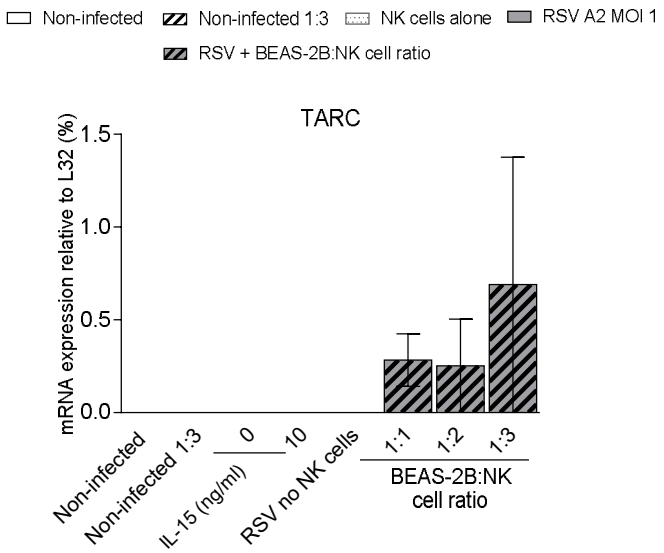


Figure 5.2. Expression of Th2 recruitment associated chemokine TARC in RSV A2 infected BEAS-2B cells co-cultured with NK cells. BEAS-2B cells were infected with RSV A2 at MOI 1 for 24hr. NK cells were isolated from healthy adult PBMCs and added at increasing ratios to BEAS-2B cells for a further 24hr. Control non-infected cultures were cultured in the absence of RSV and Palivizumab used as an anti-RSV control. Data is expressed as the mean ± SEM (n=3).

TARC mRNA expression was only observed in infected BEAS-2B cell-NK cell co-cultures (Figure 5.2). TARC mRNA expression was observed in 2/3 samples at a ratio of 1:1 and 1/3 at a cell ratios of 1:2 and 1:3. TARC mRNA expression ranged from ~0.4-2% of the housekeeping gene L32. TARC protein was not detected in any culture supernatants.

These results indicate that BEAS-2B cells do not express TARC protein, even when infected with RSV. TARC mRNA was only observed in some infected BEAS-2B-NK cell co-cultures, however these results cannot determine if expression is specific to RSV infection and/or NK cell co-culture.

5.3.1.3. Adaptive immune response cytokine BAFF

BAFF has been shown to be expressed by HBECs and AEC lines, A549 and BEAS-2B cells (145,151). BAFF expression has also been shown to be induced by IFN-γ in human intestinal epithelial cells, human decidual stromal cells and AECs (151,454,455). Therefore, to determine if NK cells influence BAFF expression by infected BEAS-2B cells, mRNA and protein were measured by qPCR and ELISA, respectively (Section 2.6.3 and 2.7).

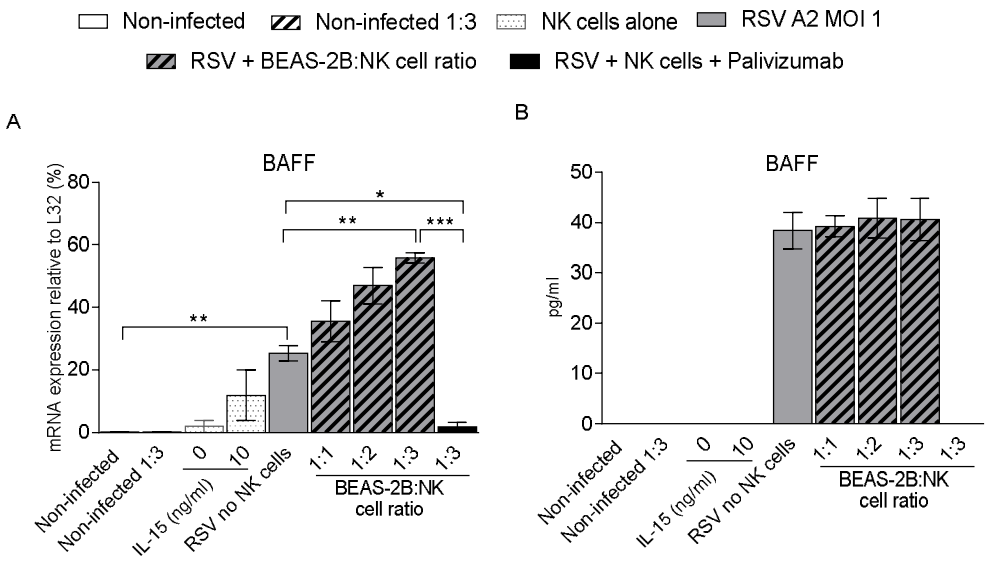


Figure 5.3. Expression of BAFF in RSV A2 infected BEAS-2B cells co-cultured with NK cells. BEAS-2B cells were infected with RSV A2 at MOI 1 for 24hr. NK cells were isolated from healthy adult PBMCs and added at increasing ratios to BEAS-2B cells for a further 24hr. Control non-infected cultures were cultured in the absence of RSV and Palivizumab used as an anti-RSV control. A) BAFF mRNA (n=3), B) BAFF protein (n=4), C) BAFF protein fold change relative to RSV control (n=4). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01, ***p<0.001).

BAFF mRNA expression was detected in non-infected BEAS-2B cells at ~0.14% of the housekeeping gene L32 level (Figure 5.3A). BAFF mRNA was detected in 1/3 NK cell samples cultured in media only and 2/3 samples cultured with 10ng/ml IL-15 at ~1.9% and ~11.92% of the housekeeping gene L32, respectively. BAFF mRNA expression from co-cultures of non-infected BEAS-2B cells with NK cells at a ratio of 1:3 was seen at ~0.2% of the housekeeping gene L32 level. RSV induced expression of BAFF mRNA ($p<0.01$) up to ~25% of the housekeeping gene L32 compared to non-infected BEAS-2B cells. BAFF mRNA was significantly increased in infected BEAS-2B cell-NK cell co-cultures at a ratio of 1:3 ($p<0.01$) to ~55% of the housekeeping gene L32 expression when compared to infected BEAS-2B cells without NK cells. Compared to infected BEAS-2B cells without NK cells, BAFF mRNA expression was ~2-fold higher at a ratio of 1:3. Palivizumab anti-RSV control significantly reduced expression of BAFF mRNA in cultures of infected BEAS-2B cells without NK cells

compared to infected BEAS-2B cells cultured with NK cells at a ratio of 1:3 ($p<0.05$). BAFF mRNA expression was significantly reduced in infected BEAS-2B cells cultured with NK cells at a ratio of 1:3 plus Palivizumab compared to infected BEAS-2B cells cultured with NK cells at a ratio of 1:3 ($p<0.001$).

BAFF protein was expressed in all cultures with RSV infection at ~40pg/ml with no significant difference observed between BEAS-2B cells cultured alone or with NK cells (Figure 5.3B).

These results indicate that BAFF mRNA can be expressed by NK cells and may be induced further by IL-15 stimulation. RSV induces BAFF mRNA expression in BEAS-2B cells and BAFF mRNA was increased with addition of NK cells to BEAS-2B cells, which could be from both cell populations.

5.3.2. Comparison of CXCL9, CXCL10 and CXCL11 expression during infection of BEAS-2B cell with RSV A2 and RSV X during NK cell co-cultures

The results in Section 3.3.12.2 show RSV X induces significantly more IL-15Rα mRNA than RSV A2. During BEAS-2B cell-NK cell co-culture, significantly more IFN-γ and TNF-α protein was observed in culture supernatants from RSV X compared to RSV A2 infected BEAS-2B cells. Although soluble or cell surface protein was not determined, taking this into consideration RSV X may induce a greater expression of IFN-γ and TNF-α inducible AEC-derived cytokines if there was an increase in IL-15/IL-15Rα complex expression. Increased IFN-γ production could enhance expression of Th1 associated chemokines such as CXCL10. As part of the second aim in this chapter and continuing from results observed in Chapter 3, expression of Th1 associated chemokines were characterised during RSV A2 and RSV X BEAS-2B cell-NK cell co-culture. Data is also shown as the fold change relative to protein present in culture supernatants of infected BEAS-2B cells without NK cells.

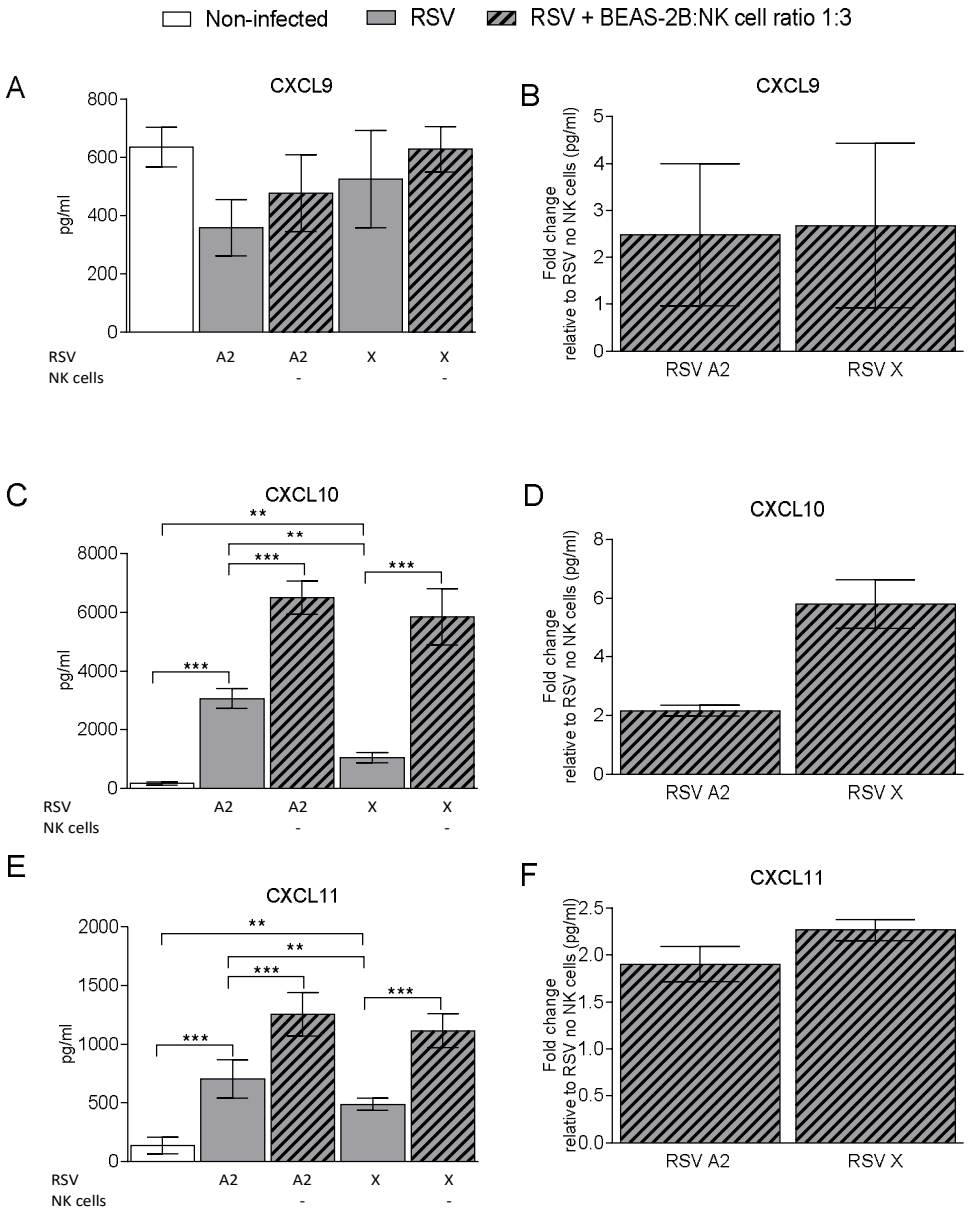


Figure 5.4. Comparison between the expression of CXCL9, CXCL10 and CXCL11 protein during RSV A2 and RSV X infection of BEAS-2B cells co-cultured with NK cells. BEAS-2B cells were infected with RSV A2 or clinical isolate, RSV X, at MOI 1 for 24hr. NK cells were isolated from healthy adult PBMCs and added at a ratio of 1:3 to BEAS-2B cells for a further 24hr. Control non-infected cultures were cultured in the absence of RSV. B, D and F show the fold change of protein compared to RSV without NK cells. A,B) CXCL9 protein (n=3), C,D) CXCL10 protein (n=3), E,F) CXCL11 protein (n=3). Data is expressed as the mean ± SEM. A, C and E (Friedman with Conover post-hoc test, *p<0.05, **p<0.01, ***p<0.01), B, D, F (Wilcoxon signed rank test).

No significant differences were observed for CXCL9 protein expression between RSV A2 and RSV X and with addition of NK cells (Figure 5.4A, B).

CXCL10 protein expression was induced by RSV A2 ($p<0.001$) and RSV X ($p<0.001$) infection to ~3000pg/ml and ~1050pg/ml, compared to control non-infected BEAS-2B cells. The increase in CXCL10 protein from infected BEAS-2B cell-NK cell co-cultures compared to those infected without NK cells was ~2-fold for RSV A2 ($p<0.001$) and ~6-fold for RSV X ($p<0.001$) (Figure 5.4C). No significant difference was observed for CXCL10 protein expression between RSV A2 and RSV X when data was expressed as protein concentration in NK cell co-culture relative to infected BEAS--2B cells without NK cells (Figure 5.4D). However, RSV X infection during BEAS-2B cell-NK cell co-culture induced ~6-fold more CXCL10 relative to infection without NK cells in comparison to RSV A2 at ~2-fold.

CXCL11 protein was significantly increased during NK cell co-culture with RSV A2 ($p<0.001$) and RSV X ($p<0.001$) infected BEAS-2B cells compared to infection without NK cells (Figure 5.4E). The increase during infected BEAS-2B cell-NK cell co-culture compared to infection without NK cells was ~2-fold for both RSV A2 and RSV X. No significant difference was observed for CXCL11 protein expression between RSV A2 and RSV X when data was expressed as NK cell co-culture relative to infection without NK cells (Figure 5.4F).

The results here indicate that RSV X induced 3 times more CXCL10 protein in culture supernatants during NK cell co-culture compared to RSV A2, although this did not reach statistical significance. Additional assays done may have provided more confidence that this results is not statistically significant.

5.3.3. Neutralisation of IFN-γ and TNF-α during BEAS-2B cell and NK cell co-culture with antibodies

To confirm that IFN-γ and TNF-α within the BEAS-2B cell-NK cell co-culture supernatants were inducing the significant increases in CXCL9, CXCL10, CXCL11 protein and BAFF mRNA seen above in Figures 5.1 and 5.3, anti-IFN-γ and anti-TNF-α neutralising antibodies were added during co-culture (Table 2.7, page 86). The BEAS-2B cell-NK cell co-culture followed the same method as in Section 3.3.8. A ratio of 1:3 gave a significant increase and highest expression of IFN-γ and TNF-α proteins compared to infected BEAS-2B cells without NK cells. Here, a BEAS-2B cell-NK cell ratio of 1:3 was used with neutralising antibodies at increasing concentrations of 10ng/ml, 100ng/ml and 1µg/ml. Antibodies were added at the same time as NK cells to the infected BEAS-2B cells. All data is expressed as relative to infected BEAS-2B cells plus NK cells, shown as 'RSV + NK cells' in Figures 5.5, 5.6 and 5.7 to remove individual assay variability. Matched isotype controls were also used at the highest concentration of 1µg/ml. The isotype control, an antibody that matches the class of the neutralising antibodies used here that should lack specificity to IFN-γ and TNF-α, should show expression of these cytokines which is comparable to infected BEAS-2B cell-NK cell co-cultures. Therefore, this specific antibody class is not inducing the response seen and results are specific to neutralisation.

5.3.3.1. Effect of anti-IFN-γ on the expression of Th1 associated chemokines CXCL9, CXCL10 and CXCL11 and adaptive immune response cytokine BAFF during BEAS-2B cell and NK cell co-culture

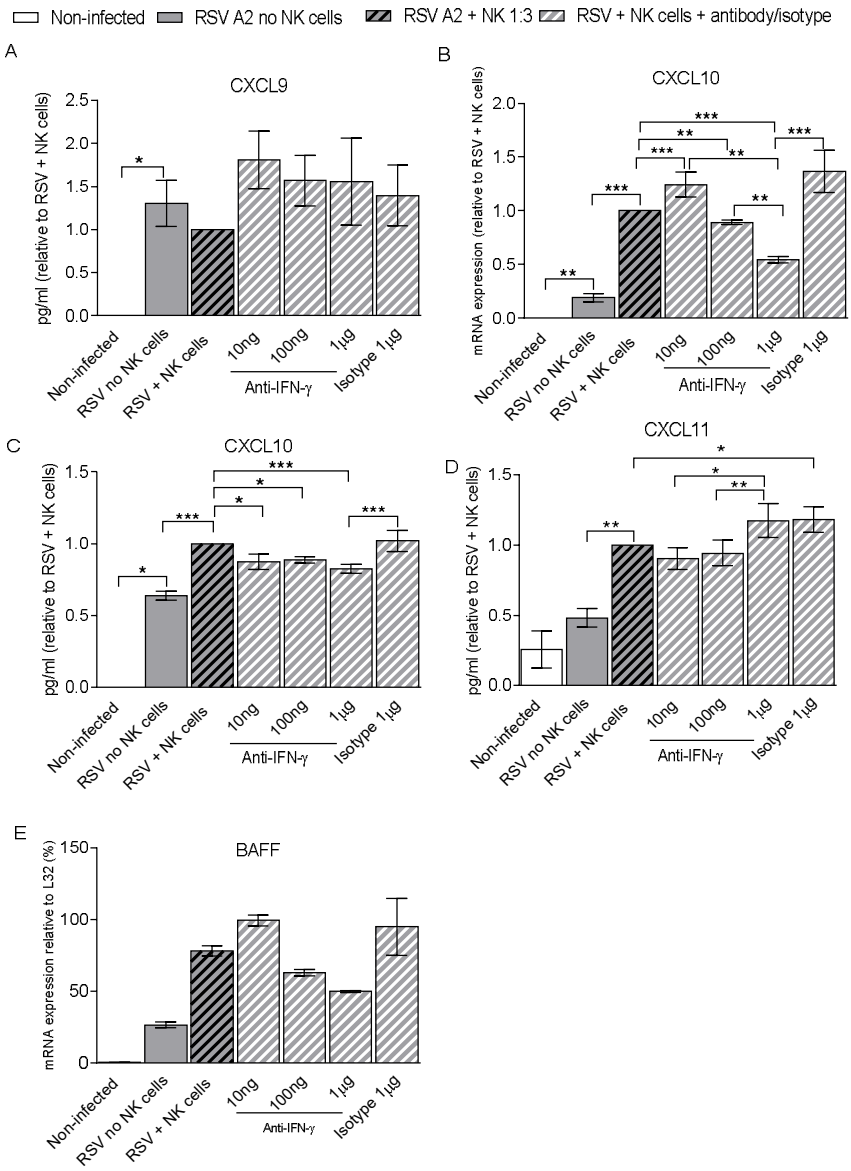


Figure 5.5. Expression of Th1 recruitment associated chemokines during BEAS-2B and NK cell co-culture with anti-IFN-γ neutralising antibody. BEAS-2B cells were infected with RSV A2 at MOI 1 for 24hr. NK cells were isolated from healthy adult PBMCs and added at ratio of 1:3 (BEAS-2B cells:NK cells) for a further 24hr, with or without anti-IFN-γ neutralising antibody at 10ng/ml, 100ng/ml and 1µg/ml or isotype control at 1µg/ml. Control non-infected cultures were cultured in the absence of RSV. Expression of A) CXCL9 protein (n=3), B) CXCL10 mRNA (n=3) and C) CXCL10 protein (n=3), D) CXCL11 protein (n=3), D) BAFF mRNA (n=2). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01, ***p<0.001).

Addition of anti-IFN-γ neutralising antibody reduced expression of CXCL10 mRNA at 100ng/ml ($p<0.001$) and 1µg/ml ($p<0.001$) and for CXCL10 protein at 10ng/ml ($p<0.05$), 100ng/ml ($p<0.05$) and 1µg/ml ($p<0.001$) (Figure 5.5B, C). The decrease in CXCL10 mRNA and protein observed was not seen with matched isotype control which showed significantly higher CXCL10 mRNA ($p<0.001$) and protein ($p<0.001$) expression compared to co-culture with 1µg/ml anti-IFN-γ antibody.

BAFF mRNA expression was measured in 2 experiments and therefore no statistical analysis was done. BAFF mRNA was not detected in control non-infected BEAS-2B cell cultures and seen at ~25% of the housekeeping gene L32 with RSV infection (Figure 5.5E). Addition of NK cells at a ratio of 1:3 increased BAFF mRNA to ~75% of the housekeeping gene L32 compared to infected BEAS-2B cells alone. BAFF mRNA followed a pattern of decrease in expression with increase in anti-IFN-γ antibody. BAFF mRNA in the isotype control was similar to that of infected BEAS-2B cells co-cultured with NK cells.

These results indicate that BAFF mRNA, CXCL10 mRNA and CXCL10 protein expression was induced by IFN-γ within the culture supernatant of infected BEAS-2B cells co-cultured with NK cells, but was not true for CXCL9 and CXCL11.

5.3.3.2. Effect of anti-TNF-α on the expression of Th1 associated chemokines CXCL9, CXCL10 and CXCL11 during BEAS-2B cell and NK cell co-culture

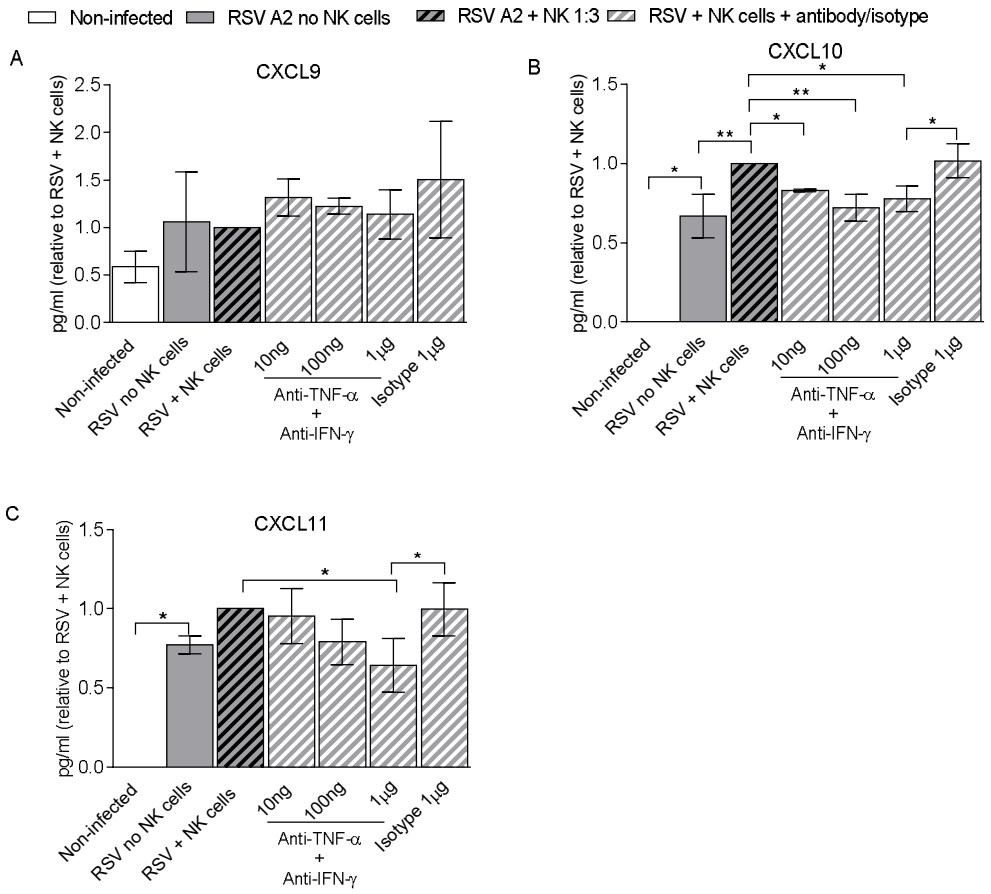


Figure 5.6. Expression of Th1 recruitment associated chemokines during BEAS-2B and NK cell co-culture with anti-TNF-α neutralising antibody. BEAS-2B cells were infected with RSV A2 at MOI 1 for 24hr. NK cells were isolated from healthy adult PBMCs and added at ratio of 1:3 (BEAS-2B cells:NK cells) for a further 24hr, with or without anti-IFN-γ neutralising antibody at 10ng/ml, 100ng/ml and 1µg/ml or isotype control at 1µg/ml. Expression of A) CXCL9 protein, B) CXCL10 protein and C) CXCL11 protein (n=3 for all). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01, ***p<0.001).

Addition of anti-TNF-α neutralising antibody only showed a reduction in CXCL10 protein levels at 10ng/ml (p<0.01), 100ng/ml (p<0.001) and 1µg/ml (p<0.001) (Figure 5.6B). The decrease in CXCL10 protein levels observed was not seen with matched isotype control which

showed significantly higher CXCL10 protein ($p<0.001$) expression compared to co-culture with 1µg/ml anti-IFN-γ antibody.

These results indicate that CXCL10 protein levels was induced by TNF-α within the culture supernatant of infected BEAS-2B cells co-cultured with NK cells, but this was not true for CXCL9 and CXCL11. For CXCL9, although a decrease in protein levels was observed with addition of anti-TNF-α neutralising antibody, the isotype also matched these levels.

5.3.3.3. Effect of anti-IFN-γ and anti-TNF-α on the expression of Th1 associated chemokines CXCL9, CXCL10 and CXCL11 during BEAS-2B cell and NK cell co-culture

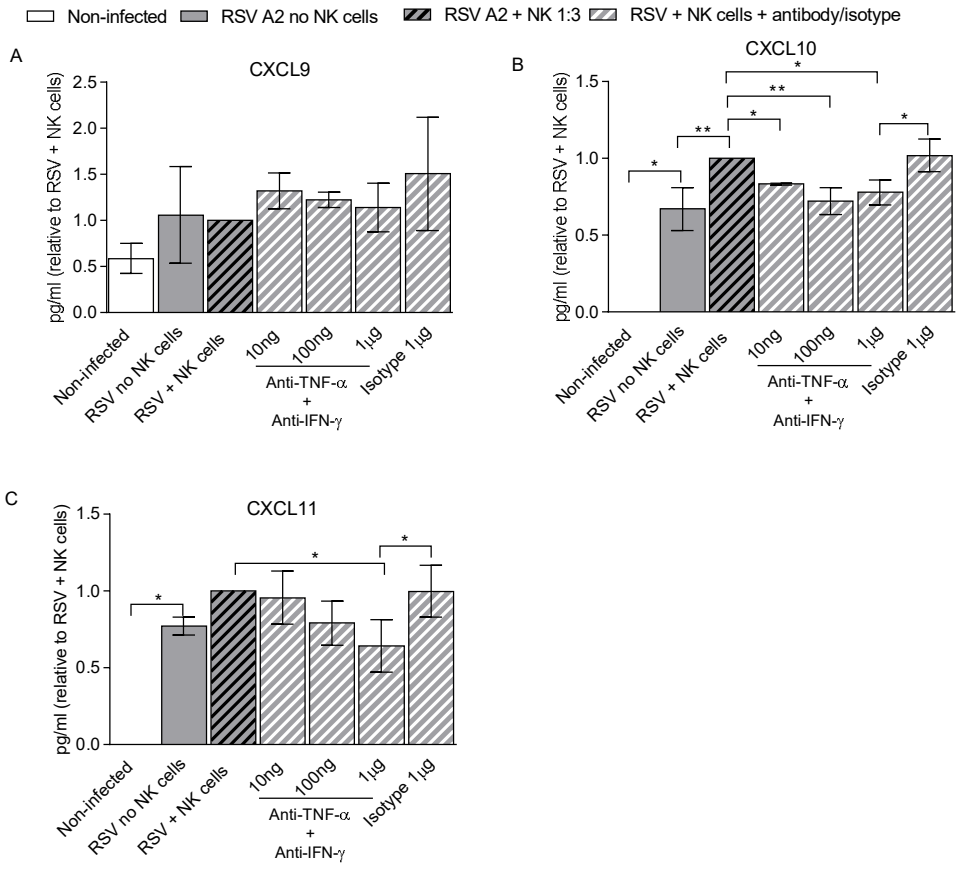


Figure 5.7. Expression of Th1 recruitment associated chemokines during BEAS-2B and NK cell co-culture with anti-IFN-γ and anti-TNF-α neutralising antibodies. BEAS-2B cells were infected with RSV A2 at MOI 1 for 24hr. NK cells were isolated from healthy adult PBMCs and added at ratio of 1:3 (BEAS-2B cells:NK cells) for a further 24hr, with or without anti-IFN-γ and anti-TNF-α neutralising antibody at 10ng/ml, 100ng/ml and 1µg/ml or isotype control at 1µg/ml. Expression of A) CXCL9 protein, B) CXCL10 protein and C) CXCL11 protein (n=3 for all). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *$p<0.05$).

Addition of both anti-IFN-γ and anti-TNF-α neutralising antibodies showed a reduction in CXCL10 protein levels at 10ng/ml ($p<0.05$), 100ng/ml ($p<0.001$) and 1µg/ml ($p<0.05$) (Figure 5.7B) and CXCL11 at 10ng/ml ($p<0.05$) (Figure 5.7C). The decrease in CXCL10 and CXCL11 protein levels observed was not seen with matched isotype control which showed significantly higher expression compared to co-culture with 1µg/ml anti-IFN-γ antibody ($p<0.05$ for both).

These results indicate that CXCL10 and CXCL11 protein expression was inhibited by both addition of anti-IFN-γ and anti-TNF-α within the culture supernatant of infected BEAS-2B cells co-cultured with NK cells, but this was not true for CXCL9.

5.3.4. Expression of chemokines, cytokines and adhesion molecules during HNAEC-NK cell co-culture

In Section 5.3.1, the expression of Th1 associated chemokines, CXCL9, CXCL10 and CXCL11, and B cell cytokine BAFF mRNA were higher in infected BEAS-2B cell–NK cell co-cultures at a ratio of 1:3 compared to RSV infected BEAS-2B cells only. It was also concluded that TARC protein was not expressed by BEAS-2B cells, with or without RSV infection and with the addition of NK cells. Then in Section 5.3.3, neutralisation assays suggested that CXCL10 protein was induced individually by IFN-γ and TNF-α, CXCL11 with both IFN-γ and TNF-α and BAFF mRNA by IFN-γ during infected BEAS-2B cell-NK cell co-culture at a ratio of 1:3. For CXCL9, this conclusion could not be reached.

In Section 4.4.3, IFN-γ was present in culture supernatants from HNAEC-NK cell co-cultures and TNF-α was present in culture supernatants from non-infected and infected HNAECs with no change seen with the addition of NK cells. Therefore, there is a potential for IFN-γ and TNF-α in HNAEC-NK cell co-culture supernatants to also induce AEC-derived cytokine expression. As part of the aim in this chapter, expression of HNAEC-derived CXCL9, CXCL10,

CXCL11, TARC and BAFF was characterised during co-culture with NK cells. The experimental approach and methodology used is the same as in Section 4.4.3 and data is expressed as relative to infected HNAECs cultures without NK cells, described in figures as 'RSV no NK cells'. This was to reduce variation in individual responses. Individual plots are also given to display exact expression values.

5.3.4.1. Th1 associated chemokines CXCL9, CXCL10 and CXCL11

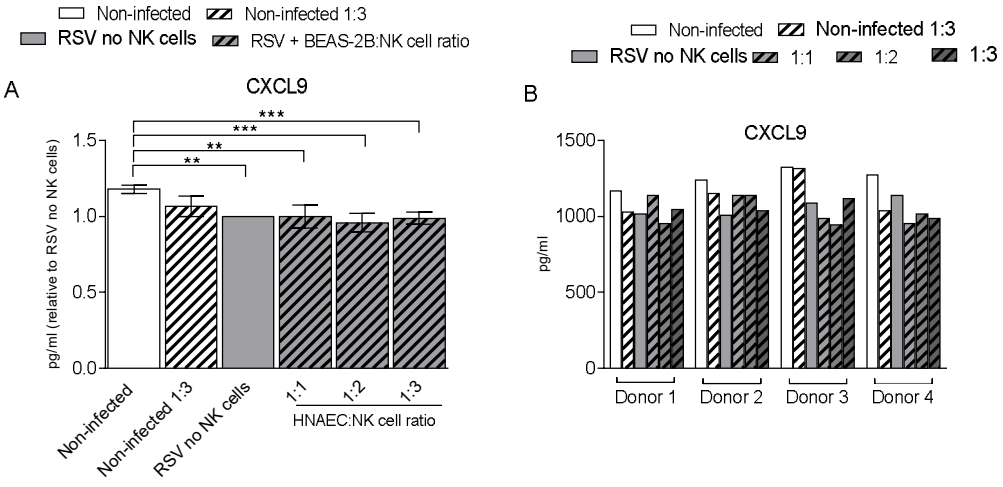


Figure 5.8. Expression of Th1 recruitment associated chemokine CXCL9 during RSV A2 infected HNAECs cocultured with NK cells. HNAECs were infected with RSV A2 at MOI 2.5 for 24hr. Donor matched NK cells were isolated from PBMCs and added at increasing ratios to HNAECs for a further 24hr. Control non-infected cultures were cultured in the absence of RSV. A) CXCL9 protein (n=4), B) individual donor expression of CXCL9 protein. Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, **p<0.01, ***p<0.001).

CXCL9 protein was found in non-infected HNAEC culture supernatants at ~1250pg/ml with a significant decrease of ~250pg/ml with RSV infection (p<0.01) (Figure 5.8A). CXCL9 protein was significantly lower in infected HNAECs co-cultured with NK cells at ratios of 1:1 (p<0.01), 1:2 (p<0.001) and 1:3 (p<0.001) compared to control non-infected HNAECs. No difference in CXCL9 protein was observed between infected HNAEC cultured alone and those co-cultured

with NK cells. Individual donor expression of CXCL9 protein is shown in Figure 5.8B, with similar expression of CXCL9 seen across all four donors and a reduction in expression with RSV infection (Figure 5.8B).

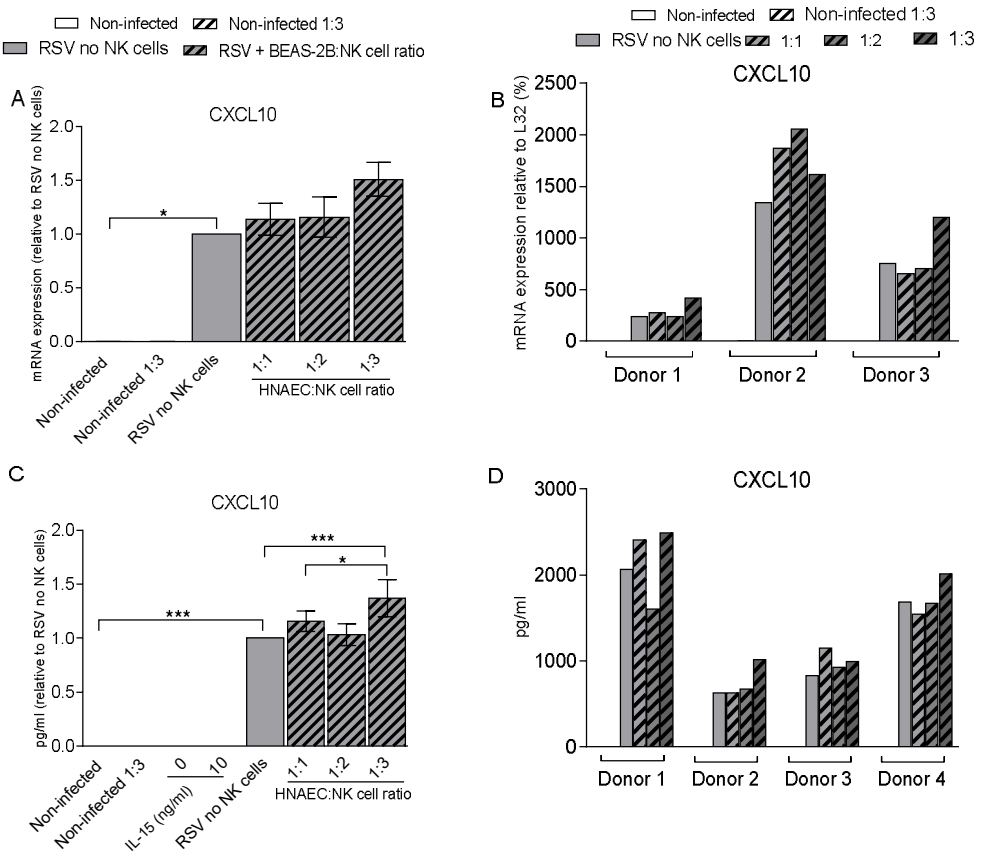


Figure 5.9. Expression of Th1 recruitment associated chemokine CXCL10 during RSV A2 infected HNAECs cocultured with NK cells. HNAECs were infected with RSV A2 at MOI 2.5 for 24hr. Donor matched NK cells were isolated from PBMCs and added at increasing ratios to HNAECs for a further 24hr. Control non-infected cultures were cultured in the absence of RSV. A) CXCL10 mRNA (n=3), B) individual donor expression of CXCL10 mRNA, C) CXCL10 protein (n=4), D) individual donor expression of CXCL10 protein. Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, ***p<0.01).

Only for CXCL10 protein was an increase in expression observed with addition of NK cells to infected HNAECs (Figure 5.9). There was a significant increase in CXCL10 protein present in infected HNAEC culture supernatants compared to control non-infected HNAECs ($p<0.001$). CXCL10 protein was significantly increased in infected HNAEC-NK cell co-culture supernatants at a ratio of 1:3 ($p<0.001$) compared to infected BEAS-2B cells without NK cells.

There was significantly higher expression of CXCL10 protein in ratio of 1:3 ($p<0.05$) compared to ratio of 1:1 (Figure 5.9C). Individual donor expression of CXCL10 protein is shown in Figure 5.13D, with addition of NK cells at a ratio of 1:3 inducing an apparent and slight increase in CXCL10 protein for all donors (Figure 5.9D).

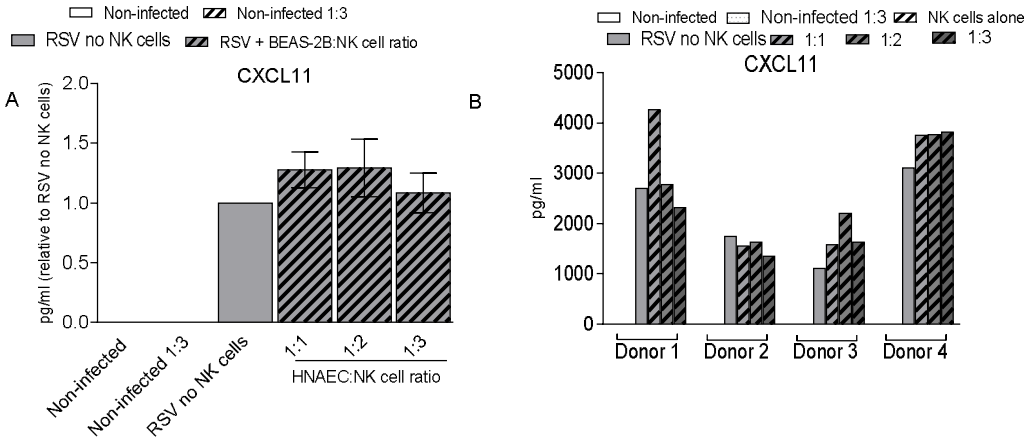


Figure 5.10. Expression of Th1 recruitment associated chemokine CXCL11 during RSV A2 infected HNAECs cocultured with NK cells. HNAECs were infected with RSV A2 at MOI 2.5 for 24hr. Donor matched NK cells were isolated from PBMCs and added at increasing ratios to HNAECs for a further 24hr. Control non-infected cultures were cultured in the absence of RSV. A) CXCL11 protein (n=4), B) individual donor expression of CXCL11 protein. Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test).

No significant differences were seen in CXCL11 protein between infected HNAECs and with NK cell co-culture (Figure 5.10).

These results indicate that only CXCL10 and not CXCL9 and CXCL11 expression was increased from infected HNAEC-NK cell co-cultures. CXCL9 protein expression was slightly reduced during RSV infection of HNAECs and with addition of NK cells. CXCL10 protein was only slightly increased during NK cell co-culture at a ratio of 1:3.

5.3.4.2. Th2 associated chemokine TARC

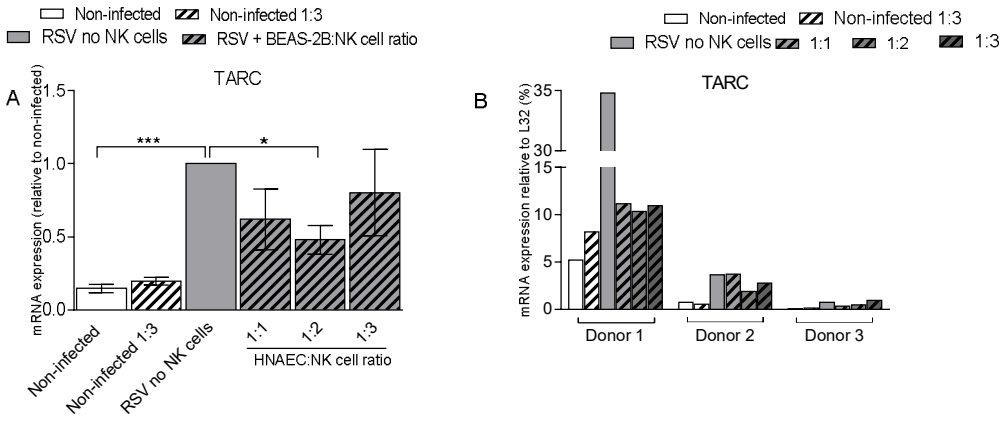


Figure 5.11. Expression of Th2 recruitment associated chemokine TARC during RSV A2 infected HNAECs cocultured with NK cells. HNAECs were infected with RSV A2 at MOI 2.5 for 24hr. NK cells were isolated from healthy adult PBMCs and added at increasing ratios to BEAS-2B cells for a further 24hr. Control non-infected cultures were cultured in the absence of RSV. A) TARC mRNA (n=3), B) individual donor expression of TARC mRNA (n=3). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, ***p<0.001).

TARC mRNA was detected in all non-infected HNAECs ranging from 0.7-5% of the housekeeping gene L32 (Figure 5.11A, B). TARC mRNA was significantly increased in infected HNAECs compared to non-infected HNAECs ($p<0.001$) (Figure 5.11A). TARC mRNA was significantly reduced with NK cell co-culture ratio of 1:2 compared to infected HNAECs without NK cells ($p<0.05$). Individual donor expression of TARC mRNA is shown in Figure 5.17B, with donor 1 basal expression being ~5-fold more than donors 2 and 3 (Figure 5.11B). RSV infection induced TARC mRNA expression by ~ 6-fold for donor 1, by ~3-fold for donor 2 and by ~ 10-fold for donor 3 compared to non-infected HNAECs. Addition of NK cells at ratios of 1:1, 1:2 and 1:3 reduced TARC mRNA expression by ~3-fold for donor 1 compared to RSV only. TARC mRNA expression was reduced by ~2-fold with NK cell ratio of 1:2 for donor 2 and at ratio 1:1 by ~2-fold for donor 3, compared to RSV only. No TARC protein was detected in culture supernatants.

These results indicate that there was great variation between TARC mRNA expressions between donor's nasal cells cultured *in vitro*. Addition of NK cells to infected HNAECs at a ratio of 1:2 reduced TARC mRNA expression.

5.3.4.3. B cell differentiation activating cytokine BAFF

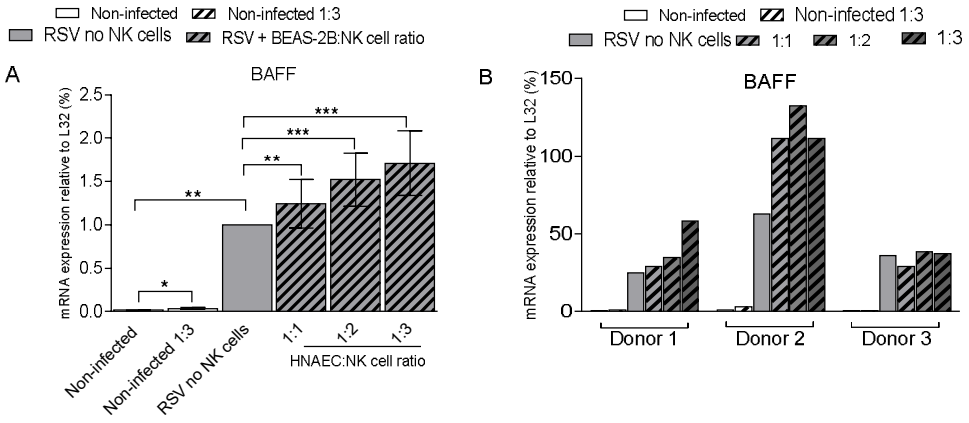


Figure 5.12. Expression of BAFF in RSV A2 infected HNAECs cocultured with NK cells. HNAECs were infected with RSV A2 at MOI 2.5 for 24hr. Donor matched NK cells were isolated from PBMCs and added at increasing ratios to HNAECs for a further 24hr. Control non-infected cultures were cultured in the absence of RSV. A) BAFF mRNA (n=3), B) individual donor expression of BAFF mRNA (n=3). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01).

There was a significant increase in BAFF mRNA expression by non-infected HNAECs co-cultured with NK cells at a ratio of 1:3 ($p<0.05$) when compared to non-infected HNAECs (Figure 5.12A). BAFF mRNA expression was significantly increased by, ~50-fold, in infected HNAECs ($p<0.01$) compared to non-infected HNAECs. BAFF mRNA was significantly increased in infected HNAEC-NK cell co-cultures at ratio of 1:1 ($p<0.01$), 1:2 ($p<0.001$) and 1:3 ($p<0.001$) when compared to infected HNAECs without NK cells. At a ratio of 1:3, BAFF mRNA expression was ~2-fold higher than that in infected HNAECs without NK cells. Individual donor expression of BAFF mRNA is shown in Figure 5.12B, with an increase in BAFF mRNA with the addition of NK cells seen for donors 1 and 2 (Figure 5.12B). Donor 3 showed no

changes in BAFF mRNA expression following the addition of NK cells. BAFF protein was not detected in any culture supernatants.

5.3.5. Effects of Th1 and Th2 cytokine environments during RSV A2 infection of BEAS-2B cells

The experiments described in this section address the final aim of the work in this chapter to determine if a primed and/or continuous cytokine environment could influence expression of inflammatory molecules during RSV infection. These experiments use the BEAS-2B cell-NK cell co-culture model. The effects of a pre-existing Th1 or Th2 cytokine environment on BEAS-2B cell cytokine and receptor expression before RSV infection was first examined. Cell surface IL-15, IL-15Rα, ICAM-1 and BAFF or soluble IL-15, IL-15/IL-15Rα, BAFF and TARC proteins in culture supernatants were measured. Finally, if these environments influence the NK cell response during BEAS-2B cell co-culture, was examined.

5.3.5.1. Effect of Th1/Th2 cytokine priming on cell surface protein expression during RSV A2 infection of BEAS-2B cells

A preliminary experiment was used to determine which cytokine/s conditions were most appropriate for inducing the expression of IL-15 and IL-15Rα in BEAS-2B cells (data not shown). Figure 5.13 illustrates the experimental design briefly, confluent BEAS-2B cells were primed with either IFN-γ (100ng/ml), TNF-α (10ng/ml) or TNF-α/IL-4/IL-13 (10ng/ml, 20ng/ml, 20ng/ml) for 24hr before RSV infection at an MOI of 1 (Table 2.10, page 86). Cytokine/s were then added again to the BEAS-2B cells after 2hr RSV inoculation for a further 48hr. Culture supernatant protein and cell surface protein expression was analysed by ELISA and flow cytometry, respectively (Section 2.7 and 2.9).

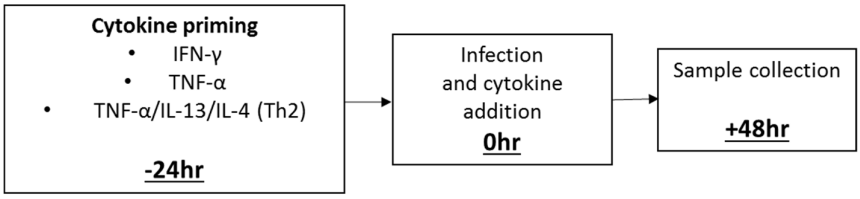


Figure 5.13. Experimental design for BEAS-2B cell cytokine priming and stimulation before and during RSV infection for protein analysis. BEAS-2B cells were primed with either IFN-γ (100ng/ml), TNF-α (10ng/ml), or TNF-α/IL-4/IL-13 (10ng/ml, 20ng/ml, 20ng/ml) for 24hr before RSV infection at an MOI of 1. Cytokine/s were then added again to the BEAS-2B cells after 2hr RSV inoculation for a further 48hr, at which RNA was collected.

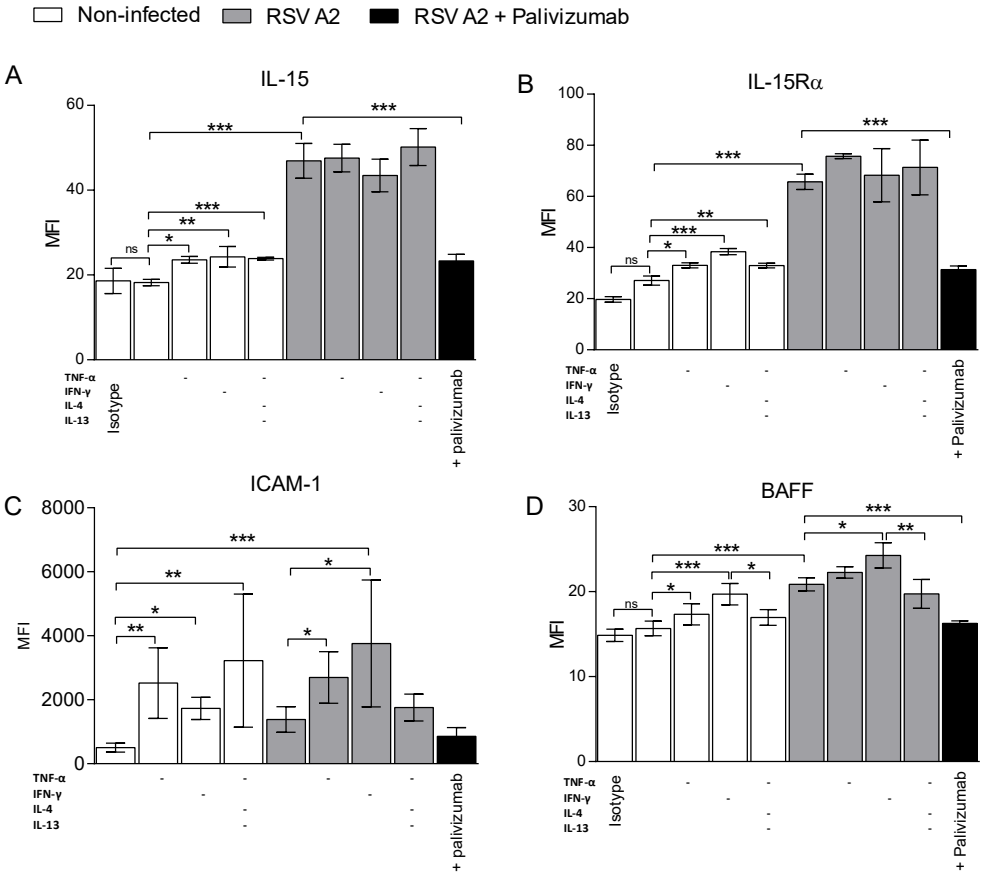


Figure 5.14. Effects of Th1 and Th2 cytokine environments on cell surface protein expression of BEAS-2B cells during RSV infection. BEAS-2B cells were primed with either IFN-γ (100ng/ml), TNF-α (10ng/ml), or TNF-α/IL-4/IL-13 (10ng/ml, 20ng/ml, 20ng/ml) for 24hr before RSV infection at an MOI of 1. Cytokine/s were then added again to the BEAS-2B cells after 2hr RSV inoculation for a further 48hr. Cell surface expression of A) IL-15, B) IL-15Rα, C) ICAM-1, D) BAFF (n=3 for all data). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01***p<0.001).

Significantly higher IL-15 MFI values were seen for BEAS-2B cells stimulated with TNF-α ($p<0.05$), IFN-γ ($p<0.01$) and TNF-α/IL-4/IL-13 ($p<0.001$) compared to control non-infected BEAS-2B cells (Figure 5.14A). RSV infected BEAS-2B cells showed significantly higher MFI values when compared to control non-infected BEAS-2B cells ($p<0.001$) and anti-RSV control ($p<0.001$). Cytokine stimulation did not change IL-15 MFI during RSV infection (Figure 5.14A).

Significantly higher MFI values were seen for BEAS-2B cells stimulated with TNF-α ($p<0.05$), IFN-γ ($p<0.001$) and TNF-α/IL-4/IL-13 ($p<0.01$) when compared to control non-infected cells (Figure 5.17B). For IFN-γ treatment, this was ~2-fold more compared to control BEAS-2B cells. RSV infected BEAS-2B cells showed significantly higher MFI values compared to control non-infected BEAS-2B cells ($p<0.001$) and or anti-RSV treated control cultures ($p<0.001$). Cytokine stimulation did not result in a change in IL-15 MFI value during RSV infection (Figure 5.14B).

Non-infected BEAS-2B cells expressed significantly more cell surface ICAM-1 when stimulated with TNF-α ($p<0.01$), IFN-γ ($p<0.05$) or TNF-α/IL-4/IL-13 ($p<0.01$) compared to control non-infected cells (Figure 5.14C). During RSV infection, BEAS-2B cells expressed significantly more cell surface ICAM-1 when stimulated with TNF-α ($p<0.05$) or IFN-γ ($p<0.05$). RSV infected BEAS-2B cells stimulated with IFN-γ expressed significantly more cell surface ICAM-1 compared to control non-infected BEAS-2B cells ($p<0.001$) (Figure 5.14C).

Significantly higher BAFF MFI values were seen for BEAS-2B cells stimulated with TNF-α ($p<0.05$) or IFN-γ ($p<0.001$) when compared to control non-infected BEAS-2B cells (Figure 5.14D). Stimulation with IFN-γ resulted in significantly higher BAFF MFI values ($p<0.05$) when compared to stimulation with Th2 cytokines. As expected RSV infected BEAS-2B cells showed a significantly higher BAFF MFI value ($p<0.001$) in comparison to control non-infected BEAS-2B cells or anti-RSV treated control cultures ($p<0.001$). Stimulation with IFN-γ before and during RSV infection resulted in a significantly increased BAFF MFI value ($p<0.05$) in comparison to that obtained by infection only or Th2 stimulated cells ($p<0.01$) (Figure 5.14D).

These results indicate that cytokine stimulation of non-infected BEAS-2B cells results in increased cell surface expression of IL-15, IL-15Rα and BAFF. This was not observed for IL-15 and IL-15Rα during RSV infection. BAFF expression was increased during RSV infection by IFN-γ treatment only. ICAM-1 expression by non-infected and infected BEAS-2B cells was increased by both TNF-α and IFN-γ treatments. Addition of Th2 associated cytokines in combination only resulted in increased ICAM-1 expression by non-infected BEAS-2B cells.

5.3.5.2. Effect of Th1/Th2 cytokine priming on expression of soluble IL-15/IL-15Rα complex, TARC and BAFF proteins during RSV A2 infection of BEAS-2B cells

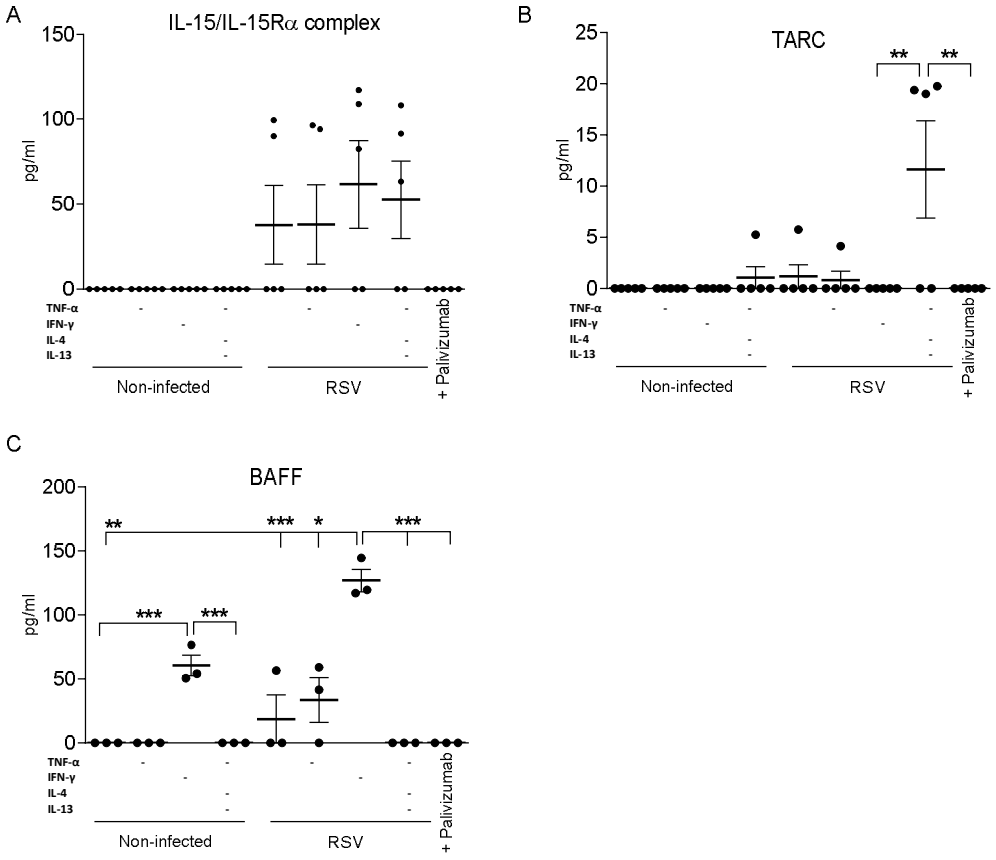


Figure 5.15. Effects of Th1 and Th2 cytokine environments on IL-15/IL-15Rα complex, TARC and BAFF protein expression in BEAS-2B cells during RSV infection. BEAS-2B cells were cultured with IFN-γ (100ng/ml), TNF-α (10ng/ml), or TNF-α/IL-4/IL-13 (10ng/ml, 20ng/ml, 20ng/ml) for 24hr, infected with RSV A2 at MOI 1 for 48hr and again cultured with cytokines. Control non-infected cultures were cultured in the absence of RSV. A) IL-15/IL-15Rα complex (n=5), B) TARC (n=5), C) BAFF (n=3). Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01***p<0.001).

IL-15/IL-15Rα complex was not detected in culture supernatants from non-infected cells with and without cytokine stimulation (Figure 5.15A). During RSV infection, IL-15/IL-15Rα complex was only detected in 2/5 samples, 2/5 samples plus TNF-α, 3/5 samples plus IFN-γ and 3/5 samples plus TNF-α/IL-4/IL-13 combination. No significance was reached at any conditions.

TARC protein was only detected in 1/5 culture supernatants from non-infected BEAS-2B cells with Th2 cytokine stimulation (Figure 2.15B). During RSV infection 1/5 samples were positive for TARC protein and 1/3 with TNF-α stimulation. TARC protein was expressed in 3/5 culture supernatants during infection and addition of IFN-γ which was significantly higher than those stimulated with IFN-γ and anti-RSV control ($p<0.01$ for each).

BAFF protein was only detected in non-infected BEAS-2B cell culture supernatants from those with IFN-γ stimulation at ~60pg/ml which was significantly higher ($p<0.001$) compared to non-infected control BEAS-2B cells and those stimulated with Th2 cytokines (Figure 2.15C). During RSV infection 1/3 samples were positive for BAFF and 2/3 following addition of TNF-α. BAFF protein was expressed at ~127pg/ml with infection and addition of IFN-γ which was significantly more than control non-infected ($p<0.01$), infected control ($p<0.001$), infected with TNF-α ($p<0.05$), infected with Th2 cytokines ($p<0.001$) and anti-RSV control ($p<0.001$) (Figure 5.15E).

These results indicate that soluble IL-15/IL-15Rα complex protein was expressed during RSV infection of BEAS-2B cells, however the detection limit of this assay may have prevented detection in all culture supernatants, such as those with expression lower than 62.5pg/ml. TARC protein was only detected in cultures with RSV infection and Th2 associated cytokine treatment. BAFF protein expression was induced by IFN-γ treatment from non-infected BEAS-2B cells and there was a synergistic increase in BAFF expression with RSV infection and IFN-γ treatment.

5.3.5.3. Th1 and Th2 cytokine environments influence NK cell responses to RSV A2 infected BEAS-2B cells

As part of the final aim of this chapter, the expression of IFN-γ and TNF-α were characterised from BEAS-2B cell-NK cell co-cultures during Th1 (IFN-γ) and Th2 (TNF-α/IL-4/IL-13) priming and/or continuous stimulation environments. The results in Figure 5.18 suggest that Th1 and Th2 cytokine environments increase both IL-15 and IL-15Rα cell surface expression, but only when BEAS-2B cells are not infected. Therefore, priming BEAS-2B cells with Th1 and Th2 cytokines may lead to different NK cell activation during co-culture with RSV infected BEAS-2B cells. To extend the analysis of inflammatory responses observed in the section above during Th1 and Th2 cytokine stimulation of BEAS-2B cells, a Th1/th2 stimulated-BEAS-2B cell-NK cell co-culture model was also used to characterise the expression of IFN-γ and TNF-α.

Figure 5.16 depicts the experimental design for results in this section. Confluent BEAS-2B cells were primed with either the Th1 associated cytokine IFN-γ (100ng/ml) or Th2 cytokine combination consisting of TNF-α/IL-4/IL-13 (10ng/ml, 20ng/ml, 20ng/ml) for 24hr before RSV infection at an MOI of 1. NK cells were then added for a further 24hrs at a ratio of 1:3 for all conditions. A set of cytokine conditions and ELISA analyses used for specific culture supernatant are shown in Figure 5.17. These conditions are detailed as NK cells cultured alone with 1) media only, 2) IL-15, 3) IL-15/IL-4/IL-13 and 4) IL-15/TNF-α/IL-4/IL-13 (Figure 5.16 and 5.17). This was to determine if Th2 cytokines could stimulate cytokine expression from NK cells without BEAS-2B cells co-culture. NK cells were also co-cultured with both non-infected and infected BEAS-2B cells. For NK cell co-culture with RSV infected BEAS-2B cells, conditions were 5) Th2 primed, 6) Th2 continuous, 7) Th1 primed, 8) Th1 continuous. For primed conditions (5, 7 and 8), NK cell were added in media only. For continuous conditions, NK cells were added in media containing cytokines to BEAS-2B cells already containing the same cytokines.

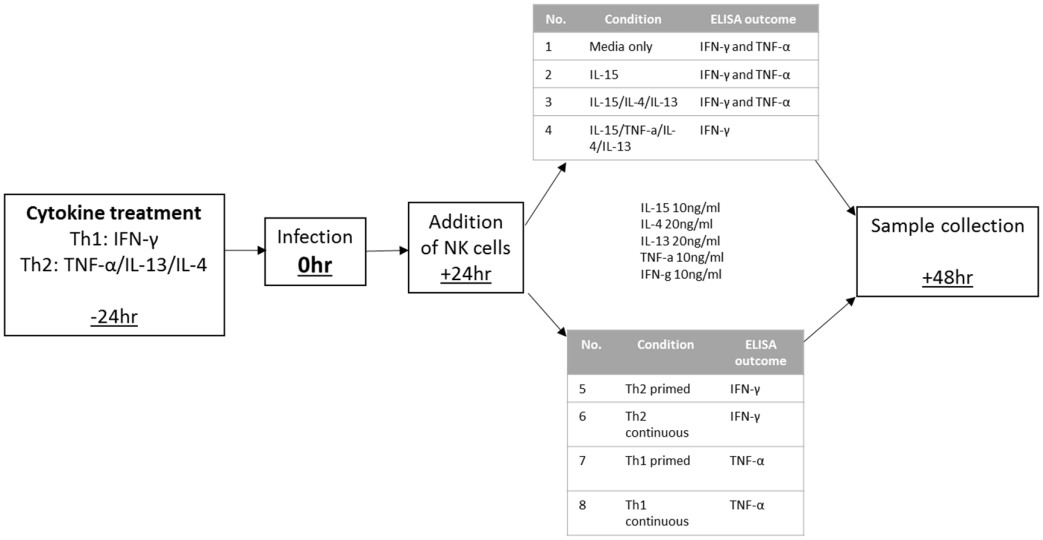
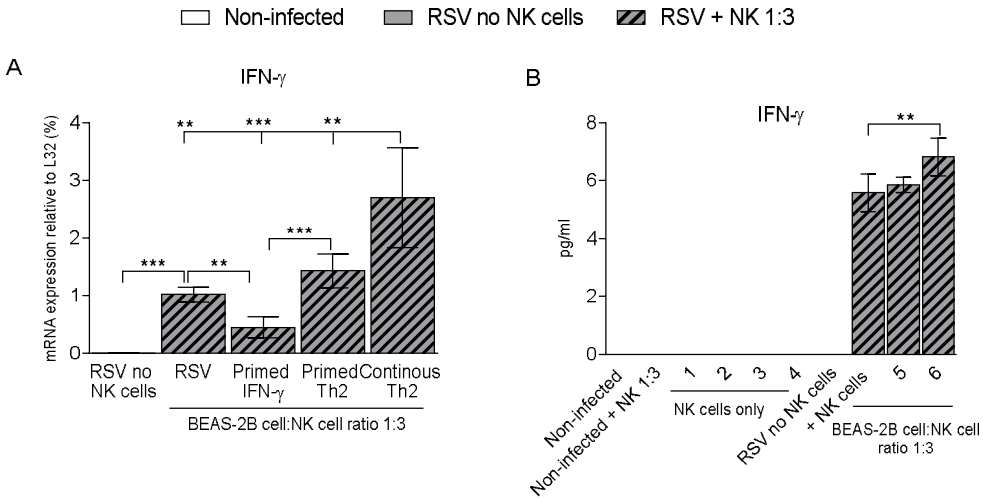


Figure 5.16. Experimental design for the influence of Th1 and Th2 cytokine environments on TNF-α and IFN-γ expression during infected BEAS-2B cell-NK cell co-culture. BEAS-2B cells were either primed with IFN-γ/Th1 or Th2 cytokines TNF-α/IL-4/IL-13 for 24hr, infected with RSV A2 at MOI 1 for 24hr and then either NK cells added with no additional cytokines (primed) or with Th2 cytokines (continuous) for 24hr. Numbers correspond to conditions used in Figure 5.15. IL-15 10ng/ml, IL-4 20ng/ml, IL-13 20ng/ml, TNF-α 10ng/ml, IFN-γ 100ng/ml.



No.	Condition	ELISA outcome
	NK cells alone	
1	Media only	IFN-γ and TNF-α
2	IL-15	IFN-γ and TNF-α
3	IL-15/IL-4/IL-13	IFN-γ and TNF-α
4	IL-15/TNF-α/IL-4/IL-13	IFN-γ
	BEAS-2B cell-NK cell co-culture	
5	Th2 primed	IFN-γ
6	Th2 continuous	IFN-γ
7	Th1 primed	TNF-α
8	Th1 continuous	TNF-α

Figure 5.17. Influence of Th1 and Th2 cytokine environments on TNF-α and IFN-γ expression during infected BEAS-2B cell-NK cell co-culture. BEAS-2B cells were either primed with IFN-γ/Th1 or Th2 cytokines TNF-α/IL-4/IL-13 for 24hr, infected with RSV A2 at MOI 1 for 24hr and then either NK cells added with no additional cytokines (primed) or with Th2 cytokines (Continuous) for 24hr. NK cells were isolated from healthy adult PBMCs and added at ratio of 1:3 to BEAS-2B cells. Control non-infected cultures were cultured in the absence of RSV. A) IFN-γ protein and B) TNF-α protein (n=3 for all data). IL-15 10ng/ml, IL-4 20ng/ml, IL-13 20ng/ml, TNF-α 10ng/ml, IFN-γ 100ng/ml. Data is expressed as the mean ± SEM (Friedman with Conover post-hoc test, *p<0.05, **p<0.01).

IFN-γ mRNA was significantly increased in infected BEAS-2B cell-NK cell co-cultures ($p<0.001$) compared to infected BEAS-2B cells alone at ~1% to the housekeeping gene L32 (Figure 5.17A). Priming BEAS-2B cells before infection with IFN-γ significantly reduced the expression of IFN-γ mRNA during co-culture ($p<0.01$) to ~0.45% to the housekeeping gene compared to co-cultures with no cytokine treatment. Priming BEAS-2B cells with Th2 cytokines did not change IFN-γ mRNA expression compared to co-culture without cytokines but was significantly higher compared to priming with IFN-γ ($p<0.001$) at ~1.4% to the housekeeping gene L32. Priming BEAS-2B cells with Th2 cytokines before RSV infection and then Th2 cytokine treatment during NK cell co-culture resulted in a significant increase in IFN-γ mRNA at ~2.7% to the housekeeping gene L32 compared to RSV only co-culture ($p<0.01$), IFN-γ priming ($p<0.001$) and Th2 priming ($p<0.01$) (Figure 5.17A).

IFN-γ protein was only detected in culture supernatants from RSV infected BEAS-2B cells co-cultured with NK cells at ~5.6pg/ml (Figure 5.17B). There was a slight but significant increase in IFN-γ protein ($p<0.01$) by ~2pg/ml in culture supernatants when BEAS-2B cells were primed and had continuous Th2 cytokine stimulus when co-cultured with NK cells compared to co-cultures where no cytokine were added.

TNF-α mRNA expression was only significantly higher in infected BEAS-2B cell-NK cell co-cultures without cytokine treatment compared to RSV infected BEAS-2B cells only ($p<0.05$) with ~2-fold higher expression (Figure 5.17C).

TNF-α protein was detected in 1/3 culture supernatants from NK cells stimulated with 10ng/ml of IL-15 (Figure 5.17D). RSV infected BEAS-2B cell cultures without NK cells expressed TNF-α protein at ~5.6pg/ml. There was a significant increase in TNF-α protein ($p<0.01$) from culture supernatants of infected BEAS-2B cells co-cultured with NK cells to ~12.9pg/ml compared infected BEAS-2B cells without NK cells. BEAS-2B cells primed with IFN-γ (Th1) showed a significant decrease in TNF-α protein ($p<0.05$) expression by ~2-fold

compared to infected BEAS-2B cell-NK cell co-cultures. BEAS-2B cells primed with Th2 cytokines showed a significant increase in TNF-α protein ($p<0.01$) expression by ~2-fold compared to NK cell co-cultures with BEAS-2B cells primed with IFN-γ at ~15.8pg/ml (Figure 5.17D).

These results indicate that priming BEAS-2B cells before RSV infection and then continuous treatment with Th2 cytokines increased IFN-γ mRNA and protein in comparison to co-cultures with no cytokine treatment. Priming with IFN-γ reduced expression of IFN-γ mRNA in comparison to co-cultures with no cytokine treatment. Priming and then continuous treatment with IFN-γ did not change TNF-α mRNA in comparison to co-cultures with no cytokine treatment. Only priming of BEAS-2B cells with IFN-γ resulted in reduced expression of TNF-α protein from co-cultures in comparison to co-cultures with no cytokine treatment.

5.4. Discussion

Having established that IFN-γ and TNF-α are expressed in culture supernatants from infected BEAS-2B cell-NK cell co-cultures and that AECs are responsive to cytokine stimulation (Sections 3.3.8 and 5.3.5), it was hypothesised that NK cells, through expression of IFN-γ and TNF-α, influence the AEC immune and inflammatory responses. Furthermore, it was hypothesised that the cytokine environment before infection also influences BEAS-2B cell activation of NK cells.

5.4.1. NK cells are influencing epithelia through expression of CXCL9, CXCL10 and CXCL11

5.4.1.1. Do NK cells influence AEC-derived expression of Th1 associated chemokines?

CXCL9 protein was expressed by infected BEAS-2B cells and during co-culture with NK cells there was a significant increase in CXCL9 protein expression at all ratios (Figure 5.4A). For HNAECs, CXCL9 protein was slightly reduced at ~100pg/ml with RSV infection and ~200pg/ml

during NK cell co-culture compared to infection only (Figure 5.14). Therefore, there is a difference in CXCL9 expression between BEAS-2B cells and HNAECs at this time point and with co-culture of NK cells. For BEAS-2B cells, the increase in CXCL9 protein with co-culture of NK cells during RSV infection was not be due to IFN-γ or TNF-α within the culture supernatants. Neutralisation assays showed that addition of anti-IFN-γ and anti-TNF-α to co-cultures did not reduced CXCL9 protein expression (Figures 5.5A, 5.6A and 5.7A). Therefore the increase in CXCL9 protein during co-culture could either be driven by a different mechanism or be NK cell derived. If not from NK cells, the neutralisation assays suggest that other molecules, not IFN-γ or TNF-α, or cell-to-cell interactions could be increased expression of CXCL9.

CXCL10 mRNA expression by infected BEAS-2B cells showed a clear increase in expression with increase in NK cell number (Figure 5.4B). Increase in expression levels also followed a dose-response relationship with increase in NK cell numbers. CXCL10 protein displayed a very similar expression pattern to CXCL10 mRNA for BEAS-2B cell co-cultures (Figure 5.4C). During co-culture with NK cells, there was a clear increase in CXCL10 protein expression with increase in NK cells. At a ratio of 1:3, ~1500pg/ml more CXCL10 protein was detected compared to infected BEAS-2B cells alone. For HNAECs, significance was only achieved for CXCL10 protein at a ratio of 1:3 compared to infected HNAECs alone (Figure 5.15). This increase in CXCL10 protein for HNAECs was not as distinct as with BEAS-2B cells. For BEAS-2B cell, the increase in CXCL10 was specific to IFN-γ and TNF-α present within the co-culture supernatant as shown by anti-IFN-γ and anti-TNF-α neutralisation assays (Figures 5.5B, 5.5C, 5.6B and 5.7B). Therefore NK cell derived IFN-γ induced an increase in BEAS-2B cell derived CXCL10 during RSV infection. During infection *in vivo*, IFN-γ and TNF-α expressed by activated NK cells may also act to enhance $CXCR3^{+}$ immune cell recruitment through a co-ordinated AEC-NK cell interactions.

CXCL11 protein, similarly to CXCL9 and CXCL10, was only expressed by BEAS-2B cells on infection and expression was also significantly increased by co-culture with NK cells at all ratios of 1:1, 1:2 and 1:3 (BEAS-2B cells:NK cells) (Figure 5.4D). Infected HNAECs did not show any difference in CXCL11 expression during NK cell co-culture (Figure 5.10). For CXCL11 expression, likewise to CXCL9 and CXCL10, BEAS-2B cells were more responsive to NK cell co-culture compared to HNAECs although this could reflect the time point and AEC-NK cell ratios used. The increase in CXCL11 expression during NK cell co-culture with infected BEAS-2B cells required the presence of IFN-γ or TNF-α alone. Use of anti-IFN-γ or anti-TNF-α alone did not reduce CXCL11 expression during NK cell co-culture (Figure 5.5D, 5.6C) and only with addition of both anti-IFN-γ and anti-TNF-α to co-cultures was expression of CXCL11 reduced (Figure 5.7C).

Overall, co-culture of NK cells for 24hr with infected BEAS-2B cells increased additional expression of all Th1 associated chemokines, but only a slight increase for CXCL10 protein was seen in HNAEC cultures. These results provide a new understanding of the role of NK cells within the lung during RSV infection possibly influencing subsequent immune responses, particularly $CXCR3^+$ immune cell recruitment, through inducing an increase in AEC-derived Th1 associated chemokine expression. This was most apparent using BEAS-2B cell-NK cell co-cultures. Further investigation of HNAECs such as longer co-culture times and increased NK cell ratios could be needed and this is discussed further below.

Some studies suggest that NK cells also express CXCL10 both intracellularly and at the cell surface (456–458). However, apparent cell surface expression may represent detection of CXCL10 bound to CXCR3 receptor on NK cells. Fauriat *et al.* suggest that NK cells themselves can express CXCL9 and CXCL10 proteins, albeit at low levels, and during co-culture with K562 cells show a significant upregulation of these protein to ~200pg/ml (387). However it cannot be conclude if the increase in expression was NK cell specific and/or in combination with

K562 cells. Flow cytometric analysis of intracellular CXCL9, CXCL10 and CXCL11 expression from NK cells during AEC-NK cell co-cultures could have clarified this issue. Nevertheless and irrespective of which cell population expresses these chemokines, an overall increase in expression is observed during co-culture and is therefore a more representative model for *in vivo* chemokine expression than infection of AECs alone. This confirms my hypothesis that NK cells influence the AEC immune and inflammatory responses, particularly CXCL10 expression.

Interestingly, CXCL10 mRNA expression was increased by ~10-fold in non-infected BEAS-2B cell-NK cell co-cultures compared to non-infected BEAS-2B cells alone. This suggests that IFN-γ and/or TNF-α protein may be present below the detection limit of 4pg/ml or direct cell-to-cell interactions induce gene expression. Other proteins may also be active within these non-infected BEAS-2B cell-NK cell co-cultures, but not examined here, which may have increased CXCL10 mRNA expression in comparison to control BEAS-2B cell basal expression. NK cells have been shown to express membrane-bound TNF-α, discussed further in Section 6.2. If membrane-bound TNF-α was present on NK cells in this co-culture model and interacted with non-infected BEAS-2B cells, this could potentially induce low levels of CXCL10 mRNA expression by BEAS-2B cells. This could also indicate that there is a basal level of AEC-NK cell communication, which may be essential for resident NK cell survival in resting lung before infection.

Sauty *et al*. reported that HBEC derived CXCL10 expression is more sensitive to IFN-γ stimulation than that of BEAS-2B cells (459). Here IFN-γ protein expression from some HNAEC-NK cell co-cultures appeared to be higher than that detected in BEAS-2B cell-NK cell co-cultures (Figures 3.11B and 4.17A). This could be explained by both the basal and RSV induced expression of IL-15 mRNA and IL-15Rα mRNA which was greater for HNAECs than for BEAS-2B cells (Chapters 3 and 4). TNF-α was also constituently expressed by HNAECs and

for donors 1 and 2, IFN-γ protein was ~60pg/ml and 40pg/ml, respectively (Figure 4.17D). Sauty *et al*. also shows that TNF-α acts with IFN-γ to synergistically induce CXCL10 expression and HNAECs have a basal expression of TNF-α at ~200-400pg/ml. The additional IFN-γ expressed during NK cell co-culture may increase further CXCL10 expression (459). Therefore here it was expected that during co-culture of infected HNAECs with donor matched NK cells a greater induction of CXCL10 would be observed compared to that of BEAS-2B cells. However, this was not the case and CXCL10 expression was more pronounced during BEAS-2B-NK cell co-cultures, as shown as a dose-response with increase in NK cells, than with HNAECs. One explanation for this could be to do with use of donor-matched NK cells. These NK cells will respond to that donor's HLA molecules (Section 1.3.2) and may be under stronger inhibitory signals when cultured with self AECs. Another explanation is the time of culture supernatant collection. Sauty *et al*. observed that IFN-γ/TNF-α stimulation of HBECs showed peak CXCL10 protein expression later than that of BEAS-2B cells (459). Although Sauty *et al*. used a different AEC cell type, HBECs, from that used here, co-culture of NK cells with infected HNAECs for a longer period of time could have provided further insight into the influence NK cells have on CXCL10 expression by HNAECs. However, the objective of this work was to determine if AEC responses are changed by NK cell co-culture and for HNAECs, this was only apparent for CXCL10. Furthermore, HNAECs were infected with RSV at an MOI of 2.5 and BEAS-2B cells at an MOI of 1. At an MOI of 2.5, CXCL10 protein expression may have reached a maximum for HNAEC cultures. Co-culture of NK cells with infected HNAECs at different MOIs would provide more clarity to increases or decreases of Th1 associated chemokines during HNAEC-NK cell co-culture.

5.4.1.2. Do NK cells influence AEC-derived expression of Th2 associated chemokine TARC?

In the BEAS-2B cell-NK cell co-culture model, TARC mRNA was only present in some co-cultures and at relatively low levels compared to housekeeping gene L32 at ~0.4-2% (Figure 5.5A). Furthermore, no TARC protein was expressed as might be expected from the low and inconsistent mRNA expression (Figure 5.5B). For HNAECs, TARC mRNA was expressed by non-infected HNAECs and expression induced by RSV infection by ~3-fold (Figure 7.17A, B). Again, TARC mRNA was relatively low at ~0.7% for donors 2 and 3 and for donor 1, 7% of housekeeping gene L32 in control HNAECs (Figure 5.17B). With co-culture of NK cells at a ratio of 1:2, TARC mRNA was significantly reduced. Alternatively, the decrease in TARC mRNA could be due to the addition of NK cells resulting in increased NK cell-derived L32 housekeeping gene mRNA levels, which is also discussed as a possible explanation of the apparent decrease in RSV N gene expression reported in Section 3.4.3.1 during AEC-NK cell co-cultures. Overall, any biological relevance of the results observed here cannot be concluded as no TARC protein was detected in culture supernatants.

Here, TARC was chosen as a Th2 associated chemokine because serum TARC levels are reported to be significantly higher in infants with RSV infection compared to other respiratory infections and may be associated with disease severity (233). TARC has also been shown to be expressed during *in vitro* RSV infection of AECs and its expression increased by AECs during stimulation with IFN-γ and TNF-α (129,453). The role of TARC during RSV infection has not been fully characterised and whether NK cells can influence its expression through AECs has also not been evaluated previously. The results from these previously published AEC *in vitro* infection studies suggest that expression of NK cell derived IFN-γ and TNF-α during co-cultures might similarly induce an increase AEC-derived TARC, as discussed above for Th1 associated chemokines but was not observed here. TARC expression may require Th2 cytokines to be present within the co-culture. Th2 cytokine treatment to HNAECs has been shown to induce TARC protein levels to ~100pg/ml with 36hr stimulation (460).

Overall, published literature and the results obtained here show different results regarding TARC expression. Expression of TARC during AEC-NK cell co-culture is inconclusive and requires further investigation.

5.4.1.3. Do NK cells influence AEC-derived expression of BAFF?

BAFF mRNA followed a similar pattern of expression as that for CXCL10 mRNA described above with expression increasing following addition of NK cells to infected BEAS-2B cells and HNAECs (Figure 5.6A and 5.12A). However BAFF protein did not follow the same pattern of expression with no increase in BAFF protein observed with increase in NK cell ratio during BEAS-2B cell co-cultures (Figure 5.6B) and for HNAECs no protein was detected in culture supernatants (Figure 5.12B).

Here, 10ng/ml IL-15 stimulated expression of BAFF mRNA in NK cells alone. Therefore, it cannot be concluded that the increase in BAFF mRNA expression observed during co-culture was exclusive to BEAS-2B or HNAECs. The increase in BAFF mRNA with increasing NK cell number may represent the activity of NK cells. Other studies have reported NK cells express BAFF (461,462). Alternatively, other results not shown here demonstrate that infected BEAS-2B cells showed increased expression of BAFF protein following TNF-α and IFN-γ treatment and therefore it is reasonable to suggest that the increase in expression, or a partial increase, was also due to IFN-γ and TNF-α stimulation of BEAS-2B cells during co-culture. Furthermore as discussed in Section 5.4.2.1, BAFF can be membrane-bound and determining BAFF expression only in culture supernatants of AEC-NK cell co-cultures does not provide a complete expression profile of BAFF protein. Overall, this data suggests that NK cells may also contribute to adaptive immunity during RSV infection and may enhance BAFF expression either producing BAFF themselves or increasing production by the airway epithelium.

5.4.1.4. Is AEC-derived protein expression increased by IFN-γ and TNF-α present in BEAS-2B cell-NK cell co-culture supernatants?

As part of the first aim in this chapter, which was to determine if the expression of CXCL9, CXCL10, CXCL11, TARC and BAFF changed during co-culture of RSV infected AECs and NK cells, it was essential to determine if the increase in these inflammatory molecules was dependent on IFN-γ and TNF-α within the co-culture model.

During treatment with anti-IFN-γ neutralising antibody CXCL10 mRNA and protein expression showed a dose-dependent decrease with increase in anti-IFN-γ neutralising antibody used (Figure 5.11B, C). BAFF mRNA showed an apparent decrease with increase in anti-IFN-γ antibody, although this is only observed in 2 experiments (Figure 5.11E). Therefore, BEAS-2B cell-derived BAFF mRNA expression may be enhanced through IFN-γ expression during NK cell co-culture. Treatment with anti-TNF-α antibody resulted in decreased CXCL9 protein, however this was also true for the isotype control and it can be concluded that the observed decrease is due to non-specific antibody effects (Figure 5.12A). For CXCL10 protein, a similar result was observed to that with anti-IFN-γ antibody and a slight but significant decrease in CXCL10 protein was observed with anti-TNF-α antibody treatment (Figure 5.12B). A combination of both anti-IFN-γ and anti-TNF-α treatment reduced CXCL10 and CXCL11 protein expression (Figure 5.13B, C). As discussed above in Section 5.4.1.1, the increase in CXCL9 protein during BEAS-2B cell-NK cell co-culture could be due to alternative stimulatory mechanisms, direct cell-to-cell interactions and/or be from NK cells. For CXCL10, either IFN-γ and/or TNF-α present in co-culture supernatants increase its expression. For CXCL11 expression, IFN-γ and TNF-α alone could induce an increase in CXCL11 expression, as shown with no changes in expression with single anti-IFN-γ or anti-TNF-α neutralisation.

Use of neutralising antibodies in this assay may not be the best method due to alternative stimulatory mechanisms and BEAS-2B cell-NK cell cellular interactions that will continue to

be present in these assays. As the effects of anti-IFN-γ antibody on CXCL10 mRNA expression was more apparent than that for protein, potentially characterising mRNA expression of CXCL9 and CXCL11 would have helped clarify changes in CXCL9 and CXCL11 during neutralisation of IFN-γ and TNF-α in more detail.

5.4.1.5. Comparison in NK cell driven, BEAS-2B cell derived Th1 associated chemokine expression between RSV strains.

Here expression of CXCL10 mRNA and protein with RSV infection and without NK cells was higher from RSV A2 than RSV X (Figure 5.9). This indicates that infection with RSV A2 alone may be able to induce a stronger expression of these Th1 associated chemokines from AECs than RSV X. The expression and induction of CXCL10 protein by RSV X during NK cell co-culture was comparable to that of RSV A2 co-culture (Figure 5.10C). Furthermore during NK cell co-culture, although not significant, culture supernatants from RSV X infected BEAS-2B cell-NK cell co-cultures had ~3-fold higher induction of CXCL10 compared to RSV A2 infected BEAS-2B-NK cell co-cultures (Figure 5.10D). No conclusion as to which strain can induce the greatest Th1 associated chemokine profile during NK cell co-culture can be made as the results were not significantly different. However it is still important to use clinical isolates for a more representative *in vivo* inflammatory profile.

Similarly to the results observed in Figure 5.4D, CXCL11 protein expression level was induced by RSV A2 infection alone and also by RSV X (Figure 5.10E). RSV A2 induced significantly more CXCL11 protein expression than RSV X by ~200pg/ml (Figure 5.10E). Co-culture of NK cells with RSV A2 and RSV X infected BEAS-2B cells induced an expected increase in CXCL11 protein level which was similar between each strain (Figure 5.10F). Overall, these results suggest that although infection of BEAS-2B cells during NK cell co-culture with RSV A2 and

RSV X resulted in both CXCL10 and CXCL11 protein expression, CXCL10 expression was more sensitive to NK cell-co-culture during RSV X infection than RSV A2.

5.4.2. Do priming and continuous cytokine environments influence cytokine expression during BEAS-2B cell-NK cell co-culture model during?

The final aim of this chapter was to determine if a priming and/or continuous cytokine environment can influence the expression of cell surface IL-15, IL-15Rα, BAFF and ICAM-1 and soluble IL-15/IL-15Rα, TARC and BAFF during BEAS-2B cell-NK cell co-culture model during. First, the influence of Th1 and Th2 cytokine treatments before and during RSV infection on expression of cell surface IL-15, IL-15Rα, ICAM-1 and BAFF and soluble IL-15/IL-15Rα complex, TARC and BAFF were measured. Secondly, if Th1 or Th2 cytokine stimulation of BEAS-2B cells before and during RSV infection could influence expression of IFN-γ and TNF-α was examined.

5.4.2.1. Th1 and Th2 cytokine environments alter expression of immune molecules by BEAS-2B cells

Some of the more interesting observations from these assays relate to the cell surface expression of IL-15 and IL-15Rα by non-infected BEAS-2B following with cytokine stimulation which has not been published in the literature. With either TNF-α, IFN-γ and TNF-α/IL-4/IL-13 in combination cell surface expression of both IL-15 and IL-15Rα were significantly increased compared to non-infected control BEAS-2B cells (Figure 5.14A, B). This was not apparent for RSV infected BEAS-2B cells which had been primed before infection and cytokines added, after viral infection, for a further 48hrs. These experiments also confirm that replicating RSV can trigger for the expression of cell surface IL-15 and IL-15Rα by BEAS-2B cells, which was not characterised in Figures 3.4 and 3.5. Furthermore, data shown in Figure 5.15A indicates that BEAS-2B cells may be able to express soluble IL-15/IL-15Rα

complex up to ~100pg/ml, which was only seen during RSV infection. Although not all samples were positive, 3/5 RSV infected BEAS-2B cells treated with IFN-γ and TNF-α/IL-4/IL-13 in combination displayed a slightly higher average concentration than those infected with RSV alone. However there was no increase in cell surface IL-15 and IL-15Rα with additional cytokine treatment during RSV infection. This was unexpected as a preliminary experiment (data not shown) indicated that IL-15Rα mRNA expression was synergistically increased by IFN-γ treatment and RSV infection. ICAM-1 and BAFF cell surface expression were induced with IFN-γ treatment and infection (Figure 5.14) and this indicates that infected BEAS-2B cells were still responsive to cytokine treatment during RSV infection and could highlight the highly controlled post transcriptional regulation that is displayed for IL-15 and IL-15Rα. Overall cytokine treatment of non-infected BEAS-2B cells increase cell surface expression of IL-15 and IL-15Rα and therefore the presence of these cytokines before infection may alter the responsiveness of NK cells.

IFN-γ may be a key regulator of AEC-derived IL-15 expression before and after RSV infection for many cell types. IFN-γ has also been shown to increase IL-15 and IL-15Rα expression in monocytes and macrophages (338). In BEAS-2B cells and A549 cells LPS, IL-1β, TNF-α and IFN-γ have been shown to induce IL-15 mRNA level with IL-15 protein only detected in cultures stimulated with IFN-γ (154,337,414,453). Stimulation with IFN-γ also followed a dose-dependent increase on IL-15 mRNA expression in BEAS-2B cells and led to increasing the half-life of IL-15 mRNA (337). Zdrenghea *et al*. also observed that IFN-γ could induce secretion of IL-15 protein alone from non-infected and RSV infected BEAS-2B cells (152). On the other hand, IFN-γ may also reduce expression of certain genes and proteins. Treatment of IFN-γ to both non-infected and RSV infected BEAS-2B cells was shown to reduce expression of soluble and cell surface MICA, a ligand for NKG2D (152). NK cells express NKG2D and IFN-γ expressed by NK cells after their activation could aid in reducing NK cell activities and cell expansion, shown as NK cells expansion only during the first few days RSV

infection. Overall this data displays that the expression of early inflammatory mediators, such as those by NK cells after RSV infection, could influence IL-15 signalling via the airway epithelium for other IL-15 responsive immune cells.

ICAM-1 cell surface expression followed that reported in the literature with IFN-γ and TNF-α individually able to induce cell surface expression in non-infected AECs and also act synergistically to enhance ICAM-1 expression during RSV infection (440,454,455). Here, IFN-γ, TNF-α and TNF-α/IL-4/IL-13 treatments induced ICAM-1 cell surface expression in non-infected BEAS-2B cells (Figure 5.14C). During RSV infection, an increase in ICAM-1 expression was seen with TNF-α and IFN-γ treatment alone, although these values were not more than that of cytokine treated non-infected BEAS-2B cells. Addition of IL-4 and IL-13 with TNF-α treatment (Th2-like environment), showed reduced ICAM-1 expression in comparison to TNF-α alone and IFN-γ treatment. Therefore possibly IL-4 and IL-13 may have inhibitory effects on TNF-α and IFN-γ induced ICAM-1 cell surface expression. The implications of this *in vivo* could be that increased immune cell adherence could be achieved in lung environments with more IFN-γ and/or TNF-α during RSV infection and this could result in increased infected cell lysis and other unknown cell-to-cell interactions. For NK cells, increase in AEC surface ICAM-1 expression could increase the strength of NK cell surface interactions allowing for a stronger activation through the IL-15/IL-15Rα complex. An increase in ICAM-1 expression was also observed with cytokine treatment of non-infected BEAS-2B cells. If these cytokine environments remain after RSV infection, this could lead to prolonged immune cell retention within the lung and could lead to pathologic effects.

As detailed above, NK cells may contribute to the adaptive immune response, both through NK cell-derived BAFF expression and also IFN-γ/TNF-α stimulation of AECs. BAFF can either be membrane-bound protein or be cleaved and act as a soluble cytokine (456). Therefore, cell surface BAFF was measured as part of this experiment. To my knowledge, here this is the

first time cell surface BAFF expression has been examined during RSV infection of AECs with treatment of Th1 and Th2 cytokines. Similarly to IL-15 and IL-15Rα, cell surface BAFF expression increased with cytokine treatment without infection compared to control non-infected BEAS-2B cells (Figure 5.14D). This was more pronounced with IFN-γ treatment. RSV induced expression of cell surface BAFF compared to control non-infected BEAS-2B cells which showed no expression of cell surface BAFF. In comparison to the results observed for IL-15 and IL-15Rα, which did not change with cytokine treatment during RSV infection, IFN-γ slightly induced BAFF cell surface expression with RSV infection, this was not seen for TNF-α or TNF-α/IL-4/IL-13 treatment (Figure 5.14D). Therefore, NK cell derived IFN-γ and TNF-α may enhance AEC-derived BAFF and co-ordinate an adaptive immune response.

Figure 5.15C details the expression of soluble BAFF protein during the same cytokine treatment. Only IFN-γ induced release of soluble BAFF protein from non-infected BEAS-2B cells and during RSV infection with IFN-γ treatment, BAFF protein expression was ~2-fold more than that from non-infected and IFN-γ treated BEAS-2B cells. This suggests IFN-γ has a synergistic effect on BAFF protein expression during RSV infection. Changes in BAFF protein expression were not observed following Th2 cytokine treatment during RSV infection. This could suggest different Th1 and Th2 environments during and after infection could alter adaptive immune responses. During infection IFN-γ could induce BAFF expression and then after virus elimination, continued IFN-γ expression could lead to sustained B cell responses and development of immunological memory. This could lead to more effective B cell responses, such as anti-RSV antibody production. Furthermore, Th2 environments during RSV infection could lead to reduced BAFF expression and reduced adaptive immune responses. IFN-γ treatment alone induced the most cell surface BAFF whereas a Th2 cytokine combination did not. This could suggest that a Th1 environment is beneficial for adaptive immune responses whereas a Th2 environment may dampen B cell activation. Overall this

further indicates that NK cell-derived IFN-γ could enhance AEC adaptive immune responses during RSV infection.

The results observed within this section have been summarised in a model of AEC induced NK cell activation shown in Figure 5.18. This model represents what could happen at the airway epithelium during RSV infection with four proposed mechanisms of IL-15 signalling reflecting the data obtained here in this work and the possible interactions between infected AECs and NK cells. Proposed mechanism number 1 details that with RSV infection there appears to be tight regulation of IL-15 and IL-15Rα expression by AECs, such low levels of IL-15 protein in culture supernatants and cell surface expression. Tight regulation of IL-15 and IL-15Rα expression could be beneficial for the airway epithelium to prevent over activation of NK cells, reduce NK cell-targeted cell lysis and/or excessive NK cell cytokine expression. However this apparent tight regulation of IL-15 and IL-15Rα expression could be an immune evasion strategy by RSV and is shown as proposed mechanism number 2. No further increase in cell surface expression of IL-15 and IL-15Rα was observed here with additional IFN-γ treatment but IFN-γ treatment did increase their expression by non-infected cells (Figure 5.14). If RSV hindered further secreted IL-15 and IL-15/IL-15Rα complex expression, this could reduce and/or slow NK cell activation and responses and other IL-15 responsive immune cells, such as T cells. Another possibility could be that during RSV infection and cytokine treatment, instead of IL-15/IL-15Rα complex being expressed at the cell surface, soluble complexes may be produced to aid in activation of IL-15 responsive immune cells not in direct contact with the infected airway epithelium as part of a co-ordinated response, shown as mechanism 3 in Figure 5.18. IL-15 has been shown to induce monocyte differentiation into DCs and regulate macrophage cytokine expression which could aid in clearance of infected cell debris and presentation of antigens to T cells (457,458). On the other hand during RSV infection NK cell derived IFN-γ, and particularly then IFN-γ expression from other immune cells during later stages of RSV infection, could reduce cell surface IL-

15/IL-15Rα complex expression as a protective mechanism to dampen NK cell responses, shown as mechanism 4. This could reduce excessive and prolonged NK cell activation which may be detrimental and complements the pattern of NK cell population expansion which rises steeply during the first 1-2 days of infection then also rapidly declines along with viral titre (Figure 1.11).

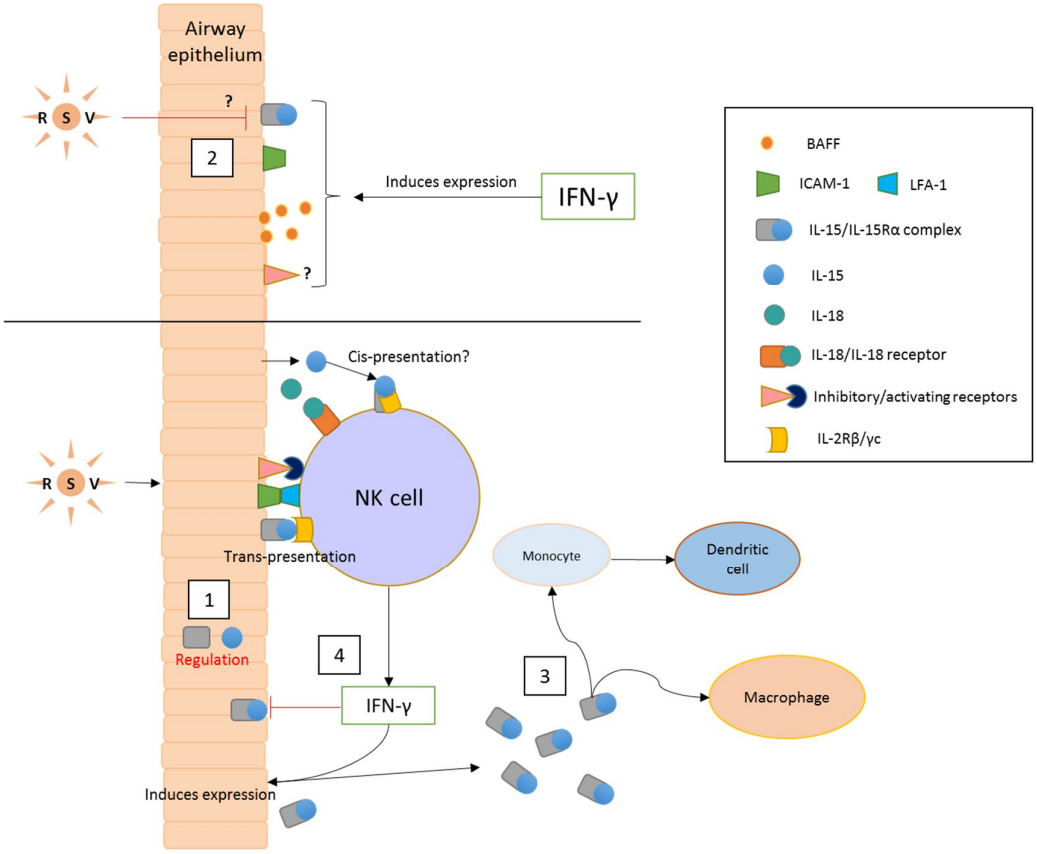


Figure 5.18. Proposed mechanisms of IL-15 signalling during RSV infection of the airway epithelium. 1) During RSV infection and IFN-γ stimulation, prevention of cell surface IL-15/IL-15Rα complex is done to reduce over activation of NK cells. 2) RSV inhibits IFN-γ induced expression of cell surface IL-15/IL-15Rα complex to reduce NK cell activation and aids in RSV spread. 3) IFN-γ induces expression of soluble IL-15/IL-15Rα complexes to act on IL-15-responsive immune cells which are not localised at the site of infection. 4) NK cell-derived IFN-γ acts back on the airway epithelium to reduce IL-15/IL-15Rα complex expression as self-protective and beneficial NK cell-activation reduction method to prevent excessive NK cell responses.

5.4.2.2. Priming BEAS-2B cells with Th1 and Th2 cytokines induces different expression of IFN-γ and TNF-α during co-culture with NK cells.

Figure 5.17 details the expression of IFN-γ during Th1/Th2 treatment of BEAS-2B cell-NK cell co-cultures. Similarly to Figure 3.11, here in Figure 5.17 IFN-γ mRNA and protein was only expressed with addition of NK cells to cultures, IFN-γ protein was only detected from co-culture supernatants, TNF-α mRNA was expressed by BEAS-2B cells and expression induced with RSV infection and TNF-α protein was expressed by RSV infected BEAS-2B cells and

increased with NK cell co-culture at a ratio of 1:3. Priming BEAS-2B cells for 24hrs before RSV infection with IFN-γ, resulted in a significant reduction in IFN-γ mRNA compared to untreated co-cultures (Figure 5.17A). For TNF-α mRNA there was an apparent decrease in TNF-α mRNA, but this was not significant (Figure 5.17C). However, a significant reduction in TNF-α protein was observed when BEAS-2B cells were primed with IFN-γ compared to untreated co-culture alone (Figure 5.17D, condition 7). Priming BEAS-2B cells with Th2 cytokine combination (TNF-α/IL-4/IL-13) and then continued cytokine stimulation after RSV infection and during NK cell co-culture induced IFN-γ mRNA expression and a slight increase in IFN-γ protein compared to untreated co-cultures (Figure 5.17A, B, condition 6). Treatment of BEAS-2B with any other conditions, both priming only and continuous stimulation, did not alter TNF-α mRNA and protein compared to untreated co-cultures (Figure 5.17C, D).

The reduction in IFN-γ mRNA, TNF-α mRNA and TNF-α protein with BEAS-2B cell IFN-γ treatment 24hrs before RSV infection and then during NK cell co-culture could suggest that IFN-γ had induced anti-viral gene and protein expression by BEAS-2B cells. RSV N gene expression would have validated this suggestion. However, TNF-α protein was unaffected when IFN-γ treatment was given before and during RSV infection during NK cell co-culture (Figure 5.19, condition 8/Th1 continuous). Here IFN-γ treatment was only for 24hrs whereas those described in Section 5.3.8.4.1 was a total of 36hrs stimulation. Therefore, it makes it difficult to compare the results from here and in the above section to describe why there was a reduction in IFN-γ mRNA with IFN-γ priming during NK cell co-culture. For instance, in the above section it was proposed that IFN-γ treatment for non-infected BEAS-2B cells enhances IL-15 and IL-15Rα cell surface expression which could possibly lead to enhanced NK cell activation before RSV infection. However this was not observed for IFN-γ and TNF-α mRNA expression during NK cell co-culture in Figure 5.19.

With continuous Th2 cytokine treatment during BEAS-2B cell-NK cell co-culture, an increase in IFN-γ mRNA was observed. The results in Figure 5.14 suggest that there was no increase in cell surface IL-15 and IL-15Rα suggesting it was not cell-surface IL-15/IL-15Rα complex driven. Soluble IL-15/IL-15Rα complex was observed with Th2 cytokine treatment (Figure 5.15) and could be influencing the increase in IFN-γ mRNA rather than through IL-15/IL-15Rα complex stimulation. These results could also be due to unknown changes in protein expression by AECs, such as activating/inhibitory receptors. Furthermore, although IFN-γ protein was not observed with NK cells treated with Th2 cytokines alone, Th2 cytokines could be acting directly on NK cells. IFN-γ mRNA was not determined here and this could have been highlighted if cytokines induced this increase in IFN-γ mRNA level.

The data described in this section is very limited and may reflect changes in other NK cell activating cytokines and/or changes in inhibitory and activating receptors expressed by BEAS-2B cells. These results may also reflect a change in NK cell functions going from cytokine production to cytotoxic responses. All of these variable shave not been examined here but would provide a much more comprehensive description of this model. It is important to note that these results are that of one single time point, using one concentration for each cytokine and lacking other cytokines that may be present within the lung. These results are however important in starting to characterise the cellular communications of AECs and NK cells both before and during RSV infection under chosen and specific cytokine environments. Overall, these results suggest that cytokine environment the airway epithelium is exposed to may alter the expression of AEC-derived NK cell stimulating molecules and thus induce a different NK cell response.

5.5. Summary

NK cell co-culture with BEAS-2B cells induces expression of CXCL9, CXCL10 and CXCL11, of which CXCL10 and CXCL11 were IFN-γ and TNF-α dependent and CXCL9 induction was dependent on another unknown stimulus. Donor matched NK cells co-cultured with HNAECs showed a slight increase in expression of CXCL10 but not for CXCL9 and CXCL11. TARC protein is not expressed during both BEAS-2B cell- and HNAEC-NK cell co-culture or by AECs alone. For HNAECs, TARC mRNA level was induced by RSV and may be reduced with co-culture of NK cells. Cell surface ICAM-1 expression is induced by RSV infection in BEAS-2B cells and is slightly reduced with co-culture of NK cells. For both BEAS-2B cell- and HNAEC-NK cell co-culture, BAFF mRNA expression is induced during co-culture and expression may be a combination from both AECs and NK cells. Between different RSV strains A2 and clinical isolate X, RSV A2 was able to induce more CXCL10 protein level during RSV infection compared to RSV X. However with RSV X infection of BEAS-2B cells during NK cell co-culture, co-culture induced an apparent greater increase in CXCL10 compared to infected BEAS-2B cells only in comparison to RSV A2. Cytokine treatment of non-infected BEAS-2B cells with IFN-γ induced cell surface expression of IL-15, IL-15Rα, ICAM-1 and BAFF. Priming BEAS-2B cells with IFN-γ before RSV infection and then during RSV infection induced the expression of cell surface ICAM-1 and BAFF and soluble BAFF and IL-15/IL-15Rα complex. Priming of BEAS-2B cells before RSV infection and then co-culture with NK cells reduced expression of IFN-γ mRNA, but not protein, an apparent decrease in TNF-α mRNA and decrease in TNF-α protein. Priming BEAS-2B cells with Th2 cytokines before RSV infection then treatment during RSV infection with NK cell co-culture induced expression of IFN-γ mRNA and protein.

Figure 5.19 details a summary of the results observed within this work here. RSV infected AECs stimulate NK cells during direct cell-contact to express IFN-γ and possibly TNF-α. IFN-γ and TNF-α present in the culture supernatant may then act back on AECs to induce further

expression of Th1 associated chemokine CXCL10. This would aid in recruitment of $CXCR3^+$ immune cells to sites of infection within the lung. Expression of CXCL9 and CXCL11 cannot be concluded here. AEC-derived BAFF expression may also be induced during NK cell co-culture, with NK cells potentially contributing to overall BAFF expression. This would aid in B cell activation and production of RSV –specific antibodies. IFN-γ and TNF-α may act synergistically to enhance expression of AEC-derived BAFF, IL-15/IL-15Rα complex and ICAM-1. Overall, co-culture between RSV infected AECs and NK cells display unique interactions which enhance both AEC-derived and NK cell-derived inflammatory responses.

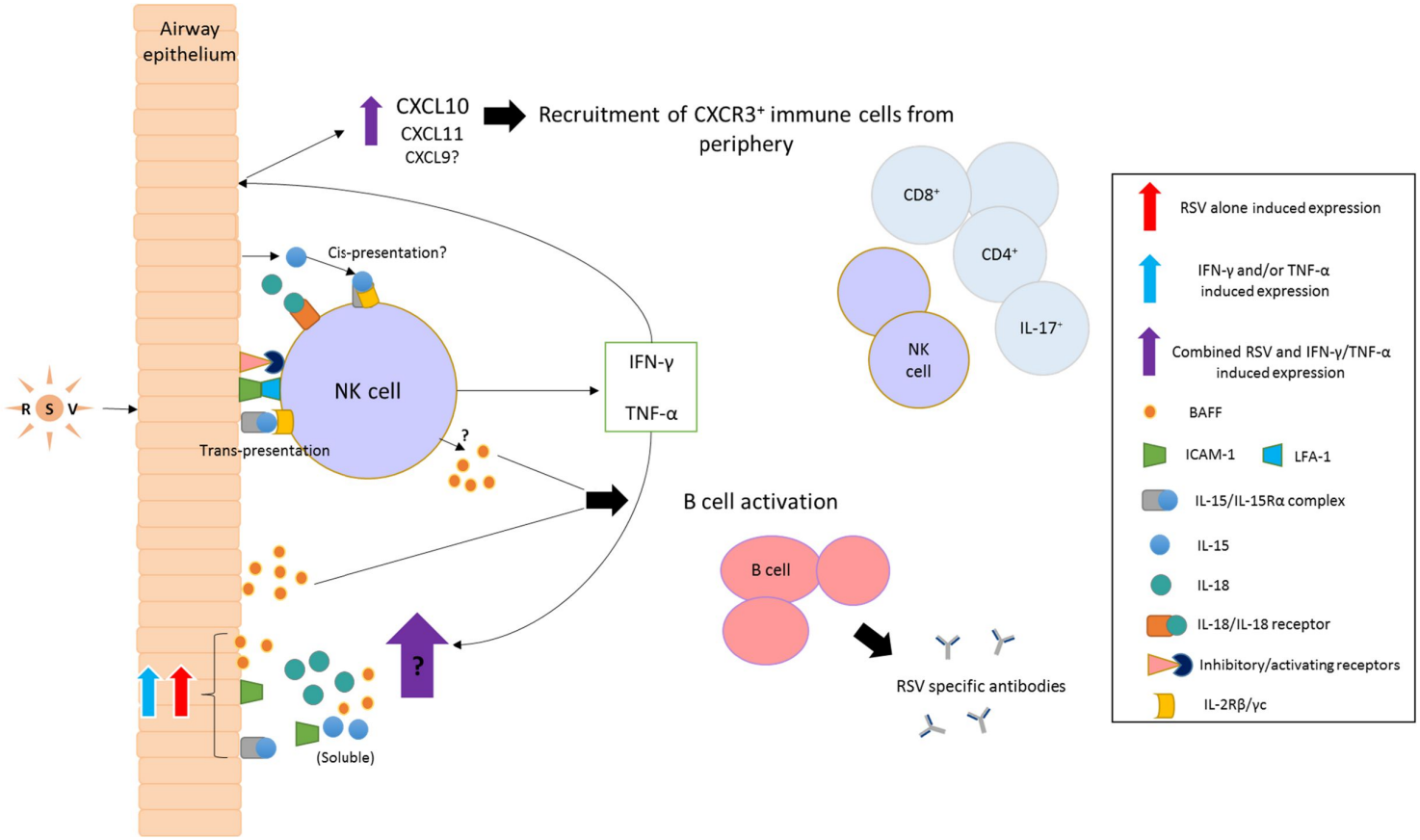


Figure 5.19. An enhanced Th1 response is observed during co-culture of RSV infected BEAS-2B cells and NK cells. RSV infected AECs express cell surface IL-15/IL-15Rα complex which through trans-presentation, and through other cellular interactions and secreted molecules such as ICAM-1 and IL-18, stimulate NK cells to express IFN-g and TNF-α. IFN-g and TNF-α present in the culture supernatant may then act back on AECs to induce further expression of Th1 chemokine CXCL10. AEC- derived BAFF may also be induced during NK cell co-culture. IFN-g and TNF-α may act synergistically to enhance expression of BAFF, IL-15/IL-15Rα complex and ICAM-1 on AECs.

Chapter 6. General discussion and future work

6.1. RSV induces AEC expression of NK cell activing cytokines IL-15, IL-18 and receptor IL-15Rα

The overall hypothesis examined in this thesis was that alone AECs, after infection, can activate NK cells by expression of NK cell-activating cytokines and the IL-15Rα receptor. The results described in Chapters 3 and 4 provide evidence to show that *in vitro* RSV infection of BEAS-2B cells and HNAECs induced expression of IL-15 and IL-15Rα. Both BEAS-2B cells and HNAECs expressed IL-15Rα at the cell surface only after RSV infection (Figures 3.6 and 4.13). Secreted IL-15 was released by infected BEAS-2B cell in culture but not by infected HNAECs. As soluble IL-15 was only expressed at low amounts by BEAS-2B cells and undetected in HNAEC cultures, this could suggest IL-15 signalling by the airway epithelium is primarily via the IL-15/IL-15Rα complex and its expression at the cell surface. Here, IL-15Rα was expressed on the cell surface of both BEAS-2B cells (Figure 3.4) and HNAECs (Figure 3.5), and IL-15 for BEAS-2B cells (Figure 4.6). These results indicate that IL-15 signalling could occur through IL-15/IL-15Rα complex provided by infected AECs. This could be beneficial allowing localised and highly controlled IL-15/IL-15Rα signalling only at the site of infection. As the expression of both IL-15 and IL-15Rα were observed at the cell surface of AECs, activation of NK cells in this co-culture model is most probably dependent on surface expression which also implies there is a need for cell-to-cell interactions between infected AECs and IL-15 responsive immune cells.

Both IL-15 and IL-15Rα were expressed inside both non-infected and infected BEAS-2B cells, whereas extracellular expression was only observed with infection (Figures 3.6 and 3.7). A possible explanation as to why intracellular IL-15Rα protein was detected in non-infected BEAS-2B cells could be explained through the expression of different isoforms, discussed in Section 1.3.3.3, in which certain IL-15Rα isoforms and other post-translational modifications

allow for IL-15 complexing and cell surface expression. This may be relevant to the results observed here as intracellular staining of IL-15Rα and IL-15 was observed in non-infected BEAS-2B cells but not at the cell surface of infected cells. However, this is only a suggestion to describe the results in this work as the intracellular control of IL-15 and IL-15Rα protein isoforms has not been explored in BEAS-2B cells and may be different from that observed in cells examined in the literature.

Expression of IL-18 by BEAS-2B cells and HNAEC IL-18 is also described in Chapters 3 and 4. IL-18 protein expression was not observed following RSV infection of BEAS-2B cells but was expressed by both non-infected and infected HNAECs, with an apparent increase in expression on infection. As mentioned in Section 3.4.1, IL-18 requires caspase-1 to convert pro-IL-18 into mature IL-18, which BEAS-2B cells have been shown to lack. A more interesting result observed here is the changes in expression of IL-18 mRNA during RSV infection. For BEAS-2B cells this included a reduction in IL-18 mRNA expression with 24hr infection compared to 4 and 8hr infection (Figure 3.2C). For HNAECs a reduction in IL-18 mRNA was observed across all MOIs used compared to non-infected HNAECs (Figure 4.4). As detailed in Section 4.4.1.2, this could be either a protective mechanism by AECs to prevent overexpression of IL-18 protein or part of RSVs immune modulation to reduce IL-18 signalling which could allow for viral spread.

Other studies have reported AECs express IL-18 protein during other viral infections. RV has been shown to induce IL-18 protein expression in HBECs, at 48hr infection with a low MOI of 0.007 inducing IL-18 to ~2000pg/ml (463). This protein concentration is much more than observed here from RSV infected HNAECs with IL-18 in culture supernatants detected at less than 150pg/ml in all donors following 48hr RSV infection at an MOI of 2.5 (Figure 4.4). This may suggest that AECs are more responsive to RV for the production of IL-18 in comparison to RSV. If so, this could be another way RSV manipulates the immune response. Lower IL-18

expression could result in reduced early NK cell activation by the airway epithelium and allow RSV to spread at low titres throughout the airways, such as upper to lower airways before NK cells are activated. However NPA analysis here suggests that there is not much difference in overall IL-18 protein present in samples from infants infected with RSV or RV (data not shown). This could suggest that *in vivo* IL-18 expression during RSV and RV infection is similar IL-18 being derived both from the airway epithelium and immune cells. Overall, IL-18 is expressed by AECs and within the airways during viral infections. Further characterisation of *in vivo* IL-18 expression following RV and RSV infections will aid in determining if IL-18 expression is related to virus specific NK cell activation levels and how this may relate to disease severity.

Other epithelial cells, such as oral epithelial cells, have also been shown to express IL-18 (464). These cells expressed IL-18 mRNA and pro-IL-18 but not the active, mature form of IL-18. Therefore epithelial barriers may be a critical store of IL-18 both at mRNA and pro-IL-18 levels. AECs may also secrete pro-IL-18 without infection and provide an alternative method of IL-18 signalling during respiratory virus infection. For example, caspase-1 has been shown to be secreted by activated monocytes and therefore have the potential to cleave pro-IL-18 if it is expressed by AECs during RSV infection (465). However pro-IL-18 protein expression by RSV infection AECs would need to be determined to conclude if this method of IL-18 signalling could occur during RSV infection.

The activity of IL-18 is also dependent on expression of IL-18BP (Section 1.3.3.2). This was not examined here at either gene expression or protein level and would have been a complimentary addition to this data set. To my knowledge, IL-18BP activity has not be assessed during RSV infection of human epithelial cells. IL-18BP gene expression has been reported to be upregulated ~1.8-4-fold in lungs of mice during RSV infection (466,467) and may be regulated *in vitro* by cytokines such as IFN-γ (468). Expression of IL-18BP could

provide a protective mechanism during inflammatory responses particularly later in infection, possibly by reducing NK cell cytotoxicity. IL-18BP knockout mice showed enhanced TNF-α expression from NK cells along with reduced IFN-γ expression, however overall the expression of IFN-γ was greater in IL-18BP deficient mice (321). This suggests that IL-18BP could also be a key regulator in NK cell responses during infection in the airway epithelium. IL-18BP protein expression has not been studied during RSV infection of AECs and could provide further information on the potential for AECs to activate NK cells.

In conclusion, RSV infected airway epithelium has the potential to activate NK cells and RSV may modulate NK cell activation and activities through expression of AEC-derived IL-15 and IL-18.

6.2. RSV infected AECs induce NK cell activation

The second section of this work was to determine if RSV infected AECs could activate NK cells. This was done using an *in vitro* AEC-NK cell co-culture model which is to the best of my knowledge the first of its kind. As described in Chapters 3 and 4, RSV infection of both BEAS-2B cells and HNAEC and then co-culture with NK cells at a ratio of 1:3 resulted in activation of NK cells determined through expression of IFN-γ and possibly TNF-α (Figures 3.11 and 4.17). IFN-γ gene expression was also highest at an NK cell co-culture ratio of 1:3 for both BEAS-2B cells and HNAECs. Use of HNAECs here was the best model to characterise AEC-NK cell interactions compared to use of BEAS-2B cells. Indeed, IFN-γ mRNA expression was ~2-fold higher from non-infected BEAS-2B cell-NK cell co-culture. This could reflect the use of donor matched NK cells which may be under the influence of additional inhibitory signals in HNAEC- compared to BEAS-2B cell- co-cultures. Direct contact was also essential for IFN-γ and TNF-α expression during BEAS-2B cell-NK cell co-cultures as shown using transwell assays (Figure 3.12). This could be indicative of the relatively low levels of IL-15 secreted by BEAS-

2B cells during RSV infection and compliments the results shown in Figures 3.4, 3.5, and 3.6 in which IL-15 and IL-15Rα were present at the cell surface of infected BEAS-2B cells only.

NK cell-specific intracellular IFN-γ protein was measured during BEAS-2B cell co-culture. This provided information that RSV infected BEAS-2B cells induced more intracellular NK cell-specific IFN-γ protein compared to NK cells cultured with non-infected BEAS-2B cells (Figure 3.15). In particular, intracellular IFN-γ protein was at approximately the same level in both NK cells stimulated with 10ng/ml IL-15 and those co-cultured with infected BEAS-2B cells. However, soluble IFN-γ protein was only detected in co-culture supernatants (Figure 3.11) suggesting that direct cell contact is required for IFN-γ protein secretion. This is an interesting finding as it indicates NK cell secretion of IFN-γ, and other proteins, may be under the influence of direct cell-to-cell contact. This may not be an exclusive characteristic of AECs but also other resident or later recruited immune cell populations as well. Within the lung, it may be better to communicate this way to control inflammatory responses and secretion of soluble factors that could lead to unnecessary and/or prolonged activation of immune cells.

TNF-α was expressed by infected BEAS-2B cells and by both non-infected and infected HNAEC (Figure 4.19) making it hard to distinguish if TNF-α protein was also expressed by NK cells during AEC-NK cell co-cultures. For BEAS-2B cells, TNF-α protein was highest at a ratio of 1:3 NK cell co-culture, however expression of TNF-α by NK cells during HNAEC co-culture was inconclusive. As HNAECs were shown to express IL-18 protein (Figure 4.4) and BEAS-2B cell did not (Figure 3.2), it was expected that more IFN-γ and TNF-α protein would be detected during NK cell co-culture as IL-18 has potent synergistic effects with IL-15 on NK cell functions (Section 1.3.3.4), although this was noticeably not the case. This could be due to stricter control of donor matched NK cell activation in co-cultures with HNAECs, although this was not determined in this work.

Lung NK cells may in general be under tighter control by inhibitory signals from the airway epithelium compared to other organs. This has been suggested to be the case for lung NK cells in mice which had a higher expression of the inhibitory receptor NKG2A and lower expression of the activating receptor CD69 compared to NK cells from other organs (261). If this is so, with potential additional RSV immune evasion mechanisms to reduce IL-18, discussed above, NK cells may not be activated as effectively as compared to other respiratory infections. Comparison between RV and RSV using this co-culture model could have provided complementary findings to the role NK cells play during RSV infection.

Within the lungs NK cell function may also be under the influence of other immune cells during RSV infection and in particular may be dependent on direct contact with both the airway epithelium and immune cells. AECs have been shown to induce differentiation of monocytes into monocyte-derived DC through IL-15 expression (469). Monocyte-derived DCs could go on to further modulate NK responses. Mature human DCs have also been shown to present IL-15 and IL-15Rα on their surface, indicting the importance of direct NK cell contact for optimum IFN-γ expression during DC-NK cell co-culture (470). Furthermore the same study observed that skin DCs (Langerhans cells) did not express cell surface IL-15Rα and even though soluble IL-15 and IL-18 was present in culture supernatants, this failed to induce NK cell activation (471). This again suggests that cell surface expression of IL-15Rα is very important in NK cell activation, such as IL-15 trans-presentation. Expression of IFN-γ was increased during direct DC-NK cell contact and this was shown to be through the interactions of DC-derived membrane-bound TNF-α and membrane TNFR2 on NK cells (431). This response was specific to direct membrane TNF-α-TNFR2 interactions and not through soluble TNF-α, suggesting other beneficial roles direct cell-to-cell contact utilise. Direct NK cell contact with macrophages has also shown to enhance IFN-γ expression during influenza infection (472). This was reported using both the NK cell line, NK-92, and isolated human NK

cells. During RSV infection in mice, mice depleted of macrophages had reduced lung NK cell numbers and those that were analysed had less CD69, indicating lower activation (166).

Overall this indicates that the airway epithelium may not only aid in the activation of NK cells during infection but also activate other immune cell populations to further enhance NK cell functions. Harker *et al.* studied acute RSV disease during secondary infection of adult mice previously also infected as neonates and reported that macrophages may enhance recruitment of NK cell to the lungs and prolong NK cell activation (166,393). Activated NK cells through these specific cellular communications could induce a series of immune cell responses. NK cell derived IFN-γ and TNF-α were also shown to induce membrane-bound IL-15, via IL-15Rα, on DCs which were then able to induce IFN-γ expression by $CD8^+$ T cells (473). The potential for NK cell derived IFN-γ to increase cell surface expression of IL-15 and IL-15Rα was explored further in Section 5.3.5.1, in which IFN-γ treatment increased cell surface expression of IL-15 and IL-15Rα on non-infected BEAS-2B cells and not infected BEAS-2B cells (Figure 5.14). So through the airway epithelium, activated NK cells may also activate other immune cells and lead to many different and unique cell-to-cell communications. This also includes interactions of the airway epithelium with other immune cell populations.

6.3. NK cells enhance expression of Th1 associated chemokines and BAFF during co-culture of RSV infected AECs

Having established that AECs after infection express NK cell activating cytokines and that in co-culture this leads to NK cell activation and IFN-γ expression, it was important to ask if in turn NK activation altered the AEC inflammatory response. For BEAS-2B cells, Th1 associated chemokines CXCL9, CXCL10 and CXCL11 were all expressed and their expression increased during NK cell co-culture (Figure 5.1). For HNAECs this was less apparent with only CXCL10 levels slightly higher with NK cell ratio at the highest ratio of 1:3 (Section 5.3.5.1). For

HNAECs, this could be due to the early time-point used and only 24hr co-culture and 48hr total RSV infection as described in more detail in Chapter 5's discussion (Section 5.3.8.1). Another study reported HNAECs and HBECs show greatest CXCL10 and CXCL11 expression at 96hrs post RSV infection (474). However, the bottom line is that expression of chemokines, in particular CXCL10, was changed indicating NK cells can influence AEC-responses in line with the hypothesis. Overall, the data presented here suggests that NK cell-derived IFN-γ during the early stages of RSV infection, before the activation and/or recruitment of $CXCR3^+$/IFN-γ expressing immune cells, may enhance AEC-derived CXCL10. Furthermore, both AECs and NK cells may contribute towards an adaptive immune response through an increase in BAFF, discussed below.

A major observation in this part of my study was increased CXCL10 expression. The results demonstrate that CXCL10 mRNA and protein expression during NK cell co-culture with BEAS-2B cells increases in proportion to the number of NK cells present. The more NK cells present, the more CXCL10 protein may be expressed *in vivo*. Indeed for Th1 associated chemokine protein expression there seemed to be an optimum infected BEAS-2B cell-NK cell ratio of 1:3. Overall this indicates how important the total number of NK cells may be as a determinant of AEC-derived chemokine expression. In humans, a lack of NK cells during respiratory viral infection may lead to inefficient and early Th1 associated chemokine expression by the airway epithelium which could lead to delayed $CXCR3^+$ immune cell infiltration. This could lead to prolonged and uncontrolled viral spread and replication. On the other hand, excessive NK cell numbers may lead to excessive immune cell infiltration and an imbalance in Th1 responses, which has been linked to RSV illness (Section 1.2.3). Therefore, a reasonable question to ask during RSV infection is whether the total number of NK cells influence RSV disease outcome.

Here, there was a clear dose-dependent increase in IFN-γ protein expression with an increase in the ratio of NK cells during direct contact with infected BEAS-2B cells (Figure 3.12A, B). Moreover, a comparison between NK cells at a ratio of 1:3 in Figures 3.12B and 3.16 show that when the total number of NK cells was increased in the transwell assay, more IFN-γ protein was detected which was ~2-fold greater. This was not observed for TNF-α which seemed to be more dependent on the ratio of BEAS-2B cells to NK cells rather than an increase in NK cell numbers. This could have implications on the Th1/2 balance as directed by NK cells through AEC-activation. For instance, the presence of more NK cells during the early stages of infection could produce a stronger Th1-repsonse through IFN-γ expression and then Th1 associated chemokine expression. Here a TNF-α, or Th2, response was not as pronounced as the IFN-γ, or Th1, response. NK cell numbers are lower in infants compared to adults and lower for infants born prematurely compared to full term (475–477). Lower NK cell numbers in infants could lead to a reduced recruitment and activation of T cells through reduced AEC-NK cell co-ordinated chemokine expression. Furthermore, T cells stimulated with PMA/ionomycin from infants were not able to illicit a strong IFN-γ and TNF-α response in comparison to adults and with increase in infant age, a positive correlation is seen for IFN-γ/TNF-α producing T cells (478). Therefore in infants, a lack of sufficient NK cell numbers could then lead to reduced T cell recruitment to the lung and could be a possible contribution towards RSV illness. Limited T cell responses and IFN-γ expression has been studied in mouse models (Section 1.2.2.1) where $CD8^+$ T cells have been shown to limit Th2-driven RSV pathology and IFN-γ reduced eosinophilia on re-infection (185,203).

A published study reported experiments in which A549 cells and HBECs were co-cultured with human PBMCs (479). This study showed a similar CXCL10 profile as those observed here in Figure 5.3. No CXCL10 protein was detected from PBMCs, A549 cells and HBECs cultured alone. CXCL10 was then expressed with the addition of IFN-γ. Then during A549/PBMC and HBEC/PBMC co-culture there was a significant induction in expression of CXCL10 with IFN-γ

stimulation. Co-cultures alone did not express CXCL10. The observations made in these studies and the results shown within this thesis indicate that a Th1 cytokine environment, or IFN-γ, co-operate to induce increased expression of CXCL0. Mouse models have shown a ~2-fold greater induction in lung CXCL10 mRNA during RSV infection compared to other chemokines making it a key chemokine to examine during RSV infection (480). In another study, mice were generated to only express IFN-γ receptors on AECs and it was observed that AECs, through IFN-γ signalling, were able to reduce mucus expression and BAL eosinophilia during allergic airway inflammation (481). This mouse model indicates that IFN-γ acting on AECs may reduce certain inflammatory responses, such as Th2 responses. In a human study in which nebulized IFN-γ was used to treat people with asthma, although small, 4/5 patients had a decrease in BAL eosinophil number (482). Furthermore, CXCL10 levels were significantly increased by IFN-γ treatment, ~1.5 fold indicating a very direct role for IFN-γ on the airway epithelium and CXCL10 expression *in vivo*.

Another role NK cells may play towards the wider immune response to RSV is the production of BAFF for the development of adaptive immune responses. For BAFF, an overall increase in mRNA was observed during co-culture but this was not reflected in levels of the soluble protein, in either BEAS-2B cell or HNAEC-NK cell co-cultures (Figures 5.6 and 5.18). This increase in BAFF mRNA could be from both IFN-γ induced AEC-derived BAFF mRNA expression and potentially NK cell derived BAFF mRNA (Section 5.3). BAFF expression has previously been shown to be induced by IFN-γ and TNF-α in BEAS-2B cells and HBECs (151). To my knowledge, this is the first time BAFF expression has been characterised during RSV infection of AECs following IFN-γ and TNF-α treatment. Furthermore, a synergistic increase in BAFF cell surface protein and secreted protein was observed during RSV infection of BEAS-2B cells with priming and continuous treatment with IFN-γ (Figure 5.14 and Figure 5.15).

AECs appear to be an important, and possibly the earliest, source of BAFF during RSV infection (145,448). However AECs may not be the only source of BAFF during RSV infection. Neutrophils and macrophages have also been shown to express BAFF with neutrophils requiring treatment of IFN-γ and IL-8 or TNF-α to express BAFF (483,484). The data here and that of the literature suggests that NK cells may not only be a contributor of BAFF themselves but aid in release of BAFF from other immune cells through expression of IFN-γ and TNF-α. This response could also be in co-ordination with the expression of airway epithelium cytokines. Another immune cell population that could benefit from BAFF expression by the airway epithelium and possibly NK cells are T cells. BAFF-R has been observed on activated T cells with BAFF possibly limiting certain T cell functions such as IFN-γ and granzyme B expression (485,486). Co-ordinated expression of BAFF through airway epithelium and NK cells interactions may therefore also influence T cell as well as B cell responses during RSV infection. Also worth noting is that a second signal maybe be needed for release of membrane-bound BAFF, such as the expression of serine proteases, which may recruited and activated through AEC-NK cell-derived chemokines and cytokines. Overall this leads to the suggestion that NK cells may be a key regulator of BAFF responses either through direct BAFF expression or indirectly by activating other immune cells to express it and/or proteases to allow for surface cleavage. This area requires further study.

6.4. Could NK cell-induced, AEC-derived CXCL10 expression contribute to RSV illness?

An important and still debated topic is whether NK cells are beneficial during RSV disease resolution or contribute to RSV bronchiolitis, such as through expression of IFN-γ. In mouse models, primary RSV infection of IFN-γ deficient mice led to severe RSV pathology upon re-challenge suggesting IFN-γ plays a critical protective role (185). However IFN-γ has also been

shown to inhibit RSV-specific antibody production in mice which would allow for repeat reinfections (400). The roles of NK cell-derived IFN-γ in humans has not been defined. In relation to the results described in this work, NK cell derived IFN-γ induced AECs to express CXCL10 (Figure 5.1).

It is known that RSV induces an increase in CXCL10 levels in BAL and serum of infants with bronchiolitis, with reduced numbers of CXCR3 positive lymphocytes in the blood suggesting recruitment of for example Th1 cells and NK cells to lungs (234,487). Other respiratory viruses have been shown to induce similar amounts of CXCL10 in serum of infants suggesting CXCL10 is a commonly expressed chemokine during respiratory viral infection (233). Influenza virus infection of HBECs cultured with IFN-γ for 24hrs induced synergistic increase in CXCL10 expression (153). The role CXCL10 plays during RSV pathogenesis remains unclear. Excessive CXCL10 protein may lead to more severe RSV illness. CXCL10 protein has been positively correlated to viral load, mucosal and plasma IgG levels and RSV severity scores in infants (233,234,487,488). However a more recent study suggested the opposite with reduced nasal fluid CXCL10 protein observed in infants with severe RSV bronchiolitis (489). This was associated with reduced viral load and IFN-γ levels and, as mentioned above, could reflect insufficient IFN-γ levels which leads to reduced CXCL10 expression and reduced $CXCR3^{+}$ immune cell infiltration. McNamara *et al*. observed that in intubated infants with severe RSV disease CXCL10 was highest at 1 day and levels decreased steadily up to 7 days (234). The CXCL10 expression profile has also been shown to correlate with viral load (488) with the NK cell population and viral load also return to base-line levels around 6 days post infection (Section 1.4). Therefore, NK cells could be a contributor to this potentially excessive CXCL10 expression through AEC-NK cell interactions and IFN-γ expression.

During human RV infection, significant increases in mucosal proteins levels of IFN-γ, CXCL11, CXCL10 and IL-15 were observed for those with asthma and not in those without asthma but

also infected with RV (447). Therefore, these cytokines and chemokines may also play a role in allergic inflammatory responses. This could also suggest a connection between AEC-derived IL-15, NK cell-derived IFN-γ, CXCL10 and CXCL11 during viral infection and a potential for them to be involved in the initiation of future respiratory diseases. This could be driven through early AEC-NK cell cellular communications. Whether RSV infection may led to the development of asthma in children has been widely debated. AEC-NK cell crosstalk may be important in the expression of these inflammatory molecules during RSV infection which may then be involved in the development of asthma. No correlation was observed between IL-18 and IFN-γ during RV infection in infants, however CXCL10 did correlate to IFN-γ and IL-12 (490). Positive correlation between serum IL-18 and IFN-γ has been associated with pulmonary sarcoidosis(491), although no correlation was observed from NPAs in the work described in Section 4.3.10. In one study, infants who required hospitalisation with RSV illness had higher NPA levels of IFN-γ, IL-15 and CXCL10 than those who were not hospitalised (439). This study also reported that those hospitalised and under 6 months old had lower IFN-γ and CXCL10. Furthermore, unlike with RSV infection, infants with RV infection did not show any difference in expression of IFN-γ, IL-15 and CXCL10 between hospitalised and non-hospitalised groups. This could indicate that IL-15, IFN-γ and CXCL10 expression levels are specific to RSV in regards to disease outcome and less so for RV.

As the airway epithelium is the initial driver of the immune response, defective AECs may also contribute to RSV disease pathology by having knock-on effects for immune cell populations which may appear to an apparent result of the examined immune cells. In a different co-culture model, HNAECs were co-cultured with monocyte-derived DCs during influenza infection (492). The aim of this study was to compare the co-culture Th1/Th2 responses of non-smokers and smokers. Here they observed that smokers with additional influenza virus infection showed reduced CXCL10 protein and increased TARC protein. This suggests that the overall Th1 and Th2 response is also dependent on the state of the airway

epithelium and therefore defective AECs may also contribute to RSV illness which ultimately begin and drive the immune response. Indeed, infants exposed to smoke have increased risk of more severe RSV illness, again indicating the lung environment before infection shapes disease outcomes (493,494). Use of cigarette smoke here in this work could have provided information on expression levels of IL-15, IL-18 and IL-15Rα by AECs and then if cigarette smoke alters NK cell responses during co-cultures.

Overall regarding the results observed in Section 5.3.5, the response of AECs and NK cells will also be influenced by cytokines present both before and during infection. A specific lung environment, such as Th1, Th2 or Th17 one, may alter AEC-NK cell interactions. NK cells themselves may therefore not be the driving force of potential IFN-γ or CXCL10-induced pathologies, but part of a wider imbalance in certain inflammatory mediators and immune cell responses.

6.5. RSV strains influence response to RSV which may influence NK cell response

Throughout this work the response to the clinical isolate RSV X was also used and compared to that to RSV A2. In Chapter 3, BEAS-2B cells infected with RSV X and co-cultured with NK cells expressed slightly more IFN-γ and TNF-α protein (Figure 3.21) which may reflect the increase in IL-15Rα mRNA observed for RSV X compared to RSV A2 (Figure 3.20). Results presented in Chapter 4 show expression of IL-15Rα mRNA is the same after infection of HNAECs with RSV A2 or RSV X, however IL-15 mRNA was ~2-fold higher after RSV X infection (Figure 4.8). Finally in Chapter 5, RSV A2 infected BEAS-2B cells alone expressed more CXCL10 protein in comparison to RSV X (Figure 5.9). However during co-culture of NK cells, RSV X induced a ~3-fold greater increase in CXCL10 expression when compared to RSV infection alone than that observed with RSV A2 (Figure 5.10). Overall these results suggest that clinical

isolates may, through increased expression of IL-15 and/or IL-15Rα, enhance NK cell activation, such as IFN-γ and TNF-α expression, which induces further CXCL10 expression from AECs. This possibility however needs more examination including measurement of cell surface IL-15 and IL-15Rα by flow cytometry comparing between RSV A2 and RSV X and then intracellular IFN-γ expression in NK cells. Different RSV strains could therefore affect the magnitude of the AEC and NK cell response and comparison between different clinical isolates should also be characterised. For example use of RSV-Long strain, which lacks the ability to suppress type I interferon induction, infected AEC-NK cell co-cultures could provide further insight into the role of RSV NS protein suppression of AEC-IL-15, -IL-18 and -IL-15Rα and how this relates to NK cell responses (126). Different cytokine expression has been observed in BALB/cJ mice between different RSV laboratory strains A2, Long and Line 19 and also between laboratory strains and clinical isolates (495,496). This indicates that although the use of clinical isolates, and not lab strains, may provide a more representative response to human RSV infection, it is still not a direct comparison to *in vivo* responses. Nevertheless, it is still important to characterise responses to RSV that is as close to human RSV infection within animal or AEC *in vitro* infection models, which clinical isolates may provide.

6.6. Model of AEC-NK cell interactions during RSV infection

A classical view on the responses to RSV infection describes the infected airway epithelium expressing cytokines and chemokines which then act on immune cells. The immune cells then co-ordinate viral elimination through expression of anti-viral molecules, killing virally infected AECs, clearing cell debris and initiating an immunological memory. The work here describes a method of co-operation and communication between the airway epithelium and early activated NK cells, which may also be applicable to other resident immune cells, which together orchestrate the immune response to RSV. This model may also suggest that an

ineffective airway epithelium response to RSV infection, such as reduced IL-15/IL-15Rα complex cell surface expression, and then response to immune cell derived cytokines, such as NK cell-derived IFN-γ and CXCL10 upregulation, could lead to enhanced or inefficient cytokine expression.

Figure 6.1 illustrates a proposed model of airway epithelium-NK cell interactions during RSV infection which draws on the results described within this work and observations reported in the literature. First, RSV induces expression of IL-15 bound to surface expressed IL-15Rα which activates resident NK cells through trans-presentation. Still unknown is whether soluble AEC-derived IL-15 can also activate NK cells via a method of cis-presentation. AEC cell surface ICAM-1 expression, induced by RSV infection, could aid in NK cell responses and/or adherence to the epithelium. Then through specific cellular communications between the airway epithelium and NK cells, NK cells expression IFN-γ and possibly TNF-α. Macrophages and DCs are also present and active during the early inflammatory response and may aid in additional cellular communications with NK cells. NK cell-derived IFN-γ and TNF-α could then act back on the airway epithelium to enhance expression of Th1 associated chemokines, in particular CXCL10, to aid in the recruitment of $CXCR3^+$ immune cells from the periphery to aid in viral clearance, including $CD4^+$, $CD8^+$ and IL-17 expressing T cells. Increased expression of BAFF from the airway epithelium by IFN-γ, and possibly also sourced from NK cells, would aid in adaptive immune responses, such as B cell activation and production of RSV-specific antibodies. The expression of NK cell-induced expression of AEC-derived BAFF, ICAM-1, IL-15/IL-15Rα complex and IL-18 requires further examination as these may be induced by RSV alone (red arrow), as an additive expression with cytokines (blue arrow), or in a synergistic fashion with cytokine stimulation (purple arrow). Overall, during RSV infection the balance between protective immunity and immunopathogenesis may be strongly influenced by AEC-NK cell and/or other resident immune cell cellular communications.

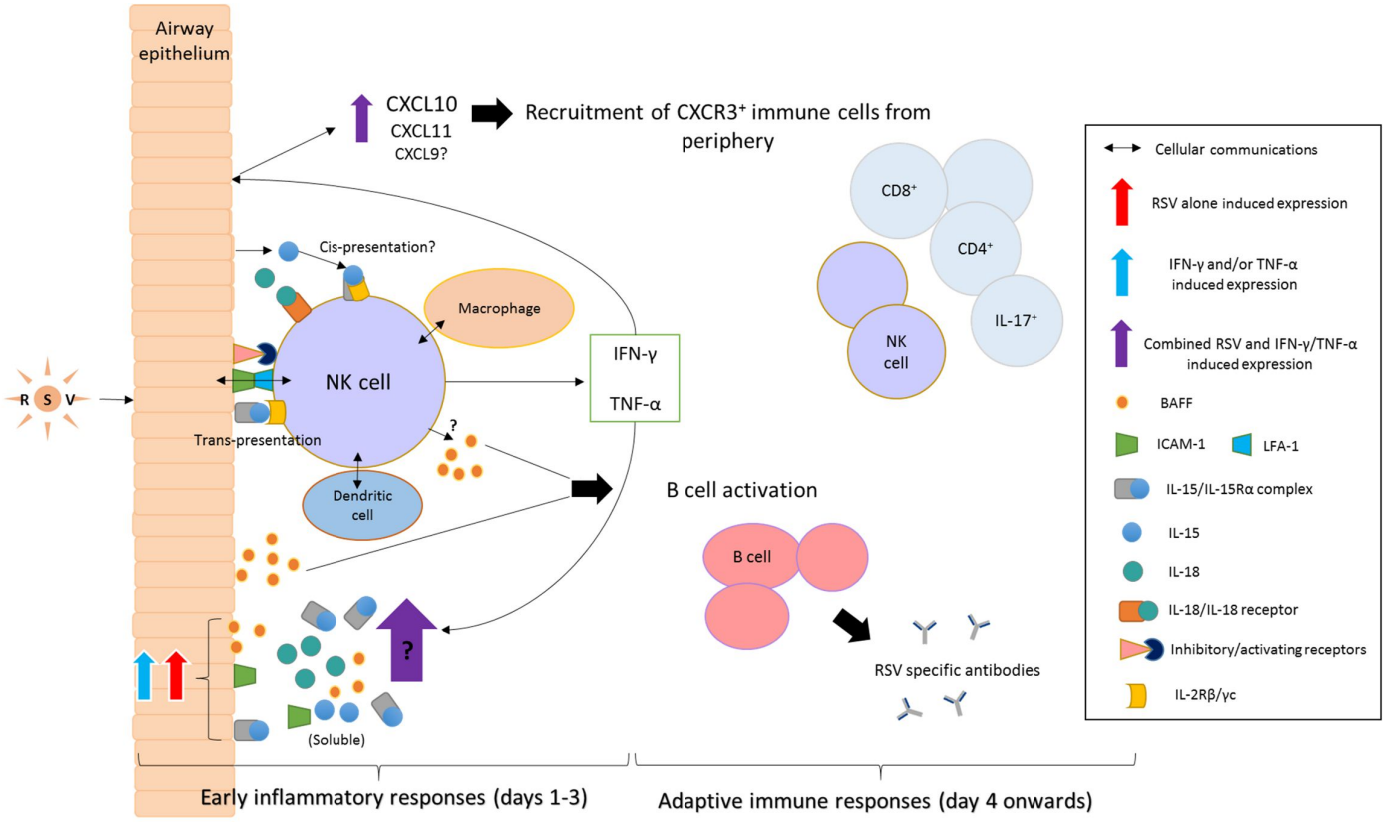


Figure 6.1. Proposed model of airway epithelium-NK cell interactions to RSV infection. Infected AECs activate NK cells through cell surface IL-15 and soluble IL-18. Activated NK cells express IFN-γ and TNF-α which can then act back on the airway epithelium to induce expression of Th1 chemokines, particularly CXCL10, and possibly other IFN-γ/TNF-α responsive genes such as BAFF. Enhanced expression of CXCL10 and BAFF driven by activated NK cells could aid in the recruitment of T cells and B cells, respectively, thus initiating the adaptive immune response. This could be through generation of RSV-specific antibodies or memory T cells. Interactions between NK cells, macrophages and DCs may also occur to further orchestrate the immune response.

6.7. Limitations and implications of an *in vitro* AEC-NK cell co-culture model

One key limitation of the experiments presented in this thesis is the lack of knowledge of NK cell cytotoxicity during these AEC co-cultures. Cytotoxicity, whilst not the focus of these experiments, is nevertheless a key feature of NK cells activity and aids in reducing viral replication. A reduction in RSV N gene expression was observed in both BEAS-2B cell and HNAEC cell NK cell co-cultures (Figures 3.10 and 4.16). This could indicate NK cell targeted lysis of infected cells reducing RSV replication. However this could also be due to the anti-viral effects of cytokines expressed during co-cultures, such as IFN-γ and/or type I interferons. The expression of other cytokines were either unaltered, such as IL-8, or increased, such as CXCL10, during co-cultures indicating that the NK cell responses was not exclusively cytotoxic. Perforin expression was determined by Liz Van Erp and is described in section 3.3.10. However these experiments used BEAS-2B cell culture supernatants only which do not reflect responses during direct AEC-NK cell co-cultures.

One potential limiting factor of the BEAS-2B cell-NK cell co-culture model was the absence IL-12 and IL-18 protein in culture supernatants and for HNAECs the lack of IL-12 which work synergistically to enhance NK cell functions and could be expressed by other immune cells during infection. DC-NK cell co-culture models show that NK cells require IL-12 and IL-18 for optimum IFN-γ expression. This suggests a limited and/or muted NK cell response could have been observed within these AEC-NK cell co-cultures lacking IL-12 and/or IL-18. However the results obtained here for HNAECs overall reflects potential *in vivo* responses and early NK cell activation, in which IFN-γ protein was expressed by NK cells during co-culture. Furthermore, IFN-γ expression in the lungs of RSV infected mice was not altered by IL-12 and/or IL-18 deficiency in the early stages of infection (497). Assuming early IFN-γ expression in this published study was to be NK cell derived, this suggests that there could be alternative

methods of NK cell activation during early stages of infection, such as direct cell-to-cell contact and IL-15/IL-15Rα complex as described in this thesis. During RSV infection, specific and tightly controlled direct cellular communications may occur between the airway epithelium and NK cells leading to IFN-γ expression. Additionally, as was discussed in Section 3.4.7, for both BEAS-2B cells and HNAECs there is a lack of knowledge about basolateral secretion of NK cell activating proteins within this study which may also affect NK cell activation and the results observed here.

Another limitation of this co-culture model is that the expression of other NK cell activating and inhibitory cytokines have not be assessed. Cytokines that have been shown to be expressed by BEAS-2B cells during RSV infection that can stimulate NK cells and have not been characterised in the work here include IL-1β, IFN-α and IFN-β (426,498). Here a focus was on cytokines that were believed to be key ones for NK cell activation. NK cell activating and inhibitory receptors will also be involved in the NK cell responses within these co-culture models and were not examined here. For instance, IFN-γ treatment alone and RSV infection was shown to increase cell surface MHC class I expression on BEAS-2B cells and HBECs after 24hr infection (152). Self- or healthy-cell expression of MHC class I molecules acts to inhibit NK cell activation (Section 1.3.2). This could be a beneficial role of IFN-γ to reduce prolonged NK cell activation and may reflect the short life-span of NK cell population expansion observed during RSV infection (Section 1.4). On the other hand, RSV was also shown to increase MHC class I molecules on BEAS-2B cells and this could be an immune evasion method RSV uses to impair immediate NK cell activation through the airway epithelium and thus allow for undetected viral spread. Therefore, the work here lacks a complete AEC-specific NK cell activating profile which would give more information and provide further understanding of AEC-NK cell communications during RSV infection. Now that key factors and time points have been defined, this model could be advanced by a more detailed examination of the changes in gene expression by both AECs and NK cells. For

instance, cells could be sorted from co-cultures and a micro array based approach, used to fully define the changes in gene expression. This would allow for analysis of proteins not characterised in this assay and aid in characterising AEC and NK cell interactions.

Another limitation is the purity of NK cells which was at or above 95%. Therefore up to 5% of the unknown cells may contribute to the responses observed here. However, RSV infected BEAS-2B cells were shown to induce expression of intracellular IFN-γ which was specific to NK cells through flow cytometric gating. Therefore, RSV infected AECs do have the ability to induce NK cell activation and expression of NK cell-derived IFN-γ.

Another consideration to make in regards to the AEC-NK cell co-culture model used here is the potential for NK cells to also be infected by RSV. RSV particles may be present from lysed AECs cells, either due to viral replication or due to NK cell lysis. A recent study has observed that NK cells contain intracellular RSV viral particles (499). Furthermore in addition with sub-neutralising RSV-antibody complexes, NK cells displayed lower activation marker receptors, increased inhibitory receptors. This suggests that NK cells with RSV infection may require higher thresholds to be activated if infected with RSV. Although, this study also showed that another NK cell activation marker, CD107a, and NK cell-derived IFN-γ expression was increased with RSV infection alone, but not the expression of perforin. In regards to the results in this work, infection of BEAS-2B cells with an RSV expressing a fluorescent protein and intracellular flow cytometry after co-culture would have determined if NK cells in this model contained viral particles. Overall, further characterisation is required for NK cell functions during RSV infection.

The use of peripheral blood NK cells here in these co-culture assays may not share the same phenotype and activity as resident lung NK cells would during RSV infection and is therefore another limitation of this model. This co-culture model may be more representative of the infiltrating NK cell response rather than that of resident NK cells which will ultimately be the

first to be activating and interact with the airway epithelium. ~90% of NK cells used in this work were $CD56^{dim}$, with the percentage of $CD56^{bright}$ NK cells being 6.8% (range 0.5-13.4) (data not shown). Both lung resident and peripheral blood NK cells show a $CD56^{dim}$ phenotype (Section 1.3.1) which may allow for a similarities between the NK cell responses observed here *in vitro* to *in vivo* responses. However resident airway NK cells may be under different levels of control compared to peripheral NK cells and if resident NK cells were used as part of this co-culture here, different results may have been observed. Use of lung or nasal NK cells would improve this co-culture model, however obtaining these specific NK cell populations and at sufficient numbers would likely to be hard to do. Therefore use of peripheral blood NK cells is the best option for this work.

Overall, both the BEAS-2B cell and HNAEC-NK cell co-culture model is less complex than whole lung infection but allows a clear dissection of the response and specific AEC-NK cell interactions not possible *in vivo* and is an improvement on single cell population cultures. This *in vitro* co-culture model is still a better match to the AEC-NK cell interactions that may occur *in vivo* in comparison to use of mouse models. Mice have a lower lung NK cell lymphocyte population percentage than for humans, ~10% compared to ~10-30% (Section 1.3.1). Mouse models may limit the true influence of lung NK cells within in humans. Other lung resident immune cells could also influence both AEC and NK cell responses and then also be involved in AEC-NK cell interactions as well, such as DCs and macrophages. This includes the potential for other NK cell activating cytokines derived from either the airway epithelium or other lung resident immune cells to be expressed and influence the NK cell response. To challenge this triple cell cultures could also be used, but this type of co-culture model was beyond the scope of the aims established for this work.

6.8. Future directions

Overall, the findings in this work describes a unique AEC-NK cell cellular communication during RSV infection. In Chapters 3 and 4 both BEAS-2B cells and HNAECs induced IFN-γ expression during NK cell co-culture. For both AECs used for co-culture models expression of NK cell specific intracellular and extracellular TNF-α protein, perforin and granzyme B expression during direct co-cultures with and without RSV infection would provide a more complete picture of NK cell responses during RSV infection. This includes a more detailed analysis of BAFF expression from NK cells, which has not been determined before during RSV infection.

Furthermore, in regards to the differences in NK cell phenotype seen for infants, comparing the cytokine and cytotoxic profile of primary AEC-donor matched NK cell co-cultures taken from infants/children and adults would detail how age affects both NK cell responses and then enhanced AEC Th1 associated chemokine and BAFF expression during co-culture. This could be extended to the responses of both AECs and NK cells between individuals including those with different airway disease, such as asthma or COPD, where there is an underlying inflammatory activity.

To improve this *in vitro* co-culture model, an ALI culture with human primary AECs and donor matched NK cells could be used. ALI culture can be seen as a more accurate representation of *in vivo* RSV infection of airway epithelium compared to submersion monolayer culture. This would be more representative of a lung environment such as having a differentiated AEC layer and polarised cytokine expression.

Another reasonable comparison to make would the AEC-NK responses during different viral infections such as influenza and RV, including RV strains A, B and C. A comparison between these viruses would determine if certain viruses create specific early AEC-NK cell driven immune profiles. If there are differences in NK cell responses and AEC-Th1/Th2 associated

chemokine expression between viruses, this could provide early viral-specific NK cell driven immune responses and could aid answering the unknown relationship between infant bronchiolitis with recurrent wheeze and the later development of asthma.

For HNAECs, expression of IL-18 protein expression was induced with RSV infection and this was not seen for BEAS-2B cells. To increase the knowledge of NK cell activating potential by primary AECs, pro-IL-18 and IL-18BP expression kinetics over time and with different MOIs should be evaluated. As discussed above, IL-18BP expression has been shown to be induced by IFN-γ in certain cell lines. The AEC-derived IL-18 signalling profile may also be extended through examination of Th1 and Th2 cytokine treatment with priming before RSV infection and continued treatment during RSV infection. This also includes expression of IL-18BP with cytokine treatment as well.

Finally, further characterisation of how different cytokine environments alter AEC-NK cell co-culture responses, such as a Th17 environment or with additional LPS stimulation, could allow for a more detailed observation of *in vivo* responses. This could be important in understanding how a stimulated airway epithelium before RSV infection induces certain resting NK cell phenotypes.

Overall this work has provided valuable knowledge to the field in regards to how RSV infected AECs and NK cells communicate to bring about a co-ordinated immune response, with a focus on IL-15/IL-15Rα complex, IFN-γ and CXCL10 expression.

References

1. Chanock R, Roizman B, Myers R. Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent (CCA): Isolation, properties and characterization. Am J Epidemiol. 1957;66(3):281–90.
2. Morris JA, Blount RE, Savage RE. Recovery of Cytopathogenic Agent from Chimpanzees with Goryza. Exp Biol Med. 1956;92(3):544–9.
3. Darniot M, Pitoiset C, Millière L, Aho-Glélé LS, Florentin E, Bour JB, et al. Different meteorological parameters influence metapneumovirus and respiratory syncytial virus activity. J Clin Virol. 2018;104:77–82.
4. Obando-Pacheco P, Justicia-Grande AJ, Rivero-Calle I, Rodríguez-Tenreiro C, Sly P, Ramilo O, et al. Respiratory Syncytial Virus Seasonality: A Global Overview. J Infect Dis. 2018;217(9):1356–64.
5. Hall CB, Walsh EE, Long CE, Schnabel KC. Immunity to and frequency of reinfection with respiratory syncytial virus. J Infect Dis. 1991;163(4):693–8.
6. Singleton R, Etchart N, Hou S, Hyland L. Inability to evoke a long-lasting protective immune response to respiratory syncytial virus infection in mice correlates with ineffective nasal antibody responses. J Virol. 2003;77(21):11303–11.
7. Ann R. Falsey, M.D., Patricia A. Hennessey, R.N., Maria A. Formica, M.S., Christopher Cox, Ph.D., and Edward E. Walsh MD. Respiratory syncytial virus infection in eldary and high-risk adults. N Engl J Med. 2005;352:1749–59.
8. Lee N, Lui GCY, Wong KT, Li TCM, Tse ECM, Chan JYC, et al. High Morbidity and Mortality in Adults Hospitalized for Respiratory Syncytial Virus Infections. Clin Infect Dis. 2013;57(8):1069–77.
9. McLaurin KK, Farr AM, Wade SW, Diakun DR, Stewart DL. Respiratory syncytial virus hospitalization outcomes and costs of full-term and preterm infants. J Perinatol. 2016;36(11):990–6.
10. Matias G, Taylor R, Haguinet F, Schuck-Paim C, Lustig R, Shinde V. Estimates of mortality attributable to influenza and RSV in the United States during 1997-2009 by influenza type or subtype, age, cause of death, and risk status. Influenza Other Respi Viruses. 2014;8(5):507–15.
11. McMorrow ML, Wemakoy EO, Tshilobo JK, Emukule GO, Mott JA, Njuguna H, et al. Severe Acute Respiratory Illness Deaths in Sub-Saharan Africa and the Role of Influenza: A Case Series From 8 Countries. J Infect Dis. 2015;212(6):853–60.
12. Cromer D, van Hoek AJ, Newall AT, Pollard AJ, Jit M. Burden of paediatric respiratory syncytial virus disease and potential effect of different immunisation strategies: a modelling and cost-effectiveness analysis for England. Lancet Public Heal. 2017;2(8):e367–74.
13. Taylor S, Taylor RJ, Lustig RL, Schuck-Paim C, Haguinet F, Webb DJ, et al. Modelling estimates of the burden of respiratory syncytial virus infection in children in the UK. BMJ Open. 2016;6(6).
14. Nolan T, Borja-Tabora C, Lopez P, Weckx L, Ulloa-Gutierrez R, Lazcano-Ponce E, et al. Prevalence and Incidence of Respiratory Syncytial Virus and Other Respiratory Viral Infections in Children Aged 6 Months to 10 Years With Influenza-like Illness Enrolled in a Randomized Trial. Clin Infect Dis An Off Publ Infect Dis Soc Am.

2015;60(11):e80-9.

15. El Saleeby CM, Bush AJ, Harrison LM, Aitken JA, Devincenzo JP. Respiratory syncytial virus load, viral dynamics, and disease severity in previously healthy naturally infected children. J Infect Dis. 2011;204(7):996–1002.

16. Mansbach JM, Piedra PA, Stevenson MD, Sullivan AF, Forgey TF, Clark S, et al. Prospective Multicenter Study of Children With Bronchiolitis Requiring Mechanical Ventilation. Pediatrics. 2012;130(3):e492–500.

17. Scheltema NM, Gentile A, Lucion F, Nokes DJ, Munywoki PK, Madhi SA, et al. Global respiratory syncytial virus-associated mortality in young children (RSV GOLD): a retrospective case series. Lancet Glob Heal. 2017;5(10):e984–91.

18. Janssen R, Bont L, Siezen CLE, Hodemaekers HM, Ermers MJ, Doornbos G, et al. Genetic Susceptibility to Respiratory Syncytial Virus Bronchiolitis Is Predominantly Associated with Innate Immune Genes. J Infect Dis. 2007;196(6):826–34.

19. Ghazaly M, Nadel S. Characteristics of children admitted to intensive care with acute bronchiolitis. Eur J Pediatr. 2018;177(6):913–20.

20. Amand C, Tong S, Kieffer A, Kyaw MH. Healthcare resource use and economic burden attributable to respiratory syncytial virus in the United States: A claims database analysis. BMC Health Serv Res. 2018;18(1).

21. Alfonso CL, Kurath G. Taxonomy of the order Mononegavirales: update 2016. Arch Virol. 2016;161(8):2351–60.

22. Hirsh S, Hindiyeh M, Kolet L, Regev L, Sherbany H, Yaary K, et al. Epidemiological changes of Respiratory Syncytial Virus (RSV) infections in Israel. PLoS One. 2014;9(3).

23. Zlateva KT, Vijgen L, Dekeersmaeker N, Naranjo C, Van Ranst M. Subgroup prevalence and genotype circulation patterns of human respiratory syncytial virus in belgium during ten successive epidemic seasons. J Clin Microbiol. 2007;45(9):3022–30.

24. Johnson PR, Spriggs MK, Olmsted RA, Collins PL. The G glycoprotein of human respiratory syncytial viruses of subgroups A and B: extensive sequence divergence between antigenically related proteins. Proc Natl Acad Sci. 1987;84(16):5625–9.

25. Hause AM, Henke DM, Avadhanula V, Shaw CA, Tapia LI, Piedra PA. Sequence variability of the respiratory syncytial virus (RSV) fusion gene among contemporary and historical genotypes of RSV/A and RSV/B. PLoS One. 2017;12(6):e0180623.

26. Bose ME, He J, Shrivastava S, Nelson MI, Bera J, Halpin RA, et al. Sequencing and analysis of globally obtained human respiratory syncytial virus a and B genomes. PLoS One. 2015;10(3):e0120098.

27. Anderson LJ, Hierholzer JC, Tsou C, Hendry RM, Fernie BF, Stone Y, et al. Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J Infect Dis. 1985;151(4):626–33.

28. Mufson MA, Orvell C, Rafnar B, Norrby E. Two distinct subtypes of human respiratory syncytial virus. J Gen Virol. 1985;66 (Pt 10(November 1985):2111–24.

29. Tripp RA, Jones LP, Haynes LM, Zheng HQ, Murphy PM, Anderson LJ. CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. Nat Immunol. 2001;2(8):732–8.

30. Johnson SM, McNally BA, Ioannidis I, Flano E, Teng MN, Oomens AG, et al. Respiratory Syncytial Virus Uses CX3CR1 as a Receptor on Primary Human Airway Epithelial Cultures. PLoS Pathog. 2015;11(12):e1005318.

31. Meng J, Hotard AL, Currier MG, Lee S, Stobart CC, Moore ML. Respiratory Syncytial Virus Attachment Glycoprotein Contribution to Infection Depends on the Specific Fusion Protein. J Virol. 2016;90(1):245–53.

32. Jeong K Il, Piepenhagen PA, Kishko M, DiNapoli JM, Groppo RP, Zhang L, et al. CX3CR1 is expressed in differentiated human ciliated airway cells and co-localizes with respiratory syncytial virus on cilia in a G protein-dependent manner. PLoS One. 2015;24(6):e0130517.

33. Marchant D, Singhera GK, Utokaparch S, Hackett TL, Boyd JH, Luo Z, et al. Toll-like receptor 4-mediated activation of p38 mitogen-activated protein kinase is a determinant of respiratory virus entry and tropism. J Virol. 2010;84(21):11359–73.

34. Krusat T, Streckert HJ. Heparin-dependent attachment of respiratory syncytial virus (RSV) to host cells. Arch Virol. 1997;142(6):1247–54.

35. Kurt-Jones EA, Popova L, Kwinn L, Haynes LM, Jones LP, Tripp RA, et al. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol. 2000;1(5):398–401.

36. Tayyari F, Marchant D, Moraes TJ, Duan W, Mastrangelo P, Hegele RG. Identification of nucleolin as a cellular receptor for human respiratory syncytial virus. Nat Med. 2011;17(9):1132–5.

37. Walsh EE, Hruska J. Monoclonal antibodies to respiratory syncytial virus proteins: identification of the fusion protein. J Virol. 1983;47(1):171–7.

38. Srinivasakumar N, Ogra PL, Flanagan TD. Characteristics of fusion of respiratory syncytial virus with HEp-2 cells as measured by R18 fluorescence dequenching assay. J Virol. 1991;65(8):4063–9.

39. McLellan JS, Yang Y, Graham BS, Kwong PD. Structure of Respiratory Syncytial Virus Fusion Glycoprotein in the Postfusion Conformation Reveals Preservation of Neutralizing Epitopes. J Virol. 2011;85(15):7788–96.

40. Johnson PR, Collins PL. The fusion glycoproteins of human respiratory syncytial virus of subgroups A and B: Sequence conservation provides a structural basis for antigenic relatedness. J Gen Virol. 1988;69(10):2623–8.

41. Behera AK, Matsuse H, Kumar M, Kong X, Lockey RF, Mohapatra SS. Blocking intercellular adhesion molecule-1 on human epithelial cells decreases respiratory syncytial virus infection. Biochem Biophys Res Commun. 2001;280(1):188–95.

42. Krzyzaniak MA, Zumstein MT, Gerez JA, Picotti P, Helenius A. Host Cell Entry of Respiratory Syncytial Virus Involves Macropinocytosis Followed by Proteolytic Activation of the F Protein. PLoS Pathog. 2013;9(4).

43. Fuentes S, Tran KC, Luthra P, Teng MN, He B. Function of the Respiratory Syncytial Virus Small Hydrophobic Protein. J Virol. 2007;81(15):8361–6.

44. McLellan JS, Ray WC, Peeples ME. Structure and function of respiratory syncytial virus surface glycoproteins. Curr Top Microbiol Immunol. 2013;372:83–104.

45. Araujo GC, Silva RHT, Scott LPB, Araujo AS, Souza FP, de Oliveira RJ. Structure and functional dynamics characterization of the ion channel of the human respiratory syncytial virus (hRSV) small hydrophobic protein (SH) transmembrane domain by

combining molecular dynamics with excited normal modes. J Mol Model. 2016;22(12).

46. Karron RA, Buonagurio DA, Georgiu AF, Whitehead SS, Adamus JE, Clements-Mann M Lou, et al. Respiratory syncytial virus (RSV) SH and G proteins are not essential for viral replication in vitro: clinical evaluation and molecular characterization of a cold-passaged. Proc Natl Acad Sci U S A. 1997;9(94):13961–6.

47. Bukreyev A, Whitehead SS, Murphy BR, Collins PL. Recombinant respiratory syncytial virus from which the entire SH gene has been deleted grows efficiently in cell culture and exhibits site-specific attenuation in the respiratory tract of the mouse. J Virol. 1997;71(12):8973–82.

48. Whitehead SS, Bukreyev A, Teng MN, Firestone CY, St Claire M, Elkins WR, et al. Recombinant respiratory syncytial virus bearing a deletion of either the NS2 or SH gene is attenuated in chimpanzees. J Virol. 1999;73(4):3438–42.

49. Teng MN, Collins PL. Identification of the Respiratory Syncytial Virus Proteins Required for Formation and Passage of Helper-Dependent Infectious Particles. J Virol. 1998;72(7):5707–16.

50. Li D, Jans DA, Bardin PG, Meanger J, Mills J, Ghildyal R. Association of Respiratory Syncytial Virus M Protein with Viral Nucleocapsids Is Mediated by the M2-1 Protein. J Virol. 2008;82(17):8863–70.

51. Mitra R, Baviskar P, Duncan-Decocq RR, Patel D, Oomens AGP. The Human Respiratory Syncytial Virus Matrix Protein Is Required for Maturation of Viral Filaments. J Virol. 2012;86(8):4432–43.

52. Blondot M-L, Dubosclard V, Fix J, Lassoued S, Aumont-Nicaise M, Bontems F, et al. Structure and functional analysis of the RNA- and viral phosphoprotein-binding domain of respiratory syncytial virus M2-1 protein. PLoS Pathog. 2012;8(5):e1002734.

53. Fearns R, Collins PL. Role of the M2-1 transcription antitermination protein of respiratory syncytial virus in sequential transcription. J Virol. 1999;73(7):5852–64.

54. Tawar RG, Duquerroy S, Vonrhein C, Varela PF, Damier-Piolle L, Castagne N, et al. Crystal structure of a nucleocapsid-like nucleoprotein-RNA complex of respiratory syncytial virus. Science (80-). 2009;326(5957):1279–83.

55. Tiong-Yip CL, Aschenbrenner L, Johnson KD, McLaughlin RE, Fan J, Challa SR, et al. Characterization of a respiratory syncytial virus L protein inhibitor. Antimicrob Agents Chemother. 2014;58(7):3867–73.

56. Hacking D, Hull J. Respiratory syncytial virus--viral biology and the host response. J Infect. 2002;45(1):18–24.

57. Maclellan K, Loney C, Yeo RP, Bhella D. The 24-angstrom structure of respiratory syncytial virus nucleocapsid protein-RNA decameric rings. J Virol. 2007;81(17):9519–24.

58. Ghildyal R. Protein-Protein Interactions in RSV Assembly: Potential Targets for Attenuating RSV Strains. Infect Disord - Drug Targets(Formerly Curr Drug Targets - Infect Disord. 2012;12(2):103–9.

59. Shaikh FY, Crowe JE. Molecular mechanisms driving respiratory syncytial virus assembly. Future Microbiol. 2013;8(1):123–31.

60. Bermingham A, Collins PL. The M2-2 protein of human respiratory syncytial virus is a

regulatory factor involved in the balance between RNA replication and transcription. Proc Natl Acad Sci. 1999;96(20):11259–64.

61. Lo MS, Brazas RM, Holtzman MJ. Respiratory Syncytial Virus Nonstructural Proteins NS1 and NS2 Mediate Inhibition of Stat2 Expression and Alpha/Beta Interferon Responsiveness. J Virol. 2005;79(14):9315–9.

62. Bossert B, Marozin S, Conzelmann K-K. Nonstructural proteins NS1 and NS2 of bovine respiratory syncytial virus block activation of interferon regulatory factor 3. J Virol. 2003;77(16):8661–8.

63. Spann KM, Tran KC, Collins PL. Effects of nonstructural proteins NS1 and NS2 of human respiratory syncytial virus on interferon regulatory factor 3, NF-kappaB, and proinflammatory cytokines. J Virol. 2005;79(9):5353–62.

64. Bagga B, Woods CW, Veldman TH, Gilbert A, Mann A, Balaratnam G, et al. Comparing influenza and RSV viral and disease dynamics in experimentally infected adults predicts clinical effectiveness of RSV antivirals. Antivir Ther. 2013;18(6):785–91.

65. Gonzàlez-Parra G, De Ridder F, Huntjens D, Roymans D, Ispas G, Dobrovolny HM. A comparison of RSV and influenza in vitro kinetic parameters reveals differences in infecting time. PLoS One. 2018;13(2):e0192645.

66. Zhang L, Peeples ME, Boucher RC, Collins PL, Pickles RJ. Respiratory Syncytial Virus Infection of Human Airway Epithelial Cells Is Polarized, Specific to Ciliated Cells, and without Obvious Cytopathology. J Virol. 2002;76(11):5654–66.

67. Welliver TPP, Garofalo RPP, Hosakote Y, Hintz KHH, Avendano L, Sanchez K, et al. Severe Human Lower Respiratory Tract Illness Caused by Respiratory Syncytial Virus and Influenza Virus Is Characterized by the Absence of Pulmonary Cytotoxic Lymphocyte Responses. J Infect Dis. 2007;15(8):1126–36.

68. Persson BD, Jaffe AB, Fearns R, Danahay H. Respiratory syncytial virus can infect basal cells and alter human airway epithelial differentiation. PLoS One. 2014;9(7):e102368.

69. Johnson JE, Gonzales RA, Olson SJ, Wright PF, Graham BS. The histopathology of fatal untreated human respiratory syncytial virus infection. Mod Pathol. 2007;20(1):108–19.

70. Villenave R, Thavagnanam S, Sarlang S, Parker J, Douglas I, Skibinski G, et al. In vitro modeling of respiratory syncytial virus infection of pediatric bronchial epithelium, the primary target of infection in vivo. Proc Natl Acad Sci U S A. 2012;109(13):5040–5.

71. Jumat MR, Yan Y, Ravi LI, Wong P, Huong TN, Li C, et al. Morphogenesis of respiratory syncytial virus in human primary nasal ciliated epithelial cells occurs at surface membrane microdomains that are distinct from cilia. Virology. 2015;484:395–411.

72. Baum A, García-Sastre A. Induction of type i interferon by RNA viruses: Cellular receptors and their substrates. Vol. 38, Amino Acids. 2010. p. 1283–99.

73. Tian B, Zhang Y, Luxon BA, Garofalo RP, Casola A, Sinha M, et al. Identification of NF-κB-dependent gene networks in respiratory syncytial virus-infected cells. J Virol. 2002;76(13):6800–14.

74. Kolli D, Velayutham T, Casola A. Host-Viral Interactions: Role of Pattern Recognition

Receptors (PRRs) in Human Pneumovirus Infections. Pathogens. 2013;2(4):232–63.

75. Durbin RK, Kotenko S V., Durbin JE. Interferon induction and function at the mucosal surface. Vol. 255, Immunological Reviews. 2013. p. 25–39.

76. McClure R, Massari P. TLR-dependent human mucosal epithelial cell responses to microbial pathogens. Front Immunol. 2014;5(386).

77. Ioannidis I, Ye F, McNally B, Willette M, Flaño E. TLR expression and induction of type I and type III interferons in primary airway epithelial cells. J Virol. 2013;87(January):3261–70.

78. Sha Q, Truong-Tran AQ, Plitt JR, Beck LA, Schleimer RP. Activation of airway epithelial cells by toll-like receptor agonists. Am J Respir Cell Mol Biol. 2004;31(3):358–64.

79. Dou Y, Zhao Y, Zhang Z yong, Mao H wei, Tu W wei, Zhao X dong. Respiratory Syncytial Virus Infection Induces Higher Toll-Like Receptor-3 Expression and TNF-α Production Than Human Metapneumovirus Infection. PLoS One. 2013;8(9):e73488.

80. Guo X, Liu T, Shi H, Wang J, Ji P, Wang H, et al. Respiratory Syncytial Virus Infection Upregulates NLRC5 and Major Histocompatibility Complex Class I Expression through RIG-I Induction in Airway Epithelial Cells. J Virol. 2015;89(15):7636–45.

81. Yoboua F, Martel A, Duval A, Mukawera E, Grandvaux N. Respiratory Syncytial Virus-Mediated NF- B p65 Phosphorylation at Serine 536 Is Dependent on RIG-I, TRAF6, and IKK. J Virol. 2010;84(14):7267–77.

82. Alexopoulou L, Holt A, Medzhitov R, Flavell R. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. Nature. 2001;413(6857):732–8.

83. Liu P, Jamaluddin M, Li K, Garofalo RP, Casola A, Brasier AR. Retinoic Acid-Inducible Gene I Mediates Early Antiviral Response and Toll-Like Receptor 3 Expression in Respiratory Syncytial Virus-Infected Airway Epithelial Cells. J Virol. 2007;81(3):1401–11.

84. Ling Z, Tran KC, Teng MN. Human Respiratory Syncytial Virus Nonstructural Protein NS2 Antagonizes the Activation of Beta Interferon Transcription by Interacting with RIG-I. J Virol. 2009;83(3):3734–42.

85. Puthothu B, Forster J, Heinzmann a, Krueger M. TLR-4 and CD14 polymorphisms in respiratory syncytial virus associated disease. Dis Markers. 2006;22(5–6):303–8.

86. Douville RN, Lissitsyn Y, Hirschfeld AF, Becker AB, Kozyrskyj AL, Liem J, et al. TLR4 Asp299Gly and Thr399ile polymorphisms: No impact on human immune responsiveness to LPS or respiratory syncytial virus. PLoS One. 2010;5(8):e12087.

87. Nuolivirta K, Törmänen S, Teräsjärvi J, Vuononvirta J, Koponen P, Korppi M, et al. Post-bronchiolitis wheezing is associated with toll-like receptor 9 rs187084 gene polymorphism. Sci Rep. 2016;6(31165).

88. Marr N, Turvey SE. Role of human TLR4 in respiratory syncytial virus-induced NF-κB activation, viral entry and replication. Innate Immun. 2012;18(6):856–65.

89. Monick MM, Yarovinsky TO, Powers LS, Butler NS, Carter AB, Gudmundsson G, et al. Respiratory Syncytial Virus Up-regulates TLR4 and Sensitizes Airway Epithelial Cells to Endotoxin. J Biol Chem. 2003;278(52):53035–44.

90. Kim TH, Lee HK. Innate immune recognition of respiratory syncytial virus infection. BMB Rep. 2014;47(4):184–91.

91. Ehl S, Bischoff R, Ostler T, Vallbracht S, Schulte-Mönting J, Poltorak A, et al. The role of Toll-like receptor 4 versus interleukin-12 in immunity to respiratory syncytial virus. Eur J Immunol. 2004;34(4):1146–53.

92. Groskreutz DJ, Monick MM, Powers LS, Yarovinsky TO, Look DC, Hunninghake GW. Respiratory Syncytial Virus Induces TLR3 Protein and Protein Kinase R, Leading to Increased Double-Stranded RNA Responsiveness in Airway Epithelial Cells. J Immunol. 2006;176(3):1733–40.

93. Rodríguez-Auad JP, Nava-Frías M, Casasola-Flores J, Johnson KM, Nava-Ruiz A, Pérez-Robles V, et al. The epidemiology and clinical characteristics of respiratory syncytial virus infection in children at a public pediatric referral hospital in Mexico. Int J Infect Dis. 2012;16(7).

94. Krilov LR, Masaquel AS, Weiner LB, Smith DM, Wade SW, Mahadevia PJ. Partial palivizumab prophylaxis and increased risk of hospitalization due to respiratory syncytial virus in a medicaid population: A retrospective cohort analysis. BMC Pediatr. 2014;14(1):1–11.

95. Paes BA, Mitchell I, Banerji A, Lanctôt KL, Langley JM. A decade of respiratory syncytial virus epidemiology and prophylaxis: Translating evidence into everyday clinical practice. Can Respir J. 2011;18(2):e10-9.

96. Blanken MO, Rovers MM, Molenaar JM, Winkler-Seinstra PL, Meijer A, Kimpen JLL, et al. Respiratory Syncytial Virus and Recurrent Wheeze in Healthy Preterm Infants. N Engl J Med. 2013;368(19):1791–9.

97. Fries L, Shinde V, Stoddard JJ, Thomas DN, Kpamegan E, Lu H, et al. Immunogenicity and safety of a respiratory syncytial virus fusion protein (RSV F) nanoparticle vaccine in older adults. Immun Ageing. 2017;14(1).

98. Battles MB, Langedijk JP, Furmanova-Hollenstein P, Chaiwatpongsakorn S, Costello HM, Kwanten L, et al. Molecular mechanism of respiratory syncytial virus fusion inhibitors. Nat Chem Biol. 2016;12(2):87–93.

99. Spina D. Epithelium smooth muscle regulation and interactions. Am J Respir Crit Care Med. 1998;158(5 Pt 3):S141-5.

100. Kim KC, Hisatsune A, Kim DJ, Miyata T. Pharmacology of airway goblet cell mucin release. J Pharmacol Sci. 2003;92(4):301–7.

101. Finkbeiner WE, Shen BQ, Widdicombe JH. Chloride secretion and function of serous and mucous cells of human airway glands. Am J Physiol. 1994;267(2 Pt 1):L206-10.

102. Hajj R, Baranek T, Le Naour R, Lesimple P, Puchelle E, Coraux C. Basal Cells of the Human Adult Airway Surface Epithelium Retain Transit-Amplifying Cell Properties. Stem Cells. 2007;25(1):139–48.

103. Parker D, Prince A. Innate immunity in the respiratory epithelium. Am J Respir Cell Mol Biol. 2011;45(2):189–201.

104. Hirota JA, Knight DA. Human airway epithelial cell innate immunity: Relevance to asthma. Vol. 24, Current Opinion in Immunology. 2012. p. 740–6.

105. Thornton DJ, Rousseau K, McGuckin MA. Structure and Function of the Polymeric Mucins in Airways Mucus. Annu Rev Physiol. 2008;70(1):459–86.

106. Bals R, Hiemstra PS. Innate immunity in the lung: How epithelial cells fight against respiratory pathogens. Eur Respir J. 2004;23(2):327–33.

107. Fahy J V., Dickey BF. Airway Mucus Function and Dysfunction. N Engl J Med. 2010;363(23):2233–47.

108. Moser C, Weiner DJ, Lysenko E, Bals R, Weiser JN, Wilson JM. β-defensin 1 contributes to pulmonary innate immunity in mice. Infect Immun. 2002;70(6):3068–72.

109. Alekseeva L, Huet D, Féménia F, Mouyna I, Abdelouahab M, Cagna A, et al. Inducible expression of beta defensins by human respiratory epithelial cells exposed to Aspergillus fumigatus organisms. BMC Microbiol. 2009;9(33).

110. Nell MJ, Sandra Tjabringa G, Vonk MJ, Hiemstra PS, Grote JJ. Bacterial products increase expression of the human cathelicidin hCAP-18/LL-37 in cultured human sinus epithelial cells. FEMS Immunol Med Microbiol. 2004;42(2):225–31.

111. Shaykhiev R. Human endogenous antibiotic LL-37 stimulates airway epithelial cell proliferation and wound closure. AJP Lung Cell Mol Physiol. 2005;289(5):L842–8.

112. Lau YE, Bowdish DME, Cosseau C, Hancock REW, Davidson DJ. Apoptosis of airway epithelial cells: Human serum sensitive induction by the cathelicidin LL-37. Am J Respir Cell Mol Biol. 2006;34(4):399–409.

113. Sano H, Nagai K, Tsutsumi H, Kuroki Y. Lactoferrin and surfactant protein A exhibit distinct binding specificity to F protein and differently modulate respiratory syncytial virus infection. Eur J Immunol. 2003;33(10):2894–902.

114. Wakabayashi H, Oda H, Yamauchi K, Abe F. Lactoferrin for prevention of common viral infections. J Infect Chemother. 2014;20(11):666–71.

115. Ellison RT, Giehl TJ. Killing of gram-negative bacteria by lactoferrin and lysozyme. J Clin Invest. 1991;88(4):1080–1091.

116. Renegar KB, Jackson GD, Mestecky J. In vitro comparison of the biologic activities of monoclonal monomeric IgA, polymeric IgA, and secretory IgA. J Immunol. 1998;160(3):1219–23.

117. Renegar KB, Small PA, Boykins LG, Wright PF. Role of IgA versus IgG in the Control of Influenza Viral Infection in the Murine Respiratory Tract. J Immunol. 2004;173(3):1978–86.

118. Polack FP. The changing landscape of respiratory syncytial virus. Vaccine. 2015;33(47):6473–8.

119. Travis SM, Conway BAD, Zabner J, Smith JJ, Anderson NN, Singh PK, et al. Activity of abundant antimicrobials of the human airway. Am J Respir Cell Mol Biol. 1999;20(5):872–9.

120. Wohlkönig A, Huet J, Looze Y, Wintjens R. Structural relationships in the lysozyme superfamily: Significant evidence for glycoside hydrolase signature motifs. PLoS One. 2010;5(11).

121. Ricciardolo FLM. Multiple roles of nitric oxide in the airways. Thorax. 2003;58(2):175–82.

122. Zanin M, Baviskar P, Webster R, Webby R. The Interaction between Respiratory Pathogens and Mucus. Vol. 19, Cell Host and Microbe. 2016. p. 159–68.

123. Kesimer M, Ford AA, Ceppe A, Radicioni G, Cao R, Davis CW, et al. Airway Mucin Concentration as a Marker of Chronic Bronchitis. N Engl J Med. 2017;377(10):911–22.

124. Rajan D, O'Keefe EL, Travers C, McCracken C, Geoghegan S, Caballero MT, et al. MUC5AC Levels Associated With Respiratory Syncytial Virus Disease Severity. Clin Infect Dis. 2018;ciy340.

125. Turner MD, Nedjai B, Hurst T, Pennington DJ. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. Vol. 1843, Biochimica et Biophysica Acta - Molecular Cell Research. 2014. p. 2563–82.

126. Schijf MA, Lukens M V., Kruijsen D, Van Uden NOP, Garssen J, Coenjaerts FEJ, et al. Respiratory syncytial virus induced type I IFN production by pDC is regulated by RSV-infected airway epithelial cells, RSV-exposed monocytes and virus specific antibodies. PLoS One. 2013;8(11).

127. Farrag MA, Almajhdi FN. Human Respiratory Syncytial Virus: Role of Innate Immunity in Clearance and Disease Progression. Viral Immunol. 2016;29(1):11–26.

128. Hasegawa K, Pérez-Losada M, Hoptay CE, Epstein S, Mansbach JM, Teach SJ, et al. RSV vs. rhinovirus bronchiolitis: Difference in nasal airway microRNA profiles and NF B signaling. Pediatr Res. 2018;83(3):606–14.

129. Zhang Y, Luxon BA, Casola A, Garofalo RP, Jamaluddin M, Brasier AR. Expression of respiratory syncytial virus-induced chemokine gene networks in lower airway epithelial cells revealed by cDNA microarrays. J Virol. 2001;75(19):9044–58.

130. Oshansky CM, Barber JP, Crabtree J, Tripp R a. Respiratory syncytial virus F and G proteins induce interleukin 1alpha, CC, and CXC chemokine responses by normal human bronchoepithelial cells. J Infect Dis. 2010;201(8):1201–7.

131. Tian M, Liu F, Wen GY, Shi SY, Chen RH, Zhao DY. Effect of variation in RANTES promoter on serum RANTES levels and risk of recurrent wheezing after RSV bronchiolitis in children from Han, Southern China. Eur J Pediatr. 2009;168(8):963–7.

132. Tregoning JS, Pribul PK, Pennycook AMJ, Hussell T, Wang B, Lukacs N, et al. The chemokine MIP1α/CCL3 determines pathology in primary RSV infection by regulating the balance of t cell populations in the murine lung. PLoS One. 2010;5(2).

133. Vareille M, Kieninger E, Edwards MR, Regamey N. The airway epithelium: Soldier in the fight against respiratory viruses. Clin Microbiol Rev. 2011;24(1):210–29.

134. Okabayashi T, Kojima T, Masaki T, Yokota S ichi, Imaizumi T, Tsutsumi H, et al. Type-III interferon, not type-I, is the predominant interferon induced by respiratory viruses in nasal epithelial cells. Virus Res. 2011;160(1–2):360–6.

135. Jewell NA, Vaghefi N, Mertz SE, Akter P, Peebles RS, Bakaletz LO, et al. Differential Type I Interferon Induction by Respiratory Syncytial Virus and Influenza A Virus In Vivo. J Virol. 2007;81(18):9790–800.

136. Villenave R, Broadbent L, Douglas I, Lyons JD, Coyle P V., Teng MN, et al. Induction and Antagonism of Antiviral Responses in Respiratory Syncytial Virus-Infected Pediatric Airway Epithelium. J Virol. 2015;89(24):12309–18.

137. Everitt AR, Clare S, McDonald JU, Kane L, Harcourt K, Ahras M, et al. Defining the range of pathogens susceptible to ifitm3 restriction using a knockout mouse model. PLoS One. 2013;8(11).

138. Guerrero-Plata A, Baron S, Poast JS, Adegboyega PA, Casola A, Garofalo RP. Activity and regulation of alpha interferon in respiratory syncytial virus and human metapneumovirus experimental infections. J Virol. 2005;79(16):10190–9.

139. Zhang W, Zhang L, Zan Y, Du N, Yang Y, Tien P. Human respiratory syncytial virus

infection is inhibited by IFN-induced transmembrane proteins. J Gen Virol. 2015;96(1):170–82.

140. Samuel CE. Antiviral actions of interferons. Vol. 14, Clinical Microbiology Reviews. 2001. p. 778–809.

141. Spann KM, Tran KC, Chi B, Rabin RL, Collins PL. Suppression of the induction of alpha, beta, and lambda interferons by the NS1 and NS2 proteins of human respiratory syncytial virus in human epithelial cells and macrophages [corrected]. J Virol. 2004;78(8):4363–9.

142. Swedan S, Musiyenko A, Barik S. Respiratory Syncytial Virus Nonstructural Proteins Decrease Levels of Multiple Members of the Cellular Interferon Pathways. J Virol. 2009;83(19):9682–93.

143. Kohlmeier JE, Cookenham T, Roberts AD, Miller SC, Woodland DL. Type I interferons regulate cytolytic activity of memory CD8+T cells in the lung airways during respiratory virus challenge. Immunity. 2010;33(1):96–105.

144. Fonceca AM, Flanagan BF, Trinick R, Smyth RL, McNamara PS. Primary airway epithelial cultures from children are highly permissive to respiratory syncytial virus infection. Thorax. 2012;67(1):42–8.

145. McNamara PS, Fonceca AM, Howarth D, Correia JB, Slupsky JR, Trinick RE, et al. Respiratory syncytial virus infection of airway epithelial cells, in vivo and in vitro, supports pulmonary antibody responses by inducing expression of the B cell differentiation factor BAFF. Thorax. 2013;68(1):76–81.

146. McCutcheon KM, Jordan R, Mawhorter ME, Noton SL, Powers JG, Fearns R, et al. The Interferon Type I/III Response to Respiratory Syncytial Virus Infection in Airway Epithelial Cells Can Be Attenuated or Amplified by Antiviral Treatment. J Virol. 2016;90(4):1705–17.

147. Whiteman SC, Spiteri MA. IFN-gamma regulation of ICAM-1 receptors in bronchial epithelial cells: Soluble ICAM-1 release inhibits human rhinovirus infection. J Inflamm. 2008;5(8).

148. Cao J, Wong CK, Yin Y, Lam CWK. Activation of human bronchial epithelial cells by inflammatory cytokines IL-27 and TNF-α: Implications for immunopathophysiology of airway inflammation. J Cell Physiol. 2010;223(3):788–97.

149. Krunkosky TM, Martin LD, Fischer BM, Voynow J a, Adler KB. Effects of TNFalpha on expression of ICAM-1 in human airway epithelial cells in vitro: oxidant-mediated pathways and transcription factors. Free Radic Biol Med. 2003;35(9):1158–67.

150. Konno S, Grindle KA, Lee WM, Schroth MK, Mosser AG, Brockman-Schneider RA, et al. Interferon-γ enhances rhinovirus-induced RANTES secretion by airway epithelial cells. Am J Respir Cell Mol Biol. 2002;26(5):594–601.

151. Kato A, Truong-Tran AQ, Scott AL, Matsumoto K, Schleimer RP. Airway epithelial cells produce B cell-activating factor of TNF family by an IFN-beta-dependent mechanism. J Immunol. 2006;177(10):7164–72.

152. Zdrenghea MT, Telcian AG, Laza-Stanca V, Bellettato CM, Edwards MR, Nikonova A, et al. RSV infection modulates IL-15 production and MICA levels in respiratory epithelial cells. Eur Respir J. 2012;39(3):712–20.

153. Oslund KL, Zhou X, Lee B, Zhu L, Duong T, Shih R, et al. Synergistic up-regulation of CXCL10 by virus and IFN γ in human airway epithelial cells. PLoS One. 2014;9(7).

154. Suzuki T, Chow CW, Downey GP. Role of innate immune cells and their products in lung immunopathology. Int J Biochem Cell Biol. 2008;40(6–7):1348–61.

155. Collin M, Mcgovern N, Haniffa M. Human dendritic cell subsets. Vol. 140, Immunology. 2013. p. 22–30.

156. Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S, et al. The nature of the principal Type 1 interferon-producing cells in human blood. Science (80-). 1999;284(5421):1835–7.

157. Gill M a, Palucka a K, Barton T, Ghaffar F, Jafri H, Banchereau J, et al. Mobilization of plasmacytoid and myeloid dendritic cells to mucosal sites in children with respiratory syncytial virus and other viral respiratory infections. J Infect Dis. 2005;191(7):1105–15.

158. de Graaff PM a, de Jong EC, van Capel TM, van Dijk ME a, Roholl PJM, Boes J, et al. Respiratory Syncytial Virus Infection of Monocyte-Derived Dendritic Cells Decreases Their Capacity to Activate CD4 T Cells. J Immunol. 2005;175(9):5904–11.

159. Johnson TR, Johnson CN, Corbett KS, Edwards GC, Graham BS. Primary human mDC1, mDC2, and pDC dendritic cells are differentially infected and activated by respiratory syncytial virus. PLoS One. 2011;6(1).

160. Smit JJ, Rudd BD, Lukacs NW. Plasmacytoid dendritic cells inhibit pulmonary immunopathology and promote clearance of respiratory syncytial virus. J Exp Med. 2006;203(5):1153–9.

161. Ruckwardt TJ, Malloy AMW, Morabito KM, Graham BS. Quantitative and Qualitative Deficits in Neonatal Lung-Migratory Dendritic Cells Impact the Generation of the CD8+ T Cell Response. PLoS Pathog. 2014;10(2).

162. Cormier S a, Shrestha B, Saravia J, Lee GI, Shen L, DeVincenzo JP, et al. Limited Type I Interferons and Plasmacytoid Dendritic Cells during Neonatal Respiratory Syncytial Virus Infection Permit Immunopathogenesis upon Reinfection. J Virol. 2014;88(16):9350–60.

163. Hussell T, Bell TJ. Alveolar macrophages: Plasticity in a tissue-specific context. Vol. 14, Nature Reviews Immunology. 2014. p. 81–93.

164. Panuska JR, Merolla R, Rebert NA, Hoffmann SP, Tsivitse P, Cirino NM, et al. Respiratory syncytial virus induces interleukin-10 by human alveolar macrophages. Suppression of early cytokine production and implications for incomplete immunity. J Clin Invest. 1995;96(5):2445–53.

165. Makris S, Bajorek M, Culley FJ, Goritzka M, Johansson C. Alveolar macrophages can control respiratory syncytial virus infection in the absence of Type I interferons. J Innate Immun. 2016;8(5):452–63.

166. Pribul PK, Harker J, Wang B, Wang H, Tregoning JS, Schwarze J, et al. Alveolar Macrophages Are a Major Determinant of Early Responses to Viral Lung Infection but Do Not Influence Subsequent Disease Development. J Virol. 2008;82(9):4441–8.

167. Kolli D, Gupta MR, Sbrana E, Velayutham TS, Chao H, Casola A, et al. Alveolar macrophages contribute to the pathogenesis of human metapneumovirus infection while protecting against respiratory syncytial virus infection. Am J Respir Cell Mol Biol. 2014;51(4):502–15.

168. Reed JL, Brewah YA, Delaney T, Welliver T, Burwell T, Benjamin E, et al. Macrophage impairment underlies airway occlusion in primary respiratory syncytial virus

bronchiolitis. J Infect Dis. 2008;198(12):1783–93.

169. Ebbo M, Crinier A, Vély F, Vivier E. Innate lymphoid cells: Major players in inflammatory diseases. Nat Rev Immunol. 2017;17(11):665–78.

170. Cortez VS, Robinette ML, Colonna M. Innate lymphoid cells: New insights into function and development. Curr Opin Immunol. 2015;32:71–7.

171. De Grove KC, Provoost S, Verhamme FM, Bracke KR, Joos GF, Maes T, et al. Characterization and quantification of innate lymphoid cell subsets in human lung. PLoS One. 2016;11(1).

172. Stier MT, Bloodworth MH, Toki S, Newcomb DC, Goleniewska K, Boyd KL, et al. Respiratory syncytial virus infection activates IL-13–producing group 2 innate lymphoid cells through thymic stromal lymphopoietin. J Allergy Clin Immunol. 2016;138(3):814–824.e11.

173. Chang Y-J, Kim HY, Albacker LA, Baumgarth N, McKenzie ANJ, Smith DE, et al. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. Nat Immunol. 2011;12(7):631–8.

174. Elemam NM, Hannawi S, Maghazachi AA. Innate lymphoid cells (ILCs) as mediators of inflammation, release of cytokines and lytic molecules. Toxins (Basel). 2017;9(12).

175. Sadik CD, Kim ND, Luster AD. Neutrophils cascading their way to inflammation. Vol. 32, Trends in Immunology. 2011. p. 452–60.

176. Yasui K, Baba A, Iwasaki Y, Kubo T, Aoyama K, Mori T, et al. Neutrophil-mediated inflammation in respiratory syncytial viral bronchiolitis. Pediatr Int. 2005;47(2):190–5.

177. Emboriadou M, Hatzistilianou M, Magnisali C, Sakelaropoulou A, Exintari M, Conti P, et al. Human neutrophil elastase in RSV bronchiolitis. Ann Clin Lab Sci. 2007;37(1):79–84.

178. Hull J, Thomson A, Kwiatkowski D. Association of respiratory syncytial virus bronchiolitis with the interleukin 8 gene region in UK families. Thorax. 2000;55(12):1023–7.

179. Olszewska-Pazdrak B, Casola A, Saito T, Alam R, Crowe SE, Mei F, et al. Cell-specific expression of RANTES, MCP-1, and MIP-1alpha by lower airway epithelial cells and eosinophils infected with respiratory syncytial virus. J Virol. 1998;72(6):4756–64.

180. Matthews SP, Tregoning JS, Coyle AJ, Hussell T, Openshaw PJM. Role of CCL11 in eosinophilic lung disease during respiratory syncytial virus infection. J Virol. 2005;79(4):2050–7.

181. Phipps S, En Lam C, Mahalingam S, Newhouse M, Ramirez R, Rosenberg HF, et al. Eosinophils contribute to innate antiviral immunity and promote clearance of respiratory syncytial virus. Blood. 2007;110(5):1578–86.

182. Ehlenfield DR, Cameron K, Welliver RC. Eosinophilia at the Time of Respiratory Syncytial Virus Bronchiolitis Predicts Childhood Reactive Airway Disease. Pediatrics. 2000;105(1):79–83.

183. Smith PK, Wang SZ, Dowling KD, Forsyth KD. Leucocyte populations in respiratory syncytial virus-induced bronchiolitis. J Paediatr Child Health. 2001;37(2):146–51.

184. Culley FJ, Pollott J, Openshaw PJM. Age at First Viral Infection Determines the Pattern of T Cell–mediated Disease during Reinfection in Adulthood. J Exp Med.

2002;196(10):1381–6.

185. Lee YM, Miyahara N, Takeda K, Prpich J, Oh A, Balhorn A, et al. IFN-γ production during initial infection determines the outcome of reinfection with respiratory syncytial virus. Am J Respir Crit Care Med. 2008;177(2):208–18.

186. You D, Marr N, Saravia J, Shrestha B, Lee GI, Turvey SE, et al. IL-4Rα on CD4 $^{+}$ T cells plays a pathogenic role in respiratory syncytial virus reinfection in mice infected initially as neonates. J Leukoc Biol. 2013;93(6):933–42.

187. Wieczorek M, Abualrous ET, Sticht J, Álvaro-Benito M, Stolzenberg S, Noé F, et al. Major histocompatibility complex (MHC) class I and MHC class II proteins: Conformational plasticity in antigen presentation. Vol. 8, Frontiers in Immunology. 2017.

188. Golubovskaya V, Wu L. Different subsets of T cells, memory, effector functions, and CAR-T immunotherapy. Cancers (Basel). 2016;8(3).

189. Geginat J, Lanzavecchia A, Sallusto F. Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. Blood. 2003;101(11):4260–6.

190. Cannon MJ, Stott EJ, Taylor G, Askonas B a. Clearance of persistent respiratory syncytial virus infections in immunodeficient mice following transfer of primed T cells. Immunology. 1987;62(1):133–8.

191. Lee S, Stokes KL, Currier MG, Sakamoto K, Lukacs NW, Celis E, et al. Vaccine-Elicited CD8+ T Cells Protect against Respiratory Syncytial Virus Strain A2-Line19F-Induced Pathogenesis in BALB/c Mice. J Virol. 2012;86(23):13016–24.

192. Weiss KA, Christiaansen AF, Fulton RB, Meyerholz DK, Varga SM. Multiple CD4+ T Cell Subsets Produce Immunomodulatory IL-10 During Respiratory Syncytial Virus Infection. J Immunol. 2011;187(6):3145–54.

193. Sun L, Cornell TT, Levine A, Berlin AA, Hinkovska-Galcheva V, Fleszar AJ, et al. Dual role of interleukin-10 in the regulation of respiratory syncitial virus (RSV)-induced lung inflammation. Clin Exp Immunol. 2013;172(2):263–79.

194. Heidema J, Lukens M V, van Maren WWC, van Dijk ME a, Otten HG, van Vught AJ, et al. CD8+ T cell responses in bronchoalveolar lavage fluid and peripheral blood mononuclear cells of infants with severe primary respiratory syncytial virus infections. J Immunol. 2007;179(12):8410–7.

195. Walsh EE, Peterson DR, Kalkanoglu AE, Lee FE-H, Falsey AR. Viral Shedding and Immune Responses to Respiratory Syncytial Virus Infection in Older Adults. J Infect Dis. 2013;207(9):1424–32.

196. Brand HK, Ferwerda G, Preijers F, De Groot R, Neeleman C, Staal FJT, et al. CD4+T-cell counts and interleukin-8 and CCL-5 plasma concentrations discriminate disease severity in children with RSV infection. Pediatr Res. 2013;73(2):187–93.

197. Raiden S, Sananez I, Remes-Lenicov F, Pandolfi J, Romero C, De Lillo L, et al. Respiratory syncytial virus (RSV) infects CD4+ T cells: Frequency of circulating CD4+ RSV+ T cells as a marker of disease severity in young children. In: Journal of Infectious Diseases. 2017. p. 1049–58.

198. Larranaga CL, Ampuero SL, Luchsinger VF, Carrion FA, Aguilar N V, Morales PR, et al. Impaired immune response in severe human lower tract respiratory infection by respiratory syncytial virus. Pediatr Infect Dis J. 2009;28(10):867–73.

199. Mariani TJ, Qiu X, Chu CY, Wang L, Thakar J, Holden-Wiltse J, et al. Dynamic changes in the CD4 T cell transcriptome are associated with disease severity during primary RSV infection in young infants. J Infect Dis. 2017;216(8):1027–37.

200. Palmer DC, Restifo NP. Suppressors of cytokine signaling (SOCS) in T cell differentiation, maturation, and function. Vol. 30, Trends in Immunology. 2009. p. 592–602.

201. Graham BS, Bunton LA, Wright PF, Karzon DT. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J Clin Invest. 1991;88(3):1026–33.

202. Knudson CJ, Hartwig SM, Meyerholz DK, Varga SM. RSV Vaccine-Enhanced Disease Is Orchestrated by the Combined Actions of Distinct CD4 T Cell Subsets. PLoS Pathog. 2015;11(3):1–23.

203. Hussell T, Baldwin CJ, O'Garra A, Openshaw PJ. CD8+ T cells control Th2-driven pathology during pulmonary respiratory syncytial virus infection. Eur J Immunol. 1997;27(12):3341–9.

204. Johnson TR, Teng MN, Collins PL, Graham BS. Respiratory syncytial virus (RSV) G glycoprotein is not necessary for vaccine-enhanced disease induced by immunization with formalin-inactivated RSV. J Virol. 2004;78(11):6024–32.

205. Olson MR, Varga SM. CD8 T cells inhibit respiratory syncytial virus (RSV) vaccine-enhanced disease. J Immunol. 2007;179(8):5415–24.

206. Ruckwardt TJ, Bonaparte KL, Nason MC, Graham BS. Regulatory T cells promote early influx of CD8+ T cells in the lungs of respiratory syncytial virus-infected mice and diminish immunodominance disparities. J Virol. 2009;83(7):3019–28.

207. Fulton RB, Meyerholz DK, Varga SM. Foxp3+ CD4 regulatory T cells limit pulmonary immunopathology by modulating the CD8 T cell response during respiratory syncytial virus infection. J Immunol. 2010;185(4):2382–92.

208. Mukherjee S, Lindell DM, Berlin AA, Morris SB, Shanley TP, Hershenson MB, et al. IL-17Induced pulmonary pathogenesis during respiratory viral infection and exacerbation of allergic disease. Am J Pathol. 2011;179(1):248–58.

209. Stoppelenburg AJ, De Roock S, Hennus MP, Bont L, Boes M. Elevated Th17 response in infants undergoing respiratory viral infection. Am J Pathol. 2014;184(5):1274–9.

210. Bystrom J, Al-Adhoubi N, Al-Bogami M, Jawad AS, Mageed RA. Th17 lymphocytes in respiratory syncytial virus infection. Vol. 5, Viruses. 2013. p. 777–91.

211. Hacimustafaoglu M, Celebi S, Aynaci E, Sinirtas M, Koksal N, Kucukerdogan A, et al. The progression of maternal RSV antibodies in the offspring. Arch Dis Child. 2004;89(1):52–3.

212. Glezen WP, Paredes a, Allison JE, Taber LH, Frank a L. Risk of respiratory syncytial virus infection for infants from low-income families in relationship to age, sex, ethnic group, and maternal antibody level. J Pediatr. 1981;98(5):708–15.

213. Jans J, Wicht O, Widjaja I, Ahout IML, De Groot R, Guichelaar T, et al. Characteristics of RSV-specific maternal antibodies in plasma of hospitalized, acute RSV patients under three months of age. PLoS One. 2017;12(1).

214. van Erp EA, van Kasteren PB, Guichelaar T, Ahout IML, de Haan CAM, Luytjes W, et al. *In Vitro* Enhancement of Respiratory Syncytial Virus Infection by Maternal Antibodies Does Not Explain Disease Severity in Infants. J Virol. 2017;91(21):e00851-

17.

215. Henderson F, Collier A, Clyde WJ, Denny F. Respiratory-syncytial-virus infections, reinfections immunity. A prospective, longitudinal study in young children. N Engl J Med. 1979;300(10):530–4.

216. Bagga B, Cehelsky JE, Vaishnaw A, Tomwilkinson T, Meyers R, Harrison LM, et al. Effect of Preexisting Serum and Mucosal Antibody on Experimental Respiratory Syncytial Virus (RSV) Challenge and Infection of Adults. J Infect Dis. 2015;212(10):1719–25.

217. Walsh EE, Falsey AR. Humoral and mucosal immunity in protection from natural respiratory syncytial virus infection in adults. J Infect Dis. 2004;190(2):373–8.

218. Walsh EE, Peterson DR, Falsey AR. Risk Factors for Severe Respiratory Syncytial Virus Infection in Elderly Persons. J Infect Dis. 2004;189(2):233–8.

219. Wright PF, Gruber WC, Peters M, Reed G, Zhu Y, Robinson F, et al. Illness Severity, Viral Shedding, and Antibody Responses in Infants Hospitalized with Bronchiolitis Caused by Respiratory Syncytial Virus. J Infect Dis. 2002;185(8):1011–8.

220. Fonceca A, McNamara P, Corriera C, Trinick R, Alturaki W, Smyth R. Human respiratory syncytial virus infection induces expression of the b cell differentiation factor BAFF in vivo and in vitro in an inteferon dependent manner. Vol. 185, American Journal of Respiratory and Critical Care Medicine. 2012. p. no pagination.

221. Raes M, Peeters V, Alliet P, Gillis P, Kortleven J, Magerman K, et al. Peripheral blood T and B lymphocyte subpopulations in infants with acute respiratory syncytial virus brochiolitis. Pediatr Allergy Immunol. 1997;8(2):97–102.

222. Reed JL, Welliver TP, Sims GP, McKinney L, Velozo L, Avendano L, et al. Innate Immune Signals Modulate Antiviral and Polyreactive Antibody Responses during Severe Respiratory Syncytial Virus Infection. J Infect Dis. 2009;199(8):1128–38.

223. Boelen A, Andeweg A, Kwakkel J, Lokhorst W, Bestebroer T, Dormans J, et al. Both immunisation with a formalin-inactivated respiratory syncytial virus (RSV) vaccine and a mock antigen vaccine induce severe lung pathology and a Th2 cytokine profile in RSV-challenged mice. Vaccine. 2000;19(7–8):982–91.

224. Sallusto F, Lanzavecchia A, MacKay CR. Chemokines and chemokine receptors in T-cell priming and Th1/Th2- mediated responses. Immunol Today. 1998;19(12):568–74.

225. Legg JP, Hussain IR, Warner JOJAJO, Johnston SL, Warner JOJAJO. Type 1 and type 2 cytokine imbalance in acute respiratory syncytial virus bronchiolitis. Am J Respir Crit Care Med. 2003;168(6 I):633–9.

226. Bermejo-Martin JF, Garcia-Arevalo MC, De Lejarazu RO, Ardura J, Eiros JM, Alonso A, et al. Predominance of Th2 cytokines, CXC chemokines and innate immunity mediators at the mucosal level during severe respiratory syncytial virus infection in children. Eur Cytokine Netw. 2007;18(3):162–7.

227. ROMÁN M, CALHOUN WJ, HINTON KL, AVENDAÑO LF, SIMON V, ESCOBAR AM, et al. Respiratory Syncytial Virus Infection in Infants Is Associated with Predominant Th-2-like Response. Am J Respir Crit Care Med. 1997;156(1):190–5.

228. Brandenburg a H, Kleinjan a, van Het Land B, Moll H a, Timmerman HH, de Swart RL, et al. Type 1-like immune response is found in children with respiratory syncytial virus infection regardless of clinical severity. J Med Virol. 2000;62(2):267–77.

229. Garofalo RP, Patti J, Hintz KA, Hill V, Ogra PL, Welliver RC. Macrophage inflammatory protein-1alpha (not T helper type 2 cytokines) is associated with severe forms of respiratory syncytial virus bronchiolitis. J Infect Dis. 2001;184(4):393–9.

230. Pancham K, Perez GF, Huseni S, Jain A, Kurdi B, Rodriguez-Martinez CE, et al. Premature infants have impaired airway antiviral IFNγ responses to human metapneumovirus compared to respiratory syncytial virus. Pediatr Res. 2015;78(4):389–94.

231. Natarajan S, Ramasamy G, Kumar NP, Babu SS, Janakiraman L. Nasopharyngeal aspirate & blood cytokine profile in infants hospitalized for respiratory syncytial virus bronchiolitis: A pilot study from south india. Indian J Med Res. 2016;144(DECEMBER):929–31.

232. Kim CK, Kim SW, Park CS, Kim B, Kang H, Koh YY. Bronchoalveolar lavage cytokine profiles in acute asthma and acute bronchiolitis. J Allergy Clin Immunol. 2003;112(1):64–71.

233. Vojvoda V, Savić Mlakar A, Jergović M, Kukuruzović M, Markovinović L, Aberle N, et al. The increased type-1 and type-2 chemokine levels in children with acute RSV infection alter the development of adaptive immune responses. Biomed Res Int. 2014;2014(Article ID 750521):11 pages.

234. McNamara PSS, Flanagan BFF, Hart CAA, Smyth RLL. Production of Chemokines in the Lungs of Infants with Severe Respiratory Syncytial Virus Bronchiolitis. J Infect Dis. 2005;191(8):1225–32.

235. Harker J a, Lee DCP, Yamaguchi Y, Wang B, Bukreyev A, Collins PL, et al. Delivery of cytokines by recombinant virus in early life alters the immune response to adult lung infection. J Virol. 2010;84(10):5294–302.

236. Zhou W, Hashimoto K, Moore ML, Elias JA, Zhu Z, Durbin J, et al. IL-13 is associated with reduced illness and replication in primary respiratory syncytial virus infection in the mouse. Microbes Infect. 2006;8(14–15):2880–9.

237. Durant LR, Makris S, Voorburg CM, Loebbermann J, Johansson C, Openshaw PJM. Regulatory T cells prevent Th2 immune responses and pulmonary eosinophilia during respiratory syncytial virus infection in mice. J Virol. 2013;87(20):10946–54.

238. Herberman R, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. Int J Cancer. 1975;16(2):216–29.

239. Kiessling R, Klein E, Wigzell H. “Natural” killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. Eur J Immunol. 1975;5(2):112–7.

240. Hall LJ, Clare S, Dougan G. NK Cells Influence Both Innate and Adaptive Immune Responses after Mucosal Immunization with Antigen and Mucosal Adjuvant. J Immunol. 2010;184(8):4327–37.

241. Simoni Y, Fehlings M, Kløverpris HN, McGovern N, Koo S-L, Loh CY, et al. Human Innate Lymphoid Cell Subsets Possess Tissue-Type Based Heterogeneity in Phenotype and Frequency. Immunity. 2016;45(1):148–61.

242. Mrózek E, Anderson P, Caligiuri M a. Role of interleukin-15 in the development of human CD56+ natural killer cells from CD34+ hematopoietic progenitor cells. Blood. 1996;87(7):2632–40.

243. Suzuki H, Duncan GS, Takimoto H, Mak TW. Abnormal development of intestinal intraepithelial lymphocytes and peripheral natural killer cells in mice lacking the IL-2 receptor beta chain. J Exp Med. 1997;185(3):499–505.

244. Lanier LL, Le a M, Civin CI, Loken MR, Phillips JH. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. J Immunol. 1986;136(12):4480–6.

245. Schumann G, Dasgupta JD. Specificity of signal transduction through CD16, TCR-CD3 and BCR receptor chains containing the tyrosine-associated activation motif. Int Immunol. 1994;6(9):1383–92.

246. Fu B, Tian Z, Wei H. Subsets of human natural killer cells and their regulatory effects. Vol. 141, Immunology. 2014. p. 483–9.

247. Fehniger TA, Shah MH, Turner MJ, VanDeusen JB, Whitman SP, Cooper MA, et al. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. J Immunol. 1999;162(8):4511–20.

248. Poli A, Michel T, Thérésine M, Andrès E, Hentges F, Zimmer J. CD56bright natural killer (NK) cells: An important NK cell subset. Immunology. 2009;126(4):458–65.

249. Beziat V, Duffy D, Quoc SN, Le Garff-Tavernier M, Decocq J, Combadiere B, et al. CD56brightCD16+ NK Cells: A Functional Intermediate Stage of NK Cell Differentiation. J Immunol. 2011;186(12):6753–61.

250. Mota G, Moldovan I, Calugaru A, Hirt M, Kozma E, Galatiuc C, et al. Interaction of human immunoglobulin G with CD16 on natural killer cells: Ligand clearance, FcγRIIIA turnover and effects of metalloproteinases on FcγRIIIA-mediated binding, signal transduction and killing. Scand J Immunol. 2004;59(3):278–84.

251. Mandelboim O, Malik P, Davis DM, Jo CH, Boyson JE, Strominger JL. Human CD16 as a lysis receptor mediating direct natural killer cell cytotoxicity. Proc Natl Acad Sci U S A. 1999;96(10):5640–4.

252. Gabrielli S, Ortolani C, Del Zotto G, Luchetti F, Canonico B, Buccella F, et al. The Memories of NK Cells: Innate-Adaptive Immune Intrinsic Crosstalk. J Immunol Res. 2016;2016(1376595).

253. Pascal V, Schleinitz N, Brunet C, Ravet S, Bonnet E, Lafarge X, et al. Comparative analysis of NK cell subset distribution in normal and lymphoproliferative disease of granular lymphocyte conditions. Eur J Immunol. 2004;34(10):2930–40.

254. Borges GF, Teixeira AMBM, Rama LMLP, Pedreiro S, Santos AMC, Massart AGM, et al. Differences in peripheral blood natural killer cell populations between elite kayakers and non-athletes. Rev Bras Med do Esporte. 2012;18(5):305–7.

255. Angelo LS, Banerjee PP, Monaco-Shawver L, Rosen JB, Makedonas G, Forbes LR, et al. Practical NK cell phenotyping and variability in healthy adults. Immunol Res. 2015;62(3):341–56.

256. Ursaciuc C, Surcel M, Huică R, Ciotaru D, Dobre M, IR P, et al. CD56dim/CD56bright NK cell subpopulations and CD16/CD57 expression correlated with tumor development stages. SEE J Immunol. 2016;2016:1–5.

257. Freeman CM, Stolberg VR, Crudgington S, Martinez FJ, Han MLK, Chensue SW, et al. Human CD56+ cytotoxic lung lymphocytes kill autologous lung cells in chronic obstructive pulmonary disease. PLoS One. 2014;9(7).

258. Gao B, Radaeva S, Park O. Liver natural killer and natural killer T cells: immunobiology and emerging roles in liver diseases. J Leukoc Biol. 2009;86(3):513–28.

259. Ivanova D, Krempels R, Ryfe J, Weitzman K, Stephenson D, Gigley JP. NK cells in mucosal defense against infection. Biomed Res Int. 2014;2014(Article ID 413982):11 pages.

260. Carrega P, Bonaccorsi I, Di Carlo E, Morandi B, Paul P, Rizzello V, et al. CD56(bright)perforin(low) noncytotoxic human NK cells are abundant in both healthy and neoplastic solid tissues and recirculate to secondary lymphoid organs via afferent lymph. J Immunol. 2014;192(8):3805–15.

261. Wang J, Li F, Zheng M, Sun R, Wei H, Tian Z. Lung natural killer cells in mice: Phenotype and response to respiratory infection. Immunology. 2012;137(1):37–47.

262. Marquardt N, Kekäläinen E, Chen P, Kvedaraite E, Wilson JN, Ivarsson MA, et al. Human lung natural killer cells are predominantly comprised of highly differentiated hypofunctional CD69−CD56dimcells. J Allergy Clin Immunol. 2017;139(4):1321–1330.e4.

263. Tomasello E, Yessaad N, Gregoire E, Hudspeth K, Luci C, Mavilio D, et al. Mapping of NKp46(+) Cells in Healthy Human Lymphoid and Non-Lymphoid Tissues. Front Immunol. 2012;3(November):344.

264. Kärre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature. 1986;319(6055):675–8.

265. Lanier LL, Phillips JH. NK cell recognition of major histocompatibility complex class I molecules. Semin Immunol. 1995;7(2):75–82.

266. Cerwenka A, Lanier LL. Ligands for natural killer cell receptors: Redundancy or specificity. Vol. 181, Immunological Reviews. 2001. p. 158–69.

267. Joyce MG, Sun PD. The Structural Basis of Ligand Recognition by Natural Killer Cell Receptors. J Biomed Biotechnol. 2011;2011:1–15.

268. Carrillo-Bustamante P, Keşmir C, de Boer RJ. The evolution of natural killer cell receptors. Vol. 68, Immunogenetics. 2016. p. 3–18.

269. Parasa VRR, Sikhamani R, Raja A. Effect of recombinant cytokines on the expression of natural killer cell receptors from patients with TB or/and HIV infection. PLoS One. 2012;7(6).

270. Long EO, Sik Kim H, Liu D, Peterson ME, Rajagopalan S. Controlling Natural Killer Cell Responses: Integration of Signals for Activation and Inhibition. Annu Rev Immunol. 2013;31(1):227–58.

271. Lanier LL. NK CELL RECOGNITION. Annu Rev Immunol. 2005;23(1):225–74.

272. Mao H, Tu W, Liu Y, Qin G, Zheng J, Chan P-L, et al. Inhibition of human natural killer cell activity by influenza virions and hemagglutinin. J Virol. 2010;84(9):4148–57.

273. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, Mandelboim O. Recognition of viral hemagglutinins by NKp44 but not by NKp30. Eur J Immunol. 2001;31(9):2680–9.

274. Hershkovitz O, Rosental B, Rosenberg LA, Navarro-Sanchez ME, Jivov S, Zilka A, et al. NKp44 receptor mediates interaction of the envelope glycoproteins from the West

Nile and dengue viruses with NK cells. J Immunol (Baltimore, Md 1950). 2009;183(4):2610–21.

275. Bloushtain N, Qimron U, Bar-Ilan A, Hershkovitz O, Gazit R, Fima E, et al. Membrane-Associated Heparan Sulfate Proteoglycans Are Involved in the Recognition of Cellular Targets by NKp30 and NKp46. J Immunol. 2004;173(4):2392–401.

276. Pogge von Strandmann E, Simhadri VR, von Tresckow B, Sasse S, Reiners KSS, Hansen HP, et al. Human Leukocyte Antigen-B-Associated Transcript 3 Is Released from Tumor Cells and Engages the NKp30 Receptor on Natural Killer Cells. Immunity. 2007;27(6):965–74.

277. Joyce MG, Tran P, Zhuravleva M a, Jaw J, Colonna M, Sun PD. Crystal structure of human natural cytotoxicity receptor NKp30 and identification of its ligand binding site. Proc Natl Acad Sci U S A. 2011;108(15):6223–8.

278. Romee R, Leong JW, Fehniger TA. Utilizing Cytokines to Function-Enable Human NK Cells for the Immunotherapy of Cancer. Scientifica (Cairo). 2014;2014:1–18.

279. Martinez J, Huang X, Yang Y. Direct action of type I IFN on NK cells is required for their activation in response to vaccinia viral infection in vivo. J Immunol. 2008;180(3):1592–7.

280. Lima M, Leander M, Santos M, Santos AH, Lau C, Queirós ML, et al. Chemokine Receptor Expression on Normal Blood CD56(+) NK-Cells Elucidates Cell Partners That Comigrate during the Innate and Adaptive Immune Responses and Identifies a Transitional NK-Cell Population. J Immunol Res. 2015;2015:839684.

281. Berahovich RD, Lai NL, Wei Z, Lanier LL, Schall TJ. Evidence for NK cell subsets based on chemokine receptor expression. J Immunol. 2006;177(11):7833–40.

282. Kumar S. NK Cell Cytotoxicity And Its Regulation By Inhibitory Receptors. Immunology. 2018;154(3):383–93.

283. Kruse PH, Matta J, Ugolini S, Vivier E. Natural cytotoxicity receptors and their ligands. Vol. 92, Immunology and Cell Biology. 2014. p. 221–9.

284. Mandelboim O, Pazmany L, Davis DM, Valés-Gómez M, Reyburn HT, Rybalov B, et al. Multiple receptors for HLA-G on human natural killer cells. Proc Natl Acad Sci U S A. 1997;94(26):14666–70.

285. Anfossi N, André P, Guia S, Falk CS, Roetynck S, Stewart CA, et al. Human NK Cell Education by Inhibitory Receptors for MHC Class I. Immunity. 2006;25(2):331–42.

286. Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song YJ, Yang L, et al. Licensing of natural killer cells by host major histocompatibility complex class I molecules. Nature. 2005;436(7051):709–13.

287. Ong EZ, Chan KR, Ooi EE. Viral Manipulation of Host Inhibitory Receptor Signaling for Immune Evasion. Vol. 12, PLoS Pathogens. 2016. p. e1005776.

288. Giri JG, Kumaki S, Ahdieh M, Friend DJ, Loomis A, Shanebeck K, et al. Identification and cloning of a novel IL-15 binding protein that is structurally related to the alpha chain of the IL-2 receptor. EMBO J. 1995;14(15):3654–63.

289. Budagian V, Bulanova E, Paus R, Bulfone-Paus S. IL-15/IL-15 receptor biology: A guided tour through an expanding universe. Cytokine Growth Factor Rev. 2006;17(4):259–80.

290. Rabinowich H, Herberman RB, Whiteside TL. Differential effects of IL12 and IL2 on

expression and function of cellular adhesion molecules on purified human natural killer cells. Cell Immunol. 1993;152(2):481–98.

291. Sharma R, Das A. IL-2 mediates NK cell proliferation but not hyperactivity. Immunol Res. 2018;66(1):151–7.

292. Son YI, Dallal RM, Mailliard RB, Egawa S, Jonak ZL, Lotze MT. Interleukin-18 (IL-18) synergizes with IL-2 to enhance cytotoxicity, interferon-γ production, and expansion of natural killer cells. Cancer Res. 2001;61(3):884–8.

293. Fehniger TA, Cooper MA, Nuovo GJ, Cella M, Facchetti F, Colonna M, et al. CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: A potential new link between adaptive and innate immunity. Blood. 2003;101(8):3052–7.

294. de Rham C, Ferrari-Lacraz S, Jendly S, Schneiter G, Dayer JM, Villard J. The proinflammatory cytokines IL-2, IL-15 and IL-21 modulate the repertoire of mature human natural killer cell receptors. Arthritis Res Ther. 2007;9(6).

295. Mehrotra PT, Donnelly RP, Wong S, Kanegane H, Geremew A, Mostowski HS, et al. Production of IL-10 by human natural killer cells stimulated with IL-2 and/or IL-12. J Immunol. 1998;

296. Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, et al. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J Exp Med. 1989;170(3):827–45.

297. Wang KS, Frank DA, Ritz J. Interleukin-2 enhances the response of natural killer cells to interleukin-12 through up-regulation of the interleukin-12 receptor and STAT4. Blood. 2000;95(10):3183–90.

298. Bacon CM, Petricoin EF, Ortaldo JR, Rees RC, Larner a C, Johnston J a, et al. Interleukin 12 induces tyrosine phosphorylation and activation of STAT4 in human lymphocytes. Proc Natl Acad Sci U S A. 1995;92(August):7307–11.

299. Ling P, Gately MK, Gubler U, Stern AS, Lin P, Hollfelder K, et al. Human IL-12 p40 homodimer binds to the IL-12 receptor but does not mediate biologic activity. J Immunol. 1995;154(1):116–27.

300. Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity. 2000;13(5):715–25.

301. Freeman BE, Raué H-P, Hill AB, Slifka MK. Cytokine-Mediated Activation of NK Cells during Viral Infection. J Virol. 2015;89(15):7922–31.

302. Lehmann D, Spanholtz J, Sturtzel C, Tordoir M, Schlechta B, Groenewegen D, et al. IL-12 directs further maturation of ex vivo differentiated NK cells with improved therapeutic potential. PLoS One. 2014;9(1).

303. Wu C -y., Wang X, Gadina M, O'Shea JJ, Presky DH, Magram J. IL-12 Receptor 2 (IL-12R 2)-Deficient Mice Are Defective in IL-12-Mediated Signaling Despite the Presence of High Affinity IL-12 Binding Sites. J Immunol. 2000;165(11):6221–8.

304. Happel KI, Dubin PJ, Zheng M, Ghilardi N, Lockhart C, Quinton LJ, et al. Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae. J Exp Med. 2005;202(6):761–9.

305. D'Andrea A, Rengaraju M, Valiante NM, Chehimi J, Kubin M, Aste M, et al. Production of natural killer cell stimulatory factor (interleukin 12) by peripheral

blood mononuclear cells. J Exp Med. 1992;176(5):1387–98.

306. Macatonia SE, Hosken NA, Litton M, Vieira P, Hsieh CS, Culpepper JA, et al. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. J Immunol . 1995;154(10):5071–9.

307. Heufler C, Koch F, Stanzl U, Topar G, Wysocka M, Trinchieri G, et al. Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-γ production by T helper 1 cells. Eur J Immunol. 1996;26(3):659–68.

308. Borg C, Jalil A, Laderach D, Maruyama K, Wakasugi H, Charrier S, et al. NK cell activation by dendritic cells (DCs) requires the formation of a synapse leading to IL-12 polarization in DCs. Blood. 2004;104(10):3267–75.

309. Sims JE, Smith DE. The IL-1 family: Regulators of immunity. Vol. 10, Nature Reviews Immunology. 2010. p. 89–102.

310. Liu B, Novick D, Kim SH, Rubinstein M. Production of a biologically active human interleukin 18 requires its prior synthesis as pro-IL-18. Cytokine. 2000;12(10):1519–25.

311. Fantuzzi G, Dinarello CA. Interleukin-18 and interleukin-1β: Two cytokine substrates for ICE (caspase-1). J Clin Immunol. 1999;19(1):1–11.

312. Dinarello CA, Novick D, Kim S, Kaplanski G. Interleukin-18 and IL-18 binding protein. Front Immunol. 2013;4(289).

313. Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, et al. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL- 18-mediated function. Immunity. 1998;9(1):143–50.

314. Novick D, Schwartsburd B, Pinkus R, Suissa D, Belzer I, Sthoeger Z, et al. A novel IL-18BP ELISA shows elevated serum IL-18BP in sepsis and extensive decrease of free IL-18. Cytokine. 2001;14(6):334–42.

315. Shan NN, Zhu XJ, Wang Q, Wang CY, Qin P, Peng J, et al. High-dose dexamethasone regulates interleukin-18 and interleukin-18 binding protein in idiopathic thrombocytopenic purpura. Haematologica. 2009;94(11):1603–7.

316. Kunikata T, Torigoe K, Ushio S, Okura T, Ushio C, Yamauchi H, et al. Constitutive and induced IL-18 receptor expression by various peripheral blood cell subsets as determined by anti-hIL-18R monoclonal antibody. Cell Immunol. 1998;189(2):135–43.

317. French AR, Holroyd EB, Yang L, Kim S, Yokoyama WM. IL-18 acts synergistically with IL-15 in stimulating natural killer cell proliferation. Cytokine. 2006;35(5–6):229–34.

318. Hyodo Y, Matsui K, Hayashi N, Tsutsui H, Kashiwamura S, Yamauchi H, et al. IL-18 up-regulates perforin-mediated NK activity without increasing perforin messenger RNA expression by binding to constitutively expressed IL-18 receptor. J Immunol. 1999;162(3):1662–8.

319. Mirjacic Martinović K, Babović N, Džodić R, Jurišić V, Matković S, Konjević G. Favorable in vitro effects of combined IL-12 and IL-18 treatment on NK cell cytotoxicity and CD25 receptor expression in metastatic melanoma patients. J Transl Med. 2015;13(1).

320. Huang Y, Lei YF, Zhang H, Zhang M, Dayton A. Role of interleukin-18 in human natural killer cell is associated with interleukin-2. Mol Immunol. 2010;47(16):2604–10.

321. Harms RZ, Creer AJ, Lorenzo-Arteaga KM, Ostlund KR, Sarvetnick NE. Interleukin (IL)-18 Binding Protein Deficiency Disrupts Natural Killer Cell Maturation and Diminishes Circulating IL-18. Front Immunol. 2017;8(1020).

322. Strengell M, Matikainen S, Sirén J, Lehtonen A, Foster D, Julkunen I, et al. IL-21 in synergy with IL-15 or IL-18 enhances IFN-gamma production in human NK and T cells. J Immunol. 2003;170(11):5464–9.

323. Mao Y, Van Hoef V, Zhang X, Wennerberg E, Lorent J, Witt K, et al. IL-15 activates mTOR and primes stress-activated gene expression leading to prolonged antitumor capacity of NK cells. Blood. 2016;128(11):1475–89.

324. Cooper MA, Bush JE, Fehniger TA, Vandeusen JB, Waite RE, Liu Y, et al. In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells. Blood. 2002;100(10):3633–8.

325. Kennedy MK, Glaccum M, Brown SN, Butz EA, Viney JL, Embers M, et al. Reversible Defects in Natural Killer and Memory CD8 T Cell Lineages in Interleukin 15–deficient Mice. J Exp Med. 2000;191(5):771–80.

326. Vosshenrich CA, Ranson T, Samson SI, Corcuff E, Colucci F, Rosmaraki EE, et al. Roles for common cytokine receptor gamma-chain-dependent cytokines in the generation, differentiation, and maturation of NK cell precursors and peripheral NK cells in vivo. J Immunol. 2005;174(3):1213–21.

327. Grabstein KH, Eisenman J, Shanebeck K, Rauch C, Srinivasan S, Fung V, et al. Cloning of a T cell growth factor that interacts with the β chain of the interleukin-2 receptor. Science (80-). 1994;264(5161):965–8.

328. Bergamaschi C, Jalah R, Kulkarni V, Rosati M, Zhang G-M, Alicea C, et al. Secretion and biological activity of short signal peptide IL-15 is chaperoned by IL-15 receptor alpha in vivo. J Immunol. 2009;183(5):3064–72.

329. Fehniger TA, Caligiuri MA. Interleukin 15: Biology and relevance to human disease. Vol. 97, Blood. 2001. p. 14–32.

330. Kurys G, Tagaya Y, Bamford R, Hanover JA, Waldmann TA. The long signal peptide isoform and its alternative processing direct the intracellular trafficking of interleukin-15. J Biol Chem. 2000;275(39):30653–9.

331. Gaggero A, Azzarone B, Andrei C, Mishal Z, Meazza R, Zappia E, et al. Differential intracellular trafficking, secretion and endosomal localization of two IL-15 isoforms. Eur J Immunol. 1999;29(4):1265–74.

332. Bamford RN, Battiata AP, Waldmann TA. IL-15: The role of translational regulation in their expression. J Leukoc Biol. 1996;59(4):476–80.

333. Kleiner G, Marcuzzi A, Zanin V, Monasta L, Zauli G. Cytokine levels in the serum of healthy subjects. Mediators Inflamm. 2013;2013(Article ID 434010):6 pages.

334. Lamana A, Ortiz AAlvaro-Gracia J, Diaz-Sanchez B, Novalbos J, Garcia-Vicuna R, Gonzalez-Alvaro I. Characterization of serum interleukin-15 in healthy volunteers and patients with early arthritis to assess its potential use as a biomarker. Eur Cytokine Netw. 2010;21(3):186–94.

335. Pérez-López A, Valadés D, Vázquez Martínez C, de Cos Blanco AI, Bujan J, García-Honduvilla N. Serum IL-15 and IL-15Rα levels are decreased in lean and obese physically active humans. Scand J Med Sci Sport. 2018;28(3):1113–20.

336. Dubois S, Mariner J, Waldmann TA, Tagaya Y. IL-15Rα recycles and presents IL-15 in

trans to neighboring cells. Immunity. 2002;17(5):537–47.

337. Lorenzen I, Dingley AJ, Jacques Y, Grötzinger J. The structure of the interleukin-15α receptor and its implications for ligand binding. J Biol Chem. 2006;281(10):6642–7.

338. Anderson DM, Kumaki S, Ahdieh M, Bertles J, Tometsko M, Loomis A, et al. Functional characterization of the human interleukin-15 receptor α chain and close linkage of IL15RA and IL2RA genes. J Biol Chem. 1995;270(50):29862–9.

339. Shinozaki M, Hirahashi J, Lebedeva T, Liew FY, Salant DJ, Maron R, et al. IL-15, a survival factor for kidney epithelial cells, counteracts apoptosis and inflammation during nephritis. J Clin Invest. 2002;109(7):951–60.

340. Sato N, Patel HJ, Waldmann TA, Tagaya Y. The IL-15/IL-15Ralpha on cell surfaces enables sustained IL-15 activity and contributes to the long survival of CD8 memory T cells. Proc Natl Acad Sci U S A. 2007;104(2):588–93.

341. Ge N, Nishioka Y, Nakamura Y, Okano Y, Yoneda K, Ogawa H, et al. Synthesis and Secretion of Interleukin-15 by Freshly Isolated Human Bronchial Epithelial Cells. Int Arch Allergy Immunol. 2004;135(3):235–42.

342. Duitman EH, Orinska Z, Bulanova E, Paus R, Bulfone-Paus S. How a cytokine is chaperoned through the secretory pathway by complexing with its own receptor: lessons from interleukin-15 (IL-15)/IL-15 receptor alpha. Mol Cell Biol. 2008;28(15):4851–61.

343. Müller JR, Waldmann TA, Kruhlak MJ, Dubois S. Paracrine and transpresentation functions of IL-15 are mediated by diverse splice versions of IL-15Rα in human monocytes and dendritic cells. J Biol Chem. 2012;287(48):40328–38.

344. Burkett PR, Koka R, Chien M, Chai S, Boone DL, Ma A. Coordinate expression and trans presentation of interleukin (IL)-15Ralpha and IL-15 supports natural killer cell and memory CD8+ T cell homeostasis. J Exp Med. 2004;200(7):825–34.

345. Mortier E, Woo T, Advincula R, Gozalo S, Ma A. IL-15Rα chaperones IL-15 to stable dendritic cell membrane complexes that activate NK cells via trans presentation. J Exp Med. 2008;205(5):1213–25.

346. Dubois S, Magrangeas F, Lehours P, Raher S, Bernard J, Boisteau O, et al. Natural splicing of exon 2 of human interleukin-15 receptor α-chain mRNA results in a shortened form with a distinct pattern of expression. J Biol Chem. 1999;274(38):26978–84.

347. Bergamaschi C, Rosati M, Jalah R, Valentin A, Kulkarni V, Alicea C, et al. Intracellular interaction of interleukin-15 with its receptor α during production leads to mutual stabilization and increased bioactivity. J Biol Chem. 2008;283(7):4189–99.

348. Chertova E, Bergamaschi C, Chertov O, Sowder R, Bear J, Roser JD, et al. Characterization and Favorable in Vivo Properties of Heterodimeric Soluble IL-15·IL-15Rα Cytokine Compared to IL-15 Monomer. J Biol Chem. 2013;288(25):18093–103.

349. Stoklasek TA, Schluns KS, Lefrancois L. Combined IL-15/IL-15R Immunotherapy Maximizes IL-15 Activity In Vivo. J Immunol. 2006;177(9):6072–80.

350. Zanoni I, Spreafico R, Bodio C, DiGioia M, Cigni C, Broggi A, et al. IL-15 cis Presentation Is Required for Optimal NK Cell Activation in Lipopolysaccharide-Mediated Inflammatory Conditions. Cell Rep. 2013;4(6):1235–49.

351. Neely GG, Robbins SM, Amankwah EK, Epelman S, Wong H, Spurrell JC, et al. Lipopolysaccharide-stimulated or granulocyte-macrophage colony-stimulating

factor-stimulated monocytes rapidly express biologically active IL-15 on their cell surface independent of new protein synthesis. J Immunol. 2001;167:5011–7.

352. Musso T, Calosso L, Zucca M. Human monocytes constitutively express membrane-bound, biologically active, and interferon-gamma-upregulated interleukin-15. Vol. 120, Blood. 2012. p. 2155.

353. Neely GG, Epelman S, Ma LL, Colarusso P, Howlett CJ, Amankwah EK, et al. Monocyte surface-bound IL-15 can function as an activating receptor and participate in reverse signaling. J Immunol. 2004;172(7):4225–34.

354. Bergamaschi C, Bear J, Rosati M, Beach RK, Alicea C, Sowder R, et al. Circulating IL-15 exists as heterodimeric complex with soluble IL-15Rα in human and mouse serum. Blood. 2012;120(1).

355. Jakobisiak M, Golab J, Lasek W. Interleukin 15 as a promising candidate for tumor immunotherapy. Cytokine Growth Factor Rev. 2011;22(2):99–108.

356. Olsen SK, Ota N, Kishishita S, Kukimoto-Niino M, Murayama K, Uchiyama H, et al. Crystal structure of the interleukin-15·interleukin-15 receptor α complex: Insights into trans and cis presentation. J Biol Chem. 2007;282(51):37191–204.

357. Imamura M, Shook D, Kamiya T, Shimasaki N, Chai SMH, Coustan-Smith E, et al. Autonomous growth and increased cytotoxicity of natural killer cells expressing membrane-bound interleukin-15. Blood. 2014;124(7):1081–8.

358. Lodolce JP, Boone DL, Chai S, Swain RE, Dassopoulos T, Trettin S, et al. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. Immunity. 1998;9(5):669–76.

359. Sandau MM, Schluns KS, Lefrancois L, Jameson SC. Cutting Edge: Transpresentation of IL-15 by Bone Marrow-Derived Cells Necessitates Expression of IL-15 and IL-15R by the Same Cells. J Immunol. 2004;173(11):6537–41.

360. Son YI, Dallal RM, Mailliard RB, Egawa S, Jonak ZL, Lotze MT. Interleukin-18 (IL-18) synergizes with IL-2 to enhance cytotoxicity, interferon-gamma production, and expansion of natural killer cells. Cancer Res. 2001;61(3):884–8.

361. Carson WE. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. J Exp Med. 1994;180(4):1395–403.

362. Srivastava S, Pelloso D, Feng H, Voiles L, Lewis D, Haskova Z, et al. Effects of interleukin-18 on natural killer cells: Costimulation of activation through Fc receptors for immunoglobulin. Cancer Immunol Immunother. 2013;62(6):1073–82.

363. Roda JM, Parihar R, Magro C, Nuovo GJ, Tridandapani S, Carson WE. Natural killer cells produce T cell-recruiting chemokines in response to antibody-coated tumor cells. Cancer Res. 2006;66(1):517–26.

364. Nielsen CM, Wolf AS, Goodier MR, Riley EM. Synergy between common γ chain family cytokines and IL-18 potentiates innate and adaptive pathways of NK cell activation. Front Immunol. 2016;7(101).

365. Leong JW, Chase JM, Romee R, Schneider SE, Sullivan RP, Cooper MA, et al. Preactivation with IL-12, IL-15, and IL-18 induces cd25 and a functional high-affinity il-2 receptor on human cytokine-induced memory-like natural killer cells. Biol Blood Marrow Transplant. 2014;20(4):463–73.

366. Chaix J, Tessmer MS, Hoebe K, Fuseri N, Ryffel B, Dalod M, et al. Cutting Edge: Priming of NK Cells by IL-18. J Immunol. 2008;181(3):1627–31.

367. Agaugué S, Marcenaro E, Ferranti B, Moretta L, Moretta A. Human natural killer cells exposed to IL-2, IL-12, IL-18, or IL-4 differently modulate priming of naive T cells by monocyte-derived dendritic cells. Blood. 2008;112(5):1776–83.

368. MA BEKB. Human natural killer cells produce abundant macrophage inflammatory protein-1alpha in response to monocyte-derived cytokines. J Clin Invest. 1996;97(12):2722–7.

369. Viel S, Marçais A, Guimaraes FSF, Loftus R, Rabilloud J, Grau M, et al. TGF-β inhibits the activation and functions of NK cells by repressing the mTOR pathway. Sci Signal. 2016;9(415).

370. Park JY, Lee SH, Yoon S-R, Park Y-J, Jung H, Kim T-D, et al. IL-15-induced IL-10 increases the cytolytic activity of human natural killer cells. Mol Cells. 2011;32(3):265–72.

371. Brady J, Carotta S, Thong RPL, Chan CJ, Hayakawa Y, Smyth MJ, et al. The Interactions of Multiple Cytokines Control NK Cell Maturation. J Immunol. 2010;185(11):6679–88.

372. Smyth MJ, Cretney E, Kelly JM, Westwood JA, Street SEA, Yagita H, et al. Activation of NK cell cytotoxicity. Mol Immunol. 2005;42(4 SPEC. ISS.):501–10.

373. Lettau M, Schmidt H, Kabelitz D, Janssen O. Secretory lysosomes and their cargo in T and NK cells. Immunol Lett. 2007;108(1):10–9.

374. Chiang SCC, Theorell J, Entesarian M, Meeths M, Mastafa M, Al-Herz W, et al. Comparison of primary human cytotoxic T-cell and natural killer cell responses reveal similar molecular requirements for lytic granule exocytosis but differences in cytokine production. Blood. 2013;121(8):1345–56.

375. Ida H, Utz PJ, Anderson P, Eguchi KK. Granzyme B and natural killer (NK) cell death. Mod Rheumatol. 2005;15(5):315–22.

376. Topham NJ, Hewitt EW. Natural killer cell cytotoxicity: How do they pull the trigger? Vol. 128, Immunology. 2009. p. 7–15.

377. Talanian R V, Yang X, Turbov J, Seth P, Ghayur T, Casiano CA, et al. Granule-mediated killing: pathways for granzyme B-initiated apoptosis. J Exp Med. 1997;186(8):1323–31.

378. Trapani JA. Granzymes: a family of lymphocyte granule serine proteases. Genome Biol. 2001;2(12).

379. Osińska I, Popko K, Demkow U. Perforin: An important player in immune response. Vol. 39, Central European Journal of Immunology. 2014. p. 109–15.

380. Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. Nat Rev Immunol. 2002;2(10):735–47.

381. Reefman E, Kay JG, Wood SM, Offenhauser C, Brown DL, Roy S, et al. Cytokine Secretion Is Distinct from Secretion of Cytotoxic Granules in NK Cells. J Immunol. 2010;184(9):4852–62.

382. Screpanti V, Wallin RPA, Grandien A, Ljunggren HG. Impact of FASL-induced apoptosis in the elimination of tumor cells by NK cells. Mol Immunol. 2005;42(4):495–9.

383. Lucin P, Jonjic S, Messerle M, Polic B, Hengel H, Koszinowski UH. Late phase inhibition of murine cytomegalovirus replication by synergistic action of interferon-

gamma and tumour necrosis factor. J Gen Virol. 1994;75(1):101–10.

384. Caron G, Delneste Y, Aubry JP, Magistrelli G, Herbault N, Blaecke a, et al. Human NK cells constitutively express membrane TNF-alpha (mTNFalpha) and present mTNFalpha-dependent cytotoxic activity. Eur J Immunol. 1999;29(11):3588–95.

385. Yu M, Shi W, Zhang J, Niu L, Chen Q, Yan D, et al. Influence of reverse signaling via membrane TNF-α on cytotoxicity of NK92 cells. Eur J Cell Biol. 2009;88(3):181–91.

386. Nieto M, Navarro F, Perez-Villar JJ, del Pozo MA, Gonzalez-Amaro R, Mellado M, et al. Roles of chemokines and receptor polarization in NK-target cell interactions. J Immunol. 1998;161(7):3330–9.

387. Fauriat C, Long EO, Ljunggren H-G, Bryceson YT, Lanier L, Bottino C, et al. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. Blood. 2010;115(11):2167–76.

388. Saito S, Kasahara T, Sakakura S, Enomoto M, Umekage H, Harada N, et al. Interleukin-8 production by CD16-CD56bright natural killer cells in the human early pregnancy decidua. Biochem Biophys Res Commun. 1994;200(1):378–83.

389. Kaiko GE, Phipps S, Angkasekwinai P, Dong C, Foster PS. NK cell deficiency predisposes to viral-induced Th2-type allergic inflammation via epithelial-derived IL-25. J Immunol. 2010;185(8):4681–90.

390. Hussell T, Openshaw PJ. IL-12-activated NK cells reduce lung eosinophilia to the attachment protein of respiratory syncytial virus but do not enhance the severity of illness in CD8 T cell-immunodeficient conditions. J Immunol. 2000;165(12):7109–15.

391. Li F, Zhu H, Sun R, Wei H, Tian Z. Natural killer cells are involved in acute lung immune injury caused by respiratory syncytial virus infection. J Virol. 2012;86(4):2251–8.

392. Hussell T, Openshaw PJ. Intracellular IFN-gamma expression in natural killer cells precedes lung CD8+ T cell recruitment during respiratory syncytial virus infection. J Gen Virol. 1998;79 (Pt 11:2593–601.

393. Harker J a, Yamaguchi Y, Culley FJ, Tregoning JS, Openshaw PJM. Delayed sequelae of neonatal respiratory syncytial virus infection are dependent on cells of the innate immune system. J Virol. 2014;88(1):604–11.

394. Openshaw PJM, Tregoning JS. Immune responses and disease enhancement during respiratory syncytial virus infection. Clin Microbiol Rev. 2005;18(3):541–55.

395. Kerrin A, Fitch P, Errington C, Kerr D, Waxman L, Riding K, et al. Differential lower airway dendritic cell patterns May reveal distinct endotypes of RSV bronchiolitis. Thorax. 2016;620–7.

396. Fawaz LM, Sharif-Askari E, Menezes J. Up-regulation of NK cytotoxic activity via IL-15 induction by different viruses: a Comparative study. J Immunol. 1999;163(8):4473–80.

397. Tripp RA, Moore D, Barskey A, Jones L, Moscatiello C, Keyserling H, et al. Peripheral blood mononuclear cells from infants hospitalized because of respiratory syncytial virus infection express T helper-1 and T helper-2 cytokines and CC chemokine messenger RNA. J Infect Dis. 2002;185(10):1388–94.

398. Pérez A, Gurbindo MD, Resino S, Aguarón Á, Muñoz-Fernández MÁ. NK cell increase in neonates from the preterm to the full-term period of gestation. Neonatology. 2007;92(3):158–63.

399. Harker JA, Godlee A, Wahlsten JL, Lee DC, Thorne LG, Sawant D, et al. Interleukin 18 coexpression during respiratory syncytial virus infection results in enhanced disease mediated by natural killer cells. J Virol. 2010;84(8):4073–82.

400. Tregoning JS, Wang BL, McDonald JU, Yamaguchi Y, Harker JA, Goritzka M, et al. Neonatal antibody responses are attenuated by interferon-gamma produced by NK and T cells during RSV infection. Proc Natl Acad Sci U S A. 2013;110(14):5576–81.

401. Fu B, Wang F, Sun R, Ling B, Tian Z, Wei H. CD11b and CD27 reflect distinct population and functional specialization in human natural killer cells. Immunology. 2011;133(3):350–9.

402. Benlahrech A, Donaghy H, Rozis G, Goodier M, Klavinskis L, Gotch F, et al. Human NK cell up-regulation of CD69, HLA-DR, interferon γ secretion and cytotoxic activity by plasmacytoid dendritic cells is regulated through overlapping but different pathways. Sensors. 2009;9(1):386–403.

403. De Colvenaer V, Taveirne S, Delforche M, De Smedt M, Vandekerckhove B, Taghon T, et al. CD27-deficient mice show normal NK-cell differentiation but impaired function upon stimulation. Immunol Cell Biol. 2011;89(7):803–11.

404. Chen Y, Wei H, Sun R, Dong Z, Zhang J, Tian Z. Increased susceptibility to liver injury in hepatitis B virus transgenic mice involves NKG2D-ligand interaction and natural killer cells. Hepatology. 2007;46(3):706–15.

405. Mistry AR, O'Callaghan CA. Regulation of ligands for the activating receptor NKG2D. Vol. 121, Immunology. 2007. p. 439–47.

406. Ebihara T, Masuda H, Akazawa T, Shingai M, Kikuta H, Ariga T, et al. Induction of NKG2D ligands on human dendritic cells by TLR ligand stimulation and RNA virus infection. Int Immunol. 2007;19(10):1145–55.

407. Luo Q, Guo X, Peng S, Luo W, Tian F, Yu P, et al. The association between MICA/MICB polymorphism and respiratory syncytial virus infection in children. Int J Immunogenet. 2017;44(6):328–336.

408. Liu H, Osterburg AR, Flury J, Huang S, McCormack FX, Cormier SA, et al. NKG2D Regulation of Lung Pathology and Dendritic Cell Function Following Respiratory Syncytial Virus Infection. J Infect Dis. 2018;jiy151.

409. Farhadi N, Lambert L, Triulzi C, Openshaw PJM, Guerra N, Culley FJ. Natural killer cell NKG2D and granzyme B are critical for allergic pulmonary inflammation. J Allergy Clin Immunol. 2014;133(3).

410. Nielsen N, Ødum N, Ursø B, Lanier LL, Spee P. Cytotoxicity of CD56 bright NK cells towards autologous activated CD4 + T cells is mediated through NKG2D, LFA-1 and TRAIL and dampened via CD94/NKG2A. PLoS One. 2012;7(2).

411. Bryceson YT, Ljunggren HG, Long EO. Minimal requirement for induction of natural cytotoxicity and intersection of activation signals by inhibitory receptors. Blood. 2009;114(13):2657–66.

412. Long X, Xie J, Zhao K, Li W, Tang W, Chen S, et al. NK cells contribute to persistent airway inflammation and AHR during the later stage of RSV infection in mice. Med Microbiol Immunol. 2016;205(5):459–70.

413. Nakamura N, Rabouille C, Watson R, Nilsson T, Hui N, Slusarewicz P, et al. Characterization of a cis-Golgi matrix protein, GM130. J Cell Biol. 1995;131(6 II):1715–26.

414. Zhao Y, Jamaluddin M, Zhang Y, Sun H, Ivanciuc T, Garofalo RP, et al. Systematic Analysis of Cell-Type Differences in the Epithelial Secretome Reveals Insights into the Pathogenesis of Respiratory Syncytial Virus–Induced Lower Respiratory Tract Infections. J Immunol. 2017;198(8):3345–64.

415. Fuentes-Mattei E, Rivera E, Gioda A, Sanchez-Rivera D, Roman-Velazquez FR, Jimenez-Velez BD. Use of human bronchial epithelial cells (BEAS-2B) to study immunological markers resulting from exposure to PM2.5organic extract from Puerto Rico. Toxicol Appl Pharmacol. 2010;243(3):381–9.

416. Ogawa H, Nishimura N, Nishioka Y, Azuma M, Yanagawa H, Sone S. Interleukin (IL)-12 gene transduction and its functional expression into human bronchial epithelial cells (BEAS-2B) by adenovirus vector. J Med Invest. 2002;49(1–2).

417. Gillette DD, Shah PA, Cremer T, Gavrilin MA, Besecker BY, Sarkar A, et al. Analysis of human bronchial epithelial cell proinflammatory response to Burkholderia cenocepacia infection: Inability to secrete IL-1 β. J Biol Chem. 2013;288(6):3691–5.

418. Bamford RN, DeFilippis AP, Azimi N, Kurys G, Waldmann TA. The 5' untranslated region, signal peptide, and the coding sequence of the carboxyl terminus of IL-15 participate in its multifaceted translational control. J Immunol. 1998;160(9):4418–26.

419. Fehniger TA, Suzuki K, Ponnappan A, VanDeusen JB, Cooper MA, Florea SM, et al. Fatal leukemia in interleukin 15 transgenic mice follows early expansions in natural killer and memory phenotype CD8+ T cells. J Exp Med. 2001;193(2):219–31.

420. Hocke AC, Hartmann IK, Eitel J, Optiz B, Scharf S, Suttorp N, et al. Subcellular expression pattern and role of IL-15 in pneumococci induced lung epithelial apoptosis. Histochem Cell Biol. 2008;130(1):165–76.

421. Martínez I, Lombardía L, García-Barreno B, Domínguez O, Melero J a, Martinez I, et al. Distinct gene subsets are induced at different time points after human respiratory syncytial virus infection of A549 cells. J Gen Virol. 2007;88(2):570–81.

422. Jayaraman A, Jackson DJ, Message SD, Pearson RM, Aniscenko J, Caramori G, et al. IL-15 complexes induce NK- and T-cell responses independent of type i IFN signaling during rhinovirus infection. Mucosal Immunol. 2014;7(5):1151–64.

423. Jang J, Kim W, Kim K, Chung SI, Shim YJ, Kim SM, et al. Lipoteichoic acid upregulates NF-κB and proinfammatory cytokines by modulating β-catenin in bronchial epithelial cells. Mol Med Rep. 2015;12(3):4720–6.

424. Park SH, Choi H-J, Lee SY, Han J-S. TLR4-mediated IRAK1 activation induces TNF-α expression via JNK-dependent NF-κB activation in human bronchial epithelial cells. Eur J Inflamm. 2015;13(3):183–95.

425. Stewart MJ, Kulkarni SB, Meusel TR, Imani F. c-Jun N-terminal kinase negatively regulates dsRNA and RSV induction of tumor necrosis factor- alpha transcription in human epithelial cells. J Interf Cytokine Res. 2006;26(8):521–33.

426. Meusel TR, Imani F. Viral Induction of Inflammatory Cytokines in Human Epithelial Cells Follows a p38 Mitogen-Activated Protein Kinase-Dependent but NF-κB-Independent Pathway. J Immunol. 2003;171(7):3768–74.

427. Gerald CL, Romberger DJ, Devasure JM, Khazanchi R, Nordgren TM, Heires AJ, et al. Alcohol Decreases Organic Dust-Stimulated Airway Epithelial TNF-Alpha Through a Nitric Oxide and Protein Kinase-Mediated Inhibition of TACE. Alcohol Clin Exp Res. 2016;40(2):273–83.

428. Choi SS, Chhabra VS, Nguyen QH, Ank BJ, Stiehm ER, Roberts RL. Interleukin-15 enhances cytotoxicity, receptor expression, and expansion of neonatal natural killer cells in long-term culture. Clin Diagn Lab Immunol. 2004;11(5):879–88.

429. Kämpfer H, Kalina U, Mühl H, Pfeilschifter J, Frank S. Counterregulation of interleukin-18 mRNA and protein expression during cutaneous wound repair in mice. J Invest Dermatol. 1999;113(3):369–74.

430. Telcian AG, Laza-Stanca V, Edwards MR, Harker JA, Wang H, Bartlett NW, et al. RSV-Induced Bronchial Epithelial Cell PD-L1 Expression Inhibits CD8+ T Cell Nonspecific Antiviral Activity. J Infect Dis. 2011;203(1):85–94.

431. Xu J, Chakrabarti AK, Tan JL, Ge L, Gambotto A, Vujanovic NL. Essential role of the TNF-TNFR2 cognate interaction in mouse dendritic cell-natural killer cell crosstalk. Blood. 2007;109(8):3333–41.

432. Bont L, Heijnen CJ, Kavelaars a, van Aalderen WM, Brus F, Draaisma JM, et al. Local interferon-gamma levels during respiratory syncytial virus lower respiratory tract infection are associated with disease severity. J Infect Dis. 2001;184(3):355–8.

433. Melendi GA, Laham FR, Monsalvo AC, Casellas JM, Israele V, Polack NR, et al. Cytokine Profiles in the Respiratory Tract During Primary Infection With Human Metapneumovirus, Respiratory Syncytial Virus, or Influenza Virus in Infants. Pediatrics. 2007;120(2):e410–5.

434. Kim CK, Callaway Z, Koh YY, Kim SH, Fujisawa T. Airway IFN-γ production during RSV bronchiolitis is associated with eosinophilic inflammation. Lung. 2012;190(2):183–8.

435. Somers CC, Ahmad N, Mejias A, Buckingham SC, Carubelli C, Katz K, et al. Effect of dexamethasone on respiratory syncytial virus-induced lung inflammation in children: Results of a randomized, placebo controlled clinical trial. Pediatr Allergy Immunol. 2009;20(5):477–85.

436. Byeon JH, Lee JC, Choi IS, Yoo Y, Park SH, Choung JT. Comparison of cytokine responses in nasopharyngeal aspirates from children with viral lower respiratory tract infections. Acta Paediatr Int J Paediatr. 2009;98(4):725–30.

437. Semple MG, Dankert HM, Ebrahimi B, Correia JB, Booth JA, Stewart JP, et al. Severe respiratory syncytial virus bronchiolitis in infants is associated with reduced airway interferon gamma and substance P. PLoS One. 2007;2(10).

438. Leahy TRR, McManus R, Doherty DGDGDG, Grealy R, Coulter T, Smyth P, et al. Interleukin-15 is associated with disease severity in viral bronchiolitis. Eur Respir J. 2016;47(1):212–22.

439. Nicholson EG, Schlegel C, Garofalo RP, Mehta R, Scheffler M, Mei M, et al. Robust cytokine and chemokine response in nasopharyngeal secretions: Association with decreased severity in children with physician diagnosed bronchiolitis. J Infect Dis. 2016;214(4):649–55.

440. Jartti T, Aakula M, Mansbach JM, Piedra PA, Bergroth E, Koponen P, et al. Hospital length-of-stay is associated with rhinovirus etiology of bronchiolitis. Pediatr Infect Dis J. 2014;33(8):829–34.

441. Mansbach JM, Piedra PA, Teach SJ, Sullivan AF, Forgey T, Clark S, et al. Prospective multicenter study of viral etiology and hospital length of stay in children with severe bronchiolitis. Arch Pediatr Adolesc Med. 2012;166(8):700–6.

442. Rebuli ME, Pawlak EA, Walsh D, Martin EM, Jaspers I. Distinguishing Human

Peripheral Blood NK Cells from CD56dimCD16dimCD69+CD103+ Resident Nasal Mucosal Lavage Fluid Cells. Sci Rep. 2018;8(1):3394.

443. Pezzulo AA, Starner TD, Scheetz TE, Traver GL, Tilley AE, Harvey B-G, et al. The air-liquid interface and use of primary cell cultures are important to recapitulate the transcriptional profile of in vivo airway epithelia. AJP Lung Cell Mol Physiol. 2011;300(1):L25–31.

444. Dvorak A, Tilley AE, Shaykhiev R, Wang R, Crystal RG. Do airway epithelium air-liquid cultures represent the in vivo airway epithelium transcriptome? Am J Respir Cell Mol Biol. 2011;44(4):465–73.

445. dos Santos CC, Han B, Andrade CF, Bai X, Uhlig S, Hubmayr R, et al. DNA microarray analysis of gene expression in alveolar epithelial cells in response to TNFalpha, LPS, and cyclic stretch. Physiol Genomics. 2004;19(3):331–42.

446. Stoeck M, Kromer W, Gekeler V. Induction of IL-15 mRNA and Protein in A549 Cells by Pro-inflammatory Cytokines. Immunobiology. 1998;199(1):14–22.

447. Hansel TT, Tunstall T, Trujillo-Torralbo MB, Shamji B, del-Rosario A, Dhariwal J, et al. A Comprehensive Evaluation of Nasal and Bronchial Cytokines and Chemokines Following Experimental Rhinovirus Infection in Allergic Asthma: Increased Interferons (IFN-γ and IFN-λ) and Type 2 Inflammation (IL-5 and IL-13). EBioMedicine. 2017;19:128–38.

448. Alturaiki W, McFarlane AJ, Rose K, Corkhill R, McNamara PS, Schwarze J, et al. Expression of the B cell differentiation factor BAFF and chemokine CXCL13 in a murine model of Respiratory Syncytial Virus infection. Cytokine. 2018;110:267–71.

449. Matsuzaki Z, Okamoto Y, Sarashina N, Ito E, Togawa K, Saito I. Induction of intercellular adhesion molecule-1 in human nasal epithelial cells during respiratory syncytial virus infection. Immunology. 1996;88(4):565–8.

450. Arnold R, Konig W. Respiratory Syncytial Virus Infection of Human Lung Endothelial Cells Enhances Selectively Intercellular Adhesion Molecule-1 Expression. J Immunol. 2005;174(11):7359–67.

451. Liu X, Qin X, Xiang Y, Liu H, Gao G, Qin L, et al. Progressive changes in inflammatory and matrix adherence of bronchial epithelial cells with persistent respiratory syncytial virus (RSV) infection (progressive changes in RSV infection). Int J Mol Sci. 2013;14(9):18024–40.

452. Monick MM, Powers LS, Hassan I, Groskreutz D, Yarovinsky TO, Barrett CW, et al. Respiratory syncytial virus synergizes with Th2 cytokines to induce optimal levels of TARC/CCL17. J Immunol. 2007;179(3):1648–58.

453. Sekiya T, Miyamasu M, Imanishi M, Yamada H, Nakajima T, Yamaguchi M, et al. Inducible Expression of a Th2-Type CC Chemokine Thymus- and Activation-Regulated Chemokine by Human Bronchial Epithelial Cells. J Immunol. 2000;165(4):2205–13.

454. Lundell AC, Nordström I, Andersson K, Lundqvist C, Telemo E, Nava S, et al. IFN type I and II induce BAFF secretion from human decidual stromal cells. Sci Rep. 2017;7(39904).

455. Woo S-J, Im J, Jeon JH, Kang S-S, Lee M-H, Yun C-H, et al. Induction of BAFF expression by IFN- via JAK/STAT signaling pathways in human intestinal epithelial cells. J Leukoc Biol. 2013;93(3):363–8.

456. Singh UP, Singh S, Singh R, Cong Y, Taub DD, Lillard JW. CXCL10-Producing Mucosal CD4 $^{+}$ T Cells, NK Cells, and NKT Cells Are Associated with Chronic Colitis in IL-10 $^{-/-}$ Mice, Which Can Be Abrogated by Anti-CXCL10 Antibody Inhibition. J Interf Cytokine Res. 2008;28(1):31–43.

457. Liu X, Gao N, Dong C, Zhou L, Mi QS, Standiford TJ, et al. Flagellin-induced expression of CXCL10 mediates direct fungal killing and recruitment of NK cells to the cornea in response to Candida albicans infection. Eur J Immunol. 2014;44(9):2667–79.

458. Singh UP, Singh R, Singh S, Karls RK, Quinn FD, Taub DD, et al. CXCL10+ T cells and NK cells assist in the recruitment and activation of CXCR3+ and CXCL11+ leukocytes during Mycobacteria-enhanced colitis. BMC Immunol. 2008;9(1):25.

459. Sauty A, Dziejman M, Taha RA, Iarossi AS, Neote K, Garcia-Zepeda EA, et al. The T cell-specific CXC chemokines IP-10, Mig, and I-TAC are expressed by activated human bronchial epithelial cells. J Immunol (Baltimore, Md 1950). 1999;162(6):3549–58.

460. Terada N, Nomura T, Kim WJ, Otsuka Y, Takahashi R, Kishi H, et al. Expression of C-C chemokine TARC in human nasal mucosa and its regulation by cytokines. Clin Exp Allergy. 2001;31(12):1923–31.

461. Suzuki K, Setoyama Y, Yoshimoto K, Tsuzaka K, Abe T, Takeuchi T. Effect of interleukin-2 on synthesis of B cell activating factor belonging to the tumor necrosis factor family (BAFF) in human peripheral blood mononuclear cells. Cytokine. 2008;44(1):44–8.

462. Cella M, Otero K, Colonna M. Expansion of human NK-22 cells with IL-7, IL-2, and IL-1 reveals intrinsic functional plasticity. Proc Natl Acad Sci. 2010;107(24):10961–6.

463. Piper SC, Ferguson J, Kay L, Parker LC, Sabroe I, Sleeman MA, et al. The Role of Interleukin-1 and Interleukin-18 in Pro-Inflammatory and Anti-Viral Responses to Rhinovirus in Primary Bronchial Epithelial Cells. PLoS One. 2013;8(5).

464. Rouabhia M, Ross G, Pagé N, Chakir J. Interleukin-18 and gamma interferon production by oral epithelial cells in response to exposure to Candida albicans or lipopolysaccharide stimulation. Infect Immun. 2002;70(12):7073–80.

465. Shamaa OR, Mitra S, Gavrilin MA, Wewers MD. Monocyte caspase-1 is released in a stable, active high molecular weight complex distinct from the unstable cell lysate-activated caspase-1. PLoS One. 2015;10(11).

466. Schuurhof A, Bont L, Pennings JLA, Hodemaekers HM, Wester PW, Buisman A, et al. Gene expression differences in lungs of mice during secondary immune responses to respiratory syncytial virus infection. J Virol. 2010;84(18):9584–94.

467. Hodemaekers H, Pennings J, Hoebee B, Janssen R, van Oosten M, Ozturk K, et al. Host Transcription Profiles upon Primary Respiratory Syncytial Virus Infection. J Virol. 2007;81(11):5958–67.

468. Hurgin V, Novick D, Rubinstein M. The promoter of IL-18 binding protein: Activation by an IFN-γ-induced complex of IFN regulatory factor 1 and CCAAT/enhancer binding protein β. Proc Natl Acad Sci U S A. 2002;99(26):16957–16962.

469. Regamey N, Obregon C, Ferrari-Lacraz S, Van Leer C, Chanson M, Nicod LP, et al. Airway epithelial IL-15 transforms monocytes into dendritic cells. Am J Respir Cell Mol Biol. 2007;37(1):75–84.

470. Ferlazzo G, Pack M, Thomas D, Paludan C, Schmid D, Strowig T, et al. Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. Proc Natl Acad Sci. 2004;101(47):16606–11.

471. Münz C, Dao T, Ferlazzo G, De Cos MA, Goodman K, Young JW. Mature myeloid dendritic cell subsets have distinct roles for activation and viability of circulating human natural killer cells. Blood. 2005;105(1):266–73.

472. Sirén J, Sareneva T, Pirhonen J, Strengell M, Veckman V, Julkunen I, et al. Cytokine and contact-dependent activation of natural killer cells by influenza A or Sendai virus-infected macrophages. J Gen Virol. 2004;85(Pt 8):2357–64.

473. Morandi B, Mortara L, Carrega P, Cantoni C, Costa G, Accolla RS, et al. NK cells provide helper signal for CD8+T cells by inducing the expression of membrane-bound IL-15 on DCs. Int Immunol. 2009;21(5):599–606.

474. Guo-Parke H, Canning P, Douglas I, Villenave R, Heaney LG, Coyle P V., et al. Relative Respiratory Syncytial Virus Cytopathogenesis in Upper and Lower Respiratory Tract Epithelium. Am J Respir Crit Care Med. 2013;188(7):842–51.

475. McDonald T, Sneed J, Valenski WR, Dockter M, Cooke R, Herrod HG. Natural killer cell activity in very low birth weight infants. Pediatr Res. 1992;31(4 Pt 1):376–80.

476. Huenecke S, Fryns E, Wittekindt B, Buxmann H, Königs C, Quaiser A, et al. Percentiles of Lymphocyte Subsets in Preterm Infants According to Gestational Age Compared to Children and Adolescents. Scand J Immunol. 2016;84(5):291–8.

477. Kent A, Scorrer T, Pollard AJ, Snape MD, Clarke P, Few K, et al. Lymphocyte subpopulations in premature infants: An observational study. Arch Dis Child Fetal Neonatal Ed. 2016;101(6):F546–51.

478. Härtel C, Adam N, Strunk T, Temming P, Müller-Steinhardt M, Schultz C. Cytokine responses correlate differentially with age in infancy and early childhood. Clin Exp Immunol. 2005;142(3):446–53.

479. Torvinen M, Campwala H, Kilty I. The role of IFN-γ in regulation of IFN-γ-inducible protein 10 (IP-10) expression in lung epithelial cell and peripheral blood mononuclear cell co-cultures. Respir Res. 2007;8(80).

480. Culley FJ, Pennycook AMJ, Tregoning JS, Hussell T, Openshaw PJM. Differential chemokine expression following respiratory virus infection reflects Th1- or Th2-biased immunopathology. J Virol. 2006;80(9):4521–7.

481. Mitchell C, Provost K, Niu N, Homer R, Cohn L. IFN-γ Acts on the Airway Epithelium To Inhibit Local and Systemic Pathology in Allergic Airway Disease. J Immunol. 2011;187(7):3815–20.

482. Boguniewicz M, Martin RJ, Martin D, Gibson U, Celniker A, Williams M, et al. The effects of nebulized recombinant interferon-γ in asthmatic airways. J Allergy Clin Immunol. 1995;95(1–2):133–5.

483. Craxton A, Magaletti D, Ryan EJ, Clark EA. Macrophage- and dendritic cell-dependent regulation of human B-cell proliferation requires the TNF family ligand BAFF. Blood. 2003;101(11):4464–71.

484. Scapini P, Carletto A, Nardelli B, Calzetti F, Roschke V, Merigo F, et al. Proinflammatory mediators elicit secretion of the intracellular B-lymphocyte stimulator pool (BLyS) that is stored in activated neutrophils: Implications for inflammatory diseases. Blood. 2005;105(2):830–7.

485. Ng LG, Sutherland APR, Newton R, Qian F, Cachero TG, Scott ML, et al. B Cell-Activating Factor Belonging to the TNF Family (BAFF)-R Is the Principal BAFF Receptor Facilitating BAFF Costimulation of Circulating T and B Cells. J Immunol. 2004;173(2):807–17.

486. Bloom DD, Reshetylo S, Nytes C, Goodsett CT, Hematti P. Blockade of BAFF Receptor BR3 on T Cells Enhances Their Activation and Cytotoxicity. J Immunother. 2018;41(5):213–23.

487. Roe MFE, Bloxham DM, Cowburn AS, O'Donnell DR. Changes in helper lymphocyte chemokine receptor expression and elevation of IP-10 during acute respiratory syncytial virus infection in infants. Pediatr Allergy Immunol. 2011;22(2):229–34.

488. Vissers M, Ahout IML, De Jonge MI, Ferwerda G. Mucosal IgG Levels Correlate Better with Respiratory Syncytial Virus Load and Inflammation than Plasma IgG Levels. Clin Vaccine Immunol. 2016;23(3):243–5.

489. Thwaites RS, Coates M, Ito K, Ghazaly M, Feather C, Abdulla F, et al. Reduced Nasal Viral Load and IFN Responses in Infants with RSV Bronchiolitis and Respiratory Failure. Am J Respir Crit Care Med. 2018;198(8):1074–2567.

490. Jackson DJ, Glanville N, Trujillo-Torralbo MB, Shamji BWH, Del-Rosario J, Mallia P, et al. Interleukin-18 is associated with protection against rhinovirus-induced colds and asthma exacerbations. Clin Infect Dis. 2015;60(10):1528–31.

491. Tanaka H, Miyazaki N, Oashi K, Teramoto S, Shiratori M, Hashimoto M, et al. IL-18 might reflect disease activity in mild and moderate asthma exacerbation. J Allergy Clin Immunol. 2001;107(2):331–6.

492. Horvath KM, Brighton LE, Zhang W, Carson JL, Jaspers I. Epithelial cells from smokers modify dendritic cell responses in the context of influenza infection. Am J Respir Cell Mol Biol. 2011;45(2):237–45.

493. DiFranza JR, Masaquel A, Barrett AM, Colosia AD, Mahadevia PJ. Systematic literature review assessing tobacco smoke exposure as a risk factor for serious respiratory syncytial virus disease among infants and young children. BMC Pediatr. 2012;12(81).

494. Bradley JP, Bacharier LB, Bonfiglio J, Schechtman KB, Strunk R, Storch G, et al. Severity of respiratory syncytial virus bronchiolitis is affected by cigarette smoke exposure and atopy: Commentary. Vol. 12, Archives de Pediatrie. 2005. p. 1153–4.

495. Lukacs NW, Moore ML, Rudd BD, Berlin AA, Collins RD, Olson SJ, et al. Differential immune responses and pulmonary pathophysiology are induced by two different strains of respiratory syncytial virus. Am J Pathol. 2006;169(3):977–86.

496. Stokes KL, Chi MH, Sakamoto K, Newcomb DC, Currier MG, Huckabee MM, et al. Differential pathogenesis of respiratory syncytial virus clinical isolates in BALB/c mice. J Virol. 2011;85(12):5782–93.

497. Wang S-Z, Bao Y-X, Rosenberger CL, Tesfaigzi Y, Stark JM, Harrod KS. IL-12p40 and IL-18 modulate inflammatory and immune responses to respiratory syncytial virus infection. J Immunol. 2004;173(6):4040–9.

498. Collins PL, Donnelly RP, Luongo C, Thayer KR, Sheikh F, Yan L, et al. Differential Responses by Human Respiratory Epithelial Cell Lines to Respiratory Syncytial Virus Reflect Distinct Patterns of Infection Control. J Virol. 2018;92(15):JVI.02202-17.

499. van Erp EA, Feyaerts D, Duijst M, Mulder HL, Wicht O, Luytjes W, et al. Respiratory

Syncytial Virus Infects Primary Neonatal and Adult Natural Killer Cells and Affects Their Antiviral Effector Function. J Infect Dis. 2018;219(5):723–33.