Mechanism of Natural Killer Cell Mediated T Cell Cycle Arrest during Human Parainfluenza Virus Type 3 Infection

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Abbreviations

Ab	Antibody
Abs	Absorbance
AO	Acridine Orange
APC	Antigen presenting cells
APC	Allphycocyanin
BCR	B cell receptor
BSA	Bovine serum albumin
cDCs	Classical dendritic cells
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxyfluorexcein diacetate succinimidyl ester
CNS	Central nervous system
cRPMI	Complete roswell park memorial institute
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EB	Ethidium bromide
pDCs	Plasmacytoid DCs
dsRNA	Double stranded ribonucleic acid
EDTA	Ethylenediamietetraacetic acid
F	Fusion protein
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate

FSC	Forward scatter
G	Gravity
НА	Hemagglutinin
HIV	Human immunodeficiency disorder
HLA	Human leukocyte antigen
HN	Hemagglutinin neuraminidase
HPIV	Human parainfluenza virus
HRP	Horseradish peroxidase
IFN	Interferon
IL	Interleukin
KIR	Killer cell immunoglobin-like receptors
L	Large RNA polymerase protein
М	Matrix protein
mAb	Monoclonal antibody
Mda	Melenoma differentiation associated genes
MHC	Major histocompatibility complex
MLR	Major histocompatibility complex
mRNA	Messenger Ribonucleic acid
NA	Neuraminidase
NCR	Natural cytotoxicity receptors
ΝΓκΒ	Nuclear factor kB
Ng	Nanograms
NO	Nitric Oxide
	XVII

NP	Nucleocapsid protein
NK	Natural killer
OD	Optical density
Р	Phosphoprotein
PAMPs	Pattern associated molecular receptor
РВМС	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerthrin
PI	Propidium Iodide
PIV	Parainfluenza virus
PKR	Protein Kinase receptor
РМА	Phorol 12-myristate B-acetate
PMNs	Polymorphonuclear cells
PRR	Pattern recognition receptor
RIG-1	Retinoic acid-inducible gene
RNA	Ribonucleic Acid
RSV	Respiratory syncytial virus
RT	Reverse transcriptase
RVIs	Respiratory viral infections
SARs	Severe acute respiratory syndrome
SSC	Side scatter
ssRNA	Single stranded Ribonucleic acid

TCID	Tissue culture infectious dose
Tcon	Conventional T cell
Th	T helper cell
TLR	Toll like receptor
Treg	Regulatory T cell
UV	Ultraviolet
VIG	Viral immunology group
vRNA	Viral Ribonucleic acid

Abstract

Here we investigate the role of NK cells in inhibiting T cell cycle during Human Parainfluenza Virus type 3 (HPIV3) infection, attributing viral Hemagglutininneuraminidase (HN) to activation of this NK cell mediated T cell regulation via the NK cell receptors NKp44 and NKp46. Having associated HN with the induction of low level IL-2 production we demonstrated that low dose IL-2 expands primary human CD56^{bright} NK cells resulting in contact dependent cellcycle arrest of T cell proliferation. While this was true for the conventional T cell population, Tregs were actually promoted in these cultures. We also demonstrate by using blocking and activating antibodies that simultaneous activation of both NKp44 and NKP46 induce NK mediated cell cycle arrest in T cells at low IL-2 but that NKp44 alone is responsible for enhanced apoptosis in T cells at high IL-2. These results not only highlight the importance of NK cells in immune regulation but also identify key human therapeutic NK-targets for the future. In addition, these results may provide further insights into the therapeutic mechanisms of low dose IL-2 in autoimmunity. Finally, the possibility of isolated HPIV3 HN as a molecule for induction of this immune regulation is under investigation.

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"There's nowhere you can be that isn't where you're meant to be." -John Lennon

1 GENERAL INTRODUCTION

1.1 RESPIRATORY VIRAL INFECTIONS

Viruses are small infectious agents, which have the ability to infect all organisms (Arpaia and Barton, 2011). They are composed of genomic material; either DNA or RNA and are obligate parasites, meaning they can only replicate within the cell of the host organism (Harper 1998).

Respiratory viral infections (RVIs) are a leading cause of death and hospitalization, especially in children, all over the world. Exacerbations include bronchiolitis, asthma, wheezing, croup, and even pneumonia, and they are attributed to both upper and lower respiratory tract infections (Pavia 2011). As already stated, acute RVIs are the leading cause of hospitalization for infants and younger children, accounting for significant morbidity worldwide. Many of these viruses also have overlapping clinical presentation, and therefore, can be difficult to diagnose and treat (Mahony et al 2011). The common RVIs are Respiratory Syncytial Virus (RSV), Parainfluenza Virus (PIV), influenza, and adenovirus (Pavia 2011). As RVIs are airborne diseases which can be transmitted via large droplets, aerosols or formites, they can spread rapidly from one individual to the next and must be monitored closely. This is especially true for influenza virus, as it has the potential to change rapidly, producing a virulent strain, which could result in a pandemic (Fauci 2006, Mahony et al 2011, Kreijtz et al 2011). Additionally, recurrent infections such as those caused by PIVs and RSVs are quite common. RVIs can persist within the host causing prolonged illness (Collins and Melero 2011, Hall 2001). These features are mainly due to virus' ability to modulate or evade the host immune system. For example, human parainfluenza virus type 3 (HPIV3), a strain of PIV, induces inhibition of T cell responses, preventing the development of immune memory (Noone et al 2008). Control of these viruses is a major goal for researchers and efforts have been made to develop new detection methods and means of combat (Mahony et al 2011). Vaccine development has been a crucial area of research in this field in recent years, with the introduction of the trivalent inactivated vaccine for influenza and antiviral agents, such as neuraminidase inhibitors, adamants-antiviral drugs such as ribavarin and fusion inhibitors (Collins and Melero 2011, Abed and Boivin 2006). Unfortunately, while these antiviral agents may inhibit certain aspects of viral replication, reinfection by the virus is not prevented. Thus, vaccines remain the most effective treatment for viral infections, as they can induce lifelong immunity to a particular viral strain (Kreitz et al 2011). The trivalent vaccine which targets influenza needs to be changed annually due to

the quick mutation rate of the virus (Doherty et al 2006). While such efforts are underway for influenza prevention, medically important respiratory viruses such as PIV an RSV have no licensed vaccines due to the failure of immune memory associated with infection (Mahony *et al* 2011, Collins and Mellero 2011, Abed and Boivin 2006). Thus, understanding immune response to these respiratory viruses is critical to the development of new and more efficient vaccines.

1.1.1 Parainfluenza viruses

Human Parainfluenza Viruses (HPIVs) are currently the second most common cause of acute respiratory illness leading to hospitalization (Schmidt *et al* 2011). They are a major cause of respiratory illness in neonates and infants, for which there is no effective vaccine, despite years of intensive efforts (Moscona 2005). These viruses are part of the paramyxovirus family, of which there are four different serotypes: HPIV 1, 2, 3 and 4 (Henrickson 2003, Chanock 2001). This study will focus on HPIV3 infection. This serotype is a major cause of lower respiratory infections in infants, which are commonly characterized by illness such a croup, pneumonia and bronchiolitis (Schmidt *et al* 2011).

1.1.1.1 Structural Organisation

Human Parainfluenza viruses are negative sense, single stranded, enveloped, RNA viruses, which encode at least six common structural proteins essential for viral replication: Nucleocapsid protein (NP), Phosphoprotein (P), Large RNA polymerase protein (L), Matrix protein (M), Hemagglutinin Neuraminidase (HN) and Fusion protein (F). Key to immunity and pathogenesis to the virus are the surface glycoproteins; F and HN proteins (Figure1.1), which are responsible for attachment and fusion to host cells (Moscona 2005). Notably, these surface proteins have been used in the failed vaccines against HPIV (Schmidt *et al* 2011). It is thought that the M protein is involved in mediating the attachment of nucleocapsids to the envelope. The L, P and NP proteins are in close association with the viral RNA (vRNA) and are closely involved in viral replication (Henrickson 2003, Chanock 2001, Hall 2001).



Figure 1.1: Structure of human parainfluenza virus (Taken from Moscona 2005). Here the general structure of HPIV is represented. Two surface glycoproteins, HN and F are present on the lipid bilayer, under which the M protein lies. The L protein, NP and P protein make up the internal RNA.

1.1.1.2 Viral Replication

Infection with HPIV is initiated by HN binding to sialic acid on cellular membrane proteins. The F protein mediates fusion between the viral envelope and the plasma membrane of the host cell (Schmidt *et al* 2011). As a result of this, the viral nucleocapsid is released into the cytoplasm of the host cell. The virus can then use the host ribosomes to translate the viral messenger RNA (mRNA) into viral proteins, which direct replication of the viral genome, firstly to a positive sense strand and then into a negative sense strand. Once replicated, RNA in encapsulated with NP, forming a new virion. Finally, these virions are released intracellularly by a budding (Henrickson 2003, Moscona 2005). In addition, during infection, F proteins are transported to cell surface where they can cause the host cell membrane to fuse with a neighbouring cell. This results in syncytia, which are multinucleated cells which result from multiple cell fusions of uninuclear cells (Figure 1.2) (Hall 2001).



Figure 1.2: Syncitial formation during HPIV3 infection (Taken from: <u>http://www.mcld.co.uk/hiv/?q=syncytia</u>). The binding of two cells via fusion proteins can results in dinucleated cells. When this occurs repeatedly, multi-nucleated cells form in a process known as syncytial formation.

1.1.1.3 Pathogenesis

HPIV3 infection occurs in the epithelial cells which line the respiratory tract. Generally, infection starts in the epithelial cells of the nose and throat, but can spread to the lower respiratory tract. It is suggested that host immune response, rather than the direct effect of infection, is the main contributor to pathogenesis (Schmidt *et al* 2011). Upon replication, inflammation of the airways or bronchiolitis occurs. Additionally, a large influx of inflammatory cells occurs at the site of infection. In most cases, the virus is non-cytophathic and can occasionally lead to persistent infections (Moscona 2005). The tissue damage associated with infection causes increased levels of mucus secretions, which obstruct airflow and cause wheezing or coughing of the patient. While reinfection throughout life is common, recovery from infection usually occurs within a few days (Henrickson 2003, Hall 2001).

1.1.1.4 Immunity to HPIV

HPIV infection induces strong humoral and cellular immune responses. Both local and systemic neutralizing antibodies (IgG and IgA) towards HPIV surface glycoproteins HN and F are found in serum and mucosa of infected patients. While reinfection with this virus is Virus-encoded common. these neutralizing antibodies eventually confer immunity. interferons (IFN antagonists) allow HPIV replication, but this IFN production and activity are not completely blocked and may still contribute to host defence (Schmidt et al 2011). In addition, cytotoxic T cells appear to be important in the clearance of HPIV from the lower respiratory tract. The induction of these cells is crucial, as for infants with severe T cell deficiency; HPIV3 can be associated with fatal cases of pneumonia (Chanock 2001). Although adequate protection appears to be mounted against HPIV3, with viral clearance occurring within days, no long lasting immunity to these viruses ever develops (Henrickson 2003). This failed memory was investigated by Noone et al (2008) and will be further explored in this study. The understanding of poor immune memory associated with HPIV3 is of importance for vaccine development, and while long lasting robust responses to PIV vaccines have been demonstrated in mouse, this appears not to be the case for PIV infections of humans (Schmidt et al 2011).

1.1.2 Influenza virus

For HPIV3 experiments described in this study, influenza viral infection was used as a control for comparison with another respiratory virus. Although influenza evades host immunity by altering its surface antigens it does induce long lasting immunological memory to the same strain in humans. Therefore, this agent serves as an excellent control virus for our studies with HPIV3. Influenza is a well-documented, highly contagious, acute respiratory disease of global importance. It is responsible for yearly epidemics in addition to sporadic pandemics, and so, is a major cause of mortality worldwide. Influenza infection is characterized by fever and chills, accompanied by headache, myalgias and a dry cough (Kuiken *et al* 2012, Wright and Webster 2001). Influenza is a member of the orthomyxoviridae family including; influenza A, B, C. Of these, influenza A is the most important clinically. Influenza A is further divided into subtypes based on the antigenic nature of their hemagglutinin (HA) and neuraminidase (NA) proteins (Kuiken *et al* 2012,

Hilleman 2002, Lamb 2001). The H_1N_1 subtype was used as the viral control for comparison to HPIV3 infection in this study.

1.1.2.1 Structural organization and replication

Strong immune memory is mounted towards influenza, so that reinfection rarely occurs with the same strain. Thus, influenza served as a good reference virus for studying HPIV3. Both are human viral infections that share the same portal of entry and target cells. While Influenza serves only as a reference virus in this study, the structure and replication of this virus are described briefly here, as immunity against this virus is of importance to this study.

Influenza A virus is an enveloped, negative, single-stranded RNA virus. It has a segmented genome which contains 8 RNA segments which encode at least 10 polypeptides. The viral envelope contains spike-like projections, or membrane glycoproteins; HA and NA (Figure 1.3). The HA binds to sialic acid receptors on the host cell, initiating receptor-mediated endocytosis and the formation of a membrane bound vesicle around the virus. Once the vesicles are formed, they fuse with endosomes, causing a lower pH and initiating conformational changes in HA. This allows the virus to fuse with cell membranes and form endocytic vesicles for release into the cytosol. From there, the viral genome enters the nucleus where it begins transcription. While six of the newly transcribed mRNAs are translated into viral structural proteins, two code for non-structural proteins. New virions are then assembled at the host membrane, and released from the cell by budding. The NA protein functions in the release of the new viral progeny from the cell, by cleaving sialic acid receptors, preventing aggregation and allowing release of the virion (Kuiken *et al* 2012, Lamb 2001).



Figure 1.3: Structure of Influenza virus A

(Taken from: <u>http://www.virology.ws/2009/04/30/structure-of-influenza-virus/</u>) This is a representative figure of an influenza A versome. HA and NA proteins are on the lipid bilayer, under which the matrix protein lies, and in which the M2 ion channel is present. Inside is the segmented negative strand RNA gene, NP and RNA polymerase

1.1.2.2 Pathogenesis

During infection, influenza virus replicates in the epithelial cells of the respiratory tract. This induces pathological changes, especially in the lower respiratory tract. Infection of these epithelial cells induces inflammation in the larynx, trachea and bronchi. This is associated with a strong influx of leukocytes. These inflammatory responses manifest clinically with symptoms such as fever, myalgia and dry cough. Most individuals recover from influenza infection within seven days (Kuiken et al 2012, Wright and Webster 2001). As strain specific clearance and memory occurs to influenza, including strong immune T cell responses, it served as a good viral comparison to HPIV3 infection. Infection of the respiratory tract may give rise to primary pneumonitis, especially in immunocompromised individuals. This may lead to secondary bacterial infections such as those caused by streptococcal and secondary infections staphylococcal bacteria. These can cause severe pulmonary complications and can be fatal (Wright and Webster 2001, Hilleman 2002).

1.2 Immunity to viruses

Viral infections are a constant challenge for immunologists, with regard to prevention and vaccine development. For example, the occurance of new influenza strains makes prevention a constant endeavour. In addition, there are newly emerging viruses such as severe acute respiratory syndrome (SARs) and those with high mutation rates such as human immunodeficiency virus (HIV), which can make prevention challenging (Kawai and Akira 2006, Doherty *et al* 2006). Additionally, viruses have also developed mechanisms by which they can modulate the host's immune system to promote survival. While the immune system recognises HPIV3, this virus induces suppression of human T cell responses, which could account for the poor immunological memory to this virus in humans (Noone *et al* 2008). Understanding these complex immune interactions between viruses and host, particularly to infections such as HPIV, which remain refractory to conventional approaches, is key in the development of vaccines.

There are two arms to the immune system; the non-specific, fast acting, innate responses and the more specific adaptive responses. These work to provide the most efficient means of protection and viral clearance (Khan 2011, Janeway 2003).

1.2.1 The innate immune system

The innate immune system is non-specific, but acts quickly and provides the first line of defense against invading pathogens. The first role of innate immunity is to limit pathogen entry, a role which is quite successful. Pathogen entry is prevented by both physical and mechanical barriers, including the skin and mucousal membranes. In addition, chemical barriers, such as digestive enzymes, contribute to innate immunity. If these surface defences are overcome by invading pathogens, they encounter innate immune cells and plasma proteins of the complement system. Plasma proteins can be activated by infection, resulting in enhaced opsoniation and phagocytosis of the pathogen (Janeway 2008, Sarma and Ward 2011).

The innate immune cells include various leukocytes, such as natural killer (NK) cells, monocytes, and dendritic cells (DCs). Phagocytic cells that engulf and destroy invading pathogens are of importance to innate immunity. These phagocytic cells include macrophages and neutrophils (Khan 2011, Tosi 2005). Innate cells express patter-recognition receptors (PRRs), which are capable of recognising conserved motifs on microbes, which are known as pattern associated molecular patterns (PAMPs). These include double stranded RNA (dsRNA) or single stranded RNA (ssRNA) produced by virally infected cells which are detected by TLR3 and TLR7 (Janeway 2008, Kawai and Akira 2006). These receptors recognise specific microbial motifs that have remained conserved over time. These patterns are not expressed by the host cells so the innate immune system only responseds to foreign or "danger" signals of the invading pathogen. Activation of innate immune cells is immediate and of a relatively short duration. Additionally, there is no immunological memory associated with innate immune responses. Toll like receptors (TLRs) are the most characterised PRRs in humans (Akira and Takeda 2004).

1.2.1.1 Pattern Recognition receptors: Toll like receptors

As previously described, when PRRs on innate immune cells bind PAMPs, innate immune responses are activated. The most characterised PRRs in humans are TLRs, with eleven identified in humans to date (Arpaia and Barton 2011). Expression of TLRs correlates with locations of innate immune response; within circulation, and epithelial cells lining respiratory, intestinal and urogenital tracts (Ashkar *et al* 2003). Some TLRs are expressed on the cell surface (TLR 1, 2, 4, 5, 6 and 11), while others are expressed intracellularly (TLR 3, 7, 8, 9). The most relevant TLRs for this study are TLR 3, 7, and 8, as these function in the detection of viruses. These TLRs have the ability to recognize nucleic acids that are not usually produced in host cells, such as dsRNA (detected by TLR3) and single-stranded RNA (ssRNA) (detected by TLR7 and human TLR8). TLR9 also recognizes nucleic acids produced in infected host cells, such as unmethylated CpG DNA of viruses and bacteria (Janeway 2008, Iwasaki and Medzhitov 2004, Kawai and Akira 2006, Heil *et al* 2004). TLRs 3, 7, 8 and 9 are localized to intracellular compartments where they can detect nucleic acids in late endosomes or lysosomes (Akira and Takeda 2004, Diebold *et al* 2004, Kawai and Akira 2006).

Once TLR stimulation has occurred, various signal transduction pathways are activated, which result in the nuclear translocation and activation of nuclear factor κB (NF- κB). This leads to cellular activation. NF- κB is a transcription factor which functions by activating the promotion of numerous genes, transcribing proteins involved in inflammatory responses such as cytokines and chemokines (Janeway 2008, O'Neil and Bowie 2007, O'Neil 2004). These proteins play an important role in immune response to viruses. Chemokines control leukocyte trafficking and migration, while cytokines activate and regulate numerous cell types, both of which are essential for viral clearance (Arpaia and Barton 2011, Coelho *et al* 2005).

There are also TLR-independent mechanisms such as retinoic acid-inducible gene (RIG-1) and melanoma differentiation associated gene 5 (Mda 5). These are RNA helicases that can bind to viral dsRNA in the cytoplasm, leading to activation of various signaling pathways, including the NF-KB pathway and secretion of type 1 interferon antiviral cytokines and other inflammatory proteins (Arpaia and Barton 2011, Janeway 2008, Kawai and Akira 2006, Akira and Takeda 2004).

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1.2.1.2 Interferons

In a viral infection, the interferons (IFNs) are the most important cytokines produced, as they have antiviral properties. Viral infection of a cell directly stimulates the production of type 1 IFNs (Wang and Fish 2012). These cytokines have antiviral properties and are secreted from many different cell types. Therefore, they are of great importance for viral clearance by the innate immune system. There are two main families of these antiviral cytokines: type 1 IFNs (IFN- α and IFN- β), and type II IFNs (IFN- γ) (Garcia-Sastre and Biron 2006, Guidotti and Chisari 2000).

Type 1 interferons comprise IFN- α and IFN- β , which share a common heterodimeric receptor (IFNAR). Viral infection of a cell directly stimulates the production of type 1 interferons, which function to inhibit viral replication. Binding of IFN- α or IFN- β to their receptor on the host cell triggers a transduction pathway (Jak/Stat) which leads to transcriptional activation of cellular genes whose products are responsible for the induction of an antiviral state. For example, IFN-induced protein kinase R (PKR) is an inactive kinase which requires dsRNA for its activation. In addition to activation by dsRNA, which is uncommon except during viral replication, PKR expression also increases 20 fold with IFN stimulation, and acts by inhibiting protein synthesis and blocking viral replication in infected cells. Additionally, IFN induces the family of dsRNA-dependent 2'-5' oligoadenylate synthase. These activate ribonuclease RNaseL, which degrades single-stranded RNAs of cellular and viral origin. IFN can also induce synthesis of the isoform of dsRNA-specific adenosine deaminiase which converts adenosine to inosine in viral and cellular RNA. This amino acid substitution results in the formation of inactive viral protein (Khan 2011, Collier and Oxford 2006, Garcia-Sastre and Biron 2006, Mayer 2003).

The binding of IFN- α and IFN- β to immune cells, such as macrophages, DCs and NK cells, activates them for killing virally infected cells (Wang and Fish 2012, Guidotti and Chisari 2000). Type 1 IFNs can lead to DC maturation, resulting in the upregulation of MHC and costimulatory molecules, on the surface of DCs, which is essential for T cell activation (Theofilopouos *et al* 2005). Additionally, type 1 IFN-induced DC maturation following viral infection, can promote cross priming (process whereby a captured extracellular antigen can be presented on MHC class 1 molecules) of antigens to CD8+ T cells, resulting in T cell activation (Le Bon *et al* 2003). Thus, type 1 IFNs play an important role in bridging innate and adaptive immune responses and directing appropriate T cell responses.

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IFN- γ is a pro-inflammatory type II IFN cytokine that can modulate many aspects of the immune response, and is considered key to antiviral immunity (Janeway 2008). This is mainly secreted from T cells and NK cells and is a critical cytokine involved in activating macrophages. Activated macrophages can produce nitric oxide (NO) and oxygen radicals, which have potent antimicrobial activity. This is also the primary cytokine involved in driving differentiation of naïve CD4+ T cells to the Th1 subset. These cells maximise the killing efficacy of macrophages, enhance proliferation of cytotoxic CD8+ cells and promote the production of opsonizing antibodies. As IFN- γ is associated with many protective functions, it is one of the most important cytokines involved in shaping or directing adaptive immune responses (Janeway 2008, Guidotti and Chisari 2000).

1.2.1.3 Cell types of the innate immune system

The cells of the innate immune system provide rapid but unspecific protection against viruses and other pathogens. These include cytotoxic lymphocytes (such as NK cells), toxin-releasing cells of myeloid origin such as mast cells, basophils and eosinophils, and the phagocytic cells of the monocyte/macrophage lineage, neutrophils and myeloid dendritic cells (DCs) (Janeway 2008).

1.2.1.3.1 Antigen presenting cells

Monocytes, macrophages and Dendritic cells (DCs), comprise a group of cells specialised in phagocytosis, antigen presentation to cells and control of immunity. Monocytes circulate in the blood, bone marrow and spleen. These cells do not proliferate in a healthy host. This subset of innate effector cells has both chemokine and pattern recognition receptors that modulate migration during infection. Monocytes function by the production of inflammatory cytokines, as well as the uptake of infected cells or toxic molecules. These cells can differentiate into DCs or macrophages during infection. When a monocyte in circulation enters damaged tissue, it undergoes a series of changes to become a macrophage. These macrophages are phagocytic cells of both lymphoid and non-lymphoid tissue. They have a broad range of PRRs, making them efficient at phagocytosis and the production of inflammatory cytokines.

Classical DCs (cDCs) are specialised antigen-processing and presenting cells, equipped with phagocytic activity when immature, and high cytokine production when mature. These cells can migrate from tissues to the T cell and B cell zones of lymphoid organs. cDCs regulated T cell responses both in healthy and infected hosts. Plasmacytoid DCs (pDCs) are relatively long lived compared to cDCs. These cells are specialized in anti-viral responses, with a high production level of type 1 interferons. Additionally, they act as APCs and control T cell responses. The generation of monocytes, macrophages and DCs, depends on the activation of PRRs by the pathogen and subsequent cytokine release. Current models propose that blood monocytes, many macrophage subsets and most DCs originate from hematopoietic stem-cell derived subsets (Janeway *et al* 2008).

Antigen presentation by these cells is crucial for the activation of T cells and overall adaptive responses. MHCs are cell surface glycoproteins which bind antigenic peptides for antigen presentation to T cells. Two classes exist; MHC class 1 which present to CD8+ T cells, and MHC class 2, which present to CD4+ T cells. In humans, the MHC gene is called the human leukocyte antigen (HLA) gene complex, where HLA-A, B and C, are the main genes of the MHC class 1 molecules and HLA-DR, DP, and DQ are the main genes of the MHC class II molecules. Internalised antigens are processed in acidic intracellular vesicles or endosomes, generating class II associated antigens, whereas antigens present in the cytosol, such as *de novo* synthesised viral proteins, are processed to generate class I associated antigens. Once processed, this MHC:peptide complex is delivered to the cell surface to present to T cells (Smith-Garvin *et al* 2009, Janeway 2008).

When APCs become activated by a pathogen, phenotypic changes occur to the cell such as this MHC upregulation as well as the upregulation of costimulatory molecules, such as CD80 and CD40. Upon presentation of antigen to a T cell, the T cell requires both MHC:peptide recognition, as well as the appropriate costimulatory signals from the APC, such as the binding of CD80 molecules on the APC to T cell CD28 molecules, for activation. Additionally, T cells express coreceptors CD4 and CD8 which bind to invariant sites on MHC molecules. This enhances cell-cell adhesion and strengthens signal transduction (Geismann *et al* 2010).

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1.2.1.3.2 Polymorphonuclear cells (PMNs) and Mast cells

Neutrophils are released from bone marrow in response to infection and are abundantly found in blood. These too are important phagocytes and express F_c receptors for antibodies and receptors for complement proteins that aid in the phagocytosis of opsonized bacteria. Unlike macrophages, neutrophils are quite short lived, possible due to the variety of lytic enzymes released during phagocytosis (Khan 2011, Janeway 2008). Eosinophils and basophils have noted importance in defence against parasitic infections and are recruited to sites of allergic inflammation. Mast cells in the tissue at these sites release many chemical mediators triggering local inflammation and blood vessel dilation in response to allergens. These mediators, when released in the host body, induce symptoms of an allergic reaction (Janeway *et al* 2005). These cell types are not considered to be of major importance to viral clearance.

1.2.1.3.3 Natural Killer cells

Natural killer (NK) cells are large granular cytotoxic lymphocytes which play an important role in innate responses, particularly in antiviral and tumour immunity. These cells account for approximately 10% of blood lymphocytes. They are produced in the bone marrow from CD34+ progenitor cells and are found mainly in blood throughout the body. NK cells can be characterised by their CD56 expression. As this marker is also expressed on some T cells, CD56+CD3- cells are usually considered NK cells (Raulet and Vance 2006, Tosi 2005).

The main function of NK cells is the recognition and killing of host cells infected with virus, as well as tumour cells. Their responses, although less specific, are more rapid than T cell responses. Unlike T cells, there is no immunological memory associated with NK cells.

When an NK cell is activated to kill a target cell, cytotoxic granules are released. These granules are usually perforin and/or granzymes. Perforin forms holes in the target cell membrane, enabling granzymes to enter, where they can activate capsases in the cell, leading to nuclease activation, DNA degradation and ultimately cell death. This is usually initiated by NK cell recognition of "missing self" or decreased MHC expression on infected cells, resulting in the NK cell mediated death of only infected cells, not healthy tissue. NK cells can kill virally infected cells by binding to target-cell-bound-IgG. NK cells attach to antibody-coated cells via their F_c receptors triggering the release of perforin, causing cell death

(Janeway 2008). NK cells also release of IFN- γ and other cytokines when activated. This is important for viral clearance and the induction of Th1 responses (Wang and Fish 2012, Wallace and Smyth 2005). During viral infection, APCs present viral antigen to NK cells, inducing NK cell activation and subsequent death of infected cells. Additionally, DC derived cytokines such as IL-4 and IL-12 can affect NK cell function (Moretta *et al* 2006).

1.2.1.3.3.1 Natural Killer cell subsets and their generation

NK cells can be found in body tissues, but are mainly found in circulation, such as in the lymph system (Lydyard *et al* 2003). They develop in the bone marrow from CD34+ progenitor cells (Malhotra and Shanker 2011). Two main subsets of NK cells have been noted which are classified according to CD56 expression.

The first are CD56^{Bright} NK cells, which express high levels of the marker CD56, and are both CD3- and CD16-. This subset makes up ~10% of all circulating NK cells or ~1% of all lymphoid cells (Caligiuri 2008). CD56^{Bright} NK cells are considered the more regulatory subset, due to their release of regulatory cytokines, such as IFN- γ upon stimulation. These cytokines contribute to immune homeostasis (Shereck *et al* 2007). The second subset is CD56^{Dim} NK cells, which make up 9% of lymphoid cells. These cells are low in CD56, and are CD16+ and CD3-. While low in cytokine release, this subset is highly cytolytic against infected or tumor cells (Maghazachi 2004). CD56^{Dim} NK cell cytotoxicity is attributed to CD16 expression (Caligiuri 2008) (Figure 1.4).

While gene profiling studies suggests more independency (Wilk 2007), others suggest placicity between NK cell types. Some have suggested that CD56^{Bright} NK cells are intermediates in the development of CD56^{Dim} NK cells (Caligiuri 2008). However, an earlier study supported a mechanism whereby IL-12, an interleukin produced by DCs, stimulates CD56^{Dim} NK cells to develop CD56^{Bright} phenotype (Maghazachi 2004).

Traditionally, NK cell definitions were confined to the innate immune system (Maghazachi 2004). They were considered "large granular lymphocytes" with cytotoxic activity towards infected or tumor cells (Krzewski and Struminger 2008). However in recent years, with the reported immune regulation function of CD56^{Bright} NK cells, their role is expanding. They are now taking centre stage as important players in immune regulation.

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Figure 1.4: Representation of both NK cell subsets: CD56^{Bright} NKs are represented here by their surface markers and high cytokine producition, and CD56^{Dim} NKs by low but high CD16. (Taken from Ritz 2005)

1.2.1.3.3.2 Natural Killer cell receptors

A variety of stimuli are required for the activation of NK cell functions and the balance of stimulatory and inhibitory signal is crucial to this. NK cells have a wide repertoire of both activating and inhibitory receptors for the detection of these signals (Figure 1.5). These inhibitory receptors are responsible for the ability of NK cells to spare normal healthy tissue from NK mediated death, but not transformed or infected cells (Janeway 2008, Raulet and Vance 2006, Kumar and M^cNerny 2005).



Figure 1.5: Representation of the wide range of NK cell activating and inhibitory receptors: Taken from: <u>http://www.sciencemag.org/content/331/6013/44/F2.expansion.html</u> The wide range of <u>NK cell receptors are represented here.</u> The balance of activating and inhibitory signals through these receptors dictates NK cell function

NK cells can recognise decreased levels of self major histocompatability complex (MHC) class 1 molecules. These molecules are highly polymoprhic glycoproteins expressed on the surface of nucleated cells. In a homeostatic state, inhibitory signals are delivered by self MHC class 1 molecules to NK cells, protecting that cell from lysis (Figure 1.5.A). However, some viruses reduce MHC class 1 molecule expression and the absence of class 1 molecules activates NK mediated killing of uninfected cells by NK cells. This is known as the "missing

self' hypothesis (Figure 1.5.B) (Vivier et al 2011, Raulet 2006, Tosi 2005, Biassoni et al 2001).



Figure 1.5 Detection of self and "missing self" by NK cells. Taken from Kumar and M^cNerny 2005. NK cells detect infected or transformed cells by the loss of MHC expression on these targets, marking these cells for NK cell mediated apoptosis

In humans, inhibitory receptors for MHC class 1 molecules include killer cell immunoglobinlike receptors (KIRs). These KIRs bind to the conserved region of class 1 MHC molecules associated with the self peptide. This provides a negative signal to the NK cells and killing of self-cell is prevented. Notably, KIR genes are highly polymorphic, meaning there is variability in KIR repertoirs between individuals (Jamil and Khakoo 2011, Vivier *et al* 2011, Janeway 2008, Kumar and M^cNerny 2005). There are also other inhibitory signals which NK cells can detect for self-tolerance. For example, NKG2A binds the non-classical MHC-class 1 molecule HLA-E. As HLA-E functions by stabilising MHC class 1, its expression is indicative of MHC-class 1 expression (Borrego *et al* 2005, Chapman *et al* 1999). However, not all inhibitory receptors bind MHC associated molecules. For example, ITIM-containing inhibitory receptors detect loss of E-cadherin, allowing NK cell detection of tumor cells (Ito *et al* 2006, Byrceson *et al* 2006). While inhibitory receptors tend to be variable in expression between different NK cells, activating receptor expression is relatively consistent. These activating receptors contribute to NK cell specificity towards the target, and the signaling mechanism they induce is much more complex than the common inhibitory receptor mechanism (Byrceson *et al* 2006). One group of activating receptors are associated with immunoreceptor tyrosine-based activation (ITAM)-containing adapter proteins, for example KIRG25, NKG2C and the NCRs. The activating signals induced via ITAM associated receptors are induced through the recruitement of tyrosine kinases and certain proteins (Lanier 2005, Bottino *et al* 2005). There is a second group of activating receptors which do not contain ITAMs, for example, NKG2D, 2B4. Ligands for NKG2D include MHC-class 1 chain-related genes A and B which are induced by stalled DNA replication and genomic stress, which are indicators of tumor, infected, or stressed cells (Vivier *et al* 2011, Byrceson *et al* 2006, Gasser *et al* 2005, Cerwnka and Lanier 2003).

The main group of activating receptors with the ability to induce NK-cell mediated death are the natural cytotoxicity receptors (NCRs), which include NKp30, NKp44, NKp46, and NKG2D (Vivier et al 2011, Biassoni 2008, Chang et al 1999). NKp46 is expressed on the surface of either resting or activated NK cells and is a major receptor for activation of NK mediated target cell killing (Weiss et al 2004, Costello et al 2002, Moretta et al 2001). This receptor is associated with binding of HPIV3 HN as well as influenza hemagluttinins. However, no functional output is associated with this binding. Outside of its role in NK viral responses, research is underway with regards to NKp46 ligands or targets in cancer (Gazit et al 2006, Arnon et al 2004, Mandelboim et al 2001). Unlike most activating receptors, NKp44 is not expressed on resting NK cells, but is upregulated by IL-2 (von Lilienfeld-Toal et al 2006). NKp44 has also been shown to bind human parainfluenza and influenza hemaglutinins. Notably, there is no NKp44 homologue in mouse (Biassoni 2009, Ho et al 2008, Arnon et al 2001,). Finally, NKp30 is expressed on both resting and activated NK cells (Biassoni 2009). This receptors binds poxvirus hemagluttinins (Jaharian et al 2011). Additionally, it is involved in both the control of tumor cell transformatin and the modulation of adaptive immune response (Moretta et al 2005). For example, NKp30 has been shown to participate in NK-DC crosstalk at inflammatory sites, and can induce death of innate DCs (Ferlazzo 2005, Ferlazzo et al 2001, 2002).

1.2.2 The adaptive immune system

The adaptive immune system is the more specific arm of immunity, and comes into place upon activation of the innate immune system. The innate immune system recognises conserved patterns or strucutres on pathogens that have remained unchanged over time. Adaptive immunity however, has evolved and continues to evolve, to recognise and remember specific pathogens, and can mount strong responses each time the same pathogen is re-encountered. The adaptive immune system can be further divided into humoral (mediated by B lymphocytes) and cell mediated (T lymphocytes). Both of these cell types express receptors with the specificity to recognise a wide range of pathogens. The large and diverse repertoire of receptors expressed by these cell types are attributed to the recombination of gene segments encoding them. To avoid responses towards host cells, lymphocytes whose receptors bind to self antigens are deleted. This is known as negative selection and is important in ensuring that only lymphocytes tolerant of host cells survive.

A low number of B cells or T cells specific to a particular pathogen are in circulation in a health host. Once a lymphocyte binds to their specific antigen, they are activated and proliferate, creating effector lymphocytes with the same specificity. This is given presentation of antigen in associtation with host MHC. In addition, memory cells to this antigen are also induced and these reside after infection in a resting state. Upon restimulation with the same antigen, these memory cells become activated, mounting a stronger and more rapid response. The development of these memory cells is usually associated with life long immunity to that particular pathogen (Janeway 2008, Morretta *et al* 2008).

1.2.2.1 B Lymphocytes

B cells originate from hematopoietic stem cells in the bone marrow. Here they mature and enter the circulatory network. B cells are of particular importance in the humoral arm of adaptive immunity, functioning by the release of antibodies, which bind specifically to a particular antigen, preventing the spread of infection to other host cells. Additionally, B cells can function as a type of antigen presenting cell (APC) (Janeway 2008).

Upon encountering a particular pathogen, membrane bound antibodies (BCRs) binds antigen, which is internalised, degraded, and presented on the cell suface. Antigen is presented as

peptides bound to MHC class II molecules. This molecule can be recognised by CD4+ T helper cells, which secrete B-cell activating cytokines such as IL-4. In addition, binding of co-stimulatory molecules on the surface of both T and B cells, such as T cell CD40 ligand (CD40L) and B cell CD40, also aid in B cell activation. When B cells are activated, they proliferate to form a clone of identical cells. These proliferate into either antibody secreting cells or memory cells (Janeway 2008, McHeyzer-Williams 2003).

Antibodies are important in adaptive immunity as they eliminate viruses or pathogens by neutralisation, opsonization and complement activation. Neutralization is the process whereby binding of antibody to particular antigens can inhibit pathogen entry to the host cell. Additionally, harmful toxins can also be neutralized if bound by antibody. Opsonization is the process where antibodies binding antigens, can mark this pathogen for recognition and ingestion by phagocytic cells. Finally, antibody attaching to pathogens can activate proteins of the complement system which leads to the increased opsonization and death of viruses or pathogens (Janeway 2008). Due to the clear importance of antibodies in the prevention of infection, the main aim of vaccination is to induce these antibody responses. With regards to respiratory viral infections, IgG and IgA are the most crucial types of antibodies required for the hosts'protection (Mahony *et al* 2011, Collier and Oxford 2006, Crowe and Williams 2003).

1.2.2.2 T lymphocytes

T cells are a diverse cell type that originate from hematopoietic stem cells in the bone marrow and mature in the thymus before entering circulation in the bloodstream. Here they recirculate between blood and peripheral lymphoid tissues until they encounter a specific antigen. Initially, there are low numbers T cells specific to a given antigen in a naïve (non-exposed) individual. Thus, it is imperative that they circulate to increase the likelihood of encountering the antigen they are specific to (Janeway 2008). These cells are of great importance in adaptive immunity, with key roles in the specific elimination of pathogens, as well as immunoregulation. T cell deficient individuals suffer more severe illnesses with respiratory viruses such as HPIV (Crowe and Williams 2003).

T lymphocytes are mobilised when they encounter APCs such as a B cell or DC which has digested and is displaying its cognate antigen, and is displaying these antigen peptides bound to MHC molecules. If this antigen particle is specific to the TCR, and the correct co stimulatory signals are also present, the T cell becomes activated. These activated T cells produce IL-2, a cytokine that promotes T cell growth and proliferation, sustaining T cell responses. If however, the T cell recognises peptide:MHC complexes in the absence of costimulation, T cell activation and proliferation does not occur. This limits the activation of autoreactive T cells and autoimmunity. Once activated, T cells proliferate and differentiate into either effector or memory cells. Overall effector cells are generally called Conventional T cells (Tcons) CD4+ T cells can acquire many different effector functions, such as cytokine production, immune memory, immunoregulation and differentiation of other immune subsets. This differentiation of naïve CD4+ T cells is dependent on APC maturation, which dictates the microenvironment, (E.G.cytokine mileau) of the activated T cells, (Smith-Garvin *et al* 2009, Janeway 2008, Appleman and Boussiotis 2003).

After activated T cells have eliminated a pathogen, they need to be switched off. Inhibitory receptors such as CTLA-4 are upregulated on T cells. This receptor has a higher affinity for CD80 than the activation molecule CD28, and binding with CD80 shuts down T cell proliferative response, avoiding immunopathology (Janeway 2008).

1.2.2.3 T cell subsets and effector functions

1.2.2.3.1 CD4+ T cells

Naïve CD4+ T cells can be differentiated into various types of effector cells depending on cytokines present. T helper cells function by driving and activating other immune cells. Activated APCs, such as DCs, secrete various cytokines in response to a particular pathogen, which can influence the polarization and differentiation of naïve CD4+ cells into specific effector T cell types (Figure 1.6).

Pro-inflammatory cytokines, such as IL-12 can direct T cell differentiation into Th1 cells (Figure 1.6). These Th1 cells are characterized by the secretion of large amounts of IFN- γ , as well as IL-2 and TNF α (Cope *et al* 2011, Watford *et al* 2008). This secreted IFN- γ can activate other immune cells, such as NK cell, which in turn kill intracellular pathogens. This T cell subsets is imperative for viral clearance. Stimulation with IL-4 can drive differentiation of naïve T cells into Th2 cells (Figure 1.6). This T cell subset secretes various cytokines

including IL-4, IL-5 and IL-13. These cells play a critical role in humoral immunity as they are important for B cell activation and secretion of antibodies (Li *et al* 2011, Janeway 2008). In recent years, Th17 cells have emerged as another subset of CD4+ helper T cells. It is thought that the developmental pathway of Th17 cells is distinct from Th1 and Th2 cells, and involves transforming growth factor (TGF)- β 1 and IL-6 (Figure 1.6). The cytokine IL-23 also plays a role in clonal expansion of this T cell subset. Th17 cells produces IL-17, which is involved in mediating inflammation and provides defence against extracellular bacteria (Maddur *et al* 2012, Janeway *et al* 2008, Dong 2006, Tato and O'Shea 2006, Mangan *et al* 2006). Other T helper sets have been identified recently, including Th9 which are defined by their production of IL-9, association with inflammatory disease and association with promotion of cellular proliferation (Jabeen and Kaplan 2012). Another newly emerged CD4+T cell subset is Th22, a producer of IL-22, which is important for clearance of bacterial pathogenesis especially from epithelial cells (Wolk *et al* 2010).

Virus specific Th1 responses play a major role in antiviral immunity through the secretion of cytokines and the activation of essential protective cells including B cells, NK cells, macrophage and CD8+ T cells (Collier and Oxford 2006). These virus specific T helper cells can also enhance antibody titres, strengthening the humoral immune response (Brown *et al* 2008).

These effector CD4+ T cells mount protection from pathogens via the secretion of large amounts of proinflammatory cytokines, thereby activating other immune cells, leading to strengthened immune response. However, this inflammatory response can damage selftissues. Therefore, the restraint of immune responses is essential to spare excessive damage to host tissue (Josefowicz *et al* 2012, Artavanais-Tsakanas *et al* 2003). Thus, the immune system contains subsets of T cells, such as regulatory T cells (Tregs), which can supress or regulate T cell responses (Josefowicz *et al* 2012, Janeway 2008, Mills 2004). Tregs are usually derived from naïve CD4+ cells stimulated with IL-10 or TGF- β (Figure 1.6). Natural regulatory T cells develop in the thymus and may represent a distinct T cell population (Schwartz 2005). They can suppress the activation of self-reactive T cells, through both cellcell contact dependent mechanism and the production of cytokines such as IL-10 and TGF- β (Cozzo *et al* 2003). Inducible Tregs are generated from naïve T cells in the periphery that have encountered antigen presented to them by partially-activated DCs. They can suppress effector T cell proliferation, via the production of immunosuppressive cytokines, such as IL-10 and TGF- β . Inducible T regulatory cells include Tr1 cells, which secrete large amounts of IL-10, and Tr3 cells, which secrete TGF- β (Mills 2004). Recent studies in humans have demonstrated Treg expansion at low dose IL-2, with clinically beneficial effects in graft-versus host disease and HCV-induced vasculitis (Koreth *et al* 2011, Saadoun *et al* 2011).



Figure 1.6: Schematic representation of the different developmental pathways of Th cells. (Taken from: (<u>http://www.csc.fi/english/csc/publications/cscnews/2009/1/allergy_research</u>). The differential generation of CD4+ T cell effector subsets from a naïve CD4+ progenitor is depicted here. The cytokines that drive this differentiation, as well as the protective role induced by each subset is clear

1.2.2.3.2 Cytotoxic T cells

CD8+ T cells contribute to the eradication of intracellular infections and to the control of many chronic infections (Masopost *et al* 2007). Activated CD8+ T cell or cytotoxic T

lymphocyte (CTL) can secrete cytokines, such as IFN- γ and can also directly kill target cells, such as virally infected or tumor cells (Janeway 2008, Collier and Oxford 2006). CTLs can induce apoptosis of the target cell, by releasing effector proteins, like perforin and granzymes (proteases), into the target cell. Perforin forms holes in the target cell membrane, enabling granzymes to enter, where they can activate capsases in the cell, leading to nuclease activation, DNA degradation and ultimately cell death (Janeway 2008). This subset represents the principle cells involved in controlling and clearing most viral infections (Khan 2011, Collier and Oxford 2006). With regards to respiratory viral infections, effector CTLs can be detected in the lungs 1 week after infection (Woodland 2003). Deficiency of CD8 T cells can be associated with delayed viral clearance and exacerbated illness (Crowe and Williams 2003, Hou *et al* 1992).

1.2.2.3.3 Memory T cells

Memory T cells represent the subset of T cells whose survival surpasses the contraction phase post infection. This subset circulates even in the absence of infection (Gerlach *et al* 2011). These memory T cells have phenotype differences to their naïve counterparts, such as upregulated expression of adhesion molecules, which induce a more efficient response if the same antigen is encountered a second time (Masopust and Picker 2012). These cells remain after a given infection, and proliferate if this pathogen is re-encountered. These memory cells increase the potential level of resting T- cells to this pathogen strain and are associated with more efficient adaptive responses.. This leads to sterilising immunity and is the basis for vaccination. CD45RO is expressed across memory T cell subsets, and serves as a marker for their identification.

1.3 Work leading to this study

As previously described, HPIV3 is a major respiratory pathogen responsible for bronchiolitis, pneumonia and croup. Initial infection with this virus occurs during infancy and early childhood, but reinfection is a common event and may occur several times even in adolescents and adults (Henrickson 2003, Chanock *et al* 2001). This would indicate a failure of immune memory. Early studies suggested that this failure to induce immune memory T cell responses may be due to limited T cell proliferation following infection with HPIV3 (Plotnick-Gliquin *et al* 2001, Sieg *et al* 1994).

Initially the Viral Immunology group (VIG) were part of a successful EU funded proposal aimed at developing a novel vaccine for HPIV3 and RSV. Part of the remit of the VIG was to establish the underlying cause of poor immunological memory to HPIV3 infections in humans. One of the main challenges facing the development of a successful vaccine in humans is that failed immunological memory appears to be species specific as these viruses often induce robust and long lived immunity in mice, rats and even hamsters. To address this, group members developed a human model based on co culture of virally infected human monocytes (APCs) with allogeneic human mixed lymphocyte reaction (MLRs). For completeness a comparative study was performed to investigate if infection of monocytes was a more natural method of generating APCs than infection of pre-primed DC formed using IL-4 or IFN- α . Researchers found that direct infection of monocytes induced mature and competent APCs (DCs) which induced distinct differences in T cell polarisation then pre-primed DCs. It was considered that the former was the most natural response to HPIV3 infection and was chosen as the most appropriate model with which to study human responses to HPIV3.

The model employed the use allogenic T cells as the precursor frequency of autologous T cells recognising HPIV3 is likely to be very low. The use of allogenic T cells provides a sufficient signal to mount a response which is qualitatively dictated by how the monocytes respond to infection.

Using the human *ex vivo* model of infection, our group showed that HPIV3 infected APCs failed to induce allogeneic mixed lymphocyte proliferation compared with influenza virus-infected APCs. However, purified CD3+ cells proliferated strongly when cultured with HPIV3 infected APCs. This indicated that a cell type within the mixed lymphocyte

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population has a role in T cell inhibition during HPIV3 infection. Decreased IL-2 levels were also associated with HPIV3 infection, compared to influenza infection. This cytokine is a growth factor for T cells (Liao *et al* 2011). Furthermore, when additional IL-2 was added to infected cultures, T cell proliferation was restored in mixed lymphocytes. When examining cell markers during infection, an increased number of cells expressing CD56, a marker for NK cells, was demonstrated. Additionally, when CD56+ cells were added to isolated CD3+ cell and HPIV3 infected APC cocultures, T cell proliferation was again inhibited. Finally, Noone *et al* demonstrated an increased number of T cells arrested in the G0/G1 phase of cell cycle during infection, but no change in apoptosis. These results indicate that during HPIV3 infection, NK cells induce T cell cycle arrest in an IL-2 dependent manner (Noone *et al* 2008). This was the first study to demonstrate regulation of T cells in controlling autoimmune disease progression has been demonstrated (Murphy 2012, Saadoun 2011, Vellilla *et al* 2008, Li *et al* 2007, Zorn *et al* 2006, Soiffer *et al* 1994).



Figure 1.8: Schematic of coculture assay. From Buffy coats from healthy donors, PBMCs were isolated. From donor one, CD14+ cells were further isolated, infected with HPIV3, and excess virus washed. From donor two, CD14+ cells were depleted and these MLs were cocultured (CD14:ML;1:5) after 24 hours.

1.4 Aims of this thesis

HPIV3 is a major cause of respiratory illness in neonates and infants. It accounts for 12% of all hospitalisations for lower respiratory tract infections and up to 30% of hospitalisations for acute respiratory illness in children in the USA alone (Schmidt *et al* 2011, Moscona 2005, Henrickson 2003, Hall 2001). It comes second only to RSV as the most important cause of lower respiratory tract infections in infants and young children. Reinfection with HPIV3 is common and poor immunological memory is a feature of this virus (Collins and Melero 2011, Hall 2001). Even in the presence of circulating neutralizing antibodies, individuals have been shown to be susceptible to reinfection (Schmidt *et al* 2011, Chanock 2001). This feature of HPIV3 has resulted in failure to design an effective vaccine against this virus.

It has been suggested that HPIV3 can reinfect and persist in a human host due to its ability to inhibit T cell proliferation (Plotnicky-Gliquin *et al* 2001). Our group has published evidence that CD56+ natural killer cells regulate T cell expansion to HPIV3 infected dendritic cells in a contact dependent mechanism that is reliant on inhibition of IL-2. This was the first study to demonstrate regulation of T cell responses by NK cells during viral infection (Noone *et al* 2008). We hypothesise that NK cell regulation of T cell proliferation during HPIV3 infection is the underlying cause of repeated infection and poor vaccine efficacy associated with this virus. This lead to a successful Health Research Board (HRB) grant proposal with the overall aim is to identify the mechanism of HPIV3 infections in humans that leads to arrested T cell cycle by NK cells.

Our initial aim was to determine which component of HPIV3 is responsible for the antiproliferative effect on T cells. Preliminary, unpublished work in our group using virosomes (empty influenza virus shells with HPIV3 HN and F proteins inserted into the influenza envelope), derived from HPIV3 compared with virosomes derived solely from influenza suggests that the viral surface glycoproteins HN and F are responsible for the antiproliferation outcome. The involvement of these glycoproteins is also circumstantially supported by the successive failure of vaccines that have included these components. As HPIV3 virosomes production has ceased, and this data could not be reproduced, neutralizing antibodies to these glycoproteins were used in this study to block the action of NK cell on T cell proliferation and cell cycle was determined.

Having identified what component of HPIV3 is responsible for the anti-proliferative effect of NK cells on T cells, we aimed to determine the molecular target on NK cells. Published data

has shown that NKp44 and NKp46 proteins recognise HA from influenza and HN from sendai virus and parainfluenza (Biassoni 2009, Mandelboim et al 2001). Although no function has been associated with this interaction, we felt that this circumstantial evidence pointed to involvement of these receptors in NK cell activation during infection. To assess the involvement of NKp44 and NKp46 in the induction of NK cell mediated T cell inhibition infection. their expression was assessed during infection. during Following this. commercially available neutralizing antibodies were used to block these receptors prior to viral coculture, and NK cell ability to induce T cell cycle arrest was assessed. Finally, activating antibodies were used to determine if the HPIV3 mediated anti-proliferative effect could be mimiced in the absence of infection. These receptors may have potential as therapeutic targets in a number of immune settings.

Our group has published evidence that IL-2 secretion is severely impaired during HPIV3 infection, and the addition of IL-2 completely abrogates NK cell mediated T cell cycle arrest. Other groups have published reports that IL-2 expanded NK cells induce T cell apoptosis (Murphy 2012, Saadoun 2011, Vellilla *et al* 2008, Li *et al* 2008, Shereck *et al* 2007, Zorn *et al* 2006, Soiffer *et al* 1994). While it is well accepted that NK cells limit T cell proliferation, studies differ in whether this is achieved by a cell cycle arrest or apoptotic mechanism. We hypothesise that the concentration of IL-2 that activates NK cell expansion, dictates the mode of NK cell mediated T cell regulation. We aim to investigate T cell death and cycling, as well as NK cell expansion, at various IL-2 concentrations. We also sought to determine if a specific NK cell subset is responsible for both modes of regulation, and if their influence is exerted equally on all effector, memory or regulatory T cell subsets.

Understanding the nature of poor proliferative responses to HPIV3 infection in humans may be crucial to the design of an effective vaccine against this important pathogen. Additionally, unravelling the regulatory immune arm targeted by the virus may provide insights and possible therapeutic strategies to control diseases with aberant immune activation. -To culture HPIV3 for use in the human ex vivo model of respiratory infection

-To identify the HPIV3 proteins involved in the induction of NK cell regulation of T cells

-To determine the receptors involved in the activation of NK cells during infection

-To investigate the role of IL-2 in dictating the mode of NK cell regulation of T cells.

-Observe specific T cell subsets under NK regulation during infection

-To determine if isolated HPIV3 glycoproteins could induce NK cell regulation of T cells

2. Effect of neutralisation of HPIV3 viral components on the activation of NK cell regulation of T cell proliferation

2.1. Introduction

Human Parainfluenza virus type 3 (HPIV3) is a significant respiratory virus that infects adults, neonates and infants (Moscona 2005). It has the capacity to infect the same individual several times during their lifetime, failing to induce a state of long lasting immunity (Henrickson 2003). The failure of immune memory has been a persistent problem in the development of vaccines to HPIV3 (Schmidt *et al* 2011). Studies have suggested that lymphocyte proliferation is reduced during HPIV3 infection (Plotnicky-Gliquin *et al* 2001). However, in mouse models, robust immunity is observed to this virus. To address this, our group previously developed a human *ex vivo* model of respiratory virus infection. They found that pre-primed DCs (Mohty *et al* 2003, Parlato *et al* 2001) may not be the most appropriate cell for used when studying viral infections, as they appear to skew immune responses. As HPIV3 is a potent inducer of DC generation, direct infection of CD14+ cells isolated from PBMCs of healthy donors represents a more natural immune response to HPIV3 infection (Noone *et al* 2007).

This model was used for proliferation studies which demonstrated that HPIV3 infected monocytes drive NK cell regulation of T cell proliferation in a mixed lymphocyte reaction (MLR). This offered an explanation for viral persistence and reoccurrence, and the poor immunological response associated with HPIV3 infection. Noone *et al* demonstrated that the NK mediated inhibition of mixed lymphocytes to HPIV3 infected APCs was IL-2 dependent, and via a non-apoptotic mechanism. It was also demonstrated that this regulation was a NK-T cell contact dependent mechanism (Noone *et al* 2008).

Six proteins comprise HPIV3 virus, and HN and F representing the two surface glycoproteins. During infection, HN functions in initiating binding to sialic acid-containing receptor molecules on the cell surface, and then the F protein mediates fusion with the plasma membrane of the cell (Moscona 2005). Unpublished work from our group demonstrated that empty virosomes expressing HPIV3 HN and F glycoproteins induced the same failure of T

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cell proliferation as observed with total virus. Notably, many unsuccessfull vaccines developed to date, made us of these surface glycoproteins.

This chapter aimed to culture a bank of HPIV3 virus for use in this study. Given successful isolation of this virus from infected vero cells, we aimed to use this cultured virus in the exvivo model of respiratory virus infection which was previously developed in the viral immunology group (Noone et al 2007) to determine reproducibility of results. Then, based on the important finding that virosomes expressing HPIV3 HN and F induced inhibited mixed lymphocyte proliferation, we aimed to identify the role played by these surface glycoproteins. These virosomes were no longer in production, and therefore experiments could not be repeated. To investigate the role of these surface glycoproteins using a different method, neutralizing antibodies to HN and F, provided by Prof Claes Orvell, were used. The neutralzing capacity of these antibodies as well as a range of other functional tests have been previously assessed and published by his colleagues (Rydbeck 1986). The effect of these neutralizing antibodies on the ability of HPIV3 infected CD14+ cells to inhibit mixed lymphocyte proliferation, upregulate CD56 expression and diminish IL-2 levels was assessed. Further investigation into the role of these HPIV3 surface glycoproteins in the activation of NK cell mediated regulation of T cells might explain the current issues and lack of immune memory associated with the development of a vaccine against HPIV3.

2.1.A The specific aims of this chapter were as follows:

-To culture a bank of HPIV3

-To confirm infectivity of this virus and determine its' TCID₅₀/ml

-To determine reproducibility of Noone et al (2008) data

-To investigate the effect of anti-HN or anti-F monoclonal antibodies on the ability of HPIV3 infected CD14+ cells to:

-inhibit proliferation of allogeneic mixed lymphocytes

-expand CD56+ cell populations

-induce NK mediated inhibition of T cell proliferation

-reduce IL-2 levels during infection

-induce T cell cycle arrest during infection

2.2 Materials and Methods

Table 2.2.1 Lists the reagents used in this study

Product	Catalogue #	Company
Vero cells	CCL-81	ATCC, Middlesex UK
Adherent Flask T25	83.1811	Sarstedt, Wexford, Ireland
Adherent Flask T75	83.1812	Sarstedt, Wexford, Ireland
Adherent Flask T175	83.1810	Sarstedt, Wexford, Ireland
DMEM	32430-100	Life technologies, Dun Laoghaire,
		Dublin
50ml non skirted sterile tube	62.547.004	Sarstedt, Wexford, Ireland
50ml skirted sterile tube	62.559.001	Sarstedt, Wexford, Ireland
Gentamycin	G1379	Sigma-Aldrich, Dublin 24, Ireland
Foetal calf serum (FCS)	S1830-500	Bio-sera, France
Ethelenediaminetetraacetic acid	AC09656	Scharlau Chemie S.A, Barcelona, Spain
(EDTA)		
HPIV3	Vr-93	ATCC, Middlesex, UK
Surcrose $(C_{12}H_{22}O_{11})$	302997J	BDH, Poole, England
Sodium Chloride (NaCl)	1.06400.5000	Merck, Darmstadt, Germany
Sodium Dihydrogen phosphate	102454R	BDH, Poole, England
(NaH ₂ PO ₄)		
Potassium Chloride (KCl)	P00200	Scharlau Chemie S.A. Barcelona, Spain
Potassium dihydrogen phosphate	1.04871.1000	Merck, Damstadt, Germany
(KH_2PO_4)		
Sterile 96-well plate (flat bottom)	142475	Nunc, Roskilde, Denmark
Crystal violet	1.15940	Merck, Darmstadt, Germany
Hanks balanced salt solution (HBSS)	24020-91	GIBCO BRL, Paisley, Scotland
HEPES buffer	15630-056	GIBCO BRL, Paisley, Scotland
Lymphoprep	1114547	Nycomed Ltd., Birmingham, England
RPMI 1640 medium	31870-025	GIBCO BRL, Paisley, Scotland
Pen/Strep	15070-063	GIBCO BRL, Paisley, Scotland
40µm cell strainer	352340	BD Biosceiences Europe, Belgium
Acridine orange	A6014	Sigma-Aldrich, Dublin 24, Ireland
Ethidium bromide	E8751	Sigma-Aldrich, Dublin 24, Ireland
Human microbeads, CD3	130-050-101	Miltenyi biotech, Gladbach, Germany
Human microbeads, CD56	130-050-401	Miltenyi biotech, Gladbach, Germany
Human microbeads, CD14	130-050-201	Miltenyi biotech, Gladbach, Germany
Bovine Serum Albumin (BSA)	A4503-50g	Sigma-Aldrich, Dublin 24, Ireland
MACs separation LS columns	130-042-401	Miltenyi Biotec, Gladbach, Germany
24-well TC plates (adherent cells)	83.18366	Sarstedt, Wexford, Ireland

24-well TC plates (suspension cells)	83.1836.500	Sarstedt, Wexford, Ireland
Anti-Human CD14-PE	12-0149-42	Bioquote, York, UK
Anti-Human CD3-APC	17-0038-73	eBioscience, Hatfield, UK
Anti-Human CD56-FITC	11-0569-42	eBioscience, Hatfield, UK
Ethanol (CH ₃ CH ₂ OH)	E-7023-	Sigma-Aldrich, Dublin 24, Ireland
	500ml	

Table 2.2.2 Lists equipment used in this study.

Equipment	Model	Company
Centrifuge	Eppendorf 5804	Eppendorf, Hamburg,
		Germany
Ultracentrifuge	L8-70M	Beckman UK
Flow Cytometer	FACs Calibur	Becton Dickinson, Oxford,
Haemocytometer	Improved, 2-grids	Neaubaurer
Irradiator	Gamma-cell 1000 Elite	Nordion, Ottowa, Canada
Midi MACs separation unit		Miltenyi Biotec, Gladbach,
		Germany
Fluorescent microscope	Eclipse E200	Nikon, USA
Inverted microscope	Eclipse TS100	Nikon, USA
Plate reader	MCC/340	Titertek Multiscan
Micro centrifuge	Heraeus Fresco 17	Thermo scientific
Steri-cycle CO_2 incubator	Hepa class 100	Bio-sciences, Dun Laoghaire,
		Ireland
Mastercycler gradient	5331	Eppendorf, Hamburg,
		Germany
Wide mini sub-cell GT	1704468	Biorad, Hercules, USA
electrophoresis system		

Cell culture was carried out in accordance with the Safety and Health Policy of Dublin City University and where appropriate, with the notification of the Health and Safety Authority and Environmental Protection Agency.

2.2.1 Viral stocks

Viral stock of HPIV3 was provide by ATCC (Middlesex, UK) with a stock concentration tissue culture infectious dose/ml (TCID₅₀/ml),(the TCID₅₀ of a virus is the dilution of virus that can be expected to infect 50% of cultured cells and is used to measure virus concentration), of 7 . Stocks of HPIV3 were also prepared internally as described in this section. Additionally, influenza A virus [Haemagglutinin 1, Neuraminidase 1 (H1N1)] was used as a reference virus for experiments described in this chapter. This was kindly provided by Robert Newman (NIBSC, Hertfordshire, UK)

2.2.2.1 Infection of Vero cells for HPIV3 propogation

Vero cells (African green monkey kidney cell line) were kindly provided by Marius Loetshcer (Berna Biotech AG, Switzerland). To propagate Vero cells from frozen cultures, cells were thawed quickly at 37° C and suspended in 10ml of Dulbecco's modification of Eagle medium (DMEM) with 10% FCS. Cells were pelleted at 10,000g for 5 minutes at room temperature, and resuspended in 5-10ml DMEM with 10% FCS. Cells were cultured in a vented adherent T25 flask at 37° C with 5% CO₂. Cells were split or cultured into a larger culture flask (T75 or T175) when a >90% confluent monolayer was reached.

For subculture, the culture medium was removed from the adherent cell layer, and replaced with 10-15mls of 1% trypsin-Ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS) and left to incubate at 37°C for 3-5 minutes. Trypsin is a proteolytic enzyme that cleaves protein bridges which attach adherent cells to the surface of tissue culture flasks (http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-

<u>enzymes/trypsin/cell-dissociation.html</u>). Thus, trypsin enables adherent cells to be collected by dislodging them from the surface of adherent flasks. After incubation with trypsin, the cells were visualized using an inverted microscope to confirm cell detachment from the flask surface. Once detachment was confirmed, 10-15ml culture medium was added to flasks to neutralize the toxic effects of the trypsin. Cells were spun at 10000g for 7 minutes, and the resulting pellet was resuspended and transferred to either a T75 or T175 (Ammerman et al 2008).

Prior to Vero cell infection with HPIV3, cells were left to grow until they were ~80% confluent in a T175 flask. A volume of 0.5ml HPIV3 with a $TCID_{50}/ml$ of 7, was diluted in sufficient serum free media. The culture medium was removed from the cells and they were washed 3 times with PBS. The HPIV3-containing serum free medium was then added to the Vero cells (T175) and left to incubate for 4 days at 37°C with 5% CO₂.

2.2.2.2 Harvesting and purifying HPIV3

Upon harvest, after 4 days of HPIV3 infection, Vero cells were subjected to 3x freeze thaw cycles to break up the cells (- 80° C). The resulting cell suspension, containing the virus and cell debris in culture medium, was transferred to 50ml tubes, and centrifuged at 4° C for 45 minutes at 2500g, to sediment the cell debris. The resulting supernatant was collected and carefully overlayed on ~5ml of a 30% sucrose solution made in 1x Phosphate buffer saline

(PBS), in ultracentrifuge tubes (sucrose made up 1/5 of the total volume added to the ultracentrifuge tubes). This sucrose solution functioned as a density gradient. The tubes were then ultracentrifuged (using a fixed angle rotor) at 5°C for 15 hours at 50,000g. The resulting delicate ultracentrifuge pellets, in addition to some of the overlay cushion sucrose solution were transferred to 50ml tubes using transfer pipettes. Some PBS was added to the tubes to dilute the sucrose and the pelleted fragments were spun down at 4°C at 1500g. The supernatant was carefully removed and the pellet was washed with PBS and centrifuged at 4°C for 10 minutes at 2500g. The final pellet or purified virus was then resuspended in ~160µl PBS (1/1000 of the volume subjected to ultracentrifugation), aliquoted and stored at - 80° C.

2.2.2.3 Calculating the concentrations of HPIV3

To calculate the concentrations of cultured HPIV3, a titration assay was carried out to determine the TCID₅₀/ml of the virus. The TCID₅₀/ml refers to the amount of virus that will produce a pathological change in 50% of the cell culture it is inoculated in. Vero cells in culture medium were plated at a concentration of 1×10^4 cells/well (100µl/well), in a flatbottomed 96 well plate and left to incubate at 37°C for 24 hours. Following this incubation, the Vero cells were confluent and ready for infection. For infection with HPIV3, virus was diluted in serum-free medium, starting with a 1/1000 dilution of the cultured virus, and diluting 1/10 down the plate (100µl virus/well of Vero cells). The titration scheme for the virus on the 96 well plate is shown in table 2.1. This plate was left to incubate for 5-6 days and then the titre was read. For this, cell culture medium was removed from wells and replaced with 50µl/well of 1% crystal violet staining solution. After 15 minutes, the staining solution was removed and the cells were air-dried. Cells were then examined by light microscope for the presence of syncytia, which are multinucleated cells. Wells were marked as positive or negative for the presence of syncytia. Syncytia form when viral fusion proteins, which are used by the virus to enter cells, cause cells to fuse together, forming one cell with many nuclei which is visible upon staining.

The $TCID_{50}$ /ml of the virus was calculated by the following equation:

Log TCID₅₀/ml =(number of positive wells, between 0 and 40) X 0.2 – log(lowest dilution, usually 10^{-3}) = 0.5

96 well plate:

No virus	10-3	10-3	10-3	10 ⁻³	10 ⁻³
No virus	10 ⁻⁴				
No virus	10-5	10 ⁻⁵	10 ⁻⁵	10 ⁻⁵	10 ⁻⁵
No virus	10-6	10-6	10-6	10-6	10-6
No virus	10-7	10-7	10-7	10-7	10-7
No virus	10 ⁻⁸	10-8	10-8	10 ⁻⁸	10 ⁻⁸
No virus	10 ⁻⁹				
No virus	10-10	10-10	10-10	10-10	10-10

Table 2.2 illustrates the plate plan for titration of the virus

2.2.3 Separation of peripheral blood mononuclear cells

Buffy coats from healthy donors were obtained from the Irish blood transfusion service (St James's hospital, Dublin). The peripheral venous blood (approximately 50ml) was mixed with 5ml of a 5% solution of the anti-coagulant EDTA and diluted 1:3 with Hank balanced salt solution (HBSS) containing 1% foetal calf serum (FCS; endotoxin free, heat inactivated for 30 minutes at 56°C) and 10mM Hepes buffer. This diluted blood was layered onto 14ml of LymphoprepTM solution (Axis-Shield, Norway) in a 50ml sterilin tube and centrifuged at 400g for 25 minutes with the accelerator and break switched off in order to prevent the mixing of separated layers. The LymphoprepTM solution with a density of 1.077g/ml provides a gradient that enables the blood components to be separated according to their density. Thus, during centrifugation, the higher density erythrocytes and PMNs or granulocytes sediment on the bottom of the tube, while the lower density mononuclear cells form a distinct cloud layer at the interface of the sample Figure 2.1. This mononuclear interphase layer of cells or buffy coat layer was then removed using a pasteur pipette and the cells were washed twice with 10ml of complete Roswell Park Memorial Institute (cRPMI)-1640 medium (supplemented with 10% FCS, 1% HEPEs, and 1% Penicillin/streptomycin solution and L-Glutamine). To remove clumps from these PBMCs, the cells were filtered through 40µm filters and washed through with cRPMI and the cell yield was calculated as described in section 2.2.4



Figure 2.2.1: Separation of PBMCs by density centrifugation. Peripheral blood was layered onto LymphoprepTM in a 50 ml sterile tube. Following centrifugation the blood components were separated into layers according to their density; with the erythrocytes and PMNs sedimenting to the bottom of the tube and PBMCs gathering at the interface between the HBSS and LymphoprepTM for isolation. Figure taken from: <u>http://pluriselect.com/buffy-coat.html</u>

2.2.4 Determination of cell viability and yield.

2.2.4.1 Background

Acridine Orange (AO) intercalates and stains double stranded DNA and fluoresces orange under halogen or ultraviolet (UV) light. However, it can only stain cells with permeable membranes, such as those in the final stages of apoptosis, and so, is useful for the detection of dead cells. Ethidium Bromide (EB) is a cell permeable dye that can bind to double stranded DNA of live cells, emitting a green fluorescence under halogen or UV light. Thus, live cells appear green using this. These dyes are useful for the detection of viable cells from a cell suspension as they will appear green, while non-viable cells will appear orange (Gill *et al* 1979).

2.2.4.2 Method

An EB/AO solution was made by adding EB stock solution (0.8ml of 4mg/ml solution) to AO stock solution (2ml of 1mg/ml solution) and then made up to 200ml with 0.85% weight/volume (w/v) sodium chloride (NaCl). Using this solution, viable cells should appear green under light microscope, while dead cells will appear orange. The improved Neubauer Haemocytometer slide was used to determine the number of cells in a defined volume. This microscopic slide contains a grid etched onto its surface and the volume of a solution in an area of the grid can be calculated from the area of the grid and the height between the grid and cover slip (0.1mm) (Figure 2.2.2). Cells to be counted were diluted in EB/AO (1/50 for PBMCs, 1/10 for specific immune subsets) and pipetted on to the slide beneath the cover slip, ensuring that the solution covered the entire surface of the counting chamber or grid. The number of live (green) and dead (orange) cells was determined by counting the number of cells in the four corners of the grid and cell yield was calculated as follows:

(Average cell number from grid corners)x(Dilution factor)x10⁴=cell number/ml

*The number 10^4 is the volume correction factor for the slide: each square is 1x1 mm and the depth is 0.1 mm.



Figure 2.2.2. Diagram of the grid of the Improved Neubauer Haemocytometer.

Grid etched onto surface of hemocytometer slide, from the volume of a solution in an area of the grid can be counted, and cells present determined by staining. (Image was reproduced from: http://www.emsdiasum.com/microscopy/products/magnifier/counting.aspx)

2.2.5 Separation and purification of cell subsets from PBMCs using microbead separation

2.2.5.1 Background

Magnetic microbead separation of cells is an efficient way of separating specific immune subsets of interest. These magnetic biodegradable microbeads are composed of iron oxide polysaccharide and are conjugated to antibodies specific to surface markers on the target cell. These conjugated antibodies are incubated with cells and can bind to particular cell surface proteins, which is useful for isolation of particular cell subsets. After incubation, this cell suspension is passed through a column, which is held by a magnet (MidiMAC) and this enables the cells with beads bound to appropriate antibody to be retained in the column as a result of magnetic forces. Unlabelled cells therefore flow through the column and can be collected as the depleted or unlabelled fraction. To remove the labelled cells from the column, the column is removed from the magnet and the cells are plunged out and collected as the enriched or labelled fraction (Miltenyi Biotech, Germany).

2.2.5.2 Method

In this chapter, CD14+ cells, Mixed lymphocytes, CD14- depleted MLs, CD3+ cells, CD56+ cells and CD56+ cell depleted MLs were used. So, CD14+, CD3+, and CD56+ anti-human microbeads were required for positive selection. Cells expressing the relevant markers for isolation attached to the magnetic column, while other cells flow through, and positively selected cells were then eluted. Flow through contains PBMCs depleted of labelled cell type. This was performed in accordance to the manufacturer's specifications (Miltenyi Biotech, Germany). Briefly, PBMCs were centrifuged at 300g for 10 minutes and resuspended in degassed MACs buffer (80µl of buffer/1x10⁷ cells), consisting of sterile PBS, supplemented with 0.5% BSA and 2mM EDTA, and incubated with 70µl microbeads for 40 minutes at 4°C. Following incubation, cells were washed with MACs buffer. The pellet was resuspended in buffer again (approximately $6ml/1x10^8$ cells). To positively select cell, LS columns were used and placed in the magnet. The column was firstly washed with 3ml MACs buffer and then resuspended cells were added to the column. The column was then rinsed 3 times with 3ml of the MACs buffer. The first fraction and 9ml run through was the selected cell depleted fraction (CD14- mixed lymphocytes or CD56- mixed lymphocytes). After all the MACs buffer had run through the column, the column was removed from magnetic force, and the positively labelled cells were flushed out of the column with a plunger (CD14+, CD56+ or CD3+ cells). NK subset isolation will be described in chapter 3.

2.2.5.3 Determining cell purity

To determine the purity of the CD14+ monocytes, the cells were stained with PE conjugated anti-CD14, and analysed by flow cytometry (Described in further detail in 2.2.10). Routinely, purity was >95% for CD14+ monocytes (Appendix 1.1). Similarly, APC-CD3 determined a routine CD3+ T cell purity of >95% (Appendix 1.2) and FITC-CD56 determined a routine CD56+ NK cell purity of >95% (Appendix 1.3).

2.2.6 Viral infection of monocytes and mixed lymphocytes

From freshly cultured PBMCs, CD14+ monocytes were purified (as described in section 2.2.5) and cultured in RPMI at a concentrations of 1×10^6 cells/ml. Cells were infected with HPIV3 at a TCID₅₀/ml of 6 or influenza virus at a TCID₅₀/ml of 7, for 2 hours at 37° C, then subsequently washed (centrifuged at 2,300g for 5 minutes) following the incubation to remove excess virus (Plotnicky-Gilquin *et al* 2001). The cells were then cultured in fresh cRPMI on a 24 well plate or cocultured for the indicated timepoints. The same protocol applies for direct ML infections.

2.2.7 Confirmation of HPIV3 infection

2.2.7.1 Background

The HPIV3 nucleocapsid protein (NP) is a stably expressed viral protein, which is essential for replication of the virus. This protein is expressed intracellularly upon infection, and makes a good marker for confirming HPIV3 infectivity (Henrickson 2003, Noone *et al* 2008). To confirm HPIV3 infectivity of CD14+ cells, RNA was isolated from virally infected CD14+ pellets, reverse transcribed (rt) into DNA and subjected to PCR to detect the expression of viral NP. The primers used in this assay, as described below, target a 526bp region of the NP, which was detectable by gel electrophoresis, after PCR amplification.

2.2.7.2 RNA isolation

Ribonucleic acid (RNA) was isolated from HPIV3 infected CD14+ cell samples using $TRIzol^{TM}$ (Invitrogen, Ireland) reagent which is a reagent that is comprised of a mixture of

guanidine thiocyanate and phenol in a mono-base solution. Cells were either frozen in TrizolTM reagent upon harvest or thawed in TrizolTM reagent. Cells were lysed by pipetting and gentle vortexing, and then left at room temperature for 15 minutes to allow NP complexes to dissociate and then vortexed in 100µl chloroform, and centrifuged at 12,000g at 4^{0} C for 15 minutes. The resulting mixture separated into 3 phases: 1) an upper aqueous phase containing RNA, 2) an interphase containing RNA, and 3) an organic phase containing proteins. The upper colourless phase was removed, to which 250µl glycogen was added to visualize the pellet for optimum yield, and cells were incubated overnight at -20°C. The RNA was isolated by spinning at 12,000g at 4°C for 10 minutes. The resulting pellet was washed with 75% ice cold ethanol. After centrifugation, ethanol was removed from this pellet, leaving ~10µl to air dry for a maximum time of 15 minutes. This pellet was resuspended in 20µl of water, and left at room temperature for 5 minutes, before storing at -80°C.

2.2.7.3 Quantification of RNA

Nucleic acids can absorb UV light at 260nm. The more light absorbed by the sample, the higher the nucleic acid concentration in the sample. Using the Beer Lambert Law, one can relate the amount of light absorbed to the absorbing molecule. The nanodrop machine uses this basis of spectrophotometry to calculate RNA concentration. A volume of 1.2µl of RNA sample was loaded on the nanodrop for absorbance reading.

2.2.7.4 DNAse1 treatment of RNA

Samples of RNA were treated with DNase 1 (MyBio, Ireland) which degrades any DNA contamination in a sample of RNA. Bench top, gloves, pipettes and racks to be used were sprayed with RNaseZap (Sigma) to eliminate natural RNA degrading enzymes. All steps for DNase1 treatment, reverse transcription and PCR were performed on ice to prevent RNA degradation. Stock RNA was made to $2\mu g$ in 8μ l, to which 1μ l of 10x reaction buffer and 1μ l DNase 1 were added. This reaction was incubated at 37° C for 15 minutes. A volume of 1μ l stop solution was added and the reaction was incubated at 70° C for 10 minutes to heat inactivate DNase 1 and put on ice in preparation for reverse transcription.

2.2.7.5 Reverse transcription of RNA

2.2.7.5.1 Background

Reverse transcription is a process in which single stranded RNA is converted or reverse transcribed into complementary DNA (cDNA), using a reverse transcriptase (RT) enzyme. This cDNA product is relatively stable and can be used as a template for PCR. Messenger RNA from samples was reverse transcribed using the protocol described below:

2.2.7.5.2 Method

Mastermix was made depending on number of samples:

1) Mastermix 1:

Reagent	Single reaction (µl)
Oligo dt	0.5
dNTP	1

A volume of 1.5μ l of Mastermix was added to each 11μ l RNA mix. The reaction was heated to 70° C for 5 minutes in the thermocycler and rapidly chilled on ice.

2) Mastermix 2:

Reagent	Single reaction (µl)
First strand buffer	4
0.1M DTT	2
RNase Inhibitor	0.5

A volume of 6.25μ l of Mastermix was added to each sample tube. The reaction was heated to 42° C for 2 minutes.

3) Mastermix3:

Reagent	Single reaction (µl)
Superscript II	0.25
DNase/RNase free water	1

A volume of 1.25μ l of mastermix was added to each sample tube. The reaction was heated to 42° C for 50 minutes. The reaction was then heated to 70° C for 15 minutes to inactivate the reaction. The resulting cDNA was used as a template for amplification by PCR.

2.2.7.6 Polymerase Chain Reaction (PCR)

2.2.7.6.1 Background

As previously mentioned, PCR is a method for synthesizing and amplifying specific DNA sequences. Firstly, the DNA is denatured by heating (95°C), which unfolds or separates the double-stranded molecule into single strands of DNA. A limited stretch of nucleotides on each side of the target gene is used to design complementary single stranded oligonucleotides (~20bp), which serve as primers (forward and reverse) for PCR reaction. These specific primers hybridize or anneal (temperature is specific to each primer pair) to opposite strands of DNA and flank the target DNA sequence to be amplified. Elongation of primers is catalyzed by a DNA polymerase enzyme such as taq polymerase (72°C). This enzyme is a thermostable polymerase and is able to withstand the high temperatures needed in PCR. These three different steps involving denaturation, primer annealing and elongation are referred to as a cycle and numberous cycles (~30-40) are required to detect the PCR product (Reece, 2004)

The HPIV3 NP was targeted for amplification for reasons previously discussed. The β -actin housekeeping gene was also targeted. Housekeeping genes are genes which are required for basic cellular function, expressed relatively consistently in all genes in the organism, serving as a positive control (Ruan and Lai 2007).

The primers used in this study to identify particular genes were designed from the complete coding sequence for each gene, found on the entrez nucleotides database (<u>http://www/ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide</u>) with the aid of the primer design software from cybergene (<u>http://www.cybergene.se/primertoos/idex/html</u>) and were ordered from MWG-Biotech AG (Germany). Primer pairs were as follows:

HPIV3 NP:

5'-TTGGAAGTGACCTGGATTAT-3' (forward)

5'GGATACAGATAAAAGGAGC-3' (reverse)

Annealing temperature=54°C, Product size=548bp

Human β-actin:

5'-TACAATGAGCTGCGTGTG-3' (forward)

5'-TGTGGCGTACAGGTCTT-3' (reverse)

Annealing temperature=55°C, Product size=619bp

2.2.7.6.2 Method

A 25µl volume reaction was set up for each sample using 2µl of cDNA prep as a template. PCR was carried out using primers specific to HPIV3 NP, as well as the housekeeping gene β -actin. Also, to check for genomic contamination, PCR amplification was performed on 100ng/ml of RNA from each sample using β -actin primers. The mastermix or reaction solution for PCR was set up as follows, and volume depended on number of samples:

Reagent	Single Reaction (µl)
Sterile water	16.5
Reaction buffer (10x)	2.5
$MgCl_2$ (50mM)	0.75
dNTP (10mM)	0.5
FP (10pmol)	1.25
RP (10pmol)	1.25
Taq	0.25

Table 2.3: Single reaction PCR mastermix

The following temperatures were set on the thermo cycler for PCR, with changes only to the annealing temperatures depending on primers being used:

- 1) $95^{\circ}C 5$ minutes
- 2) 95°C-45 seconds
 Annealing temperature -1 minute ← repeat 2) x 35 times
 72°C -1 minute
- 3) $72^{\circ}C 10$ minutes $4^{\circ}C$ -hold.

2.2.7.7 Agarose Gel Electorphoresis

Agarose gels are used to separate DNA fragments according to size, and so, the presence of target PCR product can be determined using Gel electrophoresis. A 1.5% agarose gel was made in 1xTAE buffer (40mM Tris-acetate, 2mM Na₂EDTA), with agarose dissolved by microwave. For reference, 3µl ladder was added to generally the first lane of the gel. This is a sample of defined proteins at known sizes for reference with PCR products. A volume of 6µl of PCR sample was mixed with 15µl orange loading dye, with 15µl of this sample loaded into gel. This gel was ran in a gel box for ~1hour at 90 volts and read on a gel reader.

2.2.8 Coculture assay

2.2.8.1 Background

Mixed lymphocyte reaction (MLR) is a common assay used to investigate T cell responses. In this reaction T cells (CD3+) or T cells in mixed lymphocytes from one individual are cultured with antigen presenting cells (APCs: CD14+) from a second individual. When these cells are mixed together, or cocultured, T cells will divide or proliferate in response to the foreign or non-self MHC molecules. This assay is useful for determining the functional capacity of an APC through its ability to stimulate T cells. Studies have revealed that approximately 1-10% of all T cells will respond to stimulation in this way (Janeway 2005).

2.2.8.2 Method

After a period of 20-24 hours following CD14+ cell infection, as described in section 2.2.6, cells were washed and centrifuged at 2,000g for 5 minutes. These cells were resuspended in cRPMI and seeded in 24 well cell culture adherent plates. Cocultures of CD14+ monocytes

and purified allogeneic CD3+ cells or MLRs, were performed at 1:10 ratios for all experiments. As a negative control, unstimulated mixed lymphoyctes were also cultured. As a positive control, CD3+ cells or MLs were stimulated with 50ng/ml of a polyclonal activation reagent, phorbol 12-myristate 13-acetate (PMA) and 1µg/ml anti-human CD3 mAb. This PMA directly activates protein kinase C, which in turn can activate various signalling pathways, leading to T cell activation. Anti-CD3 mAb, which binds and activates the T cell CD3 receptor, was bound to the plate, in disodium hydrogen phosphate (0.1M Na₂HPO₄-H₂HPO4 was made to 0.1M with sterile distilled water and pH to 9 with 0.1M sodium dihydrogen phosphate) binding buffer for 24 hours at 37° C, prior to stimulation. After incubation, the antibody solution was removed and wells were washed twice with sterile PBS, before the addition of cells. Additionally, allogeneic and influenza cocultures were set up for comparison. Cocultures were incubated for 5 days in total at 37° C. All samples were performed in triplicate on an adherent 24 well or 96 well cell culture plate.

2.2.9 MTS assay

2.2.9.1 Background

The CellTitre 96® Aqueous Non-Radioactive cell proliferation assay (Promega) is a colorimetric method for determining the number of viable cells in proliferation. The assay is composed of solutions of a novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phazine methosulfate;PMS). Cells bioreduce MTS into a formazan product that is soluble in tissue culture medium. The absorbance of the formazan at 490nm can be measured directly from 96-well assay plates without additional processing. The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product as measured by the level of absorbance at 490nm is directly proportional to the number of proliferating cells in culture (http://www.promega.com/~/media/files/resources/protocols/technical%20bulletins/0/celltiter

<u>%2096%20aqueous%20non-</u>

radioactive%20cell%20proliferation%20systems%20protocol.pdf?la=en)
2.2.9.2 Method

A volume of 20µl of MTS solution was cultured with $1x10^5$ cells in 100µl culture medium on a 96 well cell culture plate. Company protocol dictated that cells should be incubated with MTS solution for 1-4 hours, but as lymphoyetes may produce less formazan than other cell types, incubation was left for the full 4 hour incubation period. A volume of 100µl cell culture medium was also cultured with MTS solution and absorbance read from this was substracted from the absorbance of samples, to eliminate the background created by media. The absorbance was read at 490nm, using and ELISA plate reader (Promega).

2.2.10 Flow cytometry

2.2.10.1 Background

Flow cytometry is a process used to characterise the properties of individual cells as they pass by laser beams of light. It can provide information about the cell size, granularity and the expression of protein markers. To analyse protein expression, cells are incubated with particular monoclonal antibodies which bind to the protein of interest. These antibodies are conjugated to fluorochromes that emit light at various wavelengths after excitation in the laser beam, enabling the proteins to be detected.

After cells have been labelled with flurochrome conjugated antibodies, the cell suspension is forced, along with sheath fluid through a nozzle, which enables the cells to be individually spaced in this stream of liquid. As each cell passes the laser beam, which is usually an argon light with an excitation wavelength of 488nm, the cells scatter the laser light and the fluorochrome conjugated antibodies fluoresce at different wavelengths. The scattered light is detected by photomultiplier tubes, which can measure both the size of a cell, detected as forward scatter (FSC), and cell granularity, which is detected as side scatter (SSC). This enables one to distinguish between different cell subsets based on their size and granularity, such as macrophage, which are large and granular cells compared to lymphocytes, which are a much smaller and less granular cell population than macrophages.

The fluorescence emitted from the fluorochrome conjugated antibodies is also detected by photomultplier tubes. There are four main fluorochromes used in flow cytometry: fluorescein isothiocyanate (FITC)-which emits light at 530nm and is detected by the fluorescent detector FL1, phycoerythrin (PE)-which emits light at 578nm and is detected by FL2, and

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Allophycocyanin (APC)-which emits light at 660nm and is detected by FL4. The information from the flow cytometer (FACs [fluorescence activated cell sorter] caliber; Becton Dickinson) is then fed to a computer, where files can be retrieved for analysis by Cyflogic.

(http://probes.invitrogen.com/resources/education/tutorials/4Intro_Flow/player.html)



Figure 2.2.3 Simplified schematic displaying the principles of flow cytometry. Sample is carried through flow cytometer through sheath fluid, the fluorescent lense and detectors as well as side and forward scattered data are carried to computer system for analysis. (Image was reproduced from: (http://www.laserfocusworld.com/articles/print/volume-40/issue-8/features/optoelectronic-applications-drug-discovery/semiconductor-lasers-shed-new-light-on-flow-cytometry.html)

2.2.10.2 Method

Cells were centrifuged at 2,000g for 10 minutes and resuspended in flow cytometry buffer (FACs buffer: 1x PBS, containing 1% BSA and 0.02% sodium azide) at a concentration of $50\mu l/1x10^5$ of cells. This is an isotonic buffer and the sodium azide helps to prevent the endocytosis of monoclonal antibodies (mAb) and the BSA prevents non-specific binding of antibodies. Cells were washed again in FACs buffer. A volume of $5\mu l$ of fluorochrome labelled mAbs that are specific for a particular surface marker, were added to FACs tubes, along with the resuspended cells and incubated in the dark for 30 minutes at 4°C. Multiple

fluorochrome conjugated antibodies can be applied to the same FACs tube reaction; provided that they contain different fluorochromes and the flow cytometer has the capacity to detect them. This means that multiple markers on the same cell or even cell-type can be detected. After incubation, the cells were washed with FACs buffer and then read on the flow cytometer FACs calibur (Becton Dickinson, Oxford) and data was analysed using Cellquest software and Cyflogic. Fluorocrhome labelled isotype or control mAbs specifically matched to each of the fluorochrome labelled mAbs, were used as controls for non-specific staining of cells. As these should be in the negative fluorochrome quadrant upon acquisition, gates could be made based on isotype controls for each sample. The antibodies CD56-PE and CD3-APC were used in this study as well as their relevant isotype controls (eBioscience).

2.2.11 Cell cycle and Apoptosis assay

Analysis of a population's replication state can be achieved by labelling of nuclei with Propridium Iodide (PI) and analysis of fluorescent proportions. Cells in G0/G1 phase of cell cycle will have one copy of DNA, and emit 1x fluorescence, while cells in G2 phase will have 2x. Those cells in S phase will have intermediate fluorescence (Figure 2.2.4) (http://www.ucl.ac.uk/wibr/services/docs/cellcyc.pdf)



Figure 2.2.4: Determination of cell cycle by PI staining. Cell expression of PI is acquired at different levels on histogram analysis, with the furthest high expression peak representing cells in the G2 phase, the dipped intermediate peak represent those in the S phase, while low expression of PI indicates cells in the G0/G1 phase of cell cycle. (Image reproduced from: http://www.meduniwien.ac.at/user/johannes.schmid/PIstaining3.htm)

During apoptosis, the cell membrane phospholipid phospatidylserine (PS) is translocated from the inner to the outer leaf of the plasma membrane, thereby exposing PS to the external environment. Annexin V is an antibody to this externalised PS, and so, when conjugated to a detectable fluorescent agent, can act as a probe for cells undergoing apoptosis. As PS externalisation is an early event, compared to nuclear changes, cells in early apoptosis can be detected using this assay. Annexin-V staining is usually used in conjunction with a vital dye such as PI to differentiate between cells in early or late apoptosis. Cells that are both PI and Annexin-V positive are in late apoptosis, and can be quantified as such, (upper right quadrant). Cells are stained using Annexin V buffer and staining protocol as previously described



Figure 2.2.5 Apoptotic cells as shown by annexin-V pi staining. Cells positive for both PI and Annexin (UR) are those in late necrosis. Those expressing neither (LL) indicate viable cells and those positive for just annexin (LR) are in early apoptosis. (Image has been reproduced from: http://www.phnxflow.com/annexin.html)

2.2.12 Blocking of viral proteins using the monoclonal antibodies anti-HN and anti-F

Monoclonal antibodies against HPIV3 HN and F proteins (anti-HN and anti-F), as well as anti-canine distemper virus (viral control), were kindly provided by Claes Orvell (Karolinske University Hospital, Clinical Microbiology, Stockholm, Sweden). After 24 hour infection period, CD14+ cells were washed as previously described (Section 2.2.6). Their neutralizing effects have been previously demonstrated (Rydbeck 1986). Cells were incubated for twenty minutes with antibodies at a 1:100 or 1:250 volume ratio, and then cocultured with mixed lymphocytes or purified CD3+ cells (section 2.2.8)

2.2.13 Enzyme linked immunosorbent assay (ELISA)

2.2.13.1 Background

ELISAs can be used to quantify the amount of cytokine produced in solution. In a sandwich ELISA, a fixed quantity of capture antibody (mAb), specific for the cytokine being detected, is bound to a 96 well plate (the capture antibody is diluted in buffer, such as PBS and usually incubated overnight at 4°C). The plate is then washed to remove excess or unbound antibody and a blocking buffer, usually containing BSA, is added to prevent non-specific binding of subsequently added reagents. Samples of unknown antigen concentration and a series of recombinant cytokine standards of known concentration are added to the plate and incubated overnight at 4°C. The plate is washed again to remove any unbound antigen or cytokine and a biotinylated detection antibody for the cytokine is added and incubated (usually for 2 hours at RT). After incubation, the plate is washed and then streptavidin-horseradish-peroxidase (HRP) is added to the plate. Streptavidin binds biotin with high affinity and is conjugated to HRP, which is an enzyme that catalyses the oxidation of its substrate tetramethylbenzidinie (TMB) by hydrogen peroxide, forming a blue compound. Following streptavidin-HRP incubation the plate is washed again and TMB is added, which forms a blue colour that increases in intensity in the well, if a lot of cytokine has been bound to the well. The reaction is stopped by adding sulphuric acid to the plate, which turns the solution a yellow colour and the absorbance of light at a wavelength of 450nm is read by a plate reader (www.abcam.com/technical). Thus, the rate of colour formation is proportional to the amount of cytokine present:



Figure 2.2.6: Schematic illustrating the principles of a sandwich ELISA. The protein to be detected is captured on the plate by a capture antibody, specific to this protein. If protein to be

detected is present, the detection antibody, conjugated with Biotin is bound, with addition of streptavidin. TM substrate cleaves this with a colormetric result (Taken from: http://www.epitomics.com/products/product_info/1212/PBK--6112-1.html)

Once the absorbance at 450nm has been read, the known standard concentrations are plotted against optical density (OD) or optical absorbance, producing a standard curve (fig 2.2.7). The unknown samples with absorbance readings at 450nm can then be read from the curve to give known sample concentrations



Figure 2.2.7: Typical standard curve as used in ELISA calculation. Standards of known concentration are detected and plotted for comparison of unknown sample. The concentration of

unknown sample can then be determined from the equation of the line derived from these standards (taken from: <u>http://www.abcam.com/C1q-antibody-ab14004.html</u>)

2.2.13.2 Method

Supernatant from samples were collected at indicated time points and cytokine production was measured by ELISA kits, according to the manufacturer's instructions (R+D, UK). Samples and standards were plated either in duplicate or triplicate wells on the 96 well plate to ensure accurate quantitative results were obtained and statistical analyses could be performed on the samples. IL-2 was detected during this study.

2.2.14 Carboxyfluorescein succinimidyl ester (CFSE) incorporation

2.2.14.1 Background

Cell incorporation with CFSE is used as a method for tracking cell proliferation. The solution passively diffuses into cells and is colourless and nonflourescent until its acetate groups are cleaved by intracellular esterases to yield high fluorescence. The succinimidyl ester group

reacts with intracellular amines, forming fluorescent conjugates that are well retained and can be fixed with aldehyde fixatives. Excess unconjugated reagent and by-products passively diffuse to the extracellular medium, where they can be washed away. With each successive generation of cells, the level of fluorescence emitted per cell halves. So, the lower the fluorescence, the more proliferation a cell has undergone (solid grey, Figure 2.2.8). Cells in this low CFSE range can be gated based on their low CFSE peak and plotted as a percentage of total CFSE+ cells (http://probes.invitrogen.com/media/pis/mp34554.pdf).



Figure 2.2.8: Determination of proliferated cells by CFSE. The lower the CFSE emission from a cell, the more it has proliferated. Those cells in the grey peak above have proliferated more than the original cells or white population. The further left on the graph a population appears, the more proliferation has occurred. Image was reproduced from: http://probes.invitrogen.com/media/pis/mp34554.pdf

2.2.14.2 Method

Cells were spun and resuspended in a solution of 0.1% BSA in PBS at a final concentration of $1x10^6$ cells/ml. A volume of 5mM stock CFSE solution per $1x10^6$ cells was added to yield a final working concentration of 10µM. Cells were incubated at 37°C for 10 minutes. Staining was then quenched by the addition of 5 times the volume of ice-cold culture media to the cells. Cells were incubated for 5 minutes on ice and then pelleted by centriguation. Cells were washed in fresh culture media three times. Coculture and stimulation was setup as

required. Upon harvest, cells could be costained for other markers where appropriate. Upon acquisition, the peak of cells emitting low levels of CFSE was gated for analysis.

2.2.14 Statistical analysis

All coculture experiments represent normalized results for three donors in three independent experiments. Cyflogic and Facs Calibur software were used for FACs analysis, and plots were created using GraphPad Prism 5. Significance was calculated using Newman Keuls One way Anova: * p < 0.05; ** p < 0.01; *** p < 0.001.

2.2.15 Normalization Rationale:

For all experiments described in this thesis, three individual PBMC donors were used. Rather than show representative results, we normalized data to take all donors into account. The following is the rationale for this approach.

Normalization is a mode of analysis which is widely used for the presentation of data in clinical and preclinical literature. This is true in particular, for the presentation of data for multiple human donors in the viral immunology field (McGovern *et al* 2013, Mwimanzi *et al* 2013, Ballenbergern *et al* 2012, Kwok *et al* 2012, Bulua *et al* 2011, O'Connell *et al* 2009, Bocelli-Tyndall *et al* 2007, Lickliter *et al* 2007 Sindhu *et al* 2003, etc). Due to our intention to publish in this field and the support in relevant literature for this statistical method (too numerous to reference), it was chosen for presentation of data from multiple donors in this thesis.

Normalization is described as a statistical tool that enables the assay values from different laboratories or donors to be transformed in such a way that they are directly comparable (Karvanen 2003). This is important not only due to donor-to-donor variability of certain cell types and marker expression, but also due to the fact that assay signal responses will vary between assay runs and so, the cut point needs to be modified to take this into account (Guichelaar, T *et al* 2013, Mire-Shuis *et al* 2004). Graphpad Prism is not only an important and widely accepted statistical analysis tool, it is also used for normalization of data (Kowk *et al* 2012, Moreria-Tabaka 2012, Sinisimer *et al* 2010, Blanpain, C, 2001). Normalization using graphpad prism converts Y values from different data sets or donors to a common scale. The software guide describes this as useful when one wants to compare the shape or

position of two or more curves or donors, without being "distracted" or skewed by different maximum and minimum values. Investigators who analyse dose-response curves commonly normalize the data so all curves begin at 0% and plateau at 100%. Alternatively, data can be normalized to a specific replicate within the dataset (Graphpad Prism Guidelines), namely, the negative control of a particular donor replicate. For further information we supply a supplementary documentation providing a step by step guide to the normalisation process which accompanies the software guidelines and if the reader has access to Graphpad Prism software the entire dataset maybe accessed by right clicking on each graph in this document and selecting edit.

The three sets of experimental data used to justify normalization in this section are examples of data presented for individual donors, the average of all donor data, or normalized data for three donors. These experimental data sets will later be described in context, and their implications discussed in future results and discussion sections. Briefly, figure 2.3.6 shows changes to CD56+ cell levels in response to allogeneic HPIV3 infected CD14+ cells, and the effect of antibodies to HN and F surface glycoproteins to this. Figure 3.3.3.12 shows CD3+ cell proliferation in response to various IL-2, and the effect of antibodies to IL2 receptor components on this. Finally, figure 4.3.3 shows Treg proliferation in response to various IL-2 concentrations in the presence or absence of NK cells.

For these figures, analysis of individual donors demonstrated significance compared to media samples for each individual donor, similar to normalized results. However, averaging triplicate data from 3 donors' causes larger error bars than the normalization approach due to donor variability of baseline numbers- which is to be expected. (Figure 2.3.6.ii, Figure 3.3.12ii, and Figure 4.3.3.ii). For two of three figures, these error bars result in the loss of significance, although the trend still remains (Figure 2.3.6.ii, Figure 3.3.12ii). Rather than the approach of averaging all donor data, which is akin to pooling donor samples, we normalize results to the donor with the lowest baseline media samples, explaining the variance between the normalized range and the individual donors that express higher baseline values (Figure 2.3.6iii, Figure 3.3.12ii), and Figure 4.3.3iii). This is suitable for experiments presented in this thesis, as it is the relative increase of expression or proliferation compared to media samples that is of importance, rather than specific individual cell numbers/expression profiles. As the trend is maintained for all donors, normalization creates a representative graph that takes the relative increase/decrease of all donor values into account. This explains

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why the use of this method is found increasingly in the literature and is the format in which the thesis data will be presented for publication.

Furthermore, as a representative donor dataset is regularly another approach by which data is represented in the published literature (O'Connell *et al* 2009), the normalized version of the data is again more representative of each individual donor than the approach of averaging all donor data. Moreover normalisation should enable direct comparison with the results of identical experimentation carried out with different donors in different laboratories.

In conclusion, based on the strong support for this statistical approach in the literature and the close reflection of the individual donor data to the normalised rather than the averaged data in our hands we suggest that the normalization approach surpasses the latter as a method of presentation of this data. While the figures shown here are from results sections described later in this thesis, we felt they should still be shown with this rationale section. Their scientific implications will be described in later sections.





Figure 2.3.6i: Individual donor percentage of CD56+CD3- cells in the mixed lymphocyte population in response to antibodies to HPIV3 surface glycoproteins during infections. The percentage of CD56+CD3- cells in mixed lymphocytes was determined by flow cytometry. Donor 1 (A), Donor 2 (B) and Donor 3 (C) were analysed individually for significance compared to media sample by the Newman Keuls One Way Anova. Error bars represent standard error between triplicates. Numbers above bars represent average of each triplicate $***p \le 0.001$



Figure 2.3.6ii Average percentage of CD56+CD3- cells in the mixed lymphocyte population in response to antibodies to HPIV3 surface glycoproteins during infections. The percentage of CD56+CD3- cells in mixed lymphocytes was determined by flow cytometry. The average of triplicate samples from three individual donors was determined (9 figures averaged) and is presented above each column. Error bars represent standard error between 9 individual samples (3 from each of 3 donors). Significance was analysed using the Newman Keuls One way Anova.



Figure 2.3.6iii Normalized percentage of CD56+CD3- cells in the mixed lymphocyte population in response to antibodies to HPIV3 surface glycoproteins during infections. The percentage of CD56+CD3- cells in mixed lymphocytes was determined by flow cytometry. These were normalized to the lowest baseline media sample in the data set to account for donor baseline variability. The post-normalization number represented by columns is defined above each particular bar. Error bars represent standard error of normalized data. Data was analysed for significance compared to media sample by the Newman Keuls One Way Anova. ***p ≤ 0.001 **p ≤ 0.01



Figure 3.3.12i: Individual donor percentage of proliferated CD3+ cells in response to various IL-2R antibodies when stimulated with various IL-2 concentrations. The percentage of proliferated CD3+ cells were determined by CFSE incorporation and flow cytometry. Donor 1 (A), Donor 2 (B) and Donor 3 (C) were analysed individually for significance compared to media sample by the Newman Keuls One Way Anova. Error bars represent standard error of triplicate samples. *** $p \le 0.001$ ** $p \le 0.01$



Figure 3.3.12ii: Average percentage of proliferated CD3+ cells in response to various IL-2R antibodies when stimulated with various IL-2 concentrations. The percentage of proliferated CD3+ cells were determined by CFSE incorporation and flow cytometry. The average of triplicate samples from three individual donors was determined (9 figures averaged). Error bars represent standard error between 9 samples (3 from each of 3 donors).Significance was analysed using the Newman Keuls One way Anova.



Figure 3.3.12iii Normalized percentage of proliferated CD3+ cells in response to various IL-2R antibodies when stimulated with various IL-2 concentrations. The percentage of proliferated CD3+ cells were determined by CFSE incorporation and flow cytometry. These were normalized to the lowest baseline media sample in the data set to account for donor baseline variability. The post-normalization number represented by columns is defined above each particular bar for the "no antibody" data set as an example. Error bars represent standard error between normalized data. Data was analysed for significance compared to media sample by the Newman Keuls One Way Anova. *** $p \le 0.001$ ** $p \le 0.01$



Figure 4.3.3i: Individual donor percentage of proliferated Tregs in response to various IL-2 concentrations. The percentage of proliferated Tregs was determined by CFSE incorporation and flow cytometry. Donor 1 (A), Donor 2 (B) and Donor 3 (C) were analysed individually for significance compared to media sample by the Newman Keuls One Way Anova. Error bars represent standard error for triplicate samples .*** $p \le 0.001$ ** $p \le 0.01$



Figure 4.3.3ii: Average donor percentage of proliferated Tregs in response to various IL-2 concentrations. The percentage of proliferated Tregs was determined by CFSE incorporation and flow cytometry. The average of triplicate samples from three individual donors was determined (9 figures averaged). Significance was analysed using the Newman Keuls One way Anova. Error bars represent standard error for 9 samples (3 from each of 3 donors0 ***p \leq 0.001 * \leq 0.01



Figure 4.3.3iii: Normalized donor percentage of proliferated Tregs in response to various IL-2 concentrations. The percentage of proliferated Tregs was determined by CFSE incorporation and flow cytometry. These were normalized to the lowest baseline media sample in the data set to account for donor baseline variability. The post-normalization number represented by columns is defined above each particular bar Error bars represent standard error from each of three donors. Data was analysed for significance compared to media sample by the Newman Keuls One Way Anova. *** $p \le 0.001$ ** $p \le 0.01$

2.3 Results

2.3.1 HPIV3 infected CD14+ cells inhibit the proliferation of allogeneic mixed lymphocytes, but not isolated CD3+ cells

It has been suggested in literature that mixed lymphocytes fail to proliferate during HPIV3 infection, and with this, memory cells may fail to proliferate or clonally expand during infection (Plotnicky-Gilquine *et al* 2001). Our group has demonstrated inhibited T cell responses during infection (Noone *et al* 2008). Having determined the TCID₅₀/ml of the newly cultured HPIV3 as 9.7 (Appendix 1.4) by crystal violet assay, and confirmed its' ability to infected isolated CD14+ cells (Appendix 1.5) by PCR targeting NP expression, we examined mixed lymphocyte proliferation during infection, to determine reproducibility of previous results with this cultured virus.

From freshly cultured PBMCs, CD14+ cells were isolated and infected with either influenza or HPIV3. These were washed after a 2 hour incubation to remove excess virus. After 24 hours, these were cultured with allogeneic mixed lymphocytes. Mixed lymphocytes were also cultured alone, with uninfected CD14+ cells, or stimulated with Anti-CD3 and PMA. After 5 days of incubation, the capacity of HPIV3 infected CD14+ monocytes to stimulate allogeneic mixed lymphocyte proliferation was examined using the MTS assay (Figure 2.3.1.a). Isolated CD14+ cells infected with HPIV3 failed to induce allogeneic CD14- mixed lymphocyte cell proliferation compared with uninfected allogeneic CD14+ cells (*p \leq 0.05), influenza infected CD14+ cells or PMA and anti-CD3 stimulated mixed lymphocytes. This was in agreement with previous reports that HPIV3 infection fails to induce mixed lymphocyte responses (Noone et al 2008).

This experiment was repeated, this time using isolated allogeneic CD3+ cells rather than total mixed lymphocytes. The inhibited proliferation did not occur when purified CD3+ cells were cultured with allogeneic HPIV3 infected CD14+ cells as observed in mixed lymphocyte experiments (Figure 2.3.1.b). Thus, culturing HPIV3 infected cells with purified CD3+ T cells restores T cell proliferation. These results suggest that failed T cell responses associated with HPIV3 infection (Noone *et al* 2008), is due to the presence of a regulatory group of cells in the mixed lymphocyte population.



Figure 2.3.1: Proliferative responses of allogeneic mixed lymphocytes and purified CD3+ cells to infected CD14+ cells. Freshly isolated CD14+ cells were infected with HPIV3 or Influenza and washed after 2 hours. These were then cocultured with freshly isolated allogeneic mixed lymphocytes (A) or CD3+ cells (B) after 24 hours. Cells were also cultured with PMA and Anti-CD3 as a positive control, and media or as a negative control. After 5 days cells were harvested and cocultured with MTS solution for 4 hours. Absorbance was detected at 490nm. Results represent normalized data from 3 donors with triplicate samples. Error bars represent standard deviation between data. Significance was determined using Newman Keuls One Way Anova and was compared to CD14+ samples of that data set $p \le 0.05$, $**p \le 0.001$

2.3.2 CD56+ cells are responsible for the inhibited mixed lymphocyte proliferation during HPIV3 infection.

Previous results (Figure 2.3.1) suggesting that a regulatory group of cells exist in the mixed lymphocyte population which are responsible for inhibited CD3+ cell proliferation during HPIV3 infection. Noone *et al* (2008) showed an upregulation of CD56 during HPIV3 infection. As CD56 is a marker for NK cells, we wanted to investigate the role of these cells in regulating CD3+ cell proliferation during HPIV3 infection.

Both total mixed lymphocytes, and CD56+ cell depleted mixed lymphocytes were cultured with HPIV3 infected allogeneic CD14+ cells, and previously described controls. In MLs, HPIV3 infected CD14 cells induced significantly (*** $p\leq0.001$) inhibited proliferation compared to uninfected CD14+ cells, in addition to previously described controls. When CD56+ cells were depleted from mixed lymphocytes, proliferation was restored in the HPIV3 infected cocultures, to a similar level to influenza infection (Figure 2.3.2.a). Additionally, when CD3+ cells were purified and cultured in the presence of CD56+ cells and infected allogeneic CD14+ cells, proliferation was significantly (** $p\leq0.01$) inhibited in HPIV3 infected cultures (Figure 2.3.2.b). These results indicated that during HPIV3 infection CD56+ cells inhibit the proliferation of CD3+ cells.

Notably, even in uninfected samples (media sample, figure 2.3.2.a) there was less overall mixed lymphocyte proliferation in the presence of autologous CD56+ cells. These results are again in agreement with observations reported by Noone *et al* (2008).



Figure 2.3.2: Proliferative responses of allogeneic mixed lymphocytes and purified CD3+ cells to infected CD14+ cells in the presence of autologous CD56+ cells. Freshly isolated CD14+ cells were infected with HPIV3 or influenza and washed after 2 hours. These were then cocultured with freshly isolated allogeneice mixed lymphocytes, some of which were CD56+ depleted (A) or CD3+ cells in the presence or absence of CD56+ cells (B) after 24 hours. Cells were also cultured with PMA and Anti-CD3 as a positive control, and media as a negative control. After 5 days, cells were harvested and cocultured with MTS solution for 4 hours. Absorbance was detected at 490nm. Results represent normalized data from 3 donors, with each experiment carried out in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One Way Anova and was as compared to CD14+ sample of that data set. ***p ≤ 0.001 ** p ≤ 0.01

2.3.3 Direct mixed lymphocyte infection with HPIV3 induces mixed lymphocyte death.

For experiments described thus far, freshly isolated CD14+ monocytes were infected with virus, washed and incubated for 24 hours prior to coculture with allogeneic mixed lymphocytes or CD3+ cells. To determine if infection of CD14+ cells are absolutely necessary in this model, mixed lymphocytes were isolated and infected directly with either HPIV3 or influenza. It was hoped that this would determine whether the inhibited mixed lymphocyte proliferation observed during infection required infection of CD14+ cells. The MTS assay showed a much lower level of proliferation for both influenza and HPIV3 infected mixed lymphocytes (Figure 2.3.3.a). Furthermore, when these cells were examined using Annexin-V-PI staining, an increased level of apoptotic mixed lymphocytes were observed in directly infected mixed lymphocytes (Figure 2.3.3.b). Notably, this prism graph displays those cells in late apoptosis (UR) showing a ~4 fold increase in apoptotic directly infected cells compared to media samples. There is also however, a significant increase in cells in early apoptosis (LR).

These results indicated that for all subsequent experiments, infection of CD14+ cells is required for viral presentation to allogeneic mixed lymphocytes or CD3+ cells, as direct viral infection induces target cell death. This highlights the importance of the wash step to remove excess virus.



Figure 2.3.3. Proliferative (A) and Apoptotic (B) responses of directly infected mixed lymphocytes. Freshly isolated mixed lymphocytes were directly infected with HPIV3 or influenza and washed after 2 hours. Usual uninfected negative and positive controls were used. After 5 days, cells were harvested and cocultured with MTS solution for 4 hours (A). Absorbance was detected at 490nm. Annexin V-PI staining was also carried out and acquired by flow cytometry. Annexin-V+PI+ quadrant was gated as apoptotic and plotted as a percentage of total mixed lymphocytes. Representative flow cytometry data can be seen in Appendix 1.6. Results represent standard deviation between samples. Significance was determined using Newman Keuls One way Anova, and is as compared to media samples. *** $p \le 0.001$

2.3.4 Blocking HPIV3 HN, but not F protein, abrogates inhibition of mixed lymphocytes during HPIV3 infection.

Previous unpublished data from our group demonstrated that empty virosomes expressing HPIV3 surface glycoproteins HN and F induced the same anti-proliferative effect as HPIV3 infected CD14+ cells. Claes Orvell (Karlinska institute) kindly provided antagonistic monoclonal antibodies to HPIV3 HN and F, with previously demonstrated neutralizing effects (Rydbeck 1986). Our aim here was, using these antibodies, to further investigate the role of these viral proteins in the inhibition of mixed lymphocyte proliferation during HPIV3 infection.

Mixed lymphocytes were cultured with HPIV3 infected allogeneic CD14+ cells, or HPIV3 infected CD14+ cells which were then blocked with either anti-HN or anti-F antibodies, as well as anti-Distemper virus as an irrelevant anti-viral control. Controls described in previous experiments were also used and proliferation was analysed using the MTS assay. As we had not previously worked with these antibodies, they were used at a 1:100 and 1:250 volume ratio to cells. As several antibodies to the same proteins were provided by Prof Claus Orvell, we tested two candidates for each protein targets.

When HPIV3 HN was blocked prior to coculture with mixed lymphocytes, proliferation of mixed lymphocytes during HPIV3 infection was restored to a level of significance similar to that seen during influenza infection(** $p\leq0.01$) (Figure 2.3.6.a). This did not occur when F protein was blocked prior to coculture. This response was observed when using anti-HN at a ratio of 1:100 (Figure 2..3.4.a). Therefore, this ratio was used for Anti-HN and Anti-F for all subsequent experiments. These results suggest a role for HN for inhibited mixed lymphocyte proliferation during infection.



Figure 2.3.4: Proliferative responses of mixed lymphocytes or purified CD3+ cells to HPIV3 infected allogeneic CD14+ cells, where viral protein F or HN have been blocked. Freshly isolated CD14+ cells were infected with HPIV3 or influenza and washed after 2 hours. Prior to coculture with freshly isolated allogeneic mixed lymphocytes after 24 hours, Anti-HN, Anti-F or Anti-Distemper antibodies were incubated with cells at 1:100 or 1:250 ratios. Mixed lymphocytes were also cultured with Anti-CD3 and PMA as a positive control, and media as a negative control (A, B). After 5 days, cells were harvested and cocultured with MTS solution for 4 hours. Absorbance was detected at 490nm. Results represent normalized data from 3 donors, with each experiment carried out in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One way Anova and is as compared to CD14+ samples of the same data set. ** $p \le 0.01$ *** $p \le 0.001$.

2.3.5 Blocking HPIV3 HN, but not F protein, abrogates CD56+ cell mediated inhibition of T cells during HPIV3 infection.

We have demonstrated that when HN is blocked on HPIV3 infected CD14+ cells, mixed lymphocytes proliferate during infection. We examined proliferation of CD3+ cells cultured with CD56+ cells and infected CD14+ cells.

From freshly cultured PBMCs, CD3+ cells were isolated, and cultured with HPIV3 infected allogeneic CD14+ cells, or HPIV3 infected CD14+ cells which were then blocked with either anti-HN or anti-F antibodies, as well as anti-Distemper virus as an irrelevant anti-viral control. Additionally, CD3+ cells were cultured with uninfected CD14+ cells, and PMA and Anti-CD3. Proliferation was analysed using the MTS assay.

Mixed lymphocyte results (Figure 2.3.4) were reproducible with purified CD3+ cells in the presence of autologous CD56+ cells (Figure 2.3.5). These results suggest that HPIV3 HN is involved in stimulating CD56+ NK cells mediated inhibition of T cell proliferation.



Figure 2.3.5: Proliferative responses of CD3+CD56+ cell cocultures to HPIV3 infected allogeneic CD14+ cells, where viral protein F or HN have been blocked. Freshly isolated CD14+ cells were infected with HPIV3 or influenza and washed after 2 hours. Prior to coculture with freshly isolated allogeneic CD3+ and CD56+ cells after 24 hours, Anti-HN, Anti-F or Anti-Distemper antibodies were incubated with cells at a 1:100 ratio. These cells were also cultured with Anti-CD3 and PMA as a positive control, and media as a negative control). After 5 days, cells were harvested and cocultured with MTS solution for 4 hours. Absorbance was detected at 490nm. Results represent normalized data from 3 donors, with each experiment carried out in triplicate. Error bars represent standard deviation between replicates. Significance was determined using Newman Keuls One way Anova, and is as compared to CD14+ samples. ***p \leq 0.001.

2.3.6 Blocking HPIV3 HN abrogates NK cell marker upregulation during infection.

The up regulation of CD56 during HPIV3 infection had previously been shown in our group (Noone *et al* 2008), and we have demonstrated that CD56+ cells are responsible for inhibiting CD3+ cell proliferation during HPIV3 infection (Figure 2.3.1). Since blocking HPIV3 HN restored mixed lymphocyte proliferation during infection, the aim of this experiment was to investigate the levels of CD56+CD3- (NK) cells during infection and the effect of antibodies to HN and F on their expression.

Mixed lymphocytes were cultured with HPIV3 infected allogeneic CD14+ cells, as well as HPIV3 infected CD14+ cells which were cultured with Anti-HN, Anti-F or Anti-Distemper virus as previously described prior to coculture. Previously described controls were also used. Upon harvest, cells were stained for CD56 and CD3, so that CD56+CD3- (NK cells) cells could be gated for by flow cytometry and expressed as a percentage of total lymphocytes.

In keeping with previous results, infection with HPIV3 induced an upregulation in levels of CD56+CD3- cells compared to unstimulated mixed lymphocytes and CD14+ cell cocultures. However, when HN protein, but not F, was blocked, this upregulation of CD56+CD3- cells was abrogated (Figure 2.3.6). This suggests that HPIV3 HN protein induces expansion of NK cells during HPIV3 infection. Based on previous experiments, it is known that these expanded NK cells are responsible for the inhibition of CD3+ cell proliferation during infection.



Figure 2.3.6: Percentage of CD56+CD3- mixed lymphocytes in response to HPIV3 infection, when surface gylcoproteins were blocked. Freshly isolated CD14+ cells were infected with HPIV3 or influenza and washed after 2 hours. Prior to coculture with freshly isolated allogeneic mixed lymphocytes after 24 hours, Anti-HN, Anti-F or Anti-Distemper antibodies were incubated with cells at a ratio of 1:100. Mixed lymphocytes were also cultured with previously described controls. After 5 days, cells were harvested, stained for CD56 and CD3 and analysed by flow cytometry. Those cells in the CD56+CD3- quadrant were expressed as percentages of total lymphocytes. Representative flow cytometry data can be seen in appendix 1.7. Results represent normalized results from 3 donors, with each experiment carried out in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One way Anova, and is as compared to CD14+ sample. ***p \leq 0.001

2.3.7 Blocking HPIV3 HN restores IL-2 production during infection.

Our group previously demonstrated an inhibition of IL-2 production during HPIV3 infection (Noone *et al* 2008). Furthermore, addition of IL-2 to infected cocultures restored proliferation to normal viral response. Since IL-2 is considered a crucial growth factor for T cells (Boyman and Sprent, 2012), we re-examined the production of this cytokine during infection in relation to viral surface proteins.

Again, mixed lymphocytes were cultured with HPIV3 infected allogeneic CD14+ cells, as well as those cultured with Anti-HN, Anti-F, or Anti-Distemper. After 5 days incubation, supernatants were examined by ELISA for IL-2 concentrations (pg/ml). Consistent with Noone et al's finding, a significantly (*p \leq 0.05) reduced level of IL-2 secretion was observed in HPIV3 infected cocultures, when compared to uninfected CD14+ cells, and to a greater extent when compared to influenza infected CD14+ cells. However, IL-2 production during infection was restored to levels of significance similar to influenza (***p \leq 0.001) when HPIV3 HN, but not F, were blocked, when compared to uninfected cells (Figure 2.3.7).

These results suggest that during HPIV3 infection, the HN surface glycoprotein inhibits IL-2 production and expands NK cells, with overall inhibition of T cell proliferation.



Figure 2.3.7: IL-2 production in response to HPIV3 viral proteins. Freshly isolated CD14+ cells were infected with HPIV3 or influenza and washed after 2 hours. Prior to coculture with freshly isolated allogeneic mixed lymphocytes after 24 hours, Anti-HN, Anti-F or Anti-Distemper antibodies were incubated with cells at a ratio of 1:100. Mixed lymphocyces were also cultured with previously described controls. After 5 days, cells were harvested. Supernatants used to detect IL-2 concentrations (pg/ml) by ELISA. Results represent normalized results from 3 donors, with each experiment carried out in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One way Anova and is as compared to CD14+ sample of the same data set. * $p \le 0.05$ *** $p \le 0.001$

2.3.8 Anti- HN and Anti-F abrogate CD3+ cell cycle arrest during infection.

It has been reported that inhibited T cell numbers during HPIV3 infection is due to T cell cycle arrest in the G0/G1 phase and not apoptosis. Our group previously demonstrated that this cell cycle arrest is induced by CD56+ cells (Noone et al 2008). Our results suggest that HPIV3 HN may induce both CD56+ cell upregulation (Figure 2.3.6) and IL-2 inhibition (Figure 2.3.7) during HPIV3 infection, resulting in an overall inhibition of T cell proliferation (section 2.3.5). We wanted to investigate if blocking this viral protein restores progression of cells from G0/G1 phase T cell during infection.

Freshly isolated CD14+ cells were infected with HPIV3 and cocultured with allogeneic mixed lymphocytes, as well as viral protein blocking antibodies and previously described controls. Upon harvest, cells were stained with CD3, as well as the Annexin-V and PI staining assay. Cells expressing CD3 were gated for and the percentage of these in the G0/G1 phase of cell cycle expressed as a percentage of total CD3+ cells (Figure 2.3.8.a). Similarly, the percentage of Annexin-V+PI+ CD3+ cells were expressed as a percentage of total CD3+ cells (Figure 2.3.8.b).

Infection with HPIV3 increased the number of CD3+ cells accumulated in the G0/G1 phase of cell cycle (figure 2.3.9.a), which agrees with previously published work from the group (Noone et al, 2008). No increase in CD3+ cell death was observed during infection (figure 2.3.8.b). Blocking HPIV3 HN caused progression of CD3+ cells from the G0/G1 phase of cell cycle, as did blocking F (figure 2.3.8.a). Thus, based on this result, both of these glycoproteins may play a part in inducing CD3+ cell cycle arrest in the G0/G1 phase during infection. This is at odds with results from section 2.3.6 and 2.3.5, as no previous effect has been observed for F in the induction of T cell inhibition by NK cells. It would appear that while these results suggest that F protein may induce an accumulation of CD3+ cells in the G0/G1 phase of cell cycle, this does not translate to an inhibition of proliferation of these cells.

Concerns over proliferative data equation with cell cycle data are further addressed in chapter 3 (proliferation) and section 5.3.6 (cell cycle arrest).



Figure 2.3.8: Cell cycle(A) and Apoptotic (B) responses of directly infected mixed lymphocytes. Freshly isolated CD14+ cells were infected with HPIV3 or influenza and washed after 2 hours. Prior to coculture with freshly isolated allogeneic mixed lymphocytes after 24 hours, Anti-HN, Anti-F or Anti-Distemper antibodies were incubated with cells at a ratio of 1:100. Mixed lymphocyces were also cultured with previously described controls. After 5 days, upon harvest, cells were stained for CD3, Annexin-V and PI. Stages of cell cycle were gated in the PI histogram of CD3+ cells, and the percentage of cells in each stage expressed as a percentage of total CD3+ cells (A). CD3+ cells were plotted as Annexin-V vs PI and those in the Annexin V+PI+ quadrant plotted as a percentage of total CD3+ cells (B). Representative flow cytometry data can be seen in Appendix 1.8 (Cell cycle: Appendix 1.8.A, Apoptosis: Appendix 1.8.B). Results represent normalized data from 3 donors with each experiment carried out in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One Way Anova and is as compared to CD14+ samples ***p≤0.001.

2.3.9 Anti-HN abrogates inhibited CD3+ cell proliferation during infection.

Antibodies to HN and not F has been shown to restore CD3+ cell proliferation by MTS assay (Figure 2.3.5), and restore IL-2 levels during infection (Figure 2.3.7), antibodies to both HN and F restore CD3+ cell cycle from the G0/G1 phase. Due to this inconsistency, we wanted to revisit CD3+ cell proliferation using a different assay.

Freshly isolated CD14+ cells were infected with HPIV3 and cocultured with allogeneic CD3+ cells into which CFSE had been incorporated, as well as viral protein blocking antibodies and previously described controls. Upon flow cytometry acquisition at day 5, the proportion of CFSE+ cells in the "low CFSE-FITC" peak were gated and expressed as a percentage of total CD3+ cells.

Here, blocking HN, but not F, restored CD3+ cell proliferation during HPIV3 infection (Figure 2.3.9), which is consistent with previous results (Figure 2.3.5-2.3.7). However it does not explain the abrogation of CD3+ cell cycle arrest by antibodies to HPIV3 F protein as demonstrated previously (Figure 2.3.8). While antibody data suggests that F induces T cell cycle arrest, it is possible that other viral components are sufficient to overcome this proliferatively.


Figure 2.3.9: Proliferative responses of CD3+ cells as determined by CFSE in response to HPIV3 viral proteins. Freshly isolated CD14+ cells were infected with HPIV3 or influenza and washed after 2 hours. Prior to coculture with freshly isolated allogeneic CD3+ cells, into which CFSE had been incorporated, after 24 hours, Anti-HN, Anti-F or Anti-Distemper antibodies were incubated with cells at a ratio of 1:100. Mixed lymphocyctes were also cultured with previously described controls. After 5 days, upon acquisition by flow cytometry, cells in the low CFSE peak were gated for and expressed as a percentage of total CD3+ cells.). Representative flow cytometry data can be seen in Appendix 1.9. Results represent normalized data from 3 donors with each experiment carried out in triplicate. Error bars represent standard deviation between samples. Significance is as compared CD14+ sample. ***p \leq 0.001

2.4 Discussion

Control of response to self is of importance during infection. There are also regulatory mechanisms in place to limit collateral damage associated with infection (O'Garra et al 2004). While this regulatory response is essential in the prevention of immune mediated damage, it unfortunately comes with the forfeit of appropriate immune activation, which can lead to persistent infections (Accapazzato et al 2004). Viruses can exploit these regulatory strategies to promote their survival within the host. This aspect of immune regulation has been associated with the persistence and chronicity associated with Hepatitis C virus (HCV) and HIV (Boether et al 2005, Weiss et al 2004). Since infection with HPIV3 is associated with a lack of immune memory (Henrickson 2003) it was suggested that perhaps this virus makes use of these regulatory mechanisms (Plotnicky-Gilquin et al 2001). Indeed, our group previously demonstrated a novel mechanism by which NK cells inhibit T cell proliferation during HPIV3 infection. This regulation was shown to be via a contact dependent mechanism, inducing T cell cycle arrest, not apoptosis. Additionally, this is mediated by the inhibited IL-2 production associated with this virus (Noone et al, 2008). We cultured a stock of HPIV3 and this batch was tested by repeating experiments carried out by Noone et al. These experients confirmed the reproducibility with new virus.

While Noone et al demonstrated NK cell mediated regulation during HPIV3 infection, the viral components involved, as well as the mode of NK cell activation remained unclear. Previously unpublished data from our group demonstrated that empty virosomes expressing HPIV3 surface glycoproteins HN and F induced the same inhibited T cell proliferation as total virus. To explore the role of these proteins, we used neutralizing antibodies to HN and F (Prof Claes Orvell, Karlinska Institute) to determine their effect on NK cell mediated T cell inhibition during infection.

Although the inhibition of T cell proliferation during HPIV3 infection is accepted (Noone *et al* 2008), the viral components which activate this process remain unknown. Blocking HPIV3 surface HN, but not F, presented on CD14+ monocytes, prior to coculture with allogeneic mixed lymphocytes or CD3+ T cells, restored mixed lymphocyte proliferation during infection. Expansion of CD56+ cells has been associated with infection. These NK cells have been attributed with T cell inhibition (Noone *et al* 2008). We demonstrate that blocking HN abrogates this CD56+ cell upregulation. Furthermore, the inhibited production of IL-2 associated with HPIV3 infection is restored using antibodies to HN. This inhibited IL-2 has a

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functional role in the NK cell mediated T cell inhibition, as addition of IL-2 to cultures restores proliferation. Finally, blocking either HN or F with neutralizing antibody abrogates the increase of CD3+ cells in G0/G1 phase of cell cycle associated with infection. This comes as a surprise, as F had no impact on overall proliferation. As antibodies to F do not restore IL-2 production or abrogate CD56+CD3- cell expansion, it can only be presumed that F activates a different regulatory pathway than the HN induced IL-2 inhibition.

The interaction between HN and NK cells, and their subsequent activation of antiproliferative immune regulation, may be responsible for the inhibited immune memory associated with the virus. This needs further investigation. This finding suggests that as long as vaccines employ unmodified forms of HPIV3 HN, immune memory will not develop to them.

Although the induction of NK mediated T cell inhibition may be a negative with regards to the development of immunity to HPIV3, it may have potential as a target for the induction of immune regulation. We show NK cell mediated T cell cycle arrest associated with low doses of IL-2. Other groups have associated NK cell expansion with low dose IL-2, with subsequent beneficial effects in autoimmunity (Murphy 2012, Saadoun 2011, Zorn 2006, Soiffer 1994). The role of IL-2 in the modulation of NK cell mediated immune regulation will be further investigated in chapter 6.

Neutralizing antibody studies indicate that HN has a role in the inhibition of IL-2, the subsequent expansion of regulatory CD56+ NK cells and their induction of T cell cycle arrest. While we have demonstrated this using antibodies, it is important to revisit this using purified HN and F. The results of these studies will be discussed in chapter 6. These results represent the first association between HPIV3 HN protein, and the failure of T cell responses to this infection. This has important implications for the use of this viral protein as a vaccine candidate, as will be further discussed in the discussion section.

3 Role of IL-2 concentrations in dictating the mechanism by which NK cells regulate T cell proliferation

3.1 Introduction

It is regarded that IL-2 is imperative for T cell growth and proliferation (Gaffen and Liu 2004). Additionally, a lack of IL-2 has been associated with T cell suppression in some virus infections (Andrews *et al* 2001, Flamand *et al* 1995). Noone *et al* investigated IL-2 secretion from HPIV3 infected cocultures. A significant reduction in IL-2 production was observed in HPIV3 infected MLR cocultures compared to the other stimulated cocultures. However, T cell proliferation, which is inhibited during HPIV3 infection, was restored when exogenous IL-2 was added to cocultures of infected CD14+ cells and allogeneic mixed lymphocytes (Noone *et al* 2008). Paiardini *et al* demonstrated a similar effect on lymphocytes from HIV infected lymphocytes (Paiardini *et al* 2001). Thus, Noone *et al* concluded that this NK cell mediated inhibition of T cell proliferation was IL-2 dependent (Noone *et al* 2008). We have since demonstrated restoration of IL-2 production by using neutralizing antibodies to HN.

Expanding on their traditional role in the innate immune system, where they were considered "large granular lymphocytes" with cytotoxicity towards infected and tumor cells, an immune regulatory role for NK cells has emerged (Krzewski and Struminger 2008, Maghazachi 2004). Two main subtypes of NK cells have been identified in humans based on their expression of the surface marker CD56: CD56^{Bright} and CD56^{Dim}. It has been widely reported that CD56^{Dim} NK cells act cytotoxically on infected or tumor cells, while CD56^{Bright} NK cells are considered the more regulatory subset, due to their release of regulatory cytokines (Caligiuri 2008, Shereck et al 2007). However, definitions for this subset's function are ever changing.

During HPIV3 infection, blocking HN with neutralising antibodies restores IL-2 production. These antibodies also abrogated NK cell expansion and T cell cycle arrest. This is not the first IL-2 dependent regulatory report associated with these cells. $CD56^{Bright}$ NK cell mediated T cell inhibition has been demonstrated during Daclizumab therapy. Daclizumab is an IL-2R α antibody used in the treatment of multiple sclerosis (MS) (Martin *et al* 2010, Sheridan *et al* 2011, Bielikova *et al* 2006). Multiple sclerosis is a T-cell mediated

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autoimmune disease, with immune cells attacking the CNS and inducing damage to the neuronal myelin sheeth (Bielekova *et al* 2006). As the T cell IL-2R is mainly composed of IL-2R α , blocking this chain results in excess levels of IL-2 for NK stimulation. Authors suggest that this mimics high level IL-2 stimulation of NK cells, causing expansion, although a specific IL-2 concentration is unclear. These expanded NK cells induce CD4+ or CD8+ T cell death, with beneficial clinical effects (Martin *et al* 2010, Sheridan *et al* 2011, Bielikova *et al* 2006).

As well as inducing T cell cytotoxicity, it has also been reported that NK cells can inhibit T cell cycling. Trivedi *et al* reported that NK cells can do this via the upregulation of p21, a cell cycle inhibitor, which induces T cell cycle arrest. This regulation was not antigen –specific, and is a reversible phenomenon. Similarly, in a viral infection scenario, our group has demonstrated that CD56^{Bright} NK cell expansion occurs in association with low IL-2 concentrations during HPIV3 infection. These expanded CD56^{Bright} NK cells were associated with contact dependent T cell cycle arrest (Noone *et al* 2008). In addition, a recent study associated low dose IL-2 therapy with supressed inflammatory reactions in both graft versus host disease and viral induced vasculitis. Notably, while authors attributed this to Treg expansion, NK cell expansion was also observed here (Saadoun *et al* 2011).

As IL-2 expands CD56^{Bright} NK cells, which in some reports induce T cell death, and others T cell cycle arrest, we hypothesise that perhaps the concentration of IL-2 stimulating NK cell expansion, dictates the mode of T cell regulation observed. The aims of this chapter was to determine whether CD56+ (NK) cells expand at low and high concentrations of IL-2 and how this relates to CD3+ (T) cell expansion and proliferation. The role of specific NK cell subsets and the mode of NK cell mediated T cell regulation at various IL-2 concentrations was determined. We investigated the contact dependence of this mechanism, and aimed to learn more about stimulation through the NK IL-2Receptor, and finally question the role of T cell derived IL-2 in this phenomenon. We hypothesise that low dose IL-2 will mimic HPIV3 infection in these experiments.

3.1.A Chapter 3 aims:

The specific aims of the work described in this chapter were as follows:

-To determine the effect of IL-2 concentrations on NK populations levels, by investigating surface marker expression and cell proliferation

-To determine if IL-2 concentrations drive NK cell regulation of T cell populations by investigating surface marker expression and cell proliferation

-To investigate the specific NK subset involved in this regulatory mechanism

-To determine if IL-2 concentrations dictates mode of NK regulation of T cells

-To investigate the contact dependency of this regulatory mechanism

3.2 Materials and Methods

Table 3.2.1: Additional	reagents to table 2.2	.1 (Chapter	2) used in this study

Product	Catalogue #	Company	
Recombinant human IL-2	11340025	Immunotools, Griesothe,	
		Germany	
CD56+CD16- NK cell	130-092-661	Miltenyi Biotec, Gladbach,	
isolation kit		Germany	
CD56+CD16+ NK cell	130-092-660	Miltenyi Biotec, Gladbach,	
isolation kit		Germany	
0.2µm tissue culture inserts	136730	Nunc, Roskilde, Denmark	
(transwells)			
Leaf purified anti-human IL-	500313	Medical supply company,	
2		Dublin 15, Ireland	
Human IL-2 receptor alpha	MAB223	R&D systems Europe Ltd.,	
mAb 500µg		Oxon, UK	
Human IL-2 receptor beta	MAB224	R&D systems Europe Ltd,	
mAb 500µg		Oxon, UK	
Human Anti Ki67-PE 100	12-5699-42	Bioquote, York, UK	
tests			
Human Anti CD16-PE	12-0167-42	eBioscience, Hatfield, UK	
Human Anti-perforin-PE	12-9994-42	eBioscience, Hatfield, UK	
Human IFN-γ Duoset ELISA	DY285		
kit			
Human TGF- β Duoset	DY240	R&D systems Europe Ltd.,	
ELISA kit		Oxon, UK	
Human TNF-α Duoset	DY210	R&D systems Europe Ltd.,	
ELISA kit		Oxon, UK	
Human IL-1 β Duoset ELISA	DY201	R&D systems Europe Ltd.,	
kit		Oxon, UK	
Human IL-10 Duoset ELISA	DY217B	R&D systems Europe Ltd.,	
kıt		Oxon, UK	
Human IL-2 Duoset ELISA	DY202	R&D systems Europe Ltd.,	
kit		Oxon, UK	

3.2.1 IL-2 stimulation

The stock IL-2 was reconstituted as per manufacturer's guidelines (Miltenyi) and stored at - 80° C. For stimulations, doubling dilutions were carried out from 50-0.781µg/ml IL-2 using cell culture media as diluents. This means that 10µl of IL-2 would be 0, 0.781, 1.562, 3.125, 6.25, 12.5, 25 or 50ng/ml in the final volume of 1ml/well of cells.

3.2.2 Isolation of CD56^{Bright} and CD56^{Dim} NK cell subsets

The basis of MACs separation has already been described in section 2.2.5. Briefly, as well as previously described cell subsets, $CD56^{Bright}$ and $CD56^{Dim}$ NK cells were also purified from freshly cultured PBMCs. Isolation kits (Miltenyi Biotech) made use of the differential expression of CD16 on these cell subsets. For $CD56^{Bright}$ NK cells, CD56+CD16- cells were isolated, while for $CD56^{Dim}$ NK cell isolation, CD56+CD16+ cells were isolated. To determine cell purity of these subsets, cells were stained with FITC conjugated CD56 and PE conjugated CD16, and subsets were gated for upon acquisition. Purity was routinely >95% for both NK cell subsets (See appendix 2.1 and 2.2).

3.2.3 Cell cocultures

Both autologous and allogeneic, depending on experiment, CD3+ and CD56+ cells were isolated from freshly cultured PBMCs and cocultured at a 10:1 ratio respectively. In general, $1x10^{6}$ CD3+ cells were cultured in every well, with $1x10^{5}$ CD56+ cells. For CD56+ cell absent cultures, the same amount of CD3+ cells were used, and the volume was adjusted accordingly, giving a total volume of 1ml/well in all experimental setups. Isolated CD56^{Bright} or CD56^{Dim} NK cells were cocultures with CD3+ cells in the same way. Additionally, CD3+ cells were also cultured with CD56^{Bright} NK cells at a 100:1 ratio. These ratios are representative of physiological ratios of these cell subsets. Coculture incubations were usually 5 days in duration, unless stated otherwise.

3.2.4 Transwell cocultures

Transwell inserts function in separating cell types, allowing cytokines to pass between them, but preventing cell-cell contact. Beneath the insert in the lower chamber, $1x10^6$ CD3+ cells were cultured. CD56+ cells were cultured with IL-2 in the upper chamber of a 0.2µM pore insert in appropriate wells of a 24 well plate. As in the cocultures already described in section 3.3.2, a 1:10 ratio of CD56+:CD3+ cells was used to ensure comparable results. Cocultures were incubated for 5 days and then analysed as required.

3.2.5 IL-2 Neutralization

LeafTM Purified anti-IL-2 antibody (Biolegend UK) was used to block intracellular IL-2 during unstimulated cocultures. Company literature indicates that this antibody has been shown to neutralize IL-2 as described in literature, and its' blocking ability has been

confirmed by IL-2 ELISA. A concentration of 5µg/ml was used per coculture well for IL-2 neutralization. (http://www.biolegend.com/leaf-purified-anti-human-il-2%3Cna-le-azide-free-nale%3E-1350.html)

3.2.6 IL-2 Receptor (IL-2R) blocking

Purified polyclonal antibodies to both IL-2R α and IL-2R β (R and D systems) were used to block these receptor components prior to coculture. The neutralizing ability of these antibodies were measured by their ability to neutralize IL-2 induced proliferation in the N1186 human T cell line. A concentration of 1µg was used per 1ml culture well of cells.

(Stark et al 2005, Nolte-' Hoen et al 2006, <u>http://www.rndsystems.com/pdf/af223na.pdf</u> and http://www.rndsystems.com/pdf/af224na.pdf)

For receptor blocking, an isotype control antibody would usually be used. This would be considered important as NK cells express CD16. While we acknowledge this limit, we demonstrate that the CD16- NK cell subset are the regulators in these experiments, and induce all affects on T cells that we have observed thus far. As we saw different effects at different IL-2 concentrations for example in the presence of the same antibody, we felt it was clear that these cells were not being targeted for apoptosis.

3.2.7 Flow cytometry

Flow cytometry was carried out as described in section 2.2.10. The following fluorscenconjugated antibodies were used in this chapter: CD56-FITC, CD3-APC, Ki67-PE and CD16-PE. Isotype controls to these antibodies were also used. Annexin V-PI staining was also used as previously described (section 2.2.11) as was CFSE as described in this chapter.

3.2.8 ELISA

Detection of cytokines by ELISA was carried out as described in section 2.2.13. The following cytokines were detected by ELISA in this chapter: IFN- γ , TGF- β , IL-2, TNF- α , IL-10 and IL-13 (R+D Systems).

3.2.9 Statistical analysis

All coculture experiments represent normalized results for three donors in three independent experiments. Cyflogic and Facs Calibur software were used for FACs analysis, and plots were created using GraphPad Prism 5.Significance was calculated using Newman Keuls One way Anova: p<0.05; ** p<0.01; *** p<0.001.

3.3 Results

3.3.1 The percentage of MLs expression NK cell markers (CD56+CD3-) increases at both low and high IL-2 concentrations, and decreases at intermediate concentrations, inversely to MLs expressing T cell markers (CD3+CD56-)

We hypothesised that IL-2 expands NK cells at low and high IL-2 concentrations. This is based on our reports of CD56 upregulation at low IL-2 concentrations (Noone *et al* 2008), and many studies reporting expanded NK cells at high IL-2 (Martin *et al* 2010, Sheridan *et al* 2011, Bielikova *et al* 2006).

Mixed lymphocytes (ML) were isolated from PBMCs. These were divided into total MLs and CD56+ cell depleted MLs. Both ML and CD56+ cell depleted ML were stimulated with varying concentrations of IL-2 (described in section 3.2.1). Upon harvest, cells were stained for CD56 and CD3, as well as relevant isotypes, so that both CD56+CD3- (NK cells), and CD3+CD56- (T cells) cells could be examined by flow cytometry.

The percentage of cells in the total ML culture that were CD56+CD3- significantly increased at both low (0.781ng/ml) and high (12.5-50ng/ml) concentrations of IL-2 (Figure 3.3.1.b). Levels of these cells increased twofold compared to media samples. Notably, these cells make up 1% of mixed lymphocytes at media sample, consistent with the reported physiological level of CD56^{Bright} NK cells in vivo (Poli *et al* 2009, Caligiuri 2008).

In total ML cultures the percentage of CD3+CD56- cells significantly decrease at low (0.781ng/ml) and high (50ng/ml) IL-2 concentrations (Figure 3.3.1.a). However, when CD56+ cells were depleted from these cultures this decrease in CD3+CD56- cell numbers was abrogated, indicating that CD56+ cells cause a decrease in CD3+CD56- cells at low and high IL-2 concentrations, at which CD56+ cell markers are at their highest

Overall, these results suggest that CD56+ cells and CD3+ cells have an inverse relationship, which is dependent on IL-2 concentration. At low IL-2 concentrations, the percentage NK cell marker expressing MLs increase, with MLs expressing T cell markers decreasing only when NK cells are present. At intermediate IL-2 levels, MLs expressing NK markers decrease, the percentage of those expressing T cell markers increases. Finally at high IL-2

concentrations, the percentage of NK cell marker expressing MLs increases again, with the percentage of MLs expressing T cell markers decreasing. While this is not conculsive with regards to contraction and expansion of populations, as we would need absolute counts, we further investigate this inverse relationship, by investigating proliferation of these cell types, and investigating the mode of T cell contraction.



Figure 3.3.1. Percentage of CD56+CD3- MLs in response to IL-2 stimulation and the effect of these cells on the percentage of CD3+CD56- MLs. Freshly isolated MLs were divided into total ML and those from which CD56+ cells had been depleted. Both were stimulated with varying IL-2 concentrations (0-50ng/ml). After 5 days, cells were stained for CD56 and CD3 expression. The percentage of total ML or CD56+ cell depleted ML that were CD3+CD56- ML, were detected by flow cytometry (A). From the same acquisition, the percentage of CD56+CD3- cells could be detected (B). The percentage of cells in the target quadrant was then plotted. Representative flow cytometry graphs can be seen in Appendix 2.3. Results represent normalized data from 3 donors, in three separate experiments with each experiment in triplicate. Error bars represent standard deviation between samples. Significance was determined using the Newman Keuls One way Anova, and is as compared to media sample of the same data set. **p≤0.01 ***p≤0.001

3.3.2 CD56+ cells proliferate at high and low levels of IL-2

Having examined the percentage of CD56+CD3- and CD3+CD56- MLs in response to IL-2 concentrations, we wanted to investigate whether the decrease of CD3+CD56- MLs in the presence of CD56+ cells was reflected in cell proliferation profiles. Both CD56+ and CD3+ cells were isolated from freshly isolated PBMCs. To track proliferation, CFSE was incorporated into CD56+ cells prior to CD3+ cell coculture and IL-2 stimulation. 1x10⁶ CD3+ cells were cultured with CD56+ cells in keeping with the previously described 10:1 ratio. Upon flow cytometry acquisition at day 5, the proportion of CFSE+ cells in the "low CFSE-FITC" peak were gated and expressed as a percentage of total CD56+ cells. This served to compare proliferating CD56+ cells with the surface marker expression observed in figure 3.3.1.a to determine if the increase in expression of these surface markers at low and high IL-2 concentrations was due to cell proliferation as opposed to surface marker upregulation.

Similar to the percentage of CD56+CD3- MLs previously observed profiles in Figure 3.3.1, CD56+ cell proliferation significantly increased in response to both low (0.781-1.562ng/ml) and high (25-50ng/ml) concentrations of IL-2, with proliferation returning to levels similar to media samples at intermediate IL-2 concentrations (3.125-12.5ng/ml) (Figure 3.3.2.a).

This proliferative data reflects surface marker results (figure 3.3.1). CD56+ cells expand both at low and high IL-2 concentrations. The effect of these expanded CD56+ cells on CD3+ cell proliferation will be further investigated in this study.

CD56+CD3- Cell Proliferation



Figure 3.3.2: CD56+ cell proliferative response to IL-2 stimulation Both CD56+ and CD3+ cells were isolated from freshly isolated PBMCs. CFSE was incorporated into CD56+ cells. CD56+ cells were then cultured with CD3+ cells, and stimulated with concentrations of IL-2. Cells were harvested at day 5, and CD56+ cells in the "low CFSE peak" of the acquired histogram were gated and plotted as a percentage of total CFSE+ cells (CD56+ cells). Results represent normalized data from 3 donors. Representative flow cytometry histograms for this experiment can be seen in appendix 2.4. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One way Anova and is as compared to media sample of the same data set. **p ≤ 0.01 ***p ≤ 0.001 .

3.3.3 CD56+ cells inhibit CD3+ cell proliferation at low and high IL-2 concentrations after 5 days.

In cultures with CD56+ cells, staining with the proliferation maker Ki67 was initially used to determine CD3+ cell proliferation (Appendix 2.5). Due to discrepancies between this marker and previously published work, in addition to reports that this marker is only semiquantitative, and reported issues at low levels of proliferation, we decided to use CFSE incorporation to determine CD3+ cell proliferation (Shedlock *et al* 2010). As only one time point had been examined thus far (Day 5), we also took this opportunity to examine several time points, to ensure that Day 5 was optimum for future cocultures of the same type.

Both CD3+ and CD56+ cells were isolated from freshly isolated PBMCs, and CFSE was incorporated into CD3+ cells. These CD3+ cells were cultures both alone, and in the presence of CD56+ cells, at various IL-2 concentrations. Upon harvest and acquisition, cells in the "low CFSE-FITC" peak were gated for, and expressed as a percentage of total CFSE+ cells (CD3+ cells). On Day 3, no real change in CD3+ cell proliferation was observed under any IL-2 concentration, in either the presence or absence of CD56+ cells (Figure 3.3.3.A). From harvest on Day 4 however, the presence of CD56+ cells causes a decrease in CD3+ cell proliferation (Figure 3.3.3.B). In keeping with previous results (Figures 3.3.1 and 3.3.2) this regulation occurs to a significantly larger extent at low (0.781ng/ml) and high (50ng/ml) concentrations of IL-2 (Figure 3.3.3.C). This evens off by Day 7, although the presence of CD56+ cells still causes slightly diminished CD3+cell proliferation (Figure 3.3.3.D).

This is consistent with data published by Noone *et al*, where the H3 thymidine method was used for detection of CD3+ cell proliferation. Furthermore, the clearest regulatory results were observed at Day 5, the timepoint used for the vast majority of experiments described. Notably, as CFSE will be used as a marker for both CD56+ and CD3+ cell proliferation henceforth, this means that two cocultures will need to be set up: CD56+ cell-CFSE with CD3+ cells, and CD3+ cell-CFSE with CD56+ cells. While this means results for both cell types would not be from the same coculture well, it was always done using cells from the same donor, under the same experimental conditions, and results were always consistent between 3 separate donors.



Figure 3.3.3: Proliferative responses of CD3+ cells, in the presence and absence of autologous CD56+ cells, as detected by CFSE incorporation at various timepoints. Both CD56+ and CD3+ cells were isolated from freshly isolated PBMCs. CFSE was incorporated into CD3+ cells, which were cultured either alone, or cocultured with CD56+ cells, and stimulated with varying concentrations of IL-2. Cells were harvested at Day 3 (A), Day 4 (B), Day 5 (C), and Day 7 (D). Upon acquisition, cells in the "low CFSE peak" of the acquired histogram were gated and plotted as a percentage of total CFSE+/CD3+ cells. Representative flow cytometer graphs can be seen in appendix 2.6. Results represent normalized data from 3 donors. Error bars represent standard deviation between samples.Significance was determined using Newman Keuls One Way ANova and is as compared to media sample of the same data set. $*P \le 0.05 **p \le 0.01 ***P \le 0.001$

3.3.3.ii Further investigation of T cell proliferation, and apoptosis in experimental setup

Given that the majority of even unstimulated CD3+ cells appeared to have proliferated by day 5 in our experimental setup, we provide additional experimental data to add confidence to these results.

As previously described, CFSE was incorporated into CD3+ cells which were cultured either alone, or stimulated with 50ng/ml IL-2 or PMA and A-CD3 as a strong positive. The percentage of CD3+ cells in the "low CFSE peak" was determined at T0 (i), and accordingly from days1-5 (ii-vi), with 5 days being the incubation period chosen for the vast majority of experiments described in this project.

Firstly we provide FSC-SSC plots for each time point (A). For all time points these scatter plots seem representative of normal lymphocyte profiles. There are minimal cells outside the normal lymphocyte profile, which was gated for prior to fluorescent analysis of samples. Notably the number of cells skewing from this profile does increase by Day 4 and 5 (Av, vi), which is to be expected after this time period in incubation, especially in unstimulated samples. However this does not seem to be of a level such as to affect the overall population. Notably, the live gating strategy represented in figure 3.3.3.ii.A applies to proliferation or staining data. A larger gating strategy, taking both live and dead cells into account, was used for apoptosis analysis. A representation of apoptosis gating is shown in figure 3.3.3.ii.Bvii.

To add further confidence that we are in fact observing live cells in our experimental setup, the above was repeated, this time staining cells for Annexin-v and PI so apoptosis in these cultures could be determined at the same timepoints (B). At all timepoints the percentage of cells in late apoptosis were minimal. The level of apoptosis did increase with time, and was slightly higher with time in media samples. However, with the highest percentage of dead cells still only accounting for ~15% cells in culture (Bvi), we were confident that we were seeing the proliferation of live cells in these cultures.

Finally, actual CD3+ cell proliferation at these timepoints was analysed by CFSE incorporation (C). Upon acquisition, cells in the "low CFSE" peak were gated for, and expressed as a percentage of total CD3+ cells. At T0 (Ci), there were minimal cells in this peak (1-2%). The percentage of proliferated T cells increased with time in media, and stimulated cells. At later timepoints proliferated T cell levels of ~80% were observed,

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reflective of experiments reported thus far in this project. While proliferation of T cells in unstimulated cultures is an unconventional observation, it is clearly the case here, give CFSE, FSC-SSC and Apoptotic assay data. Inhibited proliferation observed here when stimulated with 50ngIL-2 is reflective of cell surface marker data, and MTS assay data, reported in this project. Additionally, it is the relative change to CD3 proliferation caused by NKs activated by low or high IL-2, or HPIV3 infection, that is important to our findings, and the integrity of this relative change is apparent across donors and multiple detection methods.



Figure 3.3.3.ii.A: FSC-SSC of T cells in response to 50ng/ml IL-2 or PMA+A-CD3 over a 5 day time course. T cells were isolated cultured in media alone, or were simulated with either 50ng/ml IL-2 or PMA+A-CD3, and cells were harvested at T0 (Ai), Day 1 (Aii), Day 2 (Aiii), Day 3 (Aiv), Day 4 (Av), Day 5 (Avi). At each time point FSC-SSC were analysed upon flow cyometry acquisition and assessed for normality of lymphocyte profiles. Results shown represent one triplicate from one donor.



Figure 3.3.3.ii.B: Apoptotic profiles of T cells in response to 50ng/ml IL-2 or PMA+A-CD3 over a 5 day time course. T cells were isolated cultured in media alone, or were simulated with either 50ng/ml IL-2 or PMA+A-CD3, and cells were harvested at T0 (Bi),Day 1 (Bii), Day 2 (Biii), Day 3 (Biv), Day 4 (Bv), Day 5 (Bvi). Cells were stained with Annexin-V and PI as previously described, and the percentage of cells in the upper-right quadrant displayed. Results shown represent one triplicate from one donor. The gating strategy for apoptotic analysis is also presented here (Bvii)



Cvii:

Sample:	Cell Count/ml
Media T0	10,000/ml (as seeded)
Media Day 1	11,316/ml
Media Day 2	14.575/ml
Media Day 3	25,496/ml
Media Day 4	46,454/ml
Media Day 5	65,865/ml

Figure 3.3.3.ii.C: Proliferation of T cells in response to 50ng/ml IL-2 or PMA+A-CD3 over a 5 day time course. T cells were isolated, into which CFSE was incorporated as previously described. Cells were harvested and analysed by flow cytometry at T0 (Ci), Day 1 (Cii), Day 2 (Ciii), Day 3 (Civ), Day 4 (Cv), Day 5 (Cvi). Upon acquisition cells in the low CFSE peak (gated by CFSE- cells) were determined and are displayed on histograms. Results

shown represent one triplicate from one donor. Representative cell counts per ml for each time point are presented here (Cvii), corresponding to media samples of each timepoint

3.3.4 CD56^{Bright}, not CD56^{Dim} NK marker expression mimics that of the overall CD56+ cell population.

Thus far, to investigate IL-2 mediated NK cell expansion, or regulation of CD3+ cells, CD56 and CD3 markers have been used. The cell population in the CD56+CD3- quadrant upon acquisition have been gated as CD56+ NK cells. CD56^{Bright} NK cells have shown prevalence in immune regulation in current literature as previously discussed. It remained to be determined whether CD56^{Bright} NK cells expand in response to low and high levels of IL-2. CD56^{Bright} are the more regulatory NK cell subset, with recent data suggesting a role for immune regulation of this subset, while CD56^{Dim} NK cells are the more traditional cytotoxic subset. CD16 expression can be used to differentiate between both subsets. Literature has described CD56^{Bright} NK cells as CD56+CD3-CD16-, while CD56^{Dim} NK cells are described as CD56+CD3-CD16+ (Poli et al 2009), and as this is widely reported, we based our experiments on these markers. Additionally, traditionally in our group, CD16+CD56+ cells, tend to be in the lower expression region of CD56+ cells. In later experiments, these subsets are also isolated based on their CD16 expression, based on recommendations by Miltenyi.

Freshly isolated PBMCs were stimulated with various concentrations of IL-2 as previously described. At Day 5, cells were stained with fluorescent conjugated antibodies for CD56, CD3 and CD16. Those cells that were CD56+CD3-CD16-, or CD56+CD3-CD16+ were determined by gating for CD56+CD3- cells and observing their CD16 expression. The percentage of CD56+CD3-CD16- cells, or CD56^{Bright} NK cells increased at both low (0.781ng/ml) and high (50ng/ml) concentrations of IL-2, but not intermediate levels (Figure 3.3.4). However, expression of CD56+CD3-CD16+ cells, or CD56^{Dim} NK cells did not alter with IL-2 concentrations (figure 3.3.4). While not all CD56^{Dim} NK cells express CD16, we found this system sufficient for the purpose of this experiment. By flow cytometry, we have seen that almost all our CD56^{Dim} NK cells do. Additionally, in this study we show that the CD16- NK subset are the regulatory cells at play here.

These results suggest that the response of CD56+CD3- cells observed previously (section 3.3.1) was in that of CD56^{Bright} and not CD56^{Dim} NK cell populations. It is also notable that in

media samples, $CD56^{Bright}$ NK cells were detectable as ~1% of the total ML population, which is the reported physiological frequency of $CD56^{Bright}$ NK cells. Similarly, $CD56^{Dim}$ NK cells were detectable at 8-9% of the ML population, similar to their 9-10% physiological frequency reported in literature (Poli *et al* 2009, Caligiuri 2008), adding strength to the suggestion that we are observing these NK cell subsets. To further investigate the role of these two NK subsets here, we go on to isolate these cell types in later experiments.



Figure 3.3.4: CD56+CD3-CD16- and CD56+CD3-CD16+ cell expression in response to IL-2 stimulation. Freshly isolated MLs were stimulated with varying IL-2 concentrations. After 5 days, upon harvest, cells were stained for CD56, CD3 and CD16. CD56+CD3- cells were gated and of these, the percentage that were CD16+ or CD16- were determined. For representative flow cytometry data see appendix 2.7. Results represent normalized data from 3 donors, from 3 separate experiments in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One Way Anova and is as compared to media sample of the same data set. $p \le 0.05 \ p \le 0.01$

3.3.5 CD56^{Bright}, not CD56^{Dim} NK cells are unequivocally responsible for inhibited CD3+ cell proliferation at high and low IL-2 concentrations.

Having examined the levels of cells expressing markers for $CD56^{Bright}$ and $CD56^{Dim}$ NK cells (Figure 3.3.4), it was important to investigate whether $CD56^{Bright}$ expansion at low and high IL-2 concentrations was reflected in proliferation profiles. Additionally, we wanted to investigate the effect of either NK cell subset on CD3+ cell proliferation.

From freshly isolated PBMCs, CD56^{Bright} and CD56^{Dim} NK cells were isolated using isolation kits for these subsets (Miltenyi Biotech UK), which isolate CD56+CD16- and CD56+CD16+ cells respectively. Autologous CD3+ cells were also isolated. Into either NK subset, CFSE was incorporated, before coculture of either subset with CD3+ cells and stimulation with varying IL-2 concentrations. Additionally, in a separate experimental setup, CFSE was incorporated into CD3+ cells before coculture with either CD56^{Bright} or CD56^{Dim} NK cells and IL-2 stimulation. Upon harvest and acquisition, cells in the "low CFSE-FITC" peak were gated for, and expressed as a percentage of total cells of that subset.

As with subset expression results observed in section 3.3.4, CD56^{bright} NK cell proliferation significantly increased at both low (0.781-1.562ng/ml) and high (25-50ng/ml) concentrations of IL-2. In contrast, the percentage of proliferated CD56^{Dim} NK cells did not change under any IL-2 conditions (Figure 3.3.5.A). Additionally, proliferation of CD3+ cells was inhibited in the presence of CD56^{Bright} NK cells at low (0.781-3.125ng/ml) and high (50ng/ml) concentrations of IL-2, but not in the presence of CD56^{Dim} NK cells (Figure 3.3.5.B). This confirms that the NK cell mediated inhibited CD3+ cell proliferation observed previously (Figure 3.3.3.C) was in fact induced by expanded CD56^{Bright} NK cells.

Results thus far indicate that at both low and high IL-2 concentrations, $CD56^{Bright}$ NK cells, but not $CD56^{Dim}$ NK cells expand, inducing inhibited CD3+ cell proliferation. Of interest, both subsets were cultured here at a 1:10 ratio with CD3+ cells. However, the physiological ratio of $CD56^{Bright}$ NK cells is 1:100. Therefor, this experiment was repeated with CD3+(CFSE) cells cultured with $CD56^{Bright}$ NK cells at this ratio. Overall, CD3+ cell inhibition by $CD56^{Bright}$ NK cells was enhanced at this lower ratio of NKs: T cells (1:100), and again, this inhibition occurred at low and high IL-2 concentrations (Figure 3.3.5.C). This allays concerns that $CD56^{Bright}$ saturation was a factor in CD3+ cell inhibition. While T cells



appear to be proliferating even in the absence of stimulus, this issue has been previously addressed and further investigated (section 3.3.3.ii).

Figure 3.3.5: CD56^{Bright} and CD56^{Dim} NK cell proliferative response to IL-2 and their effect on CD3+ cell proliferation. CD56^{Bright} and CD56^{Dim} NK cells were isolated from freshly isolated PBMCs, as well as CD3+ cells. CFSE was incorporated into each of these lymphocyte subsets and cocultures set up as follows: CD56^{Bright}(CFSE)+CD3+ cells, CD56^{Dim} (CFSE)+CD3+ cells (A), CD3+(CFSE)+CD56^{Bright}, CD3+(CFSE)+CD56^{Dim} (B), or finally CD3+(CFSE)+CD56^{Bright} was repeated at a 100:1 ratio of Tcells:NKs. Cells were harvested at day 5 and upon acquisition; cells in the "low CFSE peak" were gated and expressed as a percentage of total CFSE-FITC cells. Results represent normalized data from 3 donors from 3 separate experiments in triplicate. Error bars represent standard deviation between samples. For representative flow cytometry results see Appendix 2.8. Significance was determined using the Newman Keuls One Way Anova and is as compared to media samples of the same data set. **p≤0.01 ***p≤0.001

3.3.6 CD56+ cells increase the percentage of CD3+ cells maintained in the G0/G1 phase of cell cycle at low IL-2 concentrations and increase the percentage of CD3+ cell in apoptosis at high IL-2 concentrations.

We have demonstrated that CD56+ cells expand and inhibit CD3+ cell proliferation at low and high IL-2 concentrations. However, whether the concentration of IL-2 dictates the mode of regulation (ie, cell cycle arrest or apoptosis) had yet to be investigated. Additionally, we have associated low IL-2 with NK mediated T cell cycle arrest during HPIV3 infection (Noone *et al* 2008). It was necessary to determine the level of NK induced cell cycling and apoptosis in CD3+ cells at low or high dose IL-2.

From freshly isolated PBMCs, MLs and those from which CD56+ cells had been depleted, were isolated and stimulated with various IL-2 concentrations. Upon harvest, cells were stained for CD3, as well as Annexin-V-PI staining. From the CD3+ PI acquisition histogram, different stages of cell cycle could be gated and expressed as a percentage of CD3+ cells in each stage of cell cycle. Similarly, CD3+ cells were plotted as Annexin-V vs PI, and those in the Annexin-V+PI+ quadrant were plotted as a percentage of total CD3+ cells. At low IL-2 concentrations (0.781-3.125ng/ml), the percentage of CD3+ cells arrested in the G0/G1 phase of cell cycle significantly increased in the presence of CD56+ cells (Figure 3.3.6.A). Additionally, at high IL-2 concentrations (50ng/ml), a significant increase in CD3+ cell apoptosis occurs in the presence of CD56+ cells (Figure 3.3.6.B). These results are consistent with proliferative results. Importantly, this regulation was not observed in CD56+ cell depleted cultures.

From these results, we can conclude that at low IL-2 concentrations, $CD56^{Bright}$ NK cells expand and induce CD3+ cell cycle arrest. At intermediate concentrations of IL-2, $CD56^{Bright}$ NK cell populations contract, and CD3+ cell proliferation occurs. Finally, at high IL-2 concentrations, $CD56^{Bright}$ NK cells expand again, this time inducing CD3+ cell death. Therefore, this confirms the hypothesis that IL-2 concentration dictates the mode of regulation exerted on T cells by $CD56^{Bright}$ NK cells.



Figure 3.3.6: Cell cycle and apoptosis analysis of CD3+ cells, in the presence or absence of CD56+ cells, in response to IL-2. Both total ML and CD56+ cell depleted MLs were isolated. These MLs were stimulated with IL-2 concentrations. After 5 days, upon harvest, cells were stained for CD3, Annexin-V and PI. Stages of cell cycle were gated in the PI histogram of CD3+ cells, and the percentage of cells in each stage expressed as a percentage of total CD3+ cells (A). CD3+ cells were plotted as Annexin-V vs PI dot plots and those in the Annexin V+PI+ quadrant plotted as a percentage of total CD3+ cells (B). Results represent normalized data from 3. For representative flow cytometry data, see Appendix 2.9. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One way Anova and is compared to media samples. **p \leq 0.01 ***p \leq 0.001.

3.3.7 CD56+cell perform expression increases at high IL-2 concentrations.

Having determined that CD56+ cells induce CD3+ cell death at high IL-2 concentrations (Figure 3.3.6.B), it was also of interest to investigate if other physiological indicators correspond to the increased apoptosis observed at high IL-2 concentrations. To this end, we examined perforin expression by NK cells at varying IL-2 concentrations. Perforin is a cytolytic protein produced by NK cells. It elicits its effect by forming a pore in the target cell's plasma membrane, inducing cell death (Vaskioboinik *et al* 2010).

Freshly isolated PBMCs were stimulated with varying IL-2 concentrations. Upon harvest at day 5, cells were stained with fluorescent conjugated antibodies to both CD56 and perforin. Upon acquisition at day 5, the percentage of CD56+perforin+ cells (UR) was determined and expressed as a percentage of total CD56+ cells (UR+UL). At the upper range of IL-2 concentrations (12.5-50ng/ml) the percentage of CD56+ cells expressing perforin is significantly increased (Figure 3.3.7). This increase was twofold compared to media samples at 50ng/ml IL-2, the concentration at which CD3+ cell apoptosis was observed (Figure 3.3.6.B).

These results give further support that CD56+ cells induce target or CD3+ cell apoptosis at high IL-2 concentrations, as we have shown death in the target (CD3+ cells) and increased expression of cytolytic protein by NK cells. Notably, perforin is not the only cytolytic molecule produced by NK cells. Granzymes should also be taken into account, but as we observed perforin coincidently as the first cytolytic protein we examined this was a notable experiment. This also corresponds to increased CD107a, an overall marker for NK cytotoxicity, which has recently been observed in our group. Additionally, the mode of T cell death is relatively unimportant to the overall story, the key finding being that at high IL-2 concentrations NK cells opt for the T cell death mode of inhibition. Additionally, CD56^{Dim} are usually described as the cytolytic NK cell subset. However, as in introduction, recent reports have suggested CD56^{Bright} expansion at high IL-2, with beneficial effects in autoimmune conditions, due to T cell death. So while perforin expression is unconventional to the traditional role for CD56^{Bright} NK cells, it actually supports more recent clinical reports.



Figure 3.3.7: Perforin expression by CD56+ cells in response to various IL-2 concentrations. Freshly isolated MLs were stimulated with various IL-2 concentrations. Upon harvest at day 5, cells were stained for CD56 and perforin expression. When acquired, the percentage of CD56+perforin+ cells were expressed as a percentage of total CD56+ cells. Results represent normalized data from 3 donors in 3 separate experiments in triplicate.For representative flow cytometry data see Appendix 2.10. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One Way Anove and is as compared to media sample. *** $p \le 0.001$.

3.3.8 CD56+ cell regulation of CD3+ cells is contact dependent.

Our group has previously demonstrated that CD56+ cell regulation of CD3+ cell proliferation during HPIV3 infection was contact dependent (Noone *et al* 2008). To assess contact dependence of NK mediated T cell regulation in response to low and high IL-2 concentrations, CD3+ cells were separated from CD56+ cells by transwell inserts

From freshly isolated PBMCs, CD3+ and CD56+ cells were isolated. Using transwell inserts, IL-2 stimulated CD56+ cells were separated from CD3+ cells using 0.2µM pore inserts for separation. Cocultures were carried out at the same ratio of cells as previously described. Cocultures without transwell inserts were also used as a comparative control. Upon harvest at day 5, Annexin-V and PI staining was carried out as described previously (Section 3.3.6). When CD56+ cells and CD3+ cells were separated, CD56+ cell regulation of CD3+ cell proliferation was abrogated. At low levels of IL-2, CD3+ cell cycle arrest did not occur when transwell inserts were used (Figure 3.3.8.a). Similarly, at high IL-2 concentrations, CD3+ cell death did not occur when CD3+ cells and CD56+ cells and CD56+ cells were separated (Figure 3.3.8.B). Transwell inserts abrogated CD56+ cell regulation of CD3+ cells.

These results are in alignment with HPIV3 data. CD56+ cells regulate CD3+ cell cycle in a contact dependent mechanism, at low IL-2 concentrations. Additionally, these results indicate that CD56+ cells mediate CD3+ cell death at high concentrations of IL-2 in a contact dependent mechanism. Of interest however, CD56^{Bright} NK cells are reported to induce their regulatory effect via the release of regulatory cytokines rather than contact dependence (Caligiuri 2008).



Figure 3.3.8: Cell cycle and apoptosis analysis of CD3+ cells, in the presence or separation (//) of CD56+ cells. Both CD56+ and CD3+ cells were isolated from freshly isolated PBMCs. CD3+ cells were cultured with CD56+ cells and stimulated with varying IL-2 concentrations, or separated from IL-2 stimulated CD56+ cells by a 0.2µm pore insert. After 5 days, cells were stained for Annexin-V and PI. Stages of cell cycle were gated in the PI histogram of CD3+ cells, and the percentage of cells in each stage expressed as a percentage of total CD3+ cells acquired (A). CD3+ cells were plotted as Annexin-V vs PI and those in the Annexin V+PI+ quadrant plotted as a percentage of total CD3+ cells (B). Results represent normalized data from 3 donors. For representative flow cytometry data see Appendix 2.11. Error bars represent standard devation between samples. Significance was determined using Newman Keuls One way Anova and is as compared to media. ***p≤0.001.

3.3.9 CD56+ cell cytokine production is not altered by IL-2 stimulation.

Results thus far indicate that CD56+ cells regulate CD3+ cells at low and high IL-2 concentrations via a contact dependent mechanism (Figure 3.3.8). The CD56^{Bright} NK cell subset are responsible for this CD3+ cell regulation (Figure 3.3.5). As this NK subset are usually associated with immunoregulation by cytokine release, it was important to determine whether regulatory cytokines were not involved (Caligiuri 2008). The key cytokines involved in CD56^{Bright} mediated immune regulation were examined. IFN γ , as previously discussed, is important for immune regulation and antiviral responses. TGF- β is important for driving proliferation, can induce apoptosis, and regulates cell cycle via p15 and p21 regulation. IL-2 is a reported T cell growth factor while TNF α is involved in systemic inflammation. Finally, IL-10 is an anti-inflammatory cytokine, while IL-13 is involved in allergic reaction.

To investigate this, ELISAs were performed on supernatants of IL-2 MLs. In addition, CD56+ cell depleted MLs were also stimulated to establish whether the cytokines were actually secreted due to the presence of CD56+ cells. Cytokines known to be produced by NK cells were examined: IFN- γ (Figure 3.3.9.A), TGF- β (Figure 3.3.9.B), TNF- α (Figure 3.3.9.C), IL-10 (Figure 3.3.9.D), and IL-13 (Figure 3.3.9.E). For the majority of these cytokines, no real change in cytokine release occurred under any IL-2 conditions.

These results confirm that CD56+ cells do not regulate CD3+ cells by cytokine release at low and high IL-2 concentrations, and further support a contact dependent mechanism. IL-2 stimulation does seem to induce a decreased IFN- γ production by CD56+ cells, especially at intermediate IL-2 concentrations. While at odds with literature, (Fehniger et al 2003, Krzewstaik et al 2008) this may partly be explained by the fact that we have demonstrated contracting CD56+ cell populations at intermediate IL-2 concentrations. This suggests less potential for IFN- γ production. While we acknowledge that it is unclear if cytokines detected are from T cells or NK cells, as there was no change to cytokine levels this was irrelevant. If we had observed changes in a particular cytokine in response to IL-2, then we would perhaps have further investigated using intracellular staining. Additionally, as we have already shown that NK regulation of T cells is contact dependent, we expected no changes here.

TGF-b



Figure 3.3.9: NK associated cytokine secretion profile of IL-2 stimulated MLs. Freshly isolated MLs were stimulated with various IL-2 concentrations. At day 5, supernatants were harvested and used for examination by ELISA. IFN γ (A), TGF- β (B), TNF- α (C), IL-10 (D), and IL-13 (E) secretion was determined by ELISA. Results represent normalized data from 3 donors, from 3 separate experiments in triplicate. Error bars represent standard deviation between samples

3.3.10 CD3+ cells require the presence of CD56+ cells for IL-2 production

Traditionally, IL-2 has been described as a T cell autocrine growth factor (Gaffen and Liu 2004). We report that this cytokine drives NK cell regulation of T cells at low and high doses. Based on the importance of this finding we wanted to further investigate CD3+ derived IL-2 in response to the presence of CD56+ cells.

Both CD3+ and CD56+ cells were isolated from freshly cultured PBMCs. CD3+ cells were either cultured alone, or cocultured with CD56+ cells. At day 5, supernatants were cultured and used to test for the presence of IL-2. CD56+ IL-2 production was also undetectable when cultured alone (data not show). In the presence of CD56+ cells, the production of IL-2 by CD3+ cells increased almost threefold, compared to when CD3+ cells cultured alone (Figure 3.3.10).

From these results it can be suggested that the traditional role for IL-2, as a driver of T cell proliferation is not true under certain conditions. They suggest that CD3+ cells produce IL-2 as a signal for CD56+ cells, to report their current immune condition. As we have shown previously, this T cell derived IL-2 dictates NK regulation of T cells, depending on its' concentrations. However, as we use coculture here, we cannot decisively say that T cells are producing IL-2 in response to NK cells, as IL-2 may be produced by either cell type. In future experiments intracellular staining could be used. However we still feel these results give an interesting finding, with potential for future investigation.



Figure 3.3.10: IL-2 production by CD3+ cells in the presence or absence of CD56+ cells. Both CD56+ and CD3+ cells were isolated from freshly cultured PBMCs. CD3+ cells were cultured either alone, or with CD56+ cells. On day 5, supernatants were harvested and used for examination by ELISA. Error bars represent standard deviation between samples. Significance was determined using Newman keuls One Way Anova and is as compared to CD3+ cells alone. *p \leq 0.05.

3.3.11 Anti-IL-2 abrogates CD56+ cell responses to CD3+ cells

Having identified that IL-2 production is increased T cell cultures where CD56+ cells are present, compared to when cultured alone, we further investigated the requirement of CD56+ cells for CD3+ cell IL-2 production. Both CD56+ and CD3+ cells were isolated from freshly cultured PBMCs. In a defined number of CD3+ cells, CFSE was incorporated. These CD3+(CFSE) cells were cultured alone, with Anti-IL-2, with autologous CD56+ cells, and with both Anti-IL2 and CD56+ cells. Similarly, in a defined number of CD56+ cells were cultured alone, with Anti-IL-2, with autologous CD56+ cells, CFSE was incorporated, and counted for coculture. These CD56+(CFSE) cells were cultured alone, with Anti-IL-2, with autologous CD3+ cells and with both Anti-IL-2, with autologous CD3+ cells and with both Anti-IL-2, with autologous CD3+ cells and with both Anti-IL-2 and CD3+ cells. Upon harvest and acquisition at Day 5, cells in the "low CFSE-FITC" peak were gated, and expressed as a percentage of total CD3+ or CD56+ cells.

Although IL-2 is considered a growth factor for T cells, the presence of Anti-IL-2 had no real effect on CD3+ cell proliferation when cultured alone. As usual, CD56+ cells inhibited CD3+ cell proliferation even in this unstimulated environment. However, in the presence of both Anti-IL-2 and CD56+ cells, CD3+ cell proliferation was restored (Figure 3.3.11.A). The proliferation of CD56+ cells was not altered in the presence of Anti-IL-2 alone. As expected however, the presence of CD3+ cells induces increased CD56+ cell proliferation, which decreases in the presence of Anti-IL-2 (Figure 3.3.11.B). These results indicate that CD3+ cell derived IL-2 induces CD56+ cell proliferation, perhaps as a mechanism of self regulation.

From these results, where cells were not stimulated with excess IL-2, it seems this cytokine still plays a role in CD56+ cell regulation of CD3+ cells. Results thus far indicate that in the presence of CD56+ cells CD3+ cells produce IL-2 causing expanded CD56+ cell populations and conversely, inhibited CD3+ cell proliferation.



Figure 3.3.11: Proliferative responses of CD3+ and CD56+ in response to coculture and Anti-IL-2. Both CD56+ and CD3+ cells were isolated from freshly cultured PBMCs. From both cell types, a portion was separated, into which CFSE was incorporated. CD3+(CFSE) cells were cultured alone, or with CD56+ cells (A). Cells were either cultured with Anti-IL-2 or no antibody. Similarly, CD56+(CFSE) cells were cultured either alone, or with CD3+ cells (B). Cells were either cultured with Anti-IL-2 or No Antibody. Upon acquisition on Day 5, cells in the "low CFSE" peak of the acquired histogram were gated and plotted as a percentage of total CFSE+ cells.. Results represent normalized data from 3 donors from 3 separate experiments in triplicate. Error bars represent standard deviation between samples. For representative flow cytometry results see appendix 2.12. Significance was determined using Newman keuls One way Anova and is as compared to media samples of the same data set. $*\leq0.05$ *** ≤0.001
3.3.12 Stimulation of CD56+ cell IL-2Rβ, but not IL-2Rα, is essential for CD56+ cell regulation of CD3+ cell proliferation.

IL-2R α blocking antibodies used in therapy paradoxically result in higher concentrations of residual IL-2 for NK cell activation. This is due to the high expression of IL-2R α by T cell IL-2, while NK cell IL-2R is mostly composed of IL-2R β . This hypothesis may explain activation of CD56^{Bright} cells during Anti-IL-2R α therapy (Li 2006). As T cell cytotoxicity has been associated with this therapy, we can presume based on our own results (Section 3.3.6) that CD56+ cells are being activated at the upper range of IL-2 concentrations here (Martin *et al* 2010).

Both CD56+ and CD3+ cells were isolated from freshly cultured PBMCs. To track proliferation, CFSE was incorporated into CD3+ cells. Before coculture, CD56+ cells were incubated either alone, with Anti-IL-2R α , Anti-IL-2 R β or both of these antibodies. Cells were cocultured and treated with usual concentrations of IL-2. Upon harvest and acquisition at day 5, cells in the "low CFSE-FITC" peak were gated for and expressed as a percentage of total CD3+ cells. When IL-2R α was blocked, CD56+ cells still inhibited CD3+ cell proliferation at low and high IL-2 concentrations. This was not the case when IL-2R β was blocked, as CD3+ cells proliferation was restored at all IL-2 concentrations in these cultures, as well as cultures where both antibodies were used (Figure 3.3.12).

These results support those published by Martin *et al.* Activation via IL-2R α on CD56+ cells at low or high IL-2 concentrations induces CD56+ cell regulation of CD3+ cells. As treatment of IL-2R α on CD3+ cells allows a high availability of IL-2 for CD56+ cells, the cytotoxic mechanism we observe at high IL-2 concentrations is mimiced. Notably, it is difficult to mimick clinical data ex vivo with regards to determining the exact concentration of cytokine or antibody that reaches patient cells. For this reason we used a wide range of IL-2 concentrations upon setting up these experiments, and our low IL-2 dose seems to reflect clinical data with regards to immunological outcome. Notably, we draw these conclusions by our own dilution curve used. Daclizumab studies attribute expansion of NK cells to the high IL-2 levels available to NK cells during treatment. However, they do not quantify this cytokine stimulating NK cells. We chose our concentrations independently of these studies. Coincidently, we saw T cell death and NK expansion at high IL-2, as reported in these papers. Cell cycle arrest corresponded to low IL-2 as supported by our viral data.



Figure 3.3.12: Proliferative responses of CD3+ cells cultured with CD56+ cells, with blocked IL-2R components. Both CD56+ and CD3+ cells were isolated from freshly cultured PBMCs. CFSE was incorporated into CD3+ cells. CD56+ cell IL-2R α , IL-2R β , or both were blocked prior to coculture and IL-2 stimulation. Upon acquisition on Day 5, cells in the "low CFSE" peak of the acquired histogram was gated and plotted as a percentage of total CFSE+ cells. Results represent normalized data from 3 donors. For representative flow cytometry data see Appendix 2.13. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One way Anova and is as compared to media samples of the same data set. *p ≤ 0.05 ***p ≤ 0.001

3.3.13 CD56+ cell regulation of CD3+ cells also occurs in allogeneic cocultures

NK cells differentiate self from non-self by gauging the expression of MHC class 1 molecules on potential target cells. NK cells effectively lyse cells lacking expression of some or all MHC molecules, but this is abrogated if the target shares MHC class 1 molecules (Benjamin *et al*, 2010). Thus far, CD56+ cell regulation of CD3+ cells has been demonstrated using autologous cell types. To investigate the role of MHC in CD56+ cell recognition and regulation of CD3+ cells at low and high IL-2 concentrations, this experiment was repeated with allogeneic cell subsets.

From separate donors, both CD3+ and CD56+ cells were isolated from freshly cultured PBMCs. To track proliferation, CFSE was incorporated into CD3+ cells prior to coculture with allogeneic CD56+ cells and IL-2 stimulation. Upon harvest and acquisition, cells in the "low CFSE-FITC" peak were gated for, and expressed as a percentage of total CD3+ cells.

Similar to results observed with autologous cocultures (Figure 3.3.3.C), CD3+ cell proliferation was inhibited in the presence of CD56+ cells, especially at low and high concentrations of IL-2 (Figure 3.3.10). These low dose IL-2 results are representative of a HPIV3 infection scenario. Furthermore, the difference in proliferation between CD3+ cells cultured with CD56+ cells and those cultured alone, was even greater than previously observed. As regulation occurred in both autologous and allogeneic cultures, these results suggest that CD56+ cell regulation of CD3+ cells is not dependent on either allogeneic or autologous MHC. However, the involvement of MHC presentation should be further investigated in future.



Figure 3.3.13: Proliferative responses of CD3+ cells, in the presence and absence of allogeneic CD56+ cells, as detected by CFSE incorporation. Both CD56+ and CD3+ cells were isolated from freshly isolated PBMCs of two separate donors. CFSE was incorporated into CD3+ cells, which were cultured either alone, or cocultured with CD56+ cells, and stimulated with varying concentrations of IL-2. Upon acquisition on day 5, cells in the "low CFSE peak" of the acquired histogram were gated and plotted as a percentage of total CFSE+ cells, or CD3+ cells. Results represent normalized data from 3 donors in 3 separate experiments in triplicate. For representative flow cytometry data, see appendix 2.14. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One Way Anova and is as compared to media sample of the same data set. ***P \leq 0.001

3.4 Discussion

The role of NK cells, in particular CD56^{Bright} NK cells, as immune regulators is still poorly understood and at times contradictory. Published data from our group demonstrated that HPIV3 infected DCs could drive NK cell regulation of T cell proliferation in a mixed lymphocyte reaction. This regulation was associated with low IL-2 levels, with the addition of IL-2 restoring the anti-proliferative regulation (Noone *et al* 2008). Coincidentally, data emerged from patients who had received low dose IL-2 therapy, demonstrating a similar contraction of T cells. This response was consistent with improved outcome in patients with inflammatory conditions (Saadoun *et al* 2011, Soiffer *et al* 1994, Zorn *et al* 2006, Murphy 2012). This led us to speculate that low dose IL-2 may stimulate the same pathway as HPIV3 to induce the contraction of inflammatory cells. During infection, NK mediated T cell regulation was due to cell cycle arrest. Additionally, the inhibition of T cell cycle has previously been attributed to NK cell mediated upregulation of p21 (Trivedi *et al* 2005). As opposed to this low dose scenario, T cell apoptosis has been reported at high dose IL-2 (Harnack *et al* 2011, Degrate *et al* 2009, Ren *et al* 2007). We hypothesised that NK cells may regulate T cells differentially at low and high levels of IL-2.

We reveal an increase in cells expressing CD56^{Bright} markers at low and high IL-2 concentrations [(CD56+CD3-CD16+) figure 3.3.4]. This phenomenon was reflected in CD56^{Bright} NK cell proliferative responses (figure 3.3.5.a). This corresponds directly to reduced T cell numbers (figure 3.3.5.b). When CD56^{Bright} NK cells, but not CD56^{Dim} NKs were absent from culture an immediate increase in proliferating T cells was observed, even without the addition of IL-2. Here, we demonstrate a dual mechanism of CD56^{Bright} NK regulation of T cell proliferation, whereby NK cells expand and cause T cell cycle arrest at low IL-2 concentrations (figure 3.3.6.a), contract and allow T cell proliferation at intermediate IL-2 concentrations, and expand again at high IL-2 concentrations causing T cell death (figure 3.3.6.b). This high IL-2 mechanism is important in the prevention of immune induced tissue damage. These results explain for the first time, the contradicting reports with regards to NK cell regulation of T cells. We also found that both mechanisms of CD56^{Bright} NK cell regulation of T cells require direct contact with the target T cell (figure 3.3.8). This is of interest as finding T cell ligands for NK cell-receptors may provide further therapeutic targets in autoimmunity and cancer. Additionally, these results indicate that low dose IL-2 can mimic NK cell regulation of T cell cycle during HPIV3 infection.

Although the CD56^{Bright} NK cells are the effector NK subset in this study (Figure 3.3.5), no real change in their cytokine profiles were observed in response to IL-2 (Figure 3.3.9). This adds further confirmation to the contact dependency of this mechanism (Figure 3.3.8). This also highlights the ever changing role and profile of $CD56^{Bright}$, as this immune subset has largely been associated with the production of regulatory cytokines (Caligiuri 2008, Shereck *et al* 2007).

When CD56+ or CD56^{Bright} NK cells were depleted from culture an increase in proliferating T cells occurred even in the absence of IL-2. This was not enhanced by additional IL-2 (figuress 3.3.3 and 3.3.5). It appears that CD3+ T cells do not respond in a proliferative sense to IL-2 alone, but take direction from the CD56^{Bright} NK cells. This was further confirmed by demonstrating a two fold increase in IL-2 production by T cells in co-culture with NK cells, compared to T cells cultured alone (section 3.3.10). Additionally NK cell inhibition of T cells was abrogated when cultured with Anti-IL-2 (section 3.3.11). This gives a new outlook on the widely accepted view that IL-2 drives and promotes T cell proliferation (Gaffen and Liu 2004), and suggests T cells produce IL-2 as a mode of crosstalk for self-regulation via CD56^{Bright} NK cells.

IL-2 receptor alpha chain blocking antibodies (Daclizumab) have been associated with IL-2 dependent CD56+ cell expansion. Expanded NK cells were associated with cytotoxic effects towards disease causing T-cells. The IL-2 dependency of this activation is paradoxically due to the blocking of IL-2R α . Authors suggest that this is because NK cell IL-2R is mainly composed of IL-2R β . However, T cell IL-2R is mainly comprised of IL-2R α . Therefore, there is a higher availability of the NK IL-2R for IL-2 stimulation, mimicing a high dose scenario of activation (Martin *et al* 2010). We demonstrated that at low and high levels of IL-2, blocking IL-2R β abrogates the inhibition of T cell responses by NK cells, confirming the Daclizumab hypothesis, and demonstrating that NK cells are stimulated by T cell derived IL-2 through the β chain of their IL-2 receptor.

Finally, and of interest for NK cell activation, the same inhibition of T cell proliferation was observed with allogeneic or autologous NK cells. These results question the involvement for MHC presentation in NK activation here. However, this needs further investigation.

Overall these results represent a sophisticated and important control mechanism which ensures that a threshold of activation must be achieved before T cell proliferation is permitted by NK cell regulators. Similarly, cytotoxicity by apoptosis represents a more suitable control when levels of IL-2 have reached the upper limit and elimination of over reactive cells is key to regaining intermediate or low levels of immune activation. This mechanism not only supports the role of CD56^{Bright} NK cells as immune regulators, but also explains anomalies observed in current literature regarding the mode of T cell regulation carried out by NK cells. This may also have clinical implications in autoimmunity. To our knowledge, this is the first evidence for this dual mechanism of NK cell regulation of T cell responses.

4 NK cells promote the proliferation of Treg populations, but inhibit conventional T cells.

4.1 Introduction

T cells are a diverse immune subset with high specificity toward a wide array of antigens. There are several types of T cells, broadly divided into helper, regulatory and memory T cells. Memory T cells reside in circulation post infection, mounting a stronger immune response upon encountering the same pathogen again (Janeway 2008).

As previously stated, HPIV3 is an important respiratory pathogen responsible for bronchiolitis, pneumonia and croup. While initial infection occurs during infancy and early childhood, reinfection is a common event and may occur several times even in adulthood (Henrickson 2003, Chanock *et al* 2001). Although antibody responses occur, individuals are still susceptible to reinfection, indicating a failure of immune memory (Schmidt *et al* 2011). Indeed, our group has demonstrated NK cell mediated T cell cycle arrest during infection. In this study, we determined that neutralizing antibodies to the HPIV3 surface glycoprotein HN restores T cell cycle and proliferation. However, this phenomenon has only been demonstrated in the overall CD3+ cell population, but responses of specific T cell subsets have yet to be characterised. In addition, it would be of interest to determine wheter NK cells selectively inhibit memory T cell substs during infection.

We have also determined that low dose IL-2 mimics the effects of HPIV infection. Therefore, we are interested in low dose IL-2 responses given, its importance in understanding the therapeutic mechanisms of low dose IL-2 in the clinic.

In the past, regulation of Tcons has been attributed to Tregs (Mills 2004). Tregs are of importance for neutralization and suppression of the effects of T cell responses post infection, minimizing damage to local tissue. These cells are usually derived from naïve CD4+ cells stimulated with IL-2 and TGF- β . Recent studies demonstrate enhanced Treg responses at low doses of IL-2. Authors suggest that the benefits with low dose IL-2 treatment in graft versus host disease and HCV-induced vasculitis is due to these increased Tregs (Korreth *et al* 2011, Saadoun *et al* 2011). However, one study also described increased NK cell populations during low dose IL-2 treatment, but did not associate these cells with therapeutic effects (Saadoun *et al* 2011). It was therefore of interest to determine whether NK cells regulate all T

cell subsets during HPIV3 infection or at low dose IL-2, or whether NK cells can distinguish between Tcons and Tregs. We hypothesized that NK cells are selective in the T cell subsets they inhibit, inhibiting pro-inflammatory subsets at low and high IL-2, while promoting Treg levels. To investigate this hypothesis, we observed levels of various T cell subsets (Th1, Th17, Treg, memory T cells, or CD8+ T cells), during HPIV3 infection or in response to IL-2. We felt that in agreement with literature, perhaps while NKs inhibit Tcons, this is not the case for Tregs.

4.1.A The specific aims of the work described in this chapter were as follows:

-To determine if HPIV3 infection causes NK cells to selectively inhibit certain T cell subsets.

- Investigate if this is reflective of IL-2 stimulation

-To determine if NK cell promotion of cells expressing Treg markers is reflective in proliferative data

-Investigate the specific NK subset regulating Tregs

-To ensure that NK inhibition of CD3+ cells is not Treg mediated

-Determine if NK regulation of Tregs is contact dependent

4.2 Materials and methods

Product	Catalogue #	Company
Human Treg detection kit	FMC013	R&D systems Europe Ltd.,
		Oxon, UK
Human Th1 detection kit	FMC009	R&D systems Europe Ltd.,
		Oxon, UK
Human Th17 detection kit	FMC007	R&D systems Europe Ltd.,
		Oxon, UK
Anti-human CD8-PE	25-00870-41	eBioscience, Hatfield, UK
Anti-human CD45RO-PE	25-0457-41	eBioscience, Hatfield, UK
Human Treg isolation kit	130-091-301	Miltenyi Biotec, Gladbach,
-		Germany
Human IL-27 Duoset ELISA	DY2526	R&D systems Europe Ltd.,
kit		Oxon, UK
Human IL-ELISA kit	35 88-7357-88	eBioscience, Hatfield, UK

Table 4.2.1: Additional reagents to table 2.2.1 and 3.2.1, used in this study

4.2.1 Subset isolation

CD4+CD25+ cells were isolated (or depleted), using the Treg isolation kit (Miltenyi Biotec). Additionally, CD56+CD3+, CD56^{Bright}, and CD56^{Dim}cells were isolated as previously described.

4.2.2 Cocultures and stimulation

In this chapter, CD14+ cells were isolated, infected and cocultured with allogeneic CD3+ cells or MLs as previously described. Additionally, MLs, CD56+ cell depleted MLs were cultured and stimulated with IL-2. Similarly, CD3+ cells, CD3+Treg- cells and Tregs were cocultured with NK cells at previously described ratios, and stimulated with IL-2. Transwell separations between CD56+ cells and Tregs were carried out in the same way as previously described for CD56+ cells and CD3+ cells.

4.2.3 Flow cytometry

Flow cytometry was carried out as described in section 2.2.10. For detection of specific T cell subsets, multi-fluorescein detection kits were used (R and D systems). For Treg detection, antibodies to CD4, Foxp3 and CD25 were usedFor Th1 detection antibodies to Tbet, IFN- γ and IL-12R β 2 were used. Intracellular staining was required for Foxp3 and T bet, which was carried out as per manufacturers' guidelines. For Th17 detection expression of IL-17, IL-23 and IL-22 was determined. Additionally, Anti-CD8-PE and Anti-CD45RO-PE were used to detect cytotoxic and memory T cells. Anti-CD56-FITC, Anti-CD3-APC, Anti-CD16-PE were used as was CFSE incorporation as previously described.

4.2.4 Neutralization

IL-2R CD25, and intercellular IL-2 were blocked as previously described.

4.2.5 Statistical analysis

All coculture experiments represent normalized results for three donors in three independent experiments. Cyflogic and Facs Calibur software were used for FACs analysis, and plots were created using GraphPad Prism 5.Significance was calculated using Newman Keuls One way Anova: * p < 0.05; ** p < 0.01; *** p < 0.001.

4.3 Results

4.3.1 HPIV3 infected CD14+ cells increase the percentage of MLs expressing Treg markers and decrease the percentage of MLs expressing markers for various Tcon subsets.

We have demonstrated that during HPIV3 infection, antibodies to the surface glycoprotein HN abrogates NK cell mediated inhibition of CD3+ T cells. Reports have suggested that the failure of T cell responses during infection could be attributed to failed immune memory to the virus (Plotnicky-Gilquin 2001). However, memory responses to infection have yet to be determined. Therefore, it was of interest to investigate whether NK cells target specific T cell subsets during HPIV3 infection, or if they inhibit the overall CD3+ cell population

From freshly isolated PBMCs, mixed lymphocytes were isolated and cultured with HPIV3 infected allogeneic CD14+ cells, in addition to HPIV3 infected CD14+ cells which were cultured with Anti-HN, Anti-F, Anti-Distemper virus or suitable controls as previously described. Upon Day 5 acquisition, cells were stained depending on the T-cell population to be detected. Cells were stained for CD4, CD25 and intracellularly for Foxp3, and the percentage of cells positive for expression of all three markers were deemed Tregs, and expressed as a percentage of total MLs (Figure 4.3.1.A). Cells that were positive for CD3, IFN- γ , IL-12R β 2 and intracellular TBet were considered Th1 cells, which were plotted as a percentage of total MLs (Figure 4.3.1.B). Cells that were positive for CD3+IL-17+ IL-23+IL-22+ were indicative of Th17 expression, and were plotted as a percentage of total MLs (Figure 4.3.1.C). The percentage of CD8+ T cells was determined and plotted as a percentage of total MLs (Figure 4.3.1.D). Cells that were CD3+CD45RO+ represented memory T cells, as CD45RO is a memory marker. These were plotted as a percentage of total CD3+ cells (Figure 4.3.1.E). Any intracellular staining was carried out following company guidelines (R+D systems), as detection was done using muticolour subset kits.

Interestingly, HPIV3 infected CD14+ cells induced increased percentages of cells expressing Treg markers, compared to uninfected, or influenza samples. This was abrogated when viral HN was blocked (Figure 4.3.1.A). Consistent with the overall downregulation of CD3+ cell proliferation, all Tcon subsets were inhibited in the presence of HPIV3 infected monocytes compared to influenza. In particular, Th1, CD8+ and Memory T cells were significantly restored when HPIV3 HN was blocked (Figure 4.3.1 B-E).

These results demonstrated that while HPIV3 infection induces expanded Treg populations, it inhibits the overall Tcon cell population. These results are all abrogated by blocking HN. The only T cell subset supported during infection is anti-inflammatory, highlighting the intricate mechanism of immune regulation demonstrated by HPIV3. In relation to the failed immune memory associated with this virus, it is unsurprising that HPIV3 induces inhibition of memory cells. However, this is the first time it has been specifically confirmed. Additionally, and perhaps of greater interest, is the fact that this is abrogated upon neutralization of HPIV3 HN. This is an important finding for the development of vaccines to this virus, as these results suggest that HN in its' unmodified form inhibits levels of memory cells.







Figure 4.3.1: Surface marker expression of T cell subsets in response to HPIV3 infection. Freshly isolated CD14+ cells were infected with HPIV3 or influenza and washed after 2 hours. Prior to coculture with freshly isolated allogeneic mixed lymphocytes after 24 hours, Anti-HN, Anti-F or Anti-Distemper antibodies were incubated with cells at a ratio of 1:100. Mixed lymphocyctes were also cultured with previously described controls. After 5 days, cells were stained for T cell subset markers as follows: CD4+CD25+FOxp3+ cells represent Tregs (A), CD3+TBet+IFN- γ +IL-12R β 2+ represent Th1 cells (B), CD2+IL-17+IL-23+IL-22+ represent Th17 cells (C), CD3+CD8+ cells represent CD8+ T cells (D), and CD3+CD45RO+ cells represent memory T cells (E). Upon acquisition and gating, each subset was expressed as a percentage of total ML. For representative flow cytometry data see Appendix 3.1. Results represent normalized data from 3 donors for 3 separate exerpiments in triplicate. Error bars represent standard deviation between samples. Significance was determined using the Newman Keuls One Way anova and is as compared to CD14+ samples. *p≤0.05 **p≤0.01 ***p≤0.001.

4.3.2 CD56+ cells increase the percentage of MLs expressing Treg markers and contract MLs expressing markers for Tcon subsets.

We have demonstrated increased numbers of cells expressing markers for Tregs, and inhibited Tcon cell subsets during HPIV3 infection, during which low IL-2 concentrations have been demonstrated. Additionally, increased Tregs and reduced Tcons have been reported following low dose IL-2 therapy (Koreth *et al* 2011, Saadoun *et al* 2011). As low dose IL-2 mimices HPIV3 infection, we investigated the IL-2 driven effects of CD56+ cells on the response of key sub-populations of T-cells.

From freshly isolated PBMCs, MLs were isolated. From a defined number of these, CD56+ cells were depleted. Both total MLs, and CD56+ cell depleted MLs were stimulated with varying concentrations of IL-2. For Day 5 acquisition, cells were stained depending on the T-cell population to be detected. Cells were stained for CD4, CD25 and Foxp3, and the percentage of cells positive for expression of all three markers were deemed Tregs, and expressed as a percentage of total MLs (Figure 4.3.2.A). Cells that were positive for CD3, T-Bet, IFN- γ and IL-12R β 2 were considered Th1 cells, which were plotted as a percentage of total MLs (Figure 4.3.2.B). Cells which stained positive for CD3+IL-17+ IL-23+IL-22+ were indicative of TH17 expression, and were plotted as a percentage of total MLs (Figure 4.3.2.C). The percentage of CD8+ T cells was determined and plotted as a percentage of total MLs (Figure 4.3.2.D). Cells that were CD3+CD45RO+ represented memory T cells, as CD45RO is a memory marker. These were plotted as a percentage of total CD3+ cells (Figure 4.3.2.E).

Conversely to the overall Tcon population (Figure 4.3.2.B-E), CD56+ cells enhanced Treg marker expression in the mixed lymphocyte population at low dose IL-2 (Figure 4.3.2.A). This response did not occur in the absence of CD56+ cells. In keeping with what seems to be beneficial effects of low dose IL-2 treatment in humans, it seems that key cells expressing pro-inflammatory markers are reduced in the presence of CD56+ cells, but again, we see an increase in these cell subsets when CD56+ cells were absent. The proportion of both Th1 and Th17 marker expression was significantly reduced in the presence of CD56+ cells at low IL-2 concentrations. Similarly, significant reduction in Th17 marker expression, and approaching significant reduction of Th1 expression was observed at high IL-2 concentrations in the presence of CD56+ cells (Figure 4.3.2.B+C). The percentage of CD8 expressing CD3+ cells is markedly reduced in the presence of CD56+ cells (figure 4.3.2.C), and conforms to the

profile of overall CD3+ cell regulation at low and high IL-2 concentration observed previously (Chapter 3). Finally, memory responses are restored upon depletion of CD56+ cells (Figure 4.3.2.E), supporting the HPIV3 induction of inhibited memory responses (Figure 4.3.1).









Figure 4.3.2: Surface marker expression of T cell subsets in response to IL-2, in the presence and absence of CD56+ cells. From freshly cultured PBMCs, total MLs, and CD56+ cell depleted MLs were isolated. Both were stimulated with varying concentrations of IL-2 (0-50ng/ml). After 5 days, cells were stained for T cell subset markers as follows: CD4+CD25+FOxp3+ cells represent Tregs (A), CD3+TBet+IFN- γ +IL-12R β 2+ represent Th1 cells (B), CD2+IL-17+IL-23+IL-22+ represent Th17 cells (C), CD3+CD8+ cells represent CD8+ T cells (D), and CD3+CD45RO+ cells represent memory T cells. Upon acquisition and gating, each subset was expressed as a percentage of total MLs. For representative flow cytometry data see Appendix 3.2. Results represent normalized data from 3 donors, with experiments carried out in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One Way Anova and is as compared to media samples of the same data set. *p≤0.05 **p≤0.01 ***p≤0.001.

4.3.3 Treg proliferation is enhanced by CD56+ cells

We have demonstrated an increased percentage of cells expressing Treg markers during HPIV3 infection, and with low dose IL-2 in MLs. This low dose IL-2 mediated upregulation did not occur when NK cells were depleted from ML cultures. It was now necessary to establish if this NK cell promotion of Treg marker expression was reflected in the actual proliferative responses of Tregs.

Both Tregs and CD56+ cells were isolated (Miltenyi Biotech UK) from freshly cultured PBMCs. Treg purities can be seen in Appendix 3.3. To track Treg proliferation, CFSE was incorporated into Tregs prior to coculture. Tregs were cultured with CD56+ cells, or alone, and stimulated with varying concentrations of IL-2. Upon flow cytometry acquisition, the percentage of cells in the "low CFSE-FITC" peak of the histogram were gated and expressed as a percentage of total Tregs. This served to compare proliferating Tregs with the surface marker expression observed previously (Figure 4.3.2) to confirm that the increase in expression of these surface markers was due to cell proliferation as in addition to surface marker upregulation.

Tregs proliferate poorly in the absence of CD56+ cells, and demonstrate a significant increase in proliferation in the presence of CD56+ cells. Tregs cultured with CD56+ cells proliferated 10 fold compared to those cultured alone (Figure 4.3.3). We have demonstrated low dose IL-2 dependent NK cell mediated inhibition of the overall CD3+ cell population (Figure 3.3.3). While low doses of IL-2 induced upregulated Treg markers (Figure 4.3.2), increased proliferation of Tregs does not seem to be IL-2 dose dependent. This is at odds with studies that associate low dose IL-2 directly to increased Treg levels (Koreth 2011, Saadoun 2011). Additionally, authors observed increased NK cell populations at these levels but these cells were not attributed to the beneficial effects of this therapy (Saadoun 2011). Our data suggests that Treg levels are maintained by NK cells, and conventional T cells are regulated by NK cells, in response to IL-2 levels.

It is notable that while gating for the "low CFSE peak", unstained NK cells' background fluorescence would fall at this low FITC level. This would affect the absolute percentage we report. However, of note, NK cells were cocultured with Tregs at a 1:10 ratio, and so cannot be held accountable for 70% of cells being in this gate. Therefor, while we acknowledge percentages may be slightly scewed upwards, there is still a ~10fold increase in proliferation

of Tregs when NK cells were present. Treg enhancement of NK cells was also reflected in cell surface marker staining experiments. Additionally, upon looking at unstained NK cells in isolation by flow cytometry, we confirmed a small peak contributing to background but not sufficient to be held totally accountable. This rationale applies for all proliferative coculture assays.



Figure 4.3.3: Treg proliferative responses to CD56+ cells and IL-2 stimulation. Both Tregs and CD56+ cells were isolated from freshly cultured PBMCs. CFSE was incorporated into Tregs, which were either cultured alone, or with CD56+ cells, with both cultures stimulated with varying IL-2 concentrations. Upon acquisition after 5 days, cells in the "low CFSE peak" of the acquired histogram were gated and plotted as a percentage of total Tregs. For representative flow cytometry data, see Appendix 3.3. Error bars represent standard deviation between samples. Results represent normalized data from 3 donors, with experiments carried out in triplicate. Significance was determined using Newman Keuls One way Anova and is as compared to media samples. ** $p \le 0.01$ *** $p \le 0.001$.

4.3.4 Both CD56^{Bright} and CD56^{Dim} NK cells enhance Treg proliferation.

We have previously demonstrated that CD56^{Bright} NK cells, not CD56^{Dim}, inhibit proliferation of the overall CD3+ cell population, particularly at low and high IL-2 concentrations. Having determined that CD56+ cells strongly promote Treg proliferation, we wanted to determine if a specific NK subset was responsible for this.

From freshly cultured PBMCs, both CD56^{Bright} and CD56^{Dim} NK cells were isolated using isolation kits for these subsets (Miltenyi Biotech UK), which isolate CD56+CD16-(CD56^{Bright}) and CD56+CD16+ (CD56^{Dim}) NK cells respectively. Autologous Tregs were also isolated, into which CFSE was incorporated before coculture with either NK cell subset and varying IL-2 concentrations. Cells were cocultured at previously described ratios. Upon harvest and acquisition at day 5, cells in the "low CFSE-FITC" peak were gated for and expressed as a percentage of total Tregs. Results indicated that both CD56^{Bright} and CD56^{Dim} NK cells promote Treg proliferation (Figure 4.3.4.A). Similarly, CFSE was incorporated into either CD56^{Bright} or CD56^{Dim} NK cells before culture with unlabelled Tregs and IL-2 stimulation. These NK cell subsets were analyzed as described above, with cells in the "low CFSE-FITC" peak being expressed as a percentage of total cells in that subset type

Both NK cell subsets induce a high level of Treg proliferation, compared to Tregs cultured alone. A slightly higher level of Treg proliferation was induced by CD56^{Dim} NK cells. However it is notable that CD56^{Dim} NK cells were cultured with Tregs at a ratio of 10:1 while CD56^{Bright} NK cells were at a 100:1 ratio to mimic their physiological ratio to T cells, which may be a factor in this difference.

CD56^{Bright} NK cells proliferate at low and high IL-2 concentrations, while CD56^{Dim} proliferation levels remain constant (Figure 4.3.3.B). Therefore, both NK cell subset proliferation in response to IL-2 is as previously observed, and is unaffected by the T cell type they are cultured with (Figure 3.3.5).



Figure 4.3.4: Treg proliferative response to CD56^{Bright} and CD56^{Dim} NK cells, stimulated with IL-2. Tregs and CD56^{Bright} and CD56^{Dim} NK cells were isolated from freshly cultured PBMCs. CFSE was incorporated into Tregs which were cultured alone, with CD56^{Bright} or with CD56^{Dim} NK cells (A). Similarly, CFSE was incorporated into these NK cell subsets which were cultured with unlabelled Tregs (B). Cultures were stimulated with various concentrations of IL-2. Upon acquisition, cells in the "low CFSE peak" of the acquired histogram were gated and plotted as a percentage of total Tregs. For representative flow cyometry data see Appendix 3.4. Error bars represent standard deviation between samples. Results represent normalized data from 3 donors in 3 separate experiments in triplicate. Significance was determined using the Newman Keuls One Way anova and is as compared to media samples of the same data set. **p≤0.01 ***p≤0.001.

4.3.5 CD56+ cell mediated CD3+ cell inhibition is not Treg-dependent

We have demonstrated that CD56+ cells are essential for Treg proliferation (Figure 4.3.3), and that they conversely inhibit the overall CD3+ Tcon population (Figure 4.3.2 and Figure 3.3.3). Traditionally, Tregs were the immune cell attributed with regulation of T cell homeostasis, preventing autoimmune conditions. We aimed to determine if this inhibition of CD3+ cells is due to CD56+ cells directly and not inadvertently via their enhancement of Treg levels. The experiment investigating CD3+ cell proliferation with and without CD56+ cells was repeated in the absence of Tregs.

Both CD3+ and CD56+ cells were isolated form freshly cultured PBMCs. From these CD3+ cells Tregs were depleted. To track proliferation, CFSE was incorporated into this CD3+Treg- population, which were cultured both with CD56+ cells and alone, and stimulated with varying concentrations of IL-2. Upon harvest and acquisition at day 5, cells in the "low CFSE-FITC" peak were gated for, and expressed as a percentage of total CD3+Treg- cells.

In the Treg depleted CD3+ cell populations, CD56+ cells still inhibited proliferation (Figure 4.3.5). These results confirm that the inhibited T cell proliferation observed in previous experiments (Figure 3.3.3, Figure 4.3.2) was induced directly by CD56+ cells, rather than inadvertently by CD56+ cell promotion of Treg proliferation.



Figure 4.3.5: CD3+Treg- cell proliferative response to CD56+ cells and IL-2 stimulation. From freshly cultured PBMCs, CD3+ cells were isolated from which Tregs were depleted (Treg isolation kit, Miltenyi), as well as CD56+ cells. CFSE was incorporated into these CD3+Treg- cells which were cultured alone, or with CD56+ cells. Cultures were stimulated with various concentrations of IL-2. Upon acquisition after 5 days, cells in the "low CFSE peak" of the acquired histogram were gated and plotted as a percentage of total CD3+Treg- cells. For representative flow cytometry data, see Appendix 3.5. Results represent normalized data from 3 donors for 3 separate experiments in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls OneWay Anova and is as compared to media samples of the same data set $**p \le 0.01$.

4.3.6 CD56+ cells do not promote Treg proliferation via CD25.

We have determined Treg surface marker expression by staining for CD4, CD25 and Foxp3, and observed levels of cells positive for all three makers in response to IL-2 in the presence and absence of NK cells. However, there are several subsets of Tregs which are not always positive for all three of these markers. Therefore, we revisited the previous acquisition (Figure 4.3.2), analyzing each marker individually.

While Foxp3+ cells were upregulated for all NK cell+ cultures (data not shown), CD4 upregulation occurred especially at low concentrations of IL-2 (Figure 4.3.6.A), and to a greater extent with CD25+ cells (Figure 4.3.6.B). However, as this does not take into account these markers in conjunction, this may be attributed to other cell type expression.

Given the NK cell mediated promotion of Tregs, the possible IL-2 dependency of this mechanism, and the upregulation of CD25 (IL-2R α) (Figure 4.3.6.B), the α chain of the IL-2 receptor, we hypothesised that perhaps NK cells promote Tregs via CD25.

Tregs were isolated from freshly cultured PBMCs, into which CFSE was incorporated. Additionally, CD56^{Bright} and CD56^{Dim} NK cells were isolated. Prior to coculture, Treg CD25 was blocked with Anti-CD25 (R and D systems). These CD25-blocked Tregs were cultured alone, or with CD56^{Bright} or CD56^{Dim} NK cells and treated with varying IL-2 concentrations for 5 days. Upon acquisition, cells in the "low CFSE" peak (proliferated) were plotted as a percentage of total Tregs.

No real change in NK cell mediated promotion of Treg proliferation was observed when CD25 was blocked. A significant increase (over 10 fold) in Treg proliferation was still observed when cultured with either NK subset, compared to when cultured alone, implying that CD56+ cell enhancement of Treg proliferation is not mediated via CD25.



Figure 4.3.6: Expression of individual Treg markers in response to CD56+ cells and IL-2, in addition to Treg prolifeative response when antibodies to CD25 were present in cultures. Mixed lymphocytes or CD56+ cell depleted mixed lymphocytes were stimulated with varying IL-2 concentrations and cultured for 5 days. Upon harvest, cells were stained for CD4, CD25 and Foxp3. The percentage of CD25+Foxp3+ cells expressing CD4 was determined (A), as was the percentage of CD4+FOxp3 cells expressing CD25 (B). Tregs were isolated from PBMCs, as were CD56^{Bright} and CD56^{Dim} NK cells. CFSE was incorporated into Tregs which were treated with Anti-CD25, before culture alone, or with CD56^{Bright} or CD56^{Dim} NK cells and IL-2 stimulation. Upon acquisition, cells in the "low CFSE peak" of the acquired histogram were gated and plotted as a percentage of total Tregs (C). For representative flow cytometry data see Appendix 3.6. Results represent normalized data from 3 donors, with experiments carried out in triplicate. Significance was determined using Newman Keuls One Way Anova and is as compared to media samples of the same data set. ***p≤0.001

4.3.7 IL-2 is not essential for CD56+ cell enhancement of Treg proliferation.

We have previously demonstrated that low and high IL-2 induces CD56^{Bright} NK cell inhibition of the CD3+ cell population (Figure 3.3.3). Additionally, we have shown enhanced Treg proliferation in the presence of CD56+ cells. Treg marker expression was significantly higher at low IL-2 concentrations (Figure 4.3.2, Figure 4.3.6.A and 4.3.6.B). However, promotion of proliferation did not appear to be IL-2 dependent (Figure 4.3.3). This study aimed to determine the IL-2 dependency of NK cell enhancement of Treg proliferation.

Tregs were isolated from freshly cultured PBMCs, into which CFSE was incorporated. Additionally, autologous CD56+ cells were also isolated. Tregs(CFSE) were either cultured alone, with CD56+ cells, or with both Anti-IL-2 and CD56+ cells. Cells were cocultured at previously described numbers and ratios. Upon acquisition, cells in the "low CFSE" peak were expressed as a percentage of total Tregs. Similarly, CFSE was incorporated into CD56+ cells. These CD56+(CFSE) cells were cultured alone, with Tregs, or with both Anti-IL-2 and Tregs. Upon harvest, cells in the "low CFSE" peak were expressed as a percentage of total CFSE.

As previously observed, Treg proliferation significantly increased in the presence of CD56+ cells. However, a similar increase was observed when cultured with both Anti-IL-2 and CD56+ cells (Figure 4.3.6.A). In keeping with Treg prolifeation CD56+ cell proliferation significantly increased in the presence of Tregs both with and without Anti-IL-2 (Figure 4.3.6.B). These results indicate that IL-2 is not essential for CD56+ cell enhancement of Treg populations. This suggests that those results observed previously at Ong/ml IL-2 or the "media" sample are indicative of the complete data set for that experiment. It could be suggested that the increased number of cells expressing Treg markers at low IL-2 concentrations can be attributed to enhanced CD56^{Bright}NK cells at these levels. This does not however, explain why Treg numbers are maintained at intermediated IL-2 concentrations in previous experiments, as there are lower levels of CD56+ cells observed here.



Figure 4.3.7: Proliferative reponses of Tregs and CD56+ cells to coculture with Anti-IL-2. Tregs and CD56+ cells were isolated from freshly cultured PBMCs. Into a proportion of each, CFSE was incorporated. Tregs(CFSE) were cultured alone, with CD56+ cells, and with Anti-IL-2 and CD56+ cells (A). CD56+(CFSE) cells were cultured alone, with Tregs, and with Anti-IL-2 and Tregs (B). Upon acquisition at Day 5, cells in the "low CFSE peak" of the acquired histogram were gated and plotted as a percentage of total cells of that subset. For representative flow cytometry data see Appendix 3.7. Results represent normalized data from 3 donors, with experiment carried out in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One way Anova and is as compared to media sample ***p \leq 0.001.

4.3.8 CD56^{Bright} cells acquire CD56^{Dim} markers at intermediate IL-2 concentrations.

We have demonstrated CD56+ cell promotion of Tregs by both surface marker (Figure 4.3.2) and proliferative approaches (Figure 4.3.3). This occurred significantly at low IL-2 concentrations, which we hypothesise is due to enhanced CD56+ cell proliferation at these IL-2 levels, and not due to IL-2 itself (Figure 4.3.7). However, as we have demonstrated CD56^{Bright} NK cells expand at low and high IL-2 concentrations and not intermediate IL-2 concentrations, it was unclear how Tregs were maintained at intermediate IL-2 concentrations. Even if Treg promotion is not IL-2 dependent, there are less proliferating CD56^{Bright} NK cells at this subset, and CD56^{Dim} NK cells appear to be maintained throughout. Of importance, we have demonstrated that either NK cell subset can promote Treg proliferation (Figure 4.3.4).

From freshly cultured PBMCs, CD56^{Bright} (CD56+CD16-) cells were isolated using specific isolation kit (Miltenyi Biotech UK-see materials and methods). These were stimulated with various IL-2 concentrations and cultured in isolation. At day 5, cells were stained for CD56 and CD16. Both CD56+CD16- (CD56^{Bright}) and CD56+CD16+ (CD56^{Dim}) expression were examined. At intermediate IL-2 concentrations, an increase in CD56+CD16+ expression was observed, despite the initial culture being comprised of only CD56^{Bright} NK cells. While not all CD56^{Dim} NK cells express CD16, we found this system sufficient for the purpose of this experiment. By flow cytometry, we have seen that almost all our CD56^{Dim} NK cells at play here.

These results suggest that CD56^{Bright} can acquire CD56^{Dim} features in an IL-2 dependent manner. The plasticity of these cell types has been under scrutiny in recent years (Maghazachi 2004). Additionally, it might explain the maintenance of Tregs at intermediate IL-2 concentrations, as CD56^{Dim} NK cells can promote Treg proliferation.



Figure 4.3.8: CD56^{Bright} and CD56^{Dim} marker expression on isolated CD56^{Bright} NK cells stimulated with varying IL-2 concentrations. From freshly isolated MLs, CD56^{Bright} NK cells were isolated and stimulated with varying IL-2 concentrations. After 5 days, cells were stained for CD56, CD3 and CD16. CD56+CD3- cells were gated and of these, the percentage that were CD16+ or CD16- were determined. For representative flow cytometry data see appendix 3.8. Results represent normalized data from 3 donors, with experiments carried out in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One way Anova and is as compared to media sample of the same data set. *p≤0.05 **p≤0.01

4.3.9 NK cell mediated Treg proliferation is partially abrogated when separated by transwell inserts.

Both previously published data from our group (Noone *et al* 2008) and our IL-2 studies (Figure 3.3.8) demonstrated CD56+ cells inhibit CD3+ cell (Tcon) proliferation via a cell-cell contact dependent mechanism. However, we have not yet determined whether CD56+ cell promotion of Treg proliferation, is contact dependent. As both NK subsets have been shown to promote Treg proliferation (Figure 4.3.4), the contact dependence of both subsets were investigated.

From freshly cultured PBMCs, CD56^{Bright} and CD56^{Dim} NK cells were isolated, as were Tregs. To track proliferation, CFSE was incorporated into these Tregs. Tregs were cultured alone, with CD56^{Bright} NK cells or separated from CD56^{Bright} NK cells using 0.2µM pore inserts before stimulation with varying concentrations of IL-2. Similarly, Tregs were cultured alone, with CD56^{Dim} NK cells, or separated from CD56^{Dim} NK cells using 0.2µM pore inserts before stimulation with varying concentrations of IL-2. Similarly, Tregs were cultured alone, with CD56^{Dim} NK cells, or separated from CD56^{Dim} NK cells using 0.2µM pore inserts before stimulation with varying concentrations of IL-2. At day 5, cells in the "low CFSE" peak were expressed as a percentage of total Tregs.

As previously observed (Figure 4.3.4), the presence of CD56^{Bright} NK cells induced a significant increase in Treg proliferation, compared to Tregs cultured alone. When CD56^{Bright} NK cells were separated from Tregs using a transwell insert however, proliferation was still significantly higher than in those Tregs cultured alone, but significantly lower than when CD56^{Bright} NK cells were able to come into direct contact with Tregs (***p \leq 0.001) (Figure 4.3.9.A). Similarly, as previously observed, CD56^{Dim} NK cells enhanced Treg proliferation compared to Tregs cultured alone (Figure 4.3.4). Again, when separated by transwell insert, CD56^{Dim} NK cells still promoted Treg proliferation to a significantly higher level than media samples, but still is significantly lower than when in direct contact (***p \leq 0.001)(Figure 4.3.9.B). Therefor, both NK subsets can partially promote Treg proliferation, even when not in contact with them, but this proliferation is further enhanced when NK cells are in direct contact with Tregs.



Figure 4.3.9: Proliferative response of Tregs, in the presence or separation (//) of CD56^{Bright} or CD56^{Dim} NK cells. Tregs, and CD56^{Bright} and CD56^{Dim} NK cells were isolated from freshly cultured PBMCs. CFSE was incorporated into Tregs prior to coculture. Tregs were cultured alone, with CD56^{Bright} NK cells stimulated with varying IL-2 concentrations, or separated from IL-2 stimulated CD56^{Bright} NK cells by a 0.2µm pore insert (A). Tregs were also cultured alone, with CD56^{Dim} NK cells stimulated with varying concentrations of IL-2, or separated from IL-2 stimulated CD56^{Dim} NK cells by a 0.2µm pore insert (B). Upon acquisition at Day 5, cells in the "low CFSE peak" of the acquired histogram were gated and plotted as a percentage of total cells of that subset. For representative flow cytometry data see Appendix 3.9. Results represent normalized data from 3 donors, with experiments carried out in triplicate. Due to the conserved significance between all data sets, this is summarized within the text and not the graph itself, but was calculated using Newman Keuls One Way Anova. ***p≤0.001.

4.3.10 Neither cytokines promoting Treg development, or those produced by Tregs are changed in response to IL-2.

We have demonstrated that both CD56^{Bright} and CD56^{Dim} NK cells promote Treg proliferation (Figure 4.3.3), a phenomenon which is only partially abrogated upon separation of NK cells from the Treg (Figure 4.3.9). We therefore wanted to determine the role of Treg-associated cytokines in the promotion of Tregs by NK cells. Various Treg-associated cytokines were examined by ELISAs.

To investigate this, ELISAs were performed on supernatants of IL-2 stimulated Tregs, cultured alone, or with $CD56^{Bright}$ or $CD56^{Dim}$ NK cells. ELISAs were carried out on a variety of cytokines associated with Tregs; TGF- β (Figure 4.3.10.A), IL-10 (Figure 4.3.10.B), IL-35 (Figure 4.3.10C) and IL-6 (Figure 4.3.10.D).

TGF- β is associated with the induction of Foxp3+ Tregs from CD4+CD25- precursors. An over 5 fold increase in TGF- β was observed in the presence of CD56^{Bright} NK cells than Tregs cultured alone, with CD56^{Dim} cultures producing a significantly higher level of TGF- β at high levels of IL-2 (3.125ng-50ng/ml IL-2, ***p≤0.001).

Tregs produce the immunosuppressive cytokine IL-10; which is enhanced in the presence of either NK cell subset. This cytokine is produced by Tregs, and is associated with anti-inflammatory effects. As both NK cell subsets promote the proliferation of Tregs, it was expected that IL-10 production would be enhanced in their presence.

IL-35 is responsible for the expansion of Treg populations, and there was no real change to this cytokine in the presence or absence of either NK cell subset (Figure 4.3.10.C). IL-6 is attributed to shifting the Treg/Th17 balance in favour of Treg. However, NK cell do not contribute to the production of this cytokine when cultured with Tregs (Figure 4.3.10.D). Overall, cultures with $CD56^{Bright}$ NK cells seem to produce increased levels of TGF- β , which drives Tregs, and IL-10, a cytokine produced by Tregs. However, both NK cell subsets promote Treg proliferation optimally when in direct contact with these cells (Figure 4.3.9). It is notable that it is unclear whether the cytokine is being produced by NK or Treg here. We show only that the presence of NK cells causes higher levels of this cytokine. Due to the contact dependent element here, we felt no need to investigate further, but this could be done with intracellular staining.



Figure 4.3.10: Treg associated cytokine secretion profile of IL-2 stimulated Tregs cultured alone or with Nk cell subsets. Freshly isolated Tregs, Tregs and $CD56^{Bright}$ or $CD56^{Dim}$ NK cells were stimulated with various IL-2 concentrations. At day 5, supernatants were harvested and used for examination by ELISA. TGF- β (A), IL-10(B), IL-35(C) and IL-6(D) secretion was determined by ELISA. Results were plotted using Graphpad Prism 5. Results represent normalized data from 3 donors, from 3 separate experiments in triplicate.

4.4 Discussion

HPIV3 is a major respiratory pathogen affecting infants, with reinfection occurring throughout life. This is due to failure of memory associated with this virus (Plotnicky-Gliquin *et al* 2001). Our group has determined that NK cells inhibit T cell cycle during HPIV3 infection (Noone *et al* 2008). However, memory T cell responses have yet to be investigated. We show inhibited levels of cells expressing memory T cell markers during infection. This was mediated by NK cells and reflected by low dose IL-2 results. Furthermore, this inhibition of memory T cells was abrogated in the presence of neutralizing antibodies to HPIV3 surface glycoprotein HN. Failed vaccines to this virus have used this glycoprotein. Additionally, effector anti-viral T cells are inhibited during infection, and this was abrogated by Anti-HN. These results give important insights into problems with HPIV3 vaccine development, as this molecule, in its unmodified form appears to inhibit memory responses, which is the reoccurring problem with prevention of infection.

Immune regulation is key to the control of autoimmunity and excessive immune responses to foreign pathogens. This self-protective feature of the host is controlled by a complex regulatory system, consisting of immune cells such as regulatory T cells. These regulatory T cells have been considered as the most pivotal cell type in controlling immune responses (Jian and Chess 2004). We have demonstrated the exploitation of NK cell immune regulation during HPIV3 infection for the evasion of immune memory. This mechanism can also be induced by low dose II-2. Recent studies demonstrate enhanced Treg responses at low doses of IL-2, with beneficial effects in graft versus host disease and HCV-induced vasculitis (Korreth et al 2011, Saadoun et al 2011). However, increased NK cells were also observed in the vasculitis study (Saadoun et al 2011). These NK cells were not investigated for antiinflammatory function in this study, but based on our data thus far we hypothesized that these cells have been overlooked in immune regulation. We have demonstrated NK cell mediated inhibition of T cell proliferation at low IL-2 concentrations and in a parainfluenza context. Thus, our aim was to further investigate the effect of IL-2 induced NK regulation of human immune responses by examining the expression levels of key T cell subsets in response to IL-2 in the presence or absence of NK cells. The low dose IL-2 here serves to drive the same mechanism of NK mediated T cell cycle arrest as HPIV3 infection.

We found that the presence of NK cells has an important effect on how other cells respond to IL-2. At all IL-2 concentrations, a notable reduction in cells expressing markers for key proinflammatory cell subsets such as Th1 and TH17 was observed when NK cells were

present. There was a significant reduction of marker expression for both subsets at low IL-2 and again at high IL-2 (Th1 subset approaching significance). Additionally, CD8 and memory T cell expression followed similar profiles. In contrast to these subsets responses, we see an enhancement of Treg expression at low dose IL-2 in their presence and a decrease in their numbers when NK cells were removed from the culture. This data is consistent with the studies in patients receiving low dose IL-2 (Korreth *et al* 2011, Saadoun *et al* 2011). Additionally, and conversely to the overall Tcon population, the absence of NK cells induces poor proliferative responses in the Treg population. In addition, to show that this Tcon regulation relates to NK cells and not to enhanced Tregs we provide evidence that depletion of Tregs from CD3+ T cells does not affect the proliferative regulation of CD3 T cells by CD56+ NK cells.

We have demonstrated the IL-2 dependent CD56^{Bright} regulation of CD3+ cells, and increased proliferation of Tregs. Here we determine that both CD56^{Bright} and CD56^{Dim} NK cell subsets enhance the proliferation of Tregs. Having confirmed that Treg promotion is not IL-2 dependent, we suggest that the increased percentage of cells expressing Treg markers at low IL-2 concentrations is due to increased proliferation of CD56^{Bright} NK cells at these low IL-2 levels. However, this did not explain the maintenance of Treg proliferation at intermediate IL-2 concentration, when contracted CD56^{Bright} populations are observed. We show the acquisition of CD56^{Dim} markers by isolated CD56^{Bright} NK cells at intermediate IL-2. As both NK cell subsets promote Treg proliferation, this may explain the maintenance of Treg proliferation at these levels. Some individuals have suggested that CD56^{Bright} NK cells are intermediates in the development of CD56^{Dim} NK cells (Caligiuri 2008), while others report CD56^{Dim} NK cells can develop a CD56^{Bright} phenotype under IL-12 conditions (Maghazachi 2004). Our results further support this plasticity of NK cell subsets. Additionally, we demonstrate that Treg promotion of NKs is not contact dependent, although it is at its strongest when NK subsets are in contact with Tregs.

Overall, NK cells are highly selective in the T cells they regulate in response to IL-2, and they show considerable placicity in response to varying concentrations of this cytokine.

5. CD56^{bright} NKs mediate regulation of CD3+ T cell by a combination of NKp44 and NKp46 during HPIV3 infection or at low dose IL-2, or by Nkp44 alone at high dose IL-2

5.1 Introduction

HPIV3 is an important respiratory virus, to which reinfection may occur several times even in adolescents and adults due to failure of immune memory to the virus (Henrickson 2003, Chanock *et al* 2001). It has previously been suggested that HPIV3 infection might affect T cell responses, thus preventing the development of efficient and long-lasting immune responses (Plotnicky-Gliquin *et al* 2001). Our group reported NK cell mediated T cell cycle arrest during HPIV3 infection. This was associated with inhibited IL-2 concentrations (Noone *et al* 2008). Furthermore, we have demonstrated that neutralizing antibodies to the HPIV3 surface glycoprotein HN restore IL-2 levels and CD3+ T cell proliferation (Chapter 2). With this evidence regarding the HPIV3 glycoprotein involved in NK cell regulation of T cells, we sought to determine the receptors on the NK cell involved in this mechanism.

Published data has demonstrated an interaction between HPIV3 HN and NKp44 and NKP46 (Biassoni *et al* 2002, Mandelboim *et al* 2001), although no functional output for this interaction has been shown. These receptors, along with NKp30 make up the Natural cytotoxicity Receptors (NCRs), which as the name suggests, are activating receptors associated with NK cell mediated target cell death. Additionally, IL-2 stimulation is required for the expression of NKp44 (Cantoni *et al* 1999). As we have previously demonstrated that NK cells regulate T cell proliferation in an IL-2 dependent mechanism (Chapter 3), and due to the involvement of these receptors in HPIV3 HN binding, these receptor were putative candidates for NK activation during infection. We wanted to determine the role of these receptors during the immune regulation associated with HPIV3 infection, in addition to the low dose IL-2 model of infection. Having demonstrated the expression of these receptors in our viral and IL-2 model, we then used neutralizing antibodies to these NCRs to investigate their effect on NK cell mediated T cell inhibition. To further demonstrate the involvement of
these receptors in immune regulation, we also used agonistic NCR antibodies to activate NKs in the absence of viral infection or IL-2.

5.1.A The specific aims of the work described in this chapter were as follows:

-Determine if HPIV3 HN or IL-2 concentrations induce changes to NCR expression levels

-Investigate whether blocking the known interaction between HPIV3 HN and NKp44 and NKp46 affects NK cell regulation of T cell during infection

-Determine if blocking NCRs affects NK cell regulation of T cell in response to IL-2 concentrations

-To investigate if direct activation of NCRs can mimick HPIV3 infection or low dose IL-2 stimulation

5.2 Materials and methods

Product	Catalogue #	Company		
Human Anti-NKp30-PE	FAB1849P	R&D systems Europe Ltd., Oxon, UK		
Human Anti-NKp44-PE	FAB22491P	R&D systems Europe Ltd., Oxon, UK		
Human Anti-NKp46-PE	FAB1850P	R&D systems Europe Ltd., Oxon, UK		
Leaf human anti-NKp30	325203	Medical supply company, Dublin 15,		
		Ireland		
Leaf human anti-NKp44	325103	Medical supply company, Dublin 15,		
		Ireland		
Leaf human anti-NK46	331901	Medical supply company, Dublin 15,		
		Ireland		
Hum Anti-NKp44 activating	MAB22491	R&D systems Europe Ltd., Oxon, UK		
Hum Anti-NKp46 activating	MAB1850	R&D systems Europe Ltd., Oxon, UK		

Table 5.2.1: Additional reagents to table 2.2.1, 3.2.1, and 4.2.1 used in this study

5.2.1 Cell isolation and coculture

In this chapter, CD14+ cells were isolated, infected and cocultured with allogeneic CD3+ cells or MLs as previously described. Additionally, MLs, CD56+ cell depleted MLs, or CD56+ cells and CD3+ cells were cultured as previously described and stimulated with various IL-2 concentrations.

5.2.2 Flow cytometry

Flow cytometry was carried out as described in section 2.2.10. The following fluorescent conjugated antibodies were used in this chapter: Anti-NKp-30-PE, Anti-NKp44-PE, Anti-NKp46-PE, Anti-CD56-FITC, Anti-CD3-APC. The Treg detection kit (R+D systems) was again used here. Additionally, CFSE incorporation and Annexin-V and PI staining were again used as previously described.

5.2.3 NCR neutralization

LeafTM (Medical supply company) purified Anti-NKp30, Anti-NKp44, or Anti-NKp46 antibodies were used to block these receptors prior to coculture with virally infected cells, or IL-2 stimulation. These antibodies have been shown to block these receptors in solution (Stark *et al* 2005, Nolte-'t Hoen *et al* 2006). A concentration of 1µg of these antibodies was cultured per 1×10^6 NK cells/MLs prior to coculture.

5.2.4 NCR activation

Agonistic antibodies were used to activate NKp44 and NKp46 (R and D systems). Engagement of NKp44 with this agonistic antibody has been shown to induce IFN- γ production by IL-2 activated human NK cells. Anti-NKp46 agonistic activaty was measured by its ability to induce IFN- γ secretion by NK-92 human natural killer lymphoma cells (R and D systems). A concentration of 0.2µg/ 1 x 10⁶ cells was cultured with CD56+ cells or MLs.

5.2.5 ELISA

Detection of cytokines by ELISA was carried out as described in section 2.2.13. IL-2 was detected by ELISA in this chapter (R and D systems).

5.2.6 Statistical analysis

All coculture experiments represent normalized results for three donors in three independent experiments. Cyflogic and Facs Calibur software were used for FACs analysis, and plots were created using GraphPad Prism 5.Significance was calculated using Newman Keuls One way Anova: * p < 0.05; ** p < 0.01; *** p < 0.001.

5.3 Results

5.3.1 HPIV3 causes upregulation of NKp44 and NKp46.

We have identified that during HPIV3 infection, neutralising antibodies to HN abrogates NK cell mediated regulation of T cell proliferation and is associated with reduced IL-2 levels. Published data has shown that HPIV3 HN binds human NKp44 and NKp46, but not human NKp30. This is also true for influenza HN. No functional outcome for this interaction has been demonstrated. Furthermore, as the addition of IL-2 to our cultures could reverse the proliferative regulation by NK and IL-2 stimulation is required for the expression of NKp44, we hypothesised that the key mechanistic driver of this IL-2 induced dual regulation of CD3+ cell proliferation involved NKp44 and NKp46 (Biassoni, *et al* 2002, Mandelboim and Porgador 2001, Cantoni *et al* 1999). Here we examine expression of these NCRs during HPIV3 infection.

From freshly cultured PBMCs CD14+ cells were isolated and were cultured alone, or infected with influenza or HPIV3 virus, washed after 2 hours of incubation and cocultured with allogeneic MLs after 24 hours, as well as relevant controls. Upon harvest, cells were stained for CD56 and either NKp30, NKp44 or NKP46 experession. The percentage of CD56+NCR+ cells were expressed as a percentage of total CD56+ cells. While previously described positive controls were used in this experiment, we present media vs infected data for simplicity. Positive controls are included in all functional experiments which have the more important implications to the story than this provisional expression data.

Firstly, HPIV3 induced no change to the expression of NKp30 by CD56+ cells (Figure 5.3.1). However, examining these receptors serves as an appropriate control for experiments involving NKp44 and NKp46 function. Both NKp44 and NKp46 were significantly upregulated during HPIV3 infection (Figure 5.3.1). However this upregulation was significant for both receptors in HPIV3 samples. It has also been reported that IL-2 is required for NKp44 expression (Cantoni *et al* 1999) and our results have shown reduced levels of this cytokine during HPIV3 infection (Noone *et al* 2008). However, low dose IL-2 conditions appear sufficient for NKp44 expression here. As we costained with CD56, we were confident that we were observing NCR expression by NK cells and no other cell type (EG T cells). The level of NKp46 expression by CD56+ cells, even in media samples was unexpectedly low, as the majority of NK cells should express this receptor. We will further

investigate this phenomenon in our experimental setup, at various timepoints in section 5.3.5.B.



Figure 5.3.1: NCR expression levels on CD56+ cells in response to HPIV3 infection: Freshly isolated CD14+ cells were infected with HPIV3 or Influenza and washed after 2 hours. These were then cocultured with freshly isolated allogeneic MLs. Cells were also cultured with PMA and Anti-CD3 as a positive control, and media as a negative control. While previously described positive controls were used in this experiment, we present media vs infected data for simplicity. Positive controls are included in all functional experiments which have the more important implications to the story than this provisional expression data. Upon harvest cells were stained for CD56 and NCRs. The percentage of CD56+ cells that were expressing the NCR in question cells were detected by flow cytometry. For representative flow cytometry data see Appendix 4.1. Results represent normalized data from 3 donors in, with experiments carried out intriplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One Way Anova and is as compared to CD14+ sample of the same data set. **p ≤ 0.01 ***p ≤ 0.01

5.3.2 Blocking HPIV3 HN reduces upregulation of NKp44 and NKp46 during HPIV3 infection

We have shown abrogated T cell inhibition during HPIV3 infection, using neutralizing antibodies to HN. Having demonstrated that HPIV3 infection induces upregulated NKp44 and NKp46 (Figure 5.3.1), and given the known interaction between HPIV3 HN and these NCRs, we aimed to determine if blocking HN had an effect on NKp44 and NKp46 expression levels.

Mixed lymphocytes were again cultured with HPIV3 infected CD14+ cells, or HPIV3 infected cells which were blocked with either anti-HN or anti-F antibodies, as well as anti-Distemper virus as a random viral control. Controls described in previous experiments were also used and upon harvest on day 5, cells were stained for CD56 and either NKp30, NKp44 or NKp46 expression. The percentage of CD56+NCR+ cells were expressed as a percentage of total CD56+ cells.

Expression of NKp30 was again unaffected by HPIV3 infection under any conditions (Figure 5.3.2.A). Blocking HPIV3 HN protein, but not F, partially abrogated the upregulation of both NKp44 (Figure 5.3.2.B) and NKP46 (Figure 5.3.2.C) expression on CD56+ cells during HPIV3 infection. It remains unclear, if HN induces upregulation of these receptors directly, or if this is indirectly due to the low IL-2 levels associated with infection. It also remains to be determined if this interaction between virus and NK cell activates NK cells to induce T cell cycle arrest during infection or if this occurs by a different mechanism. These issues will be addressed in this study.



Figure 5.3.2: NCR expression levels on CD56+ cells in response to HPIV3 HN: Freshly isolated CD14+ cells were infected with HPIV3 or influenza and washed after 2 hours. Prior to coculture with freshly isolated allogeneic mixed lymphocytes after 24 hours, Anti-HN, Anti-F or Anti-Distemper antibodies were incubated with cells at a 1:100 ratio. After 5 days cells were harvested and stained for CD56 and NCRs. The percentage of CD56+ cells that were expressing the NCR in question cells were detected by flow cytometry. For representative flow cyometry data see Appendix 5.2. Results represent normalized data from 3 donors, with experiments carried out in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One Way Anova and is as compared to media sample. * $p \le 0.05$, ** $p \le 0.01$

5.3.3 Blocking NKp44 or NKp46 abrogates NK cell inhibition of mixed lymphocytes during HPIV3 infection

Neutralising antibodies to HPIV3 HN abrogated the upregulation of NK receptors NKp44 and NKp46 during infection (Figure 5.3.2). This interaction suggests that these receptors may be involved in activating NK cell mediated regulation of T cell proliferation during infection. To achieve this mixed lymphocyte proliferation was investigated during HPIV3 infection, using neutralising antibodies to NCRs.

Mixed lymphocytes were cultured with HPIV3 infected CD14+ cells, or were cultured with anti-NKp30, anti-NKp44, anti-NKp46 or both Anti-NKp44 and Anti-NKp46 antibodies prior to coculture with infected cells, as well as previously described controls. Mixed lymphocyte proliferation was determined by MTS assay.

As previously demonstrated, HPIV3 infection significantly inhibited mixed lymphocytes proliferation in a MLR (Figure 5.3.3), a mechanism which we know to be mediated by CD56+ cells (Figure 2.3.2). Unsurprisingly, blocking NKp30 caused the same inhibition of proliferation as total HPIV3 infection. Blocking either NKp44 or NKp46 prior to coculture restored mixed lymphocyte proliferation to HPIV3 infection to a level similar to influenza infection (Figure 5.3.3). Blocking NKp44 and NKp46 simultaneously prior to coculture resulted in an even higher level of mixed lymphocyte proliferation, suggesting that these receptors act in synergy. While the binding of HPIV3 HN to these NCRs is well documented in literature, this is the first report for the functional output of this binding.



Figure 5.3.3. Proliferative responses of allogeneic mixed lymphocytes to HPIV3 infected CD14+ cells with or without antibodies to NKp44 and NKp46: Freshly isolated CD14+ cells were infected with HPIV3 or influenza and washed after 2 hours. These were then cocultured with freshly allogeneice mixed lymphocytes, cultured alone, or with blocking Anti-NKp44 or NKp46. Cells were also cultured with PMA and Anti-CD3 as a positive control, and media as a negative control. After 5 days, cells were harvested and cocultured with MTS solution for 4 hours. Absorbance was detected at 490nm. Results represent normalized data from 3 donors for 3 separate experiments in triplicate. Significance is as compared to CD14+HPIV3 sample of same data set.. ** $p \le 0.01$ *** $p \le 0.001$

5.3.4 Blocking NKp44 or NKp46 abrogates NK cell inhibition of CD3+ cells

We have demonstrated that the interaction between HPIV3 HN and NCRs NKp44 or NKp46 activates NK cell mediated inhibition of mixed lymphocyte proliferation. To confirm this with CD3+ cells in isolation, we cultured these with infected CD14+ cells, and CD56+ cells, using a different proliferative detection method.

From freshly cultured PBMCs, CD3+ cells were isolated and incorporated with CFSE. These were cultured with HPIV3 infected CD14+ cells, and CD56+ cells which were cultured with anti-NKp44, anti-NKp46 or both antibodies prior to coculture, as well as previously described controls. Upon acquisition, cells in the "low CFSE" gate were expressed as a percentage of total CD3+ cells

Consistent with previous results, blocking NKp30 had no effect on CD3+ cell proliferation, with levels of significant downregulation similar to HPIV3 infection. Blocking either NKp44 or NKp46 prior to coculture restored CD3+ cell proliferation during HPIV3 infection to a level similar to influenza infection. Again, blocking NKp44 and NKp46 simultaneously prior to coculture resulted in an even higher level of mixed lymphocyte proliferation, suggesting that these receptors act in synergy (Figure 5.3.4).



Figure 5.3.4. Proliferative responses of CD3+ cells to HPIV3 infected allogeneic CD14+ cells with or without antibodies to NKp44 and NKp46: Freshly isolated CD14+ cells were infected with HPIV3 or influenza and washed after 2 hours. These were then cocultured with freshly allogeneic CD3+ cells into which CFSE had been incorporated, cultured alone, or with blocking Anti-NKp44 or NKp46. Cells were also cultured with PMA and Anti-CD3 as a positive control, and media as a negative control. Upon acquisition, cells in the "low CFSE" peak were expressed as a percentage of total CD3+ cells For representative flow cytometry data see Appendix 4.3. Results represent normalized data from 3 donors, with experiments carried out in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One Way Anova and is as compared to media sample. **p ≤ 0.01 ***p ≤ 0.01

5.3.5.A NKp44 and NKp46 upregulate at low levels of IL-2, while NKp44 alone upregulates at high IL-2 concentrations

We demonstrate blocking either NKp44 and NKp46 on NK cells, or HPIV3 HN restores mixed lymphocyte or T cell proliferation during infection. NK cell regulation of T cells during infection is IL-2 dependent, and can be reproduced by low doses of IL-2 in the absence of virus (Chapter 3). Additionally, NKp44 requires IL-2 stimulation for expression on NK cells (Cantoni *et al* 1999). Therefore, it was important to determine whether IL-2 has a direct effect in NK cell activation via these receptors.

Mixed lymphocytes were isolated and stimulated with varying concentrations of IL-2. At day 5, cells were stained with fluorescent conjugated Anti-CD56 and one of Anti-NKp30, Anti-NKp44 or Anti-NKp46. The percentage of CD56+ cells expressing the NCR in question was expressed as a percentage of total CD3+ cells plotted using Graph Pad prism.

In keeping with HPIV3 data (Figure 5.3.1), NKp30 expression remained unchanged at any concentration of IL-2. Expression of NKp44 was upregulated at both low and high concentrations of IL-2. However, expression of NKp46 was upregulated at low levels of IL-2, but was almost absent on CD56+ cells at high concentrations of IL-2 (Figure 5.3.5). Thus, dual expression of NKp44 and NKp46 is observed at low doses of IL-2, the phase at which NK cells induced T cell cycle arrest, while NKp44 only is upregulated at high IL-2 concentrations coincidental with NK induced T cell death. This low dose IL-2 scenario mimics the conditions of HPIV3 infection. This begs the question of the implications of the upregulation/downregulation of these receptors in response to specific IL-2 concentrations.



Figure 5.3.5.A: NCR expression levels on CD56+ cells in response to various IL-2 concentrations. From freshly cultured PBMCs, MLs were isolated and stimulated with various concentrations of IL-2. After 5 days cells were harvested and stained for CD56 and NCRs. The percentage of CD56+ cells that were expressing the NCR in question cells were detected by flow cytometry and. For representative flow cytometry data see Appendix 4.4. Results represent normalized data from 3 donors, with experiments carried out in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One Way Anova and is as compared to media sample of the same data set. $**p \le 0.01$ $***p \le 0.001$

5.3.5.B Further investigation of NKp46 expression on CD56+ cells over a 5 day timecourse

When investigating NKp46 expression on CD56+ cells in response to IL-2 concentrations (0-50ng/ml) after 5 days culture, we saw approximately 5% expression of this receptor in any experimental conditions. However, it is widely accepted that the vast majority of NK cells express this receptor. Therefore we sought to investigate expression of this receptor over a 5 day time course, to determine whether this was an anomaly in our experimental setup, or if expression of this receptor changed over time.

Freshly isolated MLs (from PBMCs), cultured in media alone, or stimulated with 50ng/ml IL-2. Cells were prepped for flow cytometry analysis at 0hours, and daily for 5 days. Cells were stained for CD56 and NKp46. Upon acquisition, CD56+ cells were gated, and NKp46 expression was gated on NKp46-PE histogram.

Upon isolation (T0; Figure 5.3.5.B.i), NKp46 was expressed on the vast majority of CD56+ cells (~95%). However, NKp46 expression on CD56+ cells decreased over time (days1-5; Figure 5.3.5.Bii-vi). At day 5, the timepoint at which cells were analyzed for this project, NKp46 expression had decreased to approximately 5%, consistent with results observed thus far. For all timepoints, stimulation with high concentrations of IL-2 (50ng/ml), was consistent with lower expression of NKp46 than media sample. This is consistent with our observation that high IL-2 downregulates NKp46 expression on CD56+ cells. These results suggest that with time in ex vivo culture, NKp46 is downregulated on CD56+ cells, and that high levels of IL-2 downregulate this receptor on NKs. This decrease of NKp46 expression with time has not been previously described. The remaining experiments in this project investigate functionality of this receptor in response to IL-2, through use of both agonistic and antagonistic antibodies, and it seems that even with its low expression after 5 days incubation, this receptor still plays an important role in the activation of NK regulation of T cells.

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Figure 5.3.5.B: NKp46 expression on CD56+ cells, in response to no, or high IL-2, at Day0-5. MLs were cultured alone, or with 50ng/ml IL-2. Cells were harvested at T0 (i), Day 1 (ii), Day 2 (iii), Day 3 (iv), Day 4 (v), Day v (vi), and stained for CD56 and NKp46. CD56+ cells were gated and these were analyzed on histograms for NKp46 expression. The percentage of CD56+ cells which are NKp46+ are displayed on histograms. Results shown represent one triplicate fromm one donor.

5.3.6 Blocking NKp44 or NKp46 abrogates CD56+ cell regulation of CD3+ cell proliferation

Having demonstrated upregulation of NKp44 and NKp46 at low IL-2 and NKp44 at high IL-2, we aimed to investigate if this differential expression had a physiological effect on the type of NK regulation choice exerted on T cells by NK cells. We therefore repeated the experiments with neutralizing antibodies to NKp44, NKp46 and NKp30, and determined their effect on T cell cycling and apoptosis.

From freshly isolated PBMCs, MLs were isolated, which were cultured with blocking antibodies to NKp30, NKp44 or NKp46, as well as an unblocked ("No Ab") control, prior to IL-2 stimulation Upon harvest, cells were stained for CD3, as well as Annexin-V-PI staining. From the CD3+ PI acquisition histogram, different stages of cell cycle could be gated and expressed as a percentage of CD3+ cells in each stage of cell cycle. Similarly, CD3+ cells were plotted as Annexin-V vs PI, and those in the Annexin-V+PI+ quadrant were plotted as a percentage of total CD3+ cells.

NKp30 had no effect on the capacity of CD56+ NKs to induce cell cycle arrest or apoptosis in these cultures. Blocking NKp44 resulted in both a loss of cell cycle arrest at low IL-2 (Fig. 5.3.6.A) and apoptotic function at high IL-2 (Fig. 5.3.6.B) in these cultures. Blocking NKp46 resulted in a loss of regulation by cell cycle arrest at low IL-2 (Fig 5.3.6.A) but apoptosis continued in these cultures at high IL-2 (Fig. 5.3.6.B). Thus, it appears that the NK cell mediated T cell cycle arrest at low IL-2 concentrations observed previously (Figure 3.3.6) is induced by the dual activation of NKp44 and NKp46 by this cytokine. Additionally, at high IL-2, the NK cell mediated T cell death observed previously (Figure 3.3.6) is via IL-2 activation of NKp44, not NKp46. This is the first association of these cytotoxic receptors with target cell cycle arrest rather than apoptosis.

While concerns over strong T cell proliferation after 5 days in unstimulated cultures have been previously addressed (chapter 3), there was still concerns that cell cycle data was at odds with this proliferation. While most T cells are proliferating after this time, the majority of these cells seem to be in the G0/G1 phase of cell cycle. This would still be true for some replicating cells. Additionally, as NK cells are present in this culture, they may contribute by background fluorescence in the low FITC-PI gate. Therefore, the actual percentage of cells in G0/G1 phase may be lower than determined. However, this is not a concern, as the real finding is the relative increase of cells in the G0/G1 phase at low levels of IL-2, and this is significant for all donors.



Figure 5.3.6: Cell cycle and apoptosis analysis of CD3+ cells cultured in MLs, or MLs in which NKp30, NKp44, or NKp46 were blocked. Mls were isolated and cultured alone, with Anti-NKp30, Anti-NKp44, or Anti-NKp46, before stimulation with various IL-2 concentrations. After 5 days, cells were stained for CD3, Annexin-V and PI. Stages of cell cycle were gated in the PI histogram of CD3+ cells, and the percentage of cells in each stage expressed as a percentage of total CD3+ cells (A). CD3+ cells were plotted as Annexin-V vs PI and those in the Annexin V+PI+ quadrant plotted as a percentage of total CD3+ cells (B). Results represent normalized data from 3 donors, with experiments carried out in triplicate. For representative flow cytometry data, see Appendix 4.5. Error

bars represent standard deviation between samples. Significance was determined by Newman Keuls One Way Anova and is as compared to media samples. * $p \le 0.05 ** p \le 0.01 *** p \le 0.001$.

5.3.7 NCR activation by agonistic antibodies mimics NK cell activation by low or high dose IL-2

Having attributed functional effects to the upregulation of NKp44 and NKp46 at low doses of IL-2, and NKp44 at high doses of IL-2, we sought to investigate if this immune mechanism could be mimiced by direct targeting of these receptors. Here we used agonistic antibodies to NCRs to activate these receptors in the absence of viral infection or low dose IL-2.

From freshly isolated PBMCs, MLs were isolated, and cultured alone, or with agonstic antbodies Anti-NKp44, Anti-NKp46 or both NKp44 and NKp46. As described in materials and methods, these antibodies have been previously assessed for NK activation by determining increased IFN- γ production by NK cells. At day 5, cells were stained for CD3, as well as Annexin-V-PI staining. From the CD3+ PI acquisition histogram, different stages of cell cycle could be gated and expressed as a percentage of CD3+ cells in each stage of cell cycle. Similarly, CD3+ cells were plotted as Annexin-V vs PI, and those in the Annexin-V+PI+ quadrant were plotted as a percentage of total CD3+ cells.

In keeping with previous results, the dual activation of NKp44 and NKp46 induced a significant increase in cell cycle arrest in T cells by NKs, mimicing regulation observed at low levels of IL-2 and HPIV3 infection (Figure 5.3.7.A). Similarly, increased cytotoxicity was observed when NKp44 alone was activated (Figure 5.3.7.B). Although activation of NKp46 alone also induced apoptosis, this cytotoxicity was abrogated when both receptors were activated representing a switch to cell cycle arrest. This suggests a compensatory mechanism between these receptors. These results strongly suggest that both NKp44 and NKp46 function as controllers by cell cycle arrest and in the absence of NKp46, NK cells become cytotoxic via NKp44 in the presence of high IL-2.



Figure 5.3.7: Cell cycle and apoptosis analysis of CD3+ cells cultured in MLs cultured with agonistic antibodies to NKp44 and NKp46. Mls were isolated and cultured alone, or with agonistic antibodies to NKp44, NKP46 and both NKp44 and NKP46. After 5 days, cells were stained for CD3, Annexin-V and PI. Stages of cell cycle were gated in the PI histogram of CD3+ cells, and the percentage of cells in each stage expressed as a percentage of total CD3+ cells (A). CD3+ cells were plotted as Annexin-V vs PI and those in the Annexin V+PI+ quadrant plotted as a percentage of total CD3+ cells (B). Results represent normalized data from 3 donors, with experiments carried out in triplicate. For representative flow cytometry data, see Appendix 4.6. Error bars represent standard deviation between samples. Significance was determined by Newman Keuls One Way Anova and is as compared to media sample. * $p \le 0.05$ ** $p \le 0.01$ *** $p \le 0.001$.

5.3.8 NKp46 is upregulated at high IL-2 concentrations when blocking antibodies to NKp44 are present

The dual activation of NKp44 and NKp46 at low levels of IL-2 activate NK cell mediated inhibition of T cell cycle, while activation of NKp44 at high IL-2 concentrations induces NK cell mediated T cell apoptosis. However, NKp46 is described as a natural cytotoxicity receptor, and so, has previously been associated with NK cell induced target cell apoptosis. We sought to determine a compensatory relationship between NKp44 and NKp46 to explain this inconsistency between our data and current literature.

Freshly isolated PBMCs were cultured with blocking antibody to NKp44, as well as an unblocked control, prior to IL-2 stimulation. At day 5, cells were stained with fluorescent conjugated antibodies to CD56 and NKp46. The percentage of CD56+ cells expressing NKp46 was determined by flow cytometry and plotted using Graph Pad prism.

When NKp44 was blocked, a significant upregulation of NKp46 was observed at high IL-2 concentrations (Figure 5.3.8). This suggests that when NKp44 is unavailable or has not been induced by IL-2 stimulation, NKp46 can upregulate in its place. This is further supported by the increased T cell cytoxicity observed when NKp46 was activated alone (Figure 5.3.7.B). This is important as NKp44 is not expressed on all NK cells, as IL-2 is essential for its' expression. Therefore, this compensatory mechanism may occur in vivo in the absence of IL-2. When IL-2 is unavailable, and therefore NKp44 is not expressed, it is plausible that NKp46 compensates for this. Again, NKp46 is expressed at much lower levels than reported in literature and this has been further investigated in this study (5.3.5.B).



Figure 5.3.8: NKp46 expression on CD56+ cells cultured in MLs in which NKp44 was blocked. Mls were isolated and cultured alone, or Anti-NKp46, before stimulation with various IL-2 concentrations. After 5 days cells were stained for CD56 and NKp46. The percentage of CD56+ cells that were expressing the NCR in question cells were detected by flow cytometry. For representative flow cytometry data see Appendix 4.7. Results represent normalized data from 3 donors, with experiments carried out in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One Way Anova and is as compared to media sample. ** $p \le 0.01$ *** $p \le 0.001$

5.3.9: NK cell enhancement of Treg proliferation is not mediated by NCRs

Results thus far indicated that NK activation via either NKp44 or NKP46 induced T cell cytotoxicity, while dual activation of these receptors activates NK cell mediated T cell cycle arrest (Figure 5.3.7). Additionally, we have demonstrated that while NK cells inhibited proliferation of the overall CD3+ cell population, they actually promote expression and proliferation of Tregs (Figures 4.3.2 and 4.3.4). It remained to be confirmed if these receptors are involved in NK promotion of Tregs.

From freshly isolated PBMCs, MLs were isolated, and cultured alone, or with agonstic antbodies Anti-NKp44, Anti-NKp46 or both Anti-NKp44 and Anti-NKp46. At day 5, cells were stained for CD4, CD25 and Foxp3, and the percentage of cells positive for expression of all three markers were deemed Tregs, and expressed as a percentage of total MLs

Activation of these receptors had no significant effect on the Treg expression in these cultures (Figure 5.3.9). Previous results also demonstrate that NK cell promotion of Tregs is not necessarily dependent on IL-2 (Figure 4.3.7). These results suggest that while NK cells are activated via NKp44 and NKp46 to inhibit Tcons, they are activated in a different way to promote Tregs, either during infection or at low doses of IL-2.



Figure 5.3.9: Treg marker expression in MLs, cultured with agonistic antibodies to NKp44 or NKp46. Mls were isolated and cultured alone, or with agonistic antibodies to NKp44, NKP46 and both NKp44 and NKP46. At day 5, cells were stained for the presence of CD4, CD25 and Foxp3 and CD4+CD25+FOxp3+ cells represent Tregs which were expressed as a percentage of total MLs. For representative flow cytometry data see Appendix 4.8. Results represent normalized data from 3 donor, with experiments carried out in triplicate. Error bars represent standard deviation between samples.

5.3.10: IL-2 production is not restored during HPIV3 infection when NCRs are blocked

We have determined that the dual activation of NKp44 and NKp46 induces NK cell mediated CD3+ cell cycle arrest at low doses of IL-2, and also when bound to HPIV3 HN. Low doses of IL-2 have also been associated with HPIV3 during infection. However, it is unclear, whether HN binding to NKp44 and NKp46 induce inhibited IL-2 during infection, or if HN induces this independently.

Mixed lymphocytes were cultured with HPIV3 infected CD14+ cells, or were cultured with anti-NKp30, anti-NKp44, anti-NKp46 or both Anti-NKp44 and Anti-NKp46 antibodies prior to coculture with infected cells, as well as previously described controls. Supernatants were kept for ELISA to examine levels of IL-2 in cultures

The inhibited IL-2 production observed during HPIV3 infection occurred even when NK NKp44 or NKp46 were blocked (Figure 5.3.10). This suggests that during infection, HPIV3 HN inhibits IL-2 production in a non-NCR associated mechanism. This is coincidental, as both viral infection and the low levels of IL-2 associated with infection both activate NK cells in the same way.



Figure 5.3.10: IL-2 production by MLs in response to HPIV3 infection, when NCRs are blocked. Freshly isolated CD14+ cells were infected with HPIV3 or influenza and washed after 2 hours. These were then cocultured with freshly allogeneice mixed lymphocytes, cultured alone, or with blocking Anti-NKp44 or NKp46. Cells were also cultured with PMA and Anti-CD3 as a positive control, and media as a negative control. After 5 days, cells were harvestedand supernatants used to detect IL-2 concentrations (pg/ml) by ELISA. Results represent normalized results from 3 donors, with experiments carried out in triplicate. Error bars represent standard deviation between samples.

5.4 Discussion

We have demonstrated that HPIV3 infection inhibits IL-2 production. This reduced IL-2 induces NK regulation of T cell proliferation. This mechanism is abrogated using neutralizing antibodies to HPIV3 HN. Coincidently, published data has demonstrated that HPIV3 HN binds NKp44 and NKp46, but not NKp30 (Biassoni et al 2002, Mandelboim et al 2001). Furthermore, the addition of IL-2 to our cultures could reverse the proliferative regulation by NK cells and IL-2 stimulation is required for expression of the NCR NKp44. From this evidence, we hypothesised that the key mechanistic drivers of this IL-2 induced regulation of CD3+ cell proliferation involved NKp44 and NKp46 (Cantoni et al 1999). We therefore examined the expression of NCRs NKp44, NKp46 and NKp30 during HPIV3 infection. We found no change to NKp30 during HPIV3 infection. However, we observed an upregulation of both NKp44 and NKp46 during HPIV3 infection (Figure 5.3.1), which was abrogated when HPIV3 HN, but not F was blocked (Figure 5.3.2). As there is no documented interaction between NKp30 and HPIV3 HN, and we have found no change in expression of this receptor during HPIV3 infection, this receptor was a good negative control for these experiments. When either NKp44 or NKp46 were blocked prior to coculture with HPV3 infected CD14s, mixed lymphocyte proliferation was restored to levels similar to influenza responses during infection (Figure 5.3.3). We also detect significant enhancement of CD3+ T cell proliferation in anti NKp44 or NKp46 treated, HPIV3 infected co-cultures where CD3+ cell proliferation was measured using CFSE incorporation (Figure 5.3.4). For both ML and CD3+ cell proliferation, restoration was further enhanced when both receptors were blocked simultaneously, suggesting that these receptors act synergistically. These results indicate that HN requires access to NK receptors NKp44 and NKp46 to drive their regulatory response toward CD3+ cells. This provides a functional rational for previous studies which demonstrates HPIV3 HN binding to these NK receptors (Biassoni et al 2002, Mandelboim et al 2001). This is a plausible mechanism for failed immunological memory in humans and may also explain the successive failures of vaccines that have involved use of this component. We suggest that the success of future vaccines against this important pathogen will require the generation of modified HN versions that retain immunogenicity but do not bind/interact with human NKp44 and NKp46.

Having determined a functional role for the interaction between HPIV3 HN and NK receptors NKp44 and NKp46, we sought to determine a role for these receptors in IL-2 dependent NK

cell regulation of T cells. We therefore examined the expression of the NCRs, NKp44, NKp46 and NKp30 in response to IL-2. Interestingly at low IL-2, both NKp44 and NKp46 are expressed during the cell cycle arrest phase of control. However, at high IL-2 NKp46 expression is almost absent from these cells with remaining high levels of NKp44, coincidental with T cell cytotoxicity. Furthermore, these receptors are weakly expressed at intermediate levels of IL-2. NKp30 expression levels remain unchanged in response to IL-2 (Figure 5.3.5). To investigate if this differential expression had a physiological effect on the type of NK regulation choice we repeated the experiments with blocking antibodies to NKp44, NKp46 and NKp30. Blocking NKp46 resulted in a loss of regulation by cell cycle arrest at low IL-2 but apoptosis continued in these cultures at high IL-2. Blocking NKp44 resulted in both a loss of cell cycle arrest at low IL-2 and apoptotic function at high IL-2 in these cultures. It is not clear why blocking these receptors appear to induce cell cycle arrest at high IL-2 except that saturation of the receptors may not have occurred in which case this effect would mimic low dose IL-2. Again, NKp30 had no effect on the capacity of CD56+ cells to induce cell cycle arrest or apoptosis in these cultures (Figure 5.3.6). To further investigate the involvement of these receptors, agonistic NCR antibodies were used to activate NKs in the absence of IL-2. In keeping with previous results, activation of NKp44 and NKp46 together induced a significant increase in cell cycle arrest of T cells by NK cells, mimicing that seen at low levels of IL-2. Similarly, increased cytotoxicity was observed when NKp44 alone was activated. Although activation of NKp46 alone also induced apoptosis, this cytotoxicity was abrogated when both receptors were activated, representing a switch to cell cycle arrest (Figure 5.3.7). These results strongly suggest that both NKp44 and NKp46 function as controllers by cell cycle arrest and in the absence of NKp46, NK cells become cytotoxic via NKp44 in the presence of high IL-2. We also demonstrate that when NKp44 is unavailable, NKp46 is upregulated, suggesting a possible compensatory role between these receptors (Figure 5.3.8). Activation of NCRs had no significan effect on Treg expression (Figure 5.3.8).

This is the first study to link these NCRs with a cell cycle arrest mechanism of control, as these receptors have been associated exclusively with cytotoxicity (Bielekova *et al* 2006, Moretta *et al* 2001). It is perhaps not surprising that these receptors are not involved in Treg enhancement by NKs as we would not expect receptors that are involved in reducing cell numbers to be likely candidates for pro-proliferative responses. In addition to giving insight into HPIV3 immune evasion, these results indicate that these receptors may be key targets in

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conditions such as autoimmunity. They also add significant mechanistic insights into the beneficial effects of low dose IL-2 (Saadoun *et al* 2011, Koreth *et al* 2011).

With these results providing further insight into the dual mechanism of T cell regulation by NK cells, we hypothesise that targeting the expression or activation of NCRs may provide another therapeutic opportunity in the control of autoimmunity and transplant rejection.

6 Role of glycoproteins in immune evasion during HPIV3 infection

6.1 Introduction

We have used neutralizing antibodies to HPIV3 surface glycoproteins and demonstrated that Anti-HN abrogates NK cell (Figure 2.3.5) and Treg expansion (Figure 4.3.1), reduced IL-2 production (Figure 2.3.7), and the subsequent T cell cycle arrest associated with infection (Figure 2.3.8). In addition, we have shown that blocking NKp44 and NKp46 also inhibits this regulatory mechanism (Figure 5.3.6).

The suggestion that HN may induce failed T cell responses is of importance, as HN is a key antigenic component necessary to the development of vaccines. We sought to isolate HPIV3 HN, and determine whether in isolation, this protein maintains its ability to induce NK cell mediated T cell cycle arrest, or if it needs to be presented to NK cells by CD14+ cells in a specific orientation. This was also of interest for F protein, as Anti-F restored cell cycle in T cells (Figure 2.3.7), but unlike Anti-HN, Anti-F did not reinstate proliferation in infected cultures (Figure 2.3.4).

In addition to our interest in HPIV3 HN from a vaccine perspective, this pathway may be an appealing target for the control of inflammatory conditions or transplant rejection. The induction of T cell cycle arrest in the conventional T cell population, as well as promotion of Tregs, could be achieved by targeting IL-2 levels, which is difficult in vivo, or by activating both NKp44 and NKp46 directly. As antibody data suggests that HPIV3 HN does both of these during infection, we wished to also identify if purified forms of HN could mimic the regulatory effects of the viral infection. As HN binds extracellular targets, this protein is also an attractive option for drug delivery.

In this study, we isolated HPIV3 HN and F, and then repeated experiments previously carried out with total HPIV3. The ability of both proteins in isolation to induce expanded NK cells, and subsequent T cell cycle arrest was studied. Additionally, their capacity to induce inhibited IL-2 levels was of importance for the consideration of HN as an anti-inflammatory molecule. If HN in isolation induced the same overall T cell inhibition, this will have an impact for current or future vaccine approaches to HPIV3.

6.2.A The specific aims of the work described in this chapter were as follows:

-To purify HPIV3 surface glycoproteins HN and F

-To investigate the ability of isolated HPIV3 HN to induce the same regulatory mechanism as viral infection:

-inhibition of ML proliferation

-induction of T cell cycle arrest

-expansion of CD56+CD3- populations

-inhibition of IL-2 production

-enhancement of Treg populations

-upregulation of NCRs NKp44 and NKp46

6.2 Materials and Methods

Table 6.2.1: Additional	reagents to	table 2.2.1,	, 3.2.1, 4.2.1,	5.2.1 used in	this study
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	1	1
Product	Catalogue #	Company
Triton-X 100	X100-100ml	Sigma-Aldrich, Dublin 24,
		Ireland
Potassium Chloride (KCl)	P9541-500G	Sigma-Aldrich, Dublin 24,
		Ireland
Dialysis tubing	D9277-100ft	Sigma-Aldrich, Dublin 24,
		Ireland
Acrlyamide/Bis-acrylamide	A2792	Sigma-Aldrich, Dublin 24,
5		Ireland
Sodium dodecyl sulphate	442442F	Sigma-Aldrich, Dublin 24.
(SDS)		Ireland
Ammonium persulfate (APS)	A3678-25G	Sigma-Aldrich Dublin 24
		Ireland
Tetramethylethylenediamine	T9281-25MI	Sigma-Aldrich Dublin 24
(TEMED)	19201 231012	Ireland
Glycine (HaNCHaCOOH)	5 00190 1000	Merck Darmstadt Germany
NuPage Av LDS buffer	NP0007	Invitrogen Dun Laoghaire
Null age 4x LDS builer	1110007	Ireland
Durbhua practained marker	NY 405160	Evendoon Combridgeshire
Rundue prestanet marker	NAA03100	Expedenti, Cambridgesille,
Coomagia bha stain	D8522	UN Sigma Aldrich Dublin 24
Coomassie blue stain	D 8322	Signa-Aldrich, Dublin 24,
	220000	Rieman Aldrich Dehlin 24
Acetic Acid	320099	Sigma-Aldrich, Dublin 24,
	G0140	
Cyanogen bromide-activated	C9142	Sigma-Aldrich, Dublin 24,
sepharose 4B	05115	Ireland
Hydrochloric acid (HCl)	07115	Sigma-Aldrich, Dublin 24,
		Ireland
Sodium acetate	S2889	Sigma-Aldrich, Dublin 24,
		Ireland
Octyl glucoside	29836-26-8	Sigma-Aldrich, Dublin 24,
		Ireland
Sodium thiocyanate	251410	Sigma-Aldrich, Dublin 24,
		Ireland
BCA protein assay	23225	Thermo scientific
Nitrocellulose membrane	66485	Pall Life Sciences, MI, USA
Full range rainbow molecular	RPN800E	GE Helathcare life sciences,
weight marker		Buckinghamshire, UK
Tween	T7949	Sigma-Aldrich, Dublin 24,
		Ireland
Supersignal enhancer	44640	Thermo Scientific
Hyperfilm ECL	RPN1674K	GE Healthcare UK Ltd,
		Buckinghamshire, UK
Kodak GBX developer	P7024	Sigma-Aldrich, Dublin 24.
r		Ireland

Kodak GBX fixer	P7167	Sigma-Aldrich,	Dublin	24,
		Ireland		
Anti-Mouse IgG-HRP Ab	HAF007	R&D systems	Europe	Ltd.,
		Oxon, UK		

Fable 6.2.2:Lists of addition	al equipment	to those in	table 2.2.2
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Equipment	Model	Company
Gel box (electrophoresis)	AE6450	MSC Ltd, Dublin 15, Ireland
Semi-dry transfer cassette	AE6675L	MSC Ltd, Dublin 15, Ireland
Mini Orbital shaker	SSM1	MSC Ltd, Dublin 15, Ireland
DNR mini-Bio Pro Bio-	MiniBIS pro	DNR, Jerusalem, Israel
imaging system		
Platform rocker	STR6	MSC Ltd, Dublin 15, Ireland

6.2.1 Viral Disruption and HN+F precipitation

HPIV3 infected CD14+ cells in 0.01M phosphate buffer, pH 7.2 (PB) were treated with an equal volume of disruption buffer (2% [vol/vol] Triton X-100 [Sigma Aldrich UK] and 2M KCl in PB) for 30 minutes at 22°C. The liberated nucleocapsids were removed by ultracentrifugation (190,000xG for 1 hour), resulting in a supernatant containing the HN, F and M proteins. Dialysis of the supernatant against 0.01M PB decreased the ionic strength, which caused precipitation of the M protein. The M protein was subsequently removed by ultracentrifugation (50,000xG for 30 minutes), which resulted in a solution that contained only the F and HN proteins (Thompson *et al* 1988).

6.2.2 Size based protein detection

6.2.2.1 SDS-PAGE Gel precipitation

Before proceeding with further isolation of viral proteins, we wanted to confirm the presence of HPIV3 HN and F at this stage. Sodium Dodecyl sulphate-Polyacrylamide Gel electrophoresis was carried out in order to separate the protein within each sample according to molecular weight (size). A separating gel was composed of 3.5ml 40% Acrylamide (Sigma Aldrich), 2.5ml Tris-HCL (pH 8.8) (Sigma Aldrich), 100µl 10% (w/v) SDS (Sigma Aldrich), 10% 100µl (w/v)fresh ammonium persulfate (APS), 4µl N, N, N', N' tetramethylethylenediaminee (TEMED) (Sigma Aldrich) and the volume was brought up to a total of 10ml with dH₂O. TEMED initiates the polymeristation reaction and therefore is added just before the gel is poured. Before adding the TEMED, the plates for the gel were

prepared by placing the seal onto the plate with the lip facing upwards, and by fastening the two plates together using bulldog clips. Once the TEMED was added, the gel was mixed and poured between the two plates using a Pasteur pipette up to $\sim 2/3$ full. Ethanol (Sigma Aldrich) was then applied to the top of the separating gel to remove any air bubbles and to stop atmospheric oxygen from inhibiting the polymerisation reaction. The gel was left to polymerase for ~ 30 minutes.

The stacking gel is cast over the separating gel and has larger pores. The stacking gel was made up with 830µl 40% Acrylamide (Sigma Aldrich), 630µl Tris-HCL (pH6.8) (Sigma Aldrich), 50µl SDS (Sigma Aldrich), 50µl 10% 10% (w/v) fresh APS and 5µl TEMED (Sigma Aldrich) and the volume was brought up to a total of 5ml with dH₂O. Before adding TEMED, and when the stacking gel was fully polymerised, the ethanol layer was poured off onto a tissue and the stacking gel was then overlaid on top of the separating gel. A 12 tooth comb was introduced into the stacking gel and the gel was allowed to polymerise for ~30 minutes. Once polymerised, the gel was transferred to the electrophoresis tank and fixed, ensuring the groove side of the gel is placed facing towards the insdie of the chamber. The central chamber was then filled with Tris-Glycine running buffer. The comb was then removed, and the wells were straightened and blown out. Air bubbles were also removed from the base of the gels by tilting the whole apparatus to one side and refilling the central chamber

6.2.2.2 Sample preparation

A 16µl volume of solumble sample was added to 8µl of NuPage 4X LDS sample buffer (Invitrogen) and the mixture was heated for 5 minutes at 90°C. A 5µl volume of Runblue Prestained marker (MyBio) was applied to the first well and 20µl of each sample was applied to the wells. The gels were run at 140V until the dye front had electrophoresed off the gel (~90minutes). The gel was then removed carefully and placed in Coomassie blue stain (10% Acetic Acid) overnight at room temperature and was then visualized under white light using a DNR mini-Bio Pro Bio-imaging system.

6.2.3 HN and F isolation by immobilising A-HN and A-F onto 4B-sepharose matrices

Antibodies to either HN or F were coupled to sepharose 4B matrices for the isolation of HPIV3 HN or F from already disrupted and dialysed samples. CNB-activated sepharose (GE

Healthcare UK) are pre activated media used for the coupling of antibodies or other large proteins containing -NH₂ groups to the sepharose media, by the cyanogen bromide method, without an intermediate spacer arm. To prepare medium (sepharose), lyophilised powder was suspended in 1mM HCl. The medium swelled immediately and was washed for 15 minutes with 1mM HCl. Antibodies were diluted to a final volume 5ml in coupling buffer (0.1M NaHCO₃ pH 8.3 containing 0.5M NaCl) and this was added to prepared medium. This solution was rotated end-over end for 1 hour at room temperature or overnight at 4°C. Excess antibody was washed away with at least 5 X volumes of coupling buffer. Any remaining active goups on sepharose were blocked by transferring the medium to 0.1M Tris-HCl buffer, pH8.0 and allowed to stand for 2 hours. This was followed by washing 3 times successively with 0.1M-Tris-HCl, 0.5M-NaCl, pH8.9 and 0.1M sodium acetate, 0.1M NaCl, pH 4 to remove uncoupled protein. The sepharose beads were packed in a column and the crude HN and F containing lysate was passed slowly over the column at a flow rate of 10ml/hour and recycled at least 5 times. The unabsorbed material was used for purification of the other glycoprotein through a corresponding antibody-coupled sepharose 4B matrix. The column was washed with 20 vol wash buffer (10mM-Tris-HCl pH 8.0, 1mM EDTA, 0.1% octylglucoside). Finally, HN and F was eluted with 3M-sodium thiocyanate, and dissolved in washing buffer. The eluted material was collected and directly concentrated in a collodion bag against several changes of 10mM-Tris-HCl, 150mM NaCl, 0.01% NaN₃, pH7.6 (Ray and Compans 1987).

6.2.4 Determination of protein concentration by BCA assay

The BCA protein assay (Thermo Scientific, Pierce), is a colorimetric and quantitative method of protein detection. A standard curve of known protein concentration is set up here for comparison with an unknown. This was done with a top working standard of 2000µg/ml BSA, as well as 1500, 1000, 750, 500, 250, 125 and 25μ g/ml. A BCA wording reagent aliquot was made by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B (50:1), (A:B). A volume of 25μ of each standard or unknown sample replicate was pipetted into a microplate well. To each well, 200µl of the working reagent was added, and the plate was mixed thoroughly on a shaker for 30 seconds. The plate was covered and incubated at 37° C for 30 minutes. Absorbance was measured at 562nm using a plate reader and unknown sample could be determined based on the standard curve. Protein concentrations were determined for both isolated HN and F.

6.2.5. Detection of isolated HPIV3 HN and F by Western Blot

6.2.5.1. Background

Western blotting is a method used to detect specific proteins in a cell lysate. Firstly, protein concentration is quantified. The proteins are then run on a polyacrylamide gele by electrophoresis and the proteins are separated according to their molecular weight. Proteins are transferred to a stable support such as nitrocellulose membrane and specific proteins are detected by primary antibodies (mAbs) that bind to their corresponding protein on the membrane. To detect the primary antibody, a secondary antibody directed toward the species specific portion of the primary antibody is incubated with the protein membrane. This antibody is commonly, a horseradish peroxidase-linked secondary Ab, which is used in conjunction with a chemiluminescent agent, which react together forming a product that produces luminescence in proportion to the amount of protein. A sensitive sheet of photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot (Janeway *et al* 2005).

6.2.5.2 Method

A protein gel was performed as previously described. Two sponges, two pieces of filter paper and an appropriate sized piece of nitrocellulose paper were soaked in cold transfer buffer (3.03g Trizma Base, 14.4g Glycine, 200mls methanol), for 10-15 minutes before use. A black and white transfer cassette was set up as follows: Black; sponge (one or two), filter paper (one or two), gel membrane, filter paper (one or two), White; sponge. Making sure there was no air bubbles between the gel and the membrane, the cassette was closed, ensuring it was tightly compacted. This was placed into buffer box, which was filled with cold transfer buffer. This box was placed in a styroform box of ice, and the transfer was run at ~100volts for 2 hours (or overnight at ~25V in a cold room). A volume of 10mls blocking solution (5% Marvell milk powder) was prepared. The nitrocellulose was placed in a container on a shaker at 30rotations per minute for 1 hour at room temperature. The blocking buffer was then removed and disposed of, and the membrane was washed with 6-8mls of TBS-Tween for 5 minutes shaking at 30rpm. This wash was repeated 3 times. The primary antibody (Anti-HN or Anti-F) was diluted 1:1000 in blocking solution. This antibody solution was added to the nitrocellulose membrane, and this was shook at 30rpm for 1 hour at room temperature, or overnight at 4°C. The primary antibody solution was then removed, and the membrane was

washed with 6-8ml of TBS-Tween for 5 minutes shaking at 30rpm. This was repeated 3 times. The secondary antibody was diluted 1:1000 in blocking solution. As these antibodies were developed in mouse, an anti-mouse secondary antibody was used. This antibody solution was added to the nitrocellulose membrane, which was placed on rotation at 30rpm for 1 hour at room temperature. Secondary antibody solution was then removed, and the membrane was washed with 6-8ml of TBS-Tween for 5 minutes shaking at 30rpm. This wash was repeated 3 times. The rest of this procedure was carried out in a dark room, with only red light. A volume of 1ml of supersignal enhancer and 1ml stable solution were added together in an epindorf. The TBS-Tween solution was removed from the nitrocellulose. The enhancer/stable solution were poured over the membrane, and left for 1 minute. Using a tweezers, the nitrocellulose membrane was placed in between the plastic inside of a hypercassette. The membrane was smoothed out to remove any air bubbles. An X-Ray film removed from it's packaging and only touching edges, the film was placed onto the plastic of the hypercassette. The lid of the cassette was closed and left for one minute. Again using a tweezers, the X-ray film was placed into the developing solution in a black tray. This was rocked back and forth for 10-30 seconds. Using the tweezers, the X-Ray film was then placed into the fixer solution in a white tray and shaken for 10-30 seconds. The X-ray film was then removed for observation of bands.

6.2.6 Cell cultures and stimulation with isolated viral proteins

CD14+ cells were isolated and infected with HPIV3 or H_1N_1 as previously described. These were washed after two hours and incubated for 24hours. Allogeneic MLs were isolated and cocultured alone, or with uninfected or infected CD14+ cells. HPIV3 HN and F were used to directly stimulated MLs at this stage. A volume of 10µl of either protein was used here. Additionally, influenza HA was used as a control. This serves as a good viral protein control as it too binds NCRs, but does not appear doe induce NK mediated T cell regulation. This protein is a hemagluttinin to which no failure of memory is associated. Therefore this served as a good viral protein control for comparison with HPIV3 HN.

6.2.7 Flow cytometry

Flow cytometry was carried out as previously described. For this chapter the following fluorescent conjugated antibodies were used: Anti-CD56-FITC, Anti-CD3-APC, Anti-
NKp30-PE, Anti-NKp44-PE, Anti-NKp46-PE. Additionally, Annexin-V PI and Treg detection staining kits were used here

6.2.8 ELISA

ELISA for cytokine detection was carried out as previously described. In this chapter IL-2 was detected.

6.2.9 Statistics

All coculture experiments represent normalized results for three donors in three independent experiments. Cyflogic and Facs Calibur software were used for FACs analysis, and plots were created using GraphPad Prism 5.Significance was calculated using Newman Keuls One way Anova: * p < 0.05; ** p < 0.01; *** p < 0.001.

6.3 Results

6.3.1 Viral precipitate from HPIV3 inhibits the proliferation of mixed lymphocytes

The presence of HN and F following viral disruption and precipitation was acertained (Appendix 5.1). Before proceeding to complete glycoprotein purification, the effects of the disrupted lysate on mixed lymphocyte proliferation were examined.

MLs were stimulated with HPIV3 viral precipitate. MLs were also cocultured with HPIV3 or influenza infected CD14+ cells, in addition to previously described controls of media, and uninfected CD14+ cells. After 5 days, the proliferative capacity of cultures was detected by MTS assay.

In agreement with results for whole viral infection, viral precipitate induced inhibited mixed lymphocyte proliferation compared to normal viral response such as influenza (approaching significance). It is important to remember, that these HPIV3 proteins are not completely isolated here, and there is a possibility that some intact viral particle may still be present. However, this gives an indication that protein functionality has been maintained thus far.



Figure 6.3.1: Proliferative responses of allogeneic mixed lymphocytes to HPIV3 viral precipitate: MLs were stimulated with viral precipitates and cocultured with allogeneic CD14+ cells. Additionally, MLs were cocultured with HPIV3 or influenza infected CD14+ cells, and previously established controls. After 5 days cells were harvested and cocultured with MTS solution for 4 hours. Absorbance was detected at 490nm. Results represent normalized data from 3 donors, with experiments carried out in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One Way Anova and is as compared to CD14+sample. ***p \leq .0.001

6.3.2: HPIV3 viral precipitate induces Cell cycle arrest in CD3+ cells

Having determined that disrupted HPIV3 with precipitated HN and F inhibited mixed lymphocyte proliferation in an MLR, it was important to confirm that a smilar CD3+ cell cycle arrest mechanism to HPIV3 virus was in operation, before isolating specific viral proteins.

MLs were isolated and cultured with or without allogeneic CD14+ cells and viral precipitates. Additionally, HPIV3 or influenza infected CD14+ controls were included. Previously described ML controls were also used. Upon harvest, cells were stained with CD3, as well as PI staining. Cells expressing CD3 were gated for and the percentage of these in the G0/G1 phase of cell cycle as determined by histogram analysis were expressed as a percentage of total CD3+ cells.

Consistent with HPIV3 infection, the precipitated viral preperations retained CD3+ cell cycle arrest in the G0/G1 phase of cell cycle (Figure 6.3.2). As protein gel suggests the presence of HPIV3 HN and F (Appendix 5.1), and having demonstrated that this precipitated disrupted HPIV3 induces similar results to whole virus, our next aim was to completely purify HPIV3 HN and F.



Figure 6.3.2: Cell cycle responses to disrupted HPIV3 virus. MLs were cocultured with allogeneic CD14+ cells and stimulated with HPIV3 viral precipitate. Additionally, MLs were cocultured with HPIV3 or influenza infected CD14+ cells, and previously established controls. After 5 days, cells were stained for CD3 and PI. Stages of cell cycle were gated in the PI histogram of CD3+ cells, and the percentage of cells in each stage expressed as a percentage of total CD3+ cell. Representative flow cytometry data can be seen in Appendix 5.2. Results represent normalized data from 3 donors with each experiment carried out in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One Way Anova and is as compared CD14+ sample. * $p \le 0.05$ *** $p \le 0.001$

6.3.3: Confirmation of Isolation of HPIV3 HN and F proteins

Having further purified HN and F from the disrupted lysate, by coupling antibodies specific to these proteins to sepharose matrices, it was necessary to confirm the presence of these proteins after this harsh isolation step.

Western blot analysis was carried out on column elutions. The mouse derived antibodies used to isolate these proteins were also used for their detection. For the Anti-HN gel (Figure 6.3.3.A), buffer, disrupted CD14+ cells, and the eluted F portion were used as controls for non-specific binding. The HN elution resulted in a band between 48 and 71 kDa markers. The expected outcome for HN was ~63kDa. Similarly, for the Anti-F gel (Figure 6.3.3.B), buffer, disrupted CD14+ cells, and the eluted HN portion were used as controls for non-specific binding. The F elution resulted in a band slightly above the 48kDa band marker. The expected outcome for F was 49kDa. No protein was detected in control lanes suggesting no non-specific binding

These results suggest the successful purification of these proteins from the disrupted virus lysate. The ability of these proteins to induce the same NK cell mediated T cell inhibition as viral infection will be investigated in this chapter.



B

Figure 6.3.3: Detection of purified HPIV3 HN and F by western blot. Purified proteins were detected by western blot using mouse derived antibodies supplied by Prof Claus Orvell. For the Anti-HN gel, controls of buffer, disrupted CD14+ cells, and the F elution were used as controls. For the Anti-F gel, controls of buffer, disrupted CD14+ cells, and the F elution were used as controls. The run blue prestain marker was used for comparison of protein sizes. The expected size outcomes were ~63kDa for HN, and ~49kDa for F.

6.3.4: Isolated HPIV3 HN, but not F, inhibits the proliferation of mixed lymphocytes

Having confirmed the isolation of HPIV3 HN and F proteins, we sought to determine whether HN protein still had the ability to inhibit mixed lymphocyte proliferation when in isolation, or if this is just induced by HN in the context of viral infection (Figure 2.3.4).

MLs were stimulated with HPIV3 HN or F, or Influenza HA. MLs were were also cultured with HPIV3 or influenza infected allogeneic CD14+ cells and previously described controls. After 5 days, the proliferative capacity of ML cultures was detected by MTS assay.

Isolated HPIV3 HN, but not F, inhibits ML proliferation to a level similar to HPIV3 infection, when compared to influenza infection, or influenza protein HA (Figure 6.3.4). These results provide evidence that isolated HN can induce the same inhibitory mechanism as observed during HPIV3 infection.



Figure 6.3.4: Proliferative responses of allogeneic mixed lymphocytes to isolated HPIV3 HN and F proteins. MLs were stimulated with HPIV3 HN or F, of influenza HA. Additionally they were cocultured with HPIV3 or influenza infected allogeneic CD14+ cells, and previously established controls. After 5 days cells were harvested and cocultured with MTS solution for 4 hours. Absorbance was detected at 490nm. Results represent normalized data from 3, with experiments carried out in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One Way Anova and is as compared to CD14+ sample. ** $p \le 0.01$

6.3.5: Isolated HPIV3 HN but not F induces Cell cycle arrest in CD3+ cells

We have previously demonstrated increased T cell cycle arrest during HPIV3 infection, which was abrogated using neutralising antibodies to HPIV3 HN or F (Figure 2.3.7). It was of further importance to investigate the ability if these proteins in isolation to induce CD3+ cell cycle arrest.

MLs were stimulated with HPIV3 HN or F, or influenza HA. Additionally, HPIV3 or influenza infected allogeneic CD14+ cells were cocultured with MLs. Previously described controls were also used. Upon harvest, cells were stained with CD3, as well as PI staining. Cells expressing CD3 were gated for and the percentage of these in the G0/G1 phase of cell cycle as determined by histogram analysis were expressed as a percentage of total CD3+ cells.

Isolated HPIV3 HN, but not F protein, increased the number of CD3+ cells maintained in the G0/G1 phase of cell cycle, to a similar level as HPIV3 infection (Figure 6.3.5). While this is reflective of Anti-HN data, it does not correlate to Anti-F data, which showed abrogated cell cycle arrest upon neutralisation of this protein during infection. Although we could detect F protein by western blot, it may have lost its immunological effects, or needs to be in the context of an APC. This suggests that HPIV3 HN is a better candidate as an anti-inflammatory molecule.



Figure 6.3.5: Cell cycle responses to isolated HPIV3 surface glycoproteins. MLs were stimulated with HPIV3 HN or F, or influenza HA. Additionally, they were cocultured with HPIV3 or influenza infected allogeneic CD14+ cells, and previously established controls. After 5 days, cells were stained for CD3 and PI. Stages of cell cycle were gated in the PI histogram of CD3+ cells, and the percentage of cells in each stage expressed as a percentage of total CD3+ cell. Representative flow cytometry data can be seen in Appendix 5.3. Results represent normalized data from 3 donors, with each experiment carried out in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One Way Anova and is compared CD14+ sample. $*p \le 0.05 **p \le 0.01 ***p \le 0.001$.

6.3.6: Isolated HPIV3 HN, but not F, induces expansion of NK (CD56+CD3-) cells

Increased percentages of CD56+ MLs during HPIV3 infection had previously been demonstrated in by group (Noone *et al* 2008). CD56 is a marker for NK cells and we have attributed these immune cells with mediating T cell cycle arrest during infection (Figure 2.3.1). Furthermore, neutralizing antibodies to HPIV3 HN abrogated this CD56+ cell expansion during infection (Figure 2.3.5). Here we investigate the ability of isolated HPIV3 HN to induce this same increase of CD56+CD3- cells.

Freshly isolated MLs were stimulated with HPIV3 HN or F, or influenza HA. They were also cocultured with HPIV3 or influenza infected allogeneic CD14, or previously described controls. Upon harvest, cells were stained for CD56 and CD3, so that CD56+CD3- (NK cells) cells could be gated for by flow cytometry and expressed as a percentage of total lymphocytes.

Isolated HPIV3 HN, but not F, induced an increased percentage of CD56+CD3- MLs, to a level of significance similar to HPIV3 infected CD14+ cells. These results suggest that when in isolation, HPIV3 HN can still induce increased levels of CD56+CD3- cells, which we have shown regulate T cell cycle. This lends further support to data suggesting that this molecule has anti-inflammatory effects. This also supports mechanistic data indicating that unmodified versions of this antigen are unsuitable for vaccine development.



Figure 6.3.6: CD56+CD3- cell expression levels in response to isolated HPIV3 surface glycoproteins. MLs were stimulated with HPIV3 HN or F, or influenza HA. Additionally, they were cocultured with HPIV3 or influenza infected allogeneic CD14+ cells. After 5 days, cells were stained for CD56 and CD3 and analysed by flow cytometry. Those cells in the CD56+CD3- quadrant were expressed as percentages of total lymphocytes. Representative flow cytometry data can be seen in appendix 5.4. Results represent normalized results from 3 donors, with experiments carried ou in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One Way Anova and is as compared to CD14+ sample. ***p ≤ 0.001

6.3.7: Isolated HPIV3 HN, but not F, inhibits IL-2 production

Our group previously demonstrated an inhibition of IL-2 production during HPIV3 infection. Furthermore, addition of IL-2 to infected cocultures restored proliferation to normal viral response (Noone *et al* 2008). Neutralizing antibodies to HPIV3 HN abrogate the inhibition of IL-2 production during infection (Figure 2.3.6). However, we have yet to determine if isolated HPIV3 HN induces the same inhibited levels of IL-2.

MLs were stimulated with HPIV3 HN or F, and influenza HA. Additionally, these cells were cocultured with HPIV3 or influenza infected allogeneic CD14+ cells, and previously described controls. After 5 days incubation, supernatants were examined by ELISA for IL-2 concentrations (pg/ml).

In agreement with antibody data (Figure 2.3.6), isolated HPIV3 HN but not F, inhibits IL-2 produciton, compared to uninfected cells (*** $p\leq0.001$), or the normal viral response of influenza (Figure 6.3.7). This is of importance as inhibited IL-2 has been associated with NK-mediated T cell cycle arrest. This gives further support to evidence that unmodified HPIV3 HN in its' wild type conformation is a poor vaccine candidate. However, while low dose IL-2 therapy has shown promise in autoimmunity, tweaking cytokine levels in vivo can be difficult. As HN inhibits IL-2 produciton, it may be a more suitable therapeutic approach than using IL-2 directly.



Figure 6.3.7: IL-2 production in response to isolated HPIV3 surface glycoproteins. MLs were stimulated with HPIV3 HN or F, or influenza HA. Additionally they were cocultured with HPIV3 or influenza infected allgeneic CD14+ cells, and previously established controls. Upon harvest, supernatants were used to detect IL-2 concentrations (pg/ml) by ELISA. Results represent normalized results from 3 donors, with experiments carried out in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One Way Anova and is as compared to CD14+ sample. ***p \leq 0.001

6.3.8: Purified HPIV3 HN, but not F, enhances Treg expression

We have demonstrated the upregulation of cells expressing Treg markers during HPIV3 infection. This Treg expansion was abrogated using neutralising antibodies to HPIV3 HN (Figure 4.3.1). It was necessary to determine whether isolated HPIV3 HN could also induce this enhanced Treg expression.

MLs were stimulated with HPIV3 HN or F, and influenza HA. Additionally they were cocultured with HPIV3 or influenza infected allogeneic CD14+ cells, or previously described controls. After 5 days, cells were stained for CD4, CD25 and intracellular Foxp3. The percentage of cells positive for expression of all three markers were deemed Tregs, and expressed as a percentage of total MLs.

In keeping with experiments thus far, the immune regulatory mechanism results observed during HPIV3 infection were reproducible with isolated HN. Cells expressing Treg markers were upregulated in the presence of HN (Figure 6.3.8). These results indicate that if unmodified versions of this protein were used in vaccines, this regulatory subset of cells will be enhanced. However, this gives further support to suggestions that this protein would be of use in the treatment of autoimmunity, as Treg enhancement by low dose IL-2 has shown clinically beneficial outcomes in autoimmune conditions (Koreth *et al* 2011, Saadoun *et al* 2011).



Figure 6.3.8:Treg surface marker expression in response to isolated HPIV3 surface glycoproteins. MLs were stimulated with HPIV3 HN or F, or influenza HA. Additionally they were cocultured with HPIV3 or influenza infected allgeneic CD14+ cells, and previously established controls. After 5 days, cells were stained for CD4, CD25, and Foxp3+ cells, with cells positive for all 3 markers representing Tregs. These were expressed as a percentage of total MLs. For representative flow cytometry data see Appendix 5.5. Results represent normalized data from 3 donors, with experiments carried out in triplicate. Error bars represent standard deviation between samples. Significance was determined using Newman Keuls One Way Anova and is as compared to CD14+ sample. ***p \leq 0.001.

6.3.9: Purified HPIV3 HN, but not F, induces upregulated NKp44 and NKp46, but not NKp30 on CD56+ cells

Given the already established interaction between NKp44 and NKp46 with HPIV3 HN (Biassoni *et al* 2002, Mandelboim and Porgador 2001), we have demonstrated upregulation of these receptors during HPIV3 infection. This upregulation was abrogated using neutralizing antibodies to the viral protein HN (Figure 5.3.2). We have also associated the dual activation of these receptors with NK mediated T cell cycle arrest during infection (Figure 5.3.6). Here we aimed to determine whether isolated HN can activated NK cells in the same way as HPIV3 infections.

MLs were stimulated with HPIV3 HN or F, and influenza HA. Additionally, these were cocultured with HPIV3 or influenza infected allogeneic CD14+ cells, and previously described controls. Upon harvest, cells were stained for CD56 and either NKp30, NKp44 or NKP46 experession. The percentage of CD56+NCR+ cells were expressed as a percentage of total CD56+ cells.

No change was observed for NKp30 expression (Figure 6.3.9.A). As previously observed with whole virus experiments (Figure 5.3.2), HPIV3 HN induces upregulation of NKp44 (Figure 6.3.9.B). Additionally, NKp46 was upregulated compared to allogeneic samples by HPIV3 HN (Figure 6.3.9.C). The simultaneous activation of these receptors has been associated with NK activation to induce T cell cycle arrest (Chapter 4). Firstly, this indicates that for viral vaccines, HN is not a good candidate unless its' interaction with NKp44 and NKp46 can be prevented. However, for autoimmunity, HN activation via these receptors and subsequent T cell cycle arrest, represents an appealing alternative to current therapies such as Daclizumab, which target T cell apoptosis. Harnessing T cell cycle rather than T cell death may have less damaging effects on the overall immune response.



Figure 6.3.9: NCR expression levels on CD56+ cells in response to isolated HPIV3 surface glycoproteins. MLs were stimulated with HPIV3 HN or F, or influenza HA. Additionally they were cocultured with HPIV3 or influenza infected allogeneic CD14+ cells, and previously established controls. Upon harvest cells were stained for CD56 and NCRs. The percentage of CD56+ cells that were expressing the NCR in question cells were detected by flow cytometry and plotted using GraphPad prism 5. For representative flow cytometry data see Appendix 5.6. Results represent normalized data from 3 donors in 3 separate experiments in triplicate. Significance is as compared to CD14+ sample of the same data set. $*p \le 0.05 ***p \le 0.001$

6.4 Discussion

In this study we successfully isolated HPIV3 surface glycoproteins HN and F (Figure 6.3.3). We have previously demonstrated that blocking antibodies to HN restore IL-2 production (Figure 2.3.6), abrogate expanded NK (Figure 2.3.5) and Treg populatations (Figure 4.3.2), and NK mediated T cell cycle arrest (Figure 2.3.7). It was important to determine if this regulatory mechanism could be reproduced by isolated protein, or if HN needed to be presented to NK cells in a particular orientation by CD14+.

Here we demonstrate that isolated HN is functional with respect to this immune evasion mechanism. We show that isolated HN induces the same inhibition of mixed lymphocyte proliferation as HPIV3 infected CD14+ cells (Figure 6.3.4), and we demonstrate that this is due to the induction of T cell cycle arrest (Figure 6.3.5). Similarly, isolated HPIV3 HN, but not F, induced expansion of CD56+CD3- cells (Figure 6.3.6), and inhibited production of IL-2 (Figure 6.3.7), to levels previously demonstrated during HPIV3 infection. Treg expression was also upregulated to levels comparable to HPIV3 infection (Figure 6.3.8). Finally, we demonstrate the upregulation of NKp44 and NKp46 when cultured with isolated HN, indicating that this isolated protein can still bind these NK receptors, activating NK mediated T cell inhibition.

We have shown that during infection, and in isolation, HPIV3 HN induces NK cell mediated T cell cycle arrest. This indicates that HN is a poor vaccine candidate for this virus. While we suggest that unmodified HPIV3 HN is a poor molecule for use in HPIV3 vaccines, it may have beneficial effects in the treatment of conditions such as autoimmunity, inflammation and transplant rejection. Current therapies such as Daclizumab (Martin *et al* 2010) target the T cell apoptosis mode of NK cell regulation, due to excess availability of IL-2 to NK cell receptors. However, HPIV3 HN inhibits IL-2. Both HN, and the low IL-2 concentrations associated with this molecule, can activate NKp44 and NKp46 simultaneously. This activates NK cell mediated T cell cycle arrest. It also induces NK cell promotion of Tregs, which have been associated with beneficial outcomes in treatment of autoimmune conditions.

7.1 Conclusion

The immune system protects the host against foreign pathogens through a complex network involving both effector and regulatory mechanisms. This system preserves healthy tissue or "self", which is essential for host survival, enabling the host to control excessive immune responses induced by foreign pathogens, thus limiting immunopathology to self tissue. Additionally, the immune system mounts memory to a pathogen upon clearance, so that a stronger and more efficient response is developed if this pathogen is re-encountered (Janeway 2008, O'Garra *et al* 2004). One pathogen that evades immune response is HPIV3, which has been associated with recurrent and sometimes persistent infections (Plotnick-Gliquin *et al* 2001). This is an important respiratory virus which is responsible for both upper and lower respiratory tract infections of adults, neonates and infants. While neutralizing antibodies to this virus are generated, recurrent infection occurs throughout life suggesting failed memory (Moscona 2005, Henrickson 2003, Chanock 2001), and it has been proposed that this is due to failed T cell proliferation (Plotnicky-Gliquin *et al* 2001). The study of immune responses to this virus is crucial to the development of an efficient vaccine that mounts life long memory to this important pathogen.

Infection with HPIV3 and preliminary vaccine studies in rodents show robust immune responses and memory to this virus. However, these vaccines have failed at human clinical trial. Our group previously sought to develop a human *ex vivo* model for the investigation of respiratory viral infections. They found that the most natural pathway of DC generation was viral infection of monocytes with no artificial treatment, as artificially generated DCs appeared to give skewed results (Noone *et al* 2007). This model was used in subsequent studies evaluating immune responses to HPIV3 infection. In this study HPIV3 was cultured and stocks harvested for use in this *ex vivo* human model.

Our group identified that T cell proliferation during infection can be restored by the purification of the CD3+ T cells from the mixed-lymphocyte population. Additionally, it was demonstrated that the inability of mixed lymphocytes to respond to virally infected DCs was IL-2 dependent and involved T cell cycle arrest. Finally, they showed that the NK cells in the CD14-CD3- component of the mixed lymphocytes were responsible for the inhibition of T cell proliferation and their influence is exerted in a contact dependent manner (Noone *et al* 2008). Additionally, empty virosomes expressing the HPIV3 surface glycoproteins HN and F had the same anti-proliferative effect on T cells in mixed lymphocyte cocultures. As these

virosomes were no longer available, and these results could not be reproduced, we used neutralizing antibodies to these proteins to investigate their role in the induction of NK cell mediated T cell inhibition. We demonstrate that the HPIV3 surface glycoprotein HN inhibits T cell proliferation during infection. Additionally, this protein induces CD56+ cell expansion and inhibited IL-2 production during infection. Finally, we report that both HN and F cause NK cell mediated T cell cycle arrest. However, this induction of cell cycle arrest by F protein is overcome in proliferative results, and as no change in NK levels was associated with this protein, we hypothesize that F inhibits T cell cycle via a different mechanism to HN. Overall, these results indicated that the major envelope proteins of HPIV3, in particular HN, induce NK cell mediated T cell suppression during HPIV3 infection. In keeping with these findings, these surface glycoproteins have been major components of failed vaccines.

We demonstrate that expansion of human CD56^{Bright}, but not CD56^{Dim}, NK cells occurs at both low and high IL-2 levels and this corresponds directly to contracting T cells. When CD56^{Bright} NK cells were depleted from culture an increase in proliferating T cells occurred even in the absence of IL-2 which was not enhanced by additional IL-2. It appears that T cells do not respond in a proliferative sense to IL-2 alone but take direction from CD56^{Bright} NK cells. To investigate the mechanism of control of T cell proliferation we assessed the level of cell cycling and apoptosis in T cell cultures in the presence or absence of NK cells. Consistent with the proliferative results, at low levels of IL-2, in the presence of NK cells, T cells were retained in the G0/G1 phase of cell cycle and underwent apoptosis at high IL-2 levels. This cell cycle arrest at low IL-2 concentrations is consistent with HPIV3 results. Both these effects were not observed in the NK-depleted cultures suggesting that these NKs excercise tight control over T cell fate choices. We determined that this NK cell control of T cells is exerted in a contact dependent mechanism, and not via the production of regulatory cytokines. Our results also raise questions about IL-2 as a T cell growth factor. No change in T cell proliferation was observed in response to this cytokine when NK cells were absent, and neutralizing antibodies to this cytokine did not affect T cell proliferation when NK cells were not present. We also demonstrate that levels of T cell derived IL-2 increase significantly when NK cells are present. From this data, we hypothesized that T cells produce IL-2 as a mode of self-regulation which is drived by NK cells. These results represent a plausible control mechanism whereby T cells are "restrained" by NK cells at low IL-2, proliferate at intermediate IL-2 concentrations, and are eliminated at high IL-2 by cytotoxic NK cells. This low dose IL-2 is reflective of HPIV3 infection. Our results indicate the overall importance of NK cells, not only for evasion of immune memory by HPIV3 infection, but as key regulators of immune response.

As increased Tregs and reduced Tcons have been reported following low dose IL-2 therapy (Koreth et al 2011, Saadoun et al 2011) we investigated the effects of NK cells on the response of key sub-population of T cells to HPIV3 infection and low dose IL-2. Of interest for HPIV3 infection, inhibited memory responses were observed at low and high IL-2 concentrations, or during HPIV3 infection. This was abrogated using neutralising antibodies to HPIV3 HN. This result supports our previous data on the role of NK cells in reduced proliferative responses to this virus as it is highly consistent with the poor immunological memory associated with HPIV3 infection in humans. Conversely to the overall CD3+ cell, but reflecting the clinical data (Saadoun et al 2011, Koreth et al 2011), NK cells actually boosted Treg populations at low dose IL-2, and this response was absent in NK depleted cultures. In keeping with the beneficial effects of low dose IL-2 treatment in humans with inflammatory conditions we find that key cells expressing pro-inflammatory markers (Th1, Th17, CD8+, memory) are reduced at low IL-2 in the presence of NK cells but again we observe an increase in these cell subsets when CD56+NK cells are removed from the cultures. While NK cells inhibit the overall CD3+ cell population, we report that Tregs proliferate poorly to IL-2 in the absence of NKs and demonstrated a significant increase in proliferation in the presence of either CD56^{Dim} or CD56^{Bright} NKs. As transwell inserts only partially inhibited this proliferation, it remains to be determined if particular cytokines are involved here. We suggest that the increased expression of Treg markers at low IL-2 concentrations is associated with the increased number of CD56^{Bright} NK cells. However, when cultured with CD56^{Bright} cells in isolation, Treg proliferation was maintained even at intermediate IL-2 concentrations. We demonstrate that at these intermediate IL-2 levels, CD56^{Bright} NK cells acquire a CD56^{Dim} phenotype which maintain Treg proliferation. Additionally, it was important to confirm that this Tcon regulation relates to NK cells and not to enhanced Tregs levels. We provide supplementary evidence illustrating that depletion of the Treg from CD3+ T cells does not affect the proliferative regulation of CD3 T cells by CD56+ NK cells. We suggest in normal immune response, a threshold level of IL-2 needs to be surpassed before T cells are able to proliferate. Until this occurs, NK cells maintain T cells in G0/G1 phase of cell cycle. Once this IL-2 threshold has been reached, T cells are allowed to proliferate. Finally, at high IL-2 concentrations, when a normal immune response to a pathogen has already occurred, NK cells appear to switch off T cell responses by apoptosis.

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This high IL-2 scenario is also made use of by several autoimmune therapeutics. Additionally HPIV3 viruses appear to target the low dose IL-2 pathway which we would also suggest is the case for patients receiving low dose IL-2.

Having investigated the viral protein responsible for NK mediated T cell regulation during HPIV3 infection, it was of interest to determine the mode of NK cell activation during infection. It is well accepted that viral hemaglutinins including HN from HPIV3 bind NK NCR's, NKp44 and NKp46 but not NKp30 (Biassoni et al 2002, Mandelboium and Porgador 2001). Additionally, IL-2 is required for the expression of NKp44 (Cantoni et al 1999), and we have shown that HPIV3 HN drives NK regulation by limiting IL-2 in the cultures, attributing this to T cell cycle arrest. We investigated if the balance of NCRs had a role in defining the mechanism of regulation during HPIV3 infection and under different IL-2 conditions. Interestingly at low IL-2, both NKp44 and NKp46 are expressed during the cell cycle arrest phase of control. We also demonstrate the dual expression of these receptors during HPIV3 infection, which mimics low dose IL-2. However, at high IL-2, NKp46 expression is almost absent from these cells with remaining high levels of NKp44 coincidental with T cell cyotoxicity by NKs. In keeping with CD56 expression, these receptors are absent at intermediate levels of IL-2. To investigate if this differential expression had a physiological effect on the type of NK regulation choice we repeated the experiments with blocking antibodies to NKp46 and NKp44. As expected, blocking NKp46 resulted in a loss of regulation by cell cycle arrest at low IL-2 but apoptosis continued in these cultures at high IL-2. Blocking NKp44 resulted in both a loss of cell cycle arrest at low IL-2 and apoptotic function at high IL-2 in these cultures. These results strongly suggest that both NKp44 and NKp46 function as controllers by cell cycle arrest and in the absence of NKp46, NK cells become cytotoxic via NKp44 in the presence of high IL-2. This is the first study to link these NCRs with a cell cycle arrest mechanism of control as these receptors had hitherto been associated exclusively with cytotoxicity (Cantoni et al 1999, Biassoni et al 2002). To further investigate the involvement of these receptors, agonistic NCR antibodies were used to activate NKs in the absence of IL-2. In keeping with previous results, activation of NKp44 and NKp46 together induced a significant increase in cell cycle arrest of T cells by NKs, mimicing that seen at low levels of IL-2. Similarly, increased cytotoxicity was observed when NKp44 alone was activated. Although activation of NKp46 alone also induced apoptosis, this cytotoxicity was abrogated when both receptors were activated representing a

switch to cell cycle arrest. It is perhaps not surprising that these receptors are not involved in Treg enhancement by NK as we would not expect receptors that are involved in reducing cell number to be likely candidates for pro-proliferative responses. Additionally, HPIV3 HN inhibits IL-2 secretion during infection. It remained unclear whether HN binding to these receptors inhibits IL-2 during infection or if HN coincidently alters IL-2 independently. Blocking NCRs during HPIV3 infection did not restore IL-2 production suggesting that HN induces IL-2 production in a non-NCR associated mechanism. During infection perhaps both HN binding these receptors and low dose IL-2 induced by this protein activate NK cells via these NCRs. The mechanism by which HN inhibits IL-2 production should be further investigated.

We suggest that the lack of an NKp44 homologue in mouse can be attributed to the strong immune responses and memory observed in this species during infection or with unmodified HN vaccines. In humans, or human models, both HPIV3 HN and low dose IL-2 activate NK cells via NKp44 and NKp46, inducing T cell cycle arrest. As the mechanism for cell cycle arrest appears to involve NK cell activation via the simultaneous activation of these receptors, we believe that the development and use of a humanised mouse model at this stage would not provide a true in vivo model system for this mechanism. Rather, here we provide highly consistent primary human data that mimics the immune outcome in patients on low dose IL-2 therapy and closely reflects the clinical infection with HPIV3 in humans. This is of importance for development of vaccines to this virus in future.

Finally, we isolated HPIV3 surface glycoproteins HN and F, and demonstrated that even in isolation, HN inhibits IL-2 responses, and subsequent NK cell mediated T cell cycle arrest in the Tcon population. Additionally, this molecule promoted Treg expression. These results highlight that HN is a poor molecule for use in the development of a vaccine to this important virus. However, we suggest that in autoimmune conditions, HN induction of T cell cycle arrest could serve as a promising therapy, and would be an alternative to current treatments which target the T cell apoptosis phase of IL-2 stimulation (Martin *et al* 2010, Sheridan *et al* 2011, Bielikova *et al* 2006).

This dual mechanism of T cell regulation by NK cells supports their emerging role as important immune regulators. We hypothesize that targeting the expression or activation of NCRs, which is achievable with isolated HPIV3 HN, may provide another therapeutic opportunity in the control of autoimmunity and transplant rejection. Overall this study

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portrays an elegant mode of regulation by human NKs which reflects the data supporting the beneficial outcome for patients on low dose IL-2 therapy, and gives key insights into failure of immune memory towards HPIV3 infection. Neutralising antibodies to HN are important to succesful immunity to this virus. Therfore, HN will have to be used in any vaccine approach. However, we believe HN in its current conformation will limit its efficacy and will in fact hinder the development of protective immunity to other components. We therefore suggest that modified versions of HN which do not drive NK mediated T cell regulation, but retain immunogenicity, should be generated. During infection, or at low dose IL-2, NK cell regulation shifts the Tcon/Treg ratio in favour of Tregs and this regulation of the Tcon population is due to the NCR's NKp44 and NKp46. Given our data and the clinical evidence for the expansion of CD56^{Bright} NK cells in several therapies involving IL-2 (Shereck et al 2007, Saadoun et al 2011, Soiffer et al 1994, Zorn et al 2006, Murphy 2012, Vellilla et al 2008, Li et al 2008), we would suggest that these CD56^{Bright} NK cells may well prove to be strong correlates of treatment efficacy and perhaps primary players in treatment benefits. Here we highlight the importance of the human model of immunity developed by Noone et al previously (2007), as this key regulatory mechanism would have been overlooked using rodent models. This questions the use of the widely accepted mouse model for the study of viral immune evasion mechanisms. Overall, our results highlight the importance of NK cells in immune regulation, and suggest that these cells have been widely overlooked as an important anti-inflammatory immune subset. It would be unsurprising if in future, they are associated with the beneficial outcome observed in patients receiving low dose IL-2 therapy. They represent an important immune target for the regulation of T cell proliferation.

7.1.1 Novel findings of this study

- 1. Identification of HPIV3 HN as the viral protein responsible for inhibiting IL-2 and inducing NK cell mediated T cell cycle arrest
- 2. Providing a functional rational for the interaction of HN with NKp44 and NKp46
- 3. Associating the dual activation of NKp44 and NKp46 with T cell cycle arrest, and not apoptosis
- 4. Associating NK cells with the promotion of Treg proliferation
- 5. The acquisition of CD56^{Dim} markers by CD56^{Bright} NK cells at intermediate IL-2 levels
- The NK cell mediated dual regulation of T cells at low and high doses of IL-2
- 7. Challenging the dogma of IL-2 as a direct growth factor for CD3+ T cells

7.1.2 Future perspectives

We suggest that HPIV3 HN inhibits T cell proliferation in humans. This is consistent with the poor immunological memory associated with HPIV3 infection, and the failure of vaccines that have involved the use of this viral component. In contrast to our findings in humans, many vaccine candidates have demonstrated excellent immunological profiles in small animal models. However, these approaches have failed to result in a licensed human vaccine for this infection. We suggest that this may be due to the differences in NK biology between these two species and perhaps more specifically that there appears to be no rodent equivalent of human NKp44 (NCBI). Therefore, from this data presented in humans, it is likely that vaccines that contain unmodified HPIV3 HN, whilst inducing robust immunological responses in small animals, will ultimately fail at inducing lasting immunity in humans. To address this, we suggest HPIV3 HN mutants that no longer bind or activate human NKp44 and NKP46 would be essential to any vaccine strategy for this virus. We propose the generation of modified HN proteins that lack this human NK cell activating capacity. It is envisaged that this outcome will provide a vaccine that may be successful at combating HPIV3 and perhaps related viruses that have remained refractory to conventional approaches. This would involve the generation of recombinant sendai virus with native HPIV3 HN and demonstrate NK cell regulation of T cell proliferation response in the established human model. Site directed mutagenesis would be used to derive sendai viruses bearing functionally mutated HN molecules.

Recombinant sendai viruses expressing mutated HPIV3 HN would be examined for the induction of NK cell regulation of T cell proliferation as described previously. Other studies in the lab have shown that sendai virus produces strong proliferation in this assay and therefore this could serve as an appropriate control for these experiments. Reports suggest that HN contact with NKp44 and NKp46 involves sialic acid moieties on the receptor. The structure of the HPIV3 HN protein has been elucidated and has enabled the identification of three arginine residues that mediate sialic acid binding through direct interactions with the sugar (Biassoni *et al* 2002, Mandelboim and Porgador 2001, Arnon *et al* 2004, Ito *et al* 2011, Noone *et al* 2007). Site directed mutagenesis would be used to derive sendai viruses bearing functionally mutated HN molecules. We propose to mutagenize the gene encoding the HN protein to substitute these critical arginine residues with alanine. The goal will be to remove appropriate sialic acid binding activity without grossly perturbing the protein structure

thereby ensuring preservation of the proteins overall antigenic properties. To ensure this we will substitute each of the arginines independently and then in combination in order to identify the minimal substitutions required to eliminate appropriate sialic acid binding activity. This mutated HN will then be tested in our human model as previously describd.

These studies will have direct health benefits for the design of better vaccines to combat HPIV3 infection in humans. However, HPIVs share the same pathogenic conundrum of poor immunological memory as RSV, and although HN is not a component of RSV, the routes of generation of immunological memory with HPIV3 may also be relevant for unravelling the immunological consequence of RSV infections in humans. Both viruses express F proteins, and while we found HN key in overall NK mediated inhibition of T cell responses during HPIV3 infection, F still induced T cell cycle arrest. This link cannot be overlooked and further investigation of the immune regulation during RSV infection could be imperative for vaccine development. This future research will enhance our knowledge of NK immune regulation, a process which has recently been identified as key to protection from autoimmunity, with further implications for transplant rejection, inflammatory disease and the development of cancer. Having identified IL-2 dependency for this NK cell immune regulation, the specific mechanisms by which IL-2 production is inhibited by HN should be investigated, as these may be of use for the induction of T cell cycle arrest in vivo. Additionally, the specific mechanism by which NK cells induce this T cell cycle arrest should be further investigated.

Appendix 1 - Chapter 2

PBMCs 11.49% Histo-1 2.8 10.58 100 1.93 8.27 180.65 Visible 10133 11.79 Histo-1 1195 113.02 Isolated CD14+ 95.76% 99.9 Visible 1369 95.92 56.09 Histo-1 1311 95.76 100.06 64.83 95.79 CD14+

Appendix 1.1: CD14+ cell purity

Cells were positively selected and stained with anti-human CD14-PE. Both total PBMCs and isolated CD14+ cells were stained for comparison. Marker was set based on unstained cells. The CD14+ cell enriched population had a purity of >95% (95.76%).

Appendix 1.2: CD3+ cell purity



Cells were positively selected and stained with anti-human CD3-APC. Both total PBMCs and isolated CD3+ cells were stained for comparison. Marker was set based on unstained cells. The CD3+ cell enriched population had a purity of >95% (95.45%).

Appendix 1.3: CD56+ cell purity



Cells were positively selected and stained with anti-human CD56-FITC. Both total PBMCs and isolated CD56+ cells were stained for comparison. Marker was set based on unstained cells. The CD56+ cell enriched population had a purity of >95% (95.54%).

Appendix 1.4: Determination of TCID₅₀/ml for cultured HPIV3.

The TCID₅₀/ml of a virus is described as the median tissue culture infective dose. This refers to the amount of the virus that will produce a pathological change in 50% of the cell culture it is inoculated in. The TCID₅₀/ml varies with each virus type (http://medical-dictionary.thefreedictionary.com/TCID50). The standard TCID₅₀/ml of HPIV3 is 6.

Vero cells were cultured with a range of viral dilutions (1:10 down a table with 1:100 as the top working concentrations). These were stained with crystal violet, examined by light microscope and scored for the presence or absence of syncytia. Syncytia are multi-nucleated cells that appear as a circular cluster. The titre was read at day 5 by removing media and replacing with crystal violet, before air drying. Wells were scored as either positive or negative for the presence of syncytia and the $TCID_{50}/ml$ of the cultured HPIV3 was determined as 9.7, using the following equation:

LogTCID₅₀/ml=(number of positive wells, between 0 and 40) x 0.2 –log(lowest dilution, usually 10^{-3}) +0.5

This meant that for infection of CD14 cells it was determined that a 1/10 dilution of the virus should be performed and a working volume of 6.2µl be used per well of 1×10^6 cells.





Crystal violet assay of HPIV3 infected vero cells. Crystal Violet assay was used to score presence of syncytia (as circled) for the calculation of $TCID_{50}/ml$ of the virus.

Image was taken on light microscope (A), and a clearer image (B) taken from: http://www.tau.ac.il/lifesci/departments/biotech/members/rozenblatt/figures.html).

Appendix 1.5 Confirmation of HPIV3 infection.

3.+20The nucleocapsid protein (NP) of HPIV3 is a stably expressed viral protein, which is essential for replication of the virus. This protein is expressed intracellularly after infection of target cell (Henrickson 2003). In our case the target cells were CD14+ monocytes. To confirm that the cultured HPIV3 could infect CD14+ monocytes, RNA was isolated from virally infected CD14+ cell pellets. This RNA was then reverse transcribed and HPIV3 NP expression was determined by PCR. The primers used in this assay, as described in section 2.2.7, target a 526 base pair (bp) region of the HPIV3 NP. This was detectable when amplified by agarose gel electrophoresis (figure 2.3.2). As a positive control, PCR for β -actin was also carried out for the same samples. This was done as β -actin is a housekeeping gene. This means that as it is required for the maintenance of basic cellular function, it is expressed in all cells of an organism and so, is a reliable control for PCR (Ruan and Lai 2007). Having confirmed HPIV3 infectivity, we can be sure that results in the following sections can be attributed to viral infection.



Agarose gel (1.5%) of HPIV3 NP PCR product. A 1.5% agarose gel on which HPIV3 NP PCR products were ran. Product size was expected to be 526bp which corresponds to the band on the gel when compared to the standardised DNA ladder.

Appendix 1.6 Apoptotic results for direct mixed lymphocyte infection with HPIV3 (Figure 2.3.3):



Appendix 1.6: Representative flow cytometry data for the direct infection of MLs with HPIV3 or Influenza (H1N1). The upper right quadrant (Annexin-V+ PI+) represents cells in late apoptosis.
Appendix 1.7 Percentage of CD56+ CD3- mixed lymphocytes during HPIV3 infection (Figure 2.3.5)



CD56

Appendix 1.7: Representative flow cytometry data for CD56+CD3- (Lower right quadrant) cells or NK cells. Those in this lower right quadrant were plotted as a percentage of total mixed lymphocytes.

Appendix 1.8.A CD3+ cell cycle when cultured with HPIV3 infected allogeneic CD14+ cells, or those with which HN or F have been blocked (Figure 2.3.7.A)



Appendix 1.8.A: Representative flow cytometry data for PI staining histograms, with the percentage of CD3+ cells arrested in the G0/G1 phase of cell cycle being represented, during HPIV3 infection, or infected cultures where HPIV3 HN had been blocked

Appendix 1.8.B CD3+ cell death when cultured with HPIV3 infected allogeneic CD14+ cells, or those with which HN or F have been blocked (Figure 2.3.7.B)



Appendix 1.8.B: Representative flow cytometry data for Annexin-V and PI staining dot plots, where those in the upper right quadrant represent cells in late apoptosis. The percentage of CD3+ cells in this quadrant was determined during HPIV3 infection, or infected cultures where HPIV3 HN had been blocked

Appendix 1.9 CD3+ cell proliferation, as determined by CFSE incorporation, when cultured with HPIV3 infected allogeneic CD14+ cells, or those with which HN or F have been blocked (Figure 2.3.8)



Appendix 1.9: CFSE was incorporated into CD3+ cells, to determine proliferative responses to HN mediated HPIV3 infection. Upon harvest, cells in the "low CFSE" peak (Above) were gated and expressed as a percentage of total CD3+ cells.

Appendix 2

Appendix 2.1: CD56^{Bright} cell purity



Appendix 2.1:CD56^{Bright} NK cells were isolated and stained with anti-human CD56-FITC, CD3-APC and CD16-PE. Quadrant marker was set based on isotype control. A purity of >95% was observed for isolation of CD56^{Bright} NK cells, with 95.38% cells CD56+CD16-(Upper left quadrant A) and 95.6% CD56+CD3- (Lower right quadrant B).



CD56^{Dim}

Appendix 2.2: CD56^{Dim} NK cells were isolated and stained with anti-human CD56-FITC, CD3-APC and CD16-PE. Quadrant marker was set based on isotype control. A purity of >95% was observed for isolation of CD56^{Dim} NK cells, with 95.2% cells CD56+CD16+ (Upper right quadrant A) and 95.18% CD56+CD3- (Lower right quadrant B).

Appendix 2.3: Percentage of CD56+CD3- (LR) and CD3+CD56- (UL) MLs in response to various IL-2 concentrations –Figure 3.3.1.



A: CD56+ cells present:

CD56



B: CD56+ cells depleted

Appendix 2.3: Representative graphs of CD56 and CD3 expression at various IL-2 concentrations. Quadrant gates were set according to isotype controls. When CD56+ cells are present (A), CD3+CD56- cells (upper left) and CD56+CD3- (lower right) can be detected, and when CD56+ cells are depleted (B), CD3+CD56- cells (upper left) are again detected.

Appendix 2.4: CD56+ cell proliferation in response to IL-2 concentrations, determined by CFSE incorporation–Figure 3.3.2.



Appendix 2.4: CFSE was incorporated into CD56+ cells to determine the proliferative effects of various IL-2 concentrations in the presence of CD3+ cells. Upon harvest, cells in the "low CFSE" peak (Above) were gated and expressed as a percentage of total CD566+ cells

Appendix 2.5: CD3+ cell proliferation in response to CD56+ cells, in reponse to IL-2 concentrations as demonstrated by Ki67 expression – Section 3.3.3



Appendix 2.5: Cells were stained for Ki67 and CD3, with Ki67+CD3+ cells (Upper right quadrant) taken as a percentage of total cells in mixed lymphocytes. Quadrant gates were set according to isotype controls. This was done for both total MLs (A) and CD56+ cell depleted MLs.

Appendix 2.6: CD3+ cell proliferation in the presence and absence of CD56+ cells, in response to IL-2 concentrations, demonstrated by CFSE incorporation –Figure 3.3.3















Appendix 2.6:CFSE was incorporated into CD3+ cells to determine the proliferative effects of various IL-2 concentrations in the presence (i) and absence (ii) of CD56+ cells after 3 (A), 4(B), 5(C), and 7(D) days. Upon harvest, cells in the "low CFSE" peak (Above) were gated and expressed as a percentage of total CD3+ cells

Appendix 2.7: Percentage of MLs expressing markers for CD56^{Bright} vs CD56^{Dim} NK cells, in response to IL-2 concentrations –Figure 3.3.4.



Appendix 2.7: Cells were stained for CD56-FITC, CD3-APC and CD16-PE. CD56+CD3cells were gated on a dot plot (with quadrant gate set according to isotype controls) and of these, CD16 expression was examined via histogram. Histogram gate was set based on unstained cells. Those that were CD16- (Histo-2) were considered CD56^{Bright} while those that were CD16+ (Histo-3) were considered CD56^{Dim}.

Appendix 2.8: Proliferation of CD56^{Bright} and CD56^{Dim} NK cells in response to IL-2 and their effect on CD3+ cells, as determined by CFSE.









Appendix 2.8: CFSE was incorporated into $CD56^{Bright}$ (Ai) or $CD56^{Dim}$ (Aii) NK cells, so that their proliferation in response to IL-2 concentrations could be determined. Upon acquisition, cells in the "low CFSE" peak were gated and expressed as a percentage of cells of that subset. Similarly, CFSE was used to track CD3+ cell proliferation when cultured alone (Bi), with $CD56^{Bright}$ NK cells (Bii), or with $CD56^{Dim}$ NK cells (Biii). Finally, CD3+ cell proliferation was again determined when cultured alone (Ci) or with $CD56^{Bright}$ NK cells at a 1:100 ratio (Ci).

Appendix 2.9: Cell cycle and Apoptotic responses of CD3+ cells in response to CD56+ cells and various IL-2 concentrations –Figure 3.3.6





Appendix 2.9: Cells were stained with Annexin-V-FITC, PI-PE and CD3-APC, to track cell cycle. CD3+ cells were gated for, and plotted on a PI-PE histogram. Cells in the "low PI" peak are those in the G0/G1 phase of cell cycle, and these were gated and expressed as a percentage of total CD3+ cells. This was determined for total MLs (Ai) and CD56+ cell depeleted MLs (Aii). CD3+ cells were also expressed in a Annexin-V-FITC vs PI-PE dot plot, with cells in the upper right quadrant (late apoptosis) expressed as a percentage of total CD3+ cells. This was again determined for both total MLs (Bi) and CD56+ cell depleted MLs.

Appendix 2.10: Perforin expression by CD56+ cells in response to varying IL-2 concentrations –Figure 3.3.7



Appendix 2.10: Upon harvest, cells were stained using Anti-CD56-FITC and Anti-Perforin-PE. Quadrant gates were set according to isotype controls. The percentage of CD56+perforin+ cells (UR) was expressed as a percentage of total CD56+ cells (UR+LR).

Appendix 2.11: Cell cycle and apoptosis results of CD3+ cells in response to various IL-2 concentrations and CD56+ cells, or those separated from CD56+ cells by transwell inserts –Figure 3.3.8



Appendix 2.11Cells were stained with Annexin-V-FITC, PI-PE and CD3-APC, to track cell cycle. CD3+ cells were gated for, and plotted on a PI-PE histogram. Cells in the "low PI" peak are those in the G0/G1 phase of cell cycle, and these were gated and expressed as a percentage of total CD3+ cells. This was carried out for CD3+ cells cultured with CD56+ cells (Ai), or those which were separated from CD56+ cells by transwell inserts (Aii). CD3+ cells were also expressed in a Annexin-V-FITC vs PI-PE dot plot, with cells in the upper right quadrant (late apoptosis) expressed as a percentage of total CD3+ cells. This was again determined for + cells cultured with CD56+ cells (Bi), or those which were separated from CD56+ cells (Bi), or those which were separated from CD56+ cells (Bi), or those which were separated from CD56+ cells (Bi), or those which were separated from CD56+ cells (Bi), or those which were separated from CD56+ cells (Bi), or those which were separated from CD56+ cells (Bi), or those which were separated from CD56+ cells (Bi), or those which were separated from CD56+ cells (Bi), or those which were separated from CD56+ cells (Bi), or those which were separated from CD56+ cells (Bi), or those which were separated from CD56+ cells (Bi)

Appendix 2.12: Proliferative responses of CD3+ and CD56+ cells in response to coculture with and without Anti-IL-2 as determined by CFSE incorporation –Figure 3.3.11



Appendix 2.12: CFSE was incorporated into CD3+ cells (A) or CD56+ cells (B). Upon acquisition, cells in the "low CFSE" peak were gated and expressed as a percentage of cells of that subset.

Appendix 2.13: Proliferative responses of CD3+ cells in response to coculture with Anti-IL-2R α or Anti-IL-2R β in the presence of CD56+ cells as determined by CFSE incorporation –Figure 3.3.12



Appendix 2.13: CFSE was incorporated into CD3+ cells and upon acquisition, cells in the "low CFSE" peak were gated and expressed as a percentage of cells of that cell type. This was determined for CD3+ cells cultured with CD56+ cells in the presence of Anti-IL-2R α (A) or Anti-IL-2R β .

Appendix 2.13: Proliferation of CD3+ cells cultured with autologous or allogeneic CD56+ cells and varying concentrations of IL-2 as determined by CFSE incorporation –Figure 3.3.13



Appendix 2.13: CFSE was incorporated into CD3+ cells and upon acquisition, cells in the "low CFSE" peak were gated and expressed as a percentage of cells of that cell type. This was determined for CD3+ cells cultured with autologous CD56+ cells (A) or allogeneic CD56+ cells (B).

Appendix 3

Appendix 3.1: Expression of markers for T cell subsets in response to HPIV3 infection in the presence or absence of CD56+ cells –Figure 4.3.1



A Appendix 3.1: (A) Expression markers for various T cell subsets were used. Cells were stained for CD4, CD25 and Foxp3, and the percentage of cells positive for expression of all three markers were deemed Tregs (A). For the purpose of these representative graphs, cells that were CD4+CD25+ were gated and of these, Foxp3 expression was examined by histogram. Histogram gate was based on unstained cells.



В

Appendix 3.1: (B) Cells were stained for CD3, TBet, IFN- γ , and IL-12R β 2, and the percentage of cells positive for expression of all three markers were deemed Th1 cells. For the purpose of representative graphs IL-12R β 2 histograms are shown for CD3+, TBet+,IFN γ + cells. Histogram gate was based on unstained cells.



Appendix 3.1: (C) Cells were stained for CD3, IL-17, IL-23, and IL-22, and the percentage of cells positive for the expression of all four markers were deemed Th17 cells. For the purpose of representative graphs, IL-17 histograms are shown nfor CD3+IL-23+IL-22 cells. Histogram gate was based on unstained cells.



D

Appendix 3.1: (D) Cells were stained with Anti-CD8-PE and those high in CD8 (Hiso 2) were gated and expressed as a percentage of total MLs. Histograms were gated according to unstained cell acquisition.



Appendix 3.1:(E) Cells were stained for CD3 and CD45RO expression. CD3+ cells were gated and CD45RO expression was plotted on histograms, with the percentage of CD45RO+ cells expressed as a percentage of total CD3+ cells. This was determined for total MLs (Ei) and ML-CD56+ cells (Eii). Histograms were gated according to unstained sample.

Appendix 3.2: Expression of markers for T cell subsets in response to varying IL-2 concentrations in the presence or absence of CD56+ cells – Figure 4.3.2



Appendix 3.2: (A) Cells were stained for CD4, CD25 and Foxp3, and the percentage of cells positive for expression of all three markers were deemed Tregs. For the purpose of these representative graphs, cells that were CD4+CD25+ were gated and of these, Foxp3 expression was examined by histogram. Histogram gate was based on unstained cells. This was done for total MLs (Ai) and ML-CD56+ cells (Aii)



Appendix 3.2: (B) Cells were stained for CD3, TBet, IFN- γ , and IL-12R β 2, and the percentage of cells positive for expression of all four markers were deemed Th1 cells. For the purpose of representative graphs IL-12R β 2 histograms are shown for CD3+, TBet+,IFN γ + cells. Histogram gate was based on unstained cells. This was done for total MLs (Bi) and ML-CD56+ cells (Bii).



Appendix 3.2: (C) Cells were stained for CD3, IL-17, IL-23, and IL-22, and the percentage of cells positive for the expression of all four markers were deemed Th17 cells. For the purpose of representative graphs, IL-17 histograms are shown nfor CD3+IL-23+IL-22 cells. Histogram gate was based on unstained cells. This was determined for total MLs (Ci) and ML-CD56+ cells (Cii)



Appendix 3.2: (**D**) Cells were stained with Anti-CD8-PE and those high in CD8 (Hiso 2) were gated and expressed as a percentage of total MLs. This was determined for both total MLs (Di) and ML-CD56 (Dii). Histograms were gated according to unstained cell acquisition.





Appendix 3.3: Purity of isolated Tregs as determined by CD4 and CD25 expression



Appendix 3.3: Isolated TRegs were stained for the presence of CD25 and CD4, and upon acquisition, CD4+CD25+ cells were expressed as a percentage of total cells. Quadrant gates were set according to isotype controls. Purity of Tregs was determined as >95% (95.78%).

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Appendix 3.4: Treg proliferative responses in the presence or absence of CD56+ cells, and IL-2 stimulation, as determined by CFSE incorporation - Figure 4.3.3.



Appendix 3.4: CFSE was incorporated into Tregs to determine the proliferative effects of various IL-2 concentrations in the presence (A) and absence (B) of CD56+ cells. Upon harvest, cells in the "low CFSE" peak (Above) were gated and expressed as a percentage of total Tregs.
Appendix 3.5 Proliferative responses of Tregs in response to coculture with either NK cell subset, and the proliferative responses of these subsets, as determined by CFSE incorporation –Figure 4.3.3







Appendix 3.5 CFSE was incorporated into Tregs to determine the proliferative effects of various IL-2 concentrations when cultured with CD56^{Bright} (Ai) or CD56^{Dim} (Aii) NK cells, or when cultured alone (Aiii). Upon harvest, cells in the "low CFSE" peak were gated and expressed as a percentage of total Tregs. Similarly, CFSE was incorporated into either CD56^{Bright} (Bi) or CD56^{Dim} (Bii) NK cells before coculture with Tregs. Upon harvest, cells in the "low CFSE" peak were gated and expressed as a percentage of total subset.

Appendix 3.6: Proliferative responses of CD3+Treg- cells in the presence or absence of CD56+ cells as determined by CFSE incorporation –Figure 4.3.5



Appendix 3.6: CFSE was incorporated into CD3+Treg- cells, to determine the proliferative effects of various IL-2 concentrations when cultured with (A) or without (B) CD56+ cells. Upon harvest, cells in the "low CFSE" peak were gated and expressed as a percentage of total CD3+Treg- cells.

Appendix 3.7: Expression of specific Treg markers in response to IL-2, in the presence or absence of IL-2, and Treg proliferative responses when cultured with Anti-CD25, when cultured with NK subsets or alone, as determined by CFSE –Figure 4.3.6





Appendix 3.7: CD56- cells were gated and CD4 expression of these cells were plotted on histograms (A). Gate was set accordig to unstained cells, and CD4+ cells were expressed as a percentage of total CD4+CD56- cells. This is true for Tregs cultured with CD56+ cells (Ai) or alone (Aii). CD56- cells were gated and CD25 expression of these cells were plotted on histograms (B). Gate was set accordig to unstained cells, and CD25+ cells were expressed as a percentage of total CD4+CD56- cells. This is true for Tregs cultured with CD56+ cells (Bi) or alone (Bii). CFSE was incorporated into Tregs to determine the proliferative effects of various IL-2 concentrations when their CD25 was blocked, when cultured with CD56^{Bright} (Ci) or CD56^{Dim} (Cii) NK cells, as well as when cultured alone (Ciii). Upon harvest, cells in the "low CFSE" peak were gated and expressed as a percentage of total Tregs

Appendix 3.8: Proliferative responses of Tregs and CD56+ cells, when cultured alone, cocultured, or cocultured with Anti-IL-2, as determined by CFSE incorporation –Figure 4.3.7



Appendix 3.8: CFSE was incorporated into Tregs to determine the proliferative effects of culture alone, with CD56+ cells, or CD56+ cells and Anti-IL2 (A). Upon harvest, cells in the "low CFSE" peak were gated and expressed as a percentage of total Tregs. CFSE was also incorporated into CD56+ cells to determine the proliferative effects of culture alone, with Tregs, or Tregs and Anti-IL2 (B).). Upon harvest, cells in the "low CFSE" peak were gated and expressed as a percentage of total CD56+ cells and Anti-IL2 (B).

Appendix 3.9: CD56^{Bright} vs CD56^{Dim} marker expression on isolated CD56^{Bright} NK cells, as determined by CD16 expression, in response to various IL-2 concentrations –Figure 4.3.8



Appendix 3.9: $CD56^{Bright}$ NK cells which had been cultured with various IL-2 concentrations were stained for CD56-FITC, CD3-APC and CD16-PE. CD56+CD3- cells were gated on a dot plot (with quadrant gate set according to isotype controls) and of these, CD16 expression was examined via histogram. Histogram gate was set based on unstained cells. Those that were CD16- (Histo-1) were considered CD56^{Bright} while those that were CD16+ (Histo-2) were considered CD56^{Dim}.

Appendix 3.10: Proliferatvie responses of Tregs, when in contact with, or separated from, either NK cell subset, at various IL-2 concentrations, as determined by CFSE incorporation –Figure 4.3.9



CFSE was incorporated into Tregs to determine the proliferative effects of culture with either CD56^{Bright} (Ai) or CD56^{Dim} NK cells, or when separated from CD56^{Bright} (Aii) or CD56^{Dim} (Bii) NK cells. Upon harvest, cells in the "low CFSE" peak were gated and expressed as a percentage of total Tregs.

Appendix 4

Appendix 4.1: Expression of NCRs on CD56+ cells during HPIV3 infection -Figure 5.3.1



Appendix 4.1: Cells were stained with Anti-CD56-FITC, and NKp30-PE (A), NKp44-PE (B) or NKP46 PE (C). Upon acquisition, CD56+ cells were gated and of these NCR expression was examined. The percentage of NCR+ cells were expressed as a percentage of total CD56+ cells. Histogram gates were set according to unstained cells.

Appendix 4.2: Expression of NCRs on CD56+ cells in response to HPIV3 viral proteins HN and F –Figure 5.3.2



Appendix 4.2: Cells were stained with Anti-CD56-FITC, and NKp30-PE (A), NKp44-PE (B) or NKP46 PE (C). Upon acquisition, CD56+ cells were gated and of these NCR expression was examined. The percentage of NCR+ cells were expressed as a percentage of total CD56+ cells. Histogram gates were set according to unstained cells.

Appendix 4.3: Proliferative responses of CD3+cells cultured with CD56+ cells and HPIV3 infected CD14+ cells, cultured with NCR blocking antibodies, as determined by CFSE incorporation –Figure 5.3.4



Appendix 4.3: CFSE was incorporated into CD3+ cells which were cultured with CD56+ cells and HPIV3 infected allogeneic CD14+ cells. These were cultured either with no antibody, or blocking antibodies to NKp30, NKp44 and NKp46. Proliferated CD3+ cells were gated and expressed as a percentage of total T cells.

Appendix 4.4: Expression of NCRs on CD56+ cells in response to various IL-2 concentrations –Figure 5.3.5



Appendix 4.4: Cells were stained with Anti-CD56-FITC, and NKp30-PE (A), NKp44-PE (B) or NKP46 PE (C). Upon acquisition, CD56+ cells were gated and of these NCR expression was examined. The percentage of NCR+ cells were expressed as a percentage of total CD56+ cells. Histogram gates were set according to unstained cells.

Appendix 4.5: Cell cycle and Apoptotic responses of CD3+ cells in MLs, cultured alone or with NCR blocking antibodies and various IL-2 concentrations –Figure 5.3.6





Appendix 4.5: Cells were stained with Annexin-V-FITC, PI-PE and CD3-APC, to track cell cycle. CD3+ cells were gated for, and plotted on a PI-PE histogram. Cells in the "low PI" peak are those in the G0/G1 phase of cell cycle, and these were gated and expressed as a percentage of total CD3+ cells. This was determined for MLs with no antibody (Ai), or those cultured with Anti-NKp30 (Aii), Anti-NKp44 (Aiii) or Anti-NKp46 (Aiv). CD3+ cells were also expressed in a Annexin-V-FITC vs PI-PE dot plot, with cells in the upper right quadrant (late apoptosis) expressed as a percentage of total CD3+ cells, or those cultured with no antibody (Bi), or those cultured with Anti-NKp46 (Biv).

Appendix 4.6: Cell cycle and Apoptotic responses of CD3+ cells in MLs, cultured alone, or with agonistic antibodies to NKp44 and NKp46 –Figure 5.3.7



A



Appendix 4.6: Cells were stained with Annexin-V-FITC, PI-PE and CD3-APC, to track cell cycle. CD3+ cells were gated for, and plotted on a PI-PE histogram. Cells in the "low PI" peak are those in the G0/G1 phase of cell cycle, and these were gated and expressed as a percentage of total CD3+ cells (A). CD3+ cells were also expressed in a Annexin-V-FITC vs PI-PE dot plot, with cells in the upper right quadrant (late apoptosis) expressed as a percentage of total CD3+ cells (B).

Appendix 4.7: NKp46 expression on CD56+ cells in response to various IL-2 concentrations when cultured in MLs where NKp44 was blocked –Figure 5.3.8



Appendix 4.7: Cells were stained with Anti-CD56-FITC, and NKP46 PE. Upon acquisition, CD56+ cells were gated and of these NKp46 expression was examined. The percentage of NJp46+ cells were expressed as a percentage of total CD56+ cells. Histogram gates were set according to unstained cells. This was determined for MLs cultured alone (A) or those cultured with Anti-NKp44 (B).

Appendix 4.8: Expression of markers for Tregs in MLs, cultured with agonistic antibodies Anti-NKp44 or Anti-NKp46 – Figure 5.3.9



Appendix 4.8: Cells were stained for CD4, CD25 and Foxp3, and the percentage of cells positive for expression of all three markers were deemed Tregs. For the purpose of these representative graphs, cells that were CD4+CD25+ were gated and of these, Foxp3 expression was examined by histogram. Histogram gate was based on unstained cells.

Appendix 5

Appendix 5.1: Protein gel of Disrupted HPIV3 infected CD14+ cells prior to specific viral protein isolation

After virus disruption and HPIV3 HN and F percipitation (Seciton 6.2.1), we wanted to confirm the presence of these proteins prior to specific HN and F isolation by sepharose conjugated antibody. HPIV3 HN is 562 Amino Acids (AA), or 62.44kDa, while F is 439 AAs or 48.77kDa. The product from viral disruption and a protein size marker ladder were run on protein gel.

While various proteins were visable on gel (presumably from disrupted CD14+ cells), there was bands of HN and F sizes (Arrows). While this does not say absultuely that HN and F are among precipitated proteins here, we felt it was a good enough indicator to procede with specific protein isolation, at which point western blot would be used for more specific detection of these proteins.



Appendix 5.2: Cell cycle responses of CD3+ cells in MLs in response to disrupted HPIV3 virus with precipitated HN and F –Figure 6.3.2



Appendix 5.2: Cells were stained with PI-PE and CD3-APC, to track cell cycle. CD3+ cells were gated for, and plotted on a PI-PE histogram. Cells in the "low PI" peak are those in the G0/G1 phase of cell cycle, and these were gated and expressed as a percentage of total CD3+ cells.

Appendix 5.3: Cell cycle responses of CD3+ cells in MLs in response to isolated HPIV3 HN and F –Figure 6.3.5



Appendix 5.3: Cells were stained with PI-PE and CD3-APC, to track cell cycle. CD3+ cells were gated for, and plotted on a PI-PE histogram. Cells in the "low PI" peak are those in the G0/G1 phase of cell cycle, and these were gated and expressed as a percentage of total CD3+ cells.

Appendix 5.4: Expression of CD56+ CD3- cells in response to isolated HPIV3 HPIV3 HN and F –Figure 6.3.6



Appendix 5.4: Representative flow cytometry data for CD56+CD3- (Lower right quadrant) cells or NK cells. Those in this lower right quadrant were plotted as a percentage of total mixed lymphocytes.

Appendix 5.5: Expression of markers for Tregs in response to isolated HPIV3 surface glycoproteins –Figure 6.3.8



Appendix 5.5:For the purpose of these representative graphs, cells that were CD4+CD25+ were gated and of these, Foxp3 expression was examined by histogram. These were expressed as a percentage of total Tregs. Histogram gate was based on unstained cells.

Appendix 5.6: NCR expression on CD56+ cell s in response to HPIV3 surface glycoproteins –Figure 6.3.9



Appendix 5.6: Cells were stained with Anti-CD56-FITC, and NKp30-PE (A), NKp44-PE (B) or NKP46 PE (C). Upon acquisition, CD56+ cells were gated and of these NCR expression was examined. The percentage of NCR+ cells were expressed as a percentage of total CD56+ cells. Histogram gates were set according to unstained cells.

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