

Human helper T cell responses to RSV infection

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Doctor of Philosophy

Aleks Kamer Guvenel

Respiratory Infections Section
National Heart and Lung Institute
Faculty of Medicine
Imperial College London
London W2 1PG

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Declaration

I hereby certify that the work in this thesis is my own and was written by myself. Where other sources of information have been used, they have been acknowledged.

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Abstract

Respiratory Syncytial virus (RSV) remains one of the most important causes of severe respiratory infection worldwide. Natural immunity is only partially protective, and no safe and effective vaccine has so far been found. CD4⁺ T cells play an essential role in shaping cellular and humoral immune responses. They have been shown to provide signals that are necessary for full differentiation of CD8⁺ T cells and B cells, as well as exhibiting direct anti-viral functions. However, their role in human RSV infection is poorly understood. Among the reasons for this are restrictions on sampling during the course of human infection and a resultant lack of tools to allow the study of antigen-specific CD4⁺ T cell responses. Further understanding of their role in the generation of effective immunity is essential for the development of better vaccines.

Using an established experimental human challenge model, we inoculated 49 healthy adult volunteers with live, virulent RSV (Memphis 37). We analysed viral load by quantitative PCR (qPCR) on nasal lavage samples. Subjects were deemed infected if RSV was detectable on two consecutive days between day 2 and day 10 post inoculation. RSV infection developed in 26/49 (53%) of volunteers. In those infected, viral load peaked at day 7 and occurred at the same time as the peak symptoms score. Analysis of peripheral blood mononuclear cells (PBMCs) and BAL cells revealed the peak of polyclonal CD4⁺ T cell responses at day 10, characterised by the expression of the activation marker CD38 and proliferation marker Ki-67. At this time-point, RSV-specific CD4⁺ T cells were producing Th1 cytokines IFN- γ and TNF- α . To understand this response in greater detail, we generated a custom RSV M37 peptide library covering the complete RSV proteome. Using IFN- γ ELISpot, candidate epitopes were found in the F, G, M and N proteins. Of these, the most immunodominant CD4⁺ T cell epitopes were within the major surface glycoproteins F and G. Their class II restriction was established by depletion of CD8⁺ T cells from PBMCs and were confirmed by functional analysis of PBMCs from HLA class II matched donors. Using these, MHC class II-peptide tetramers using F and G peptides were constructed for the first time. RSV-specific CD4⁺ T cells were detectable in PBMCs of infected subjects at day 10, and exhibited the phenotypic

markers of effector and effector memory T cells. These were highly activated but showed less evidence of proliferation than RSV-specific CD8⁺ T cells generated at the same time.

Thus, the T cell response to RSV in healthy adults displays broad specificity but immunodominant CD4⁺ T cells primarily recognize peptides from the surface proteins F and G. Our studies imply the importance of Th1 and follicular helper T cells in the response to infection and provide novel tools that will allow further characterisation of RSV-specific CD4⁺ T cells in peripheral blood and lower airway.

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List of Abbreviations

A

APC	Antigen presenting cell
ASC	Antibody-secreting cell
AUC	Area under curve

B

BAL	Bronchoalveolar lavage
Bcl-6	B-cell lymphoma 6

C

CCL	Chemokine C-C motif ligand
cDC	Conventional dendritic cell
cDNA	Complimentary DNA
CFSE	Carboxyfluorescein succinimidyl ester
CLIP	Class II-associated invariant chain peptide
CX3C	C-X-3C chemokine (fractalkine)
CX3CR	C-X-3C chemokine receptor
CXCL	Chemokine CXC motif ligand
CXCR	CXC chemokine receptor 5

D

DC	Dendritic cell
DMSO	Dimethylsulfoxide

E

EDTA	Ethylenediaminetetraacetic aci
ELISpot	Enzyme-linked immunospot

F

FACS	fluorescence-activated cell sorting
FCS	Fetal calf serum
FDC	Follicular dendritic cell
FI-RSV	Formalin inactivated respiratory syncytial virus
FoxP3	Forkhead box P3

G

GATA3 GATA binding protein 3

GC Germinal centre

H

HLA Human leukocyte antigen

HUVEC Human umbilical vein endothelial cells

I

ICOS Inducible costimulator

IFITM Interferon-inducible transmembrane

IFN Interferon

IFN- γ Interferon gamma

IFNAR Type I interferon α/β receptor

IL Interleukin

ISG Interferon-stimulated gene

L

LRTI Lower respiratory tract infection

M

MACS Magnetic-activated cell sorting

MHC Major histocompatibility complex

MIMIC Modular immune in vitro construct

MOI Multiplicity of infection

mRNA Messenger RNA

N

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

NK Natural killer (cell)

NS1/2 Non-structural protein 1/2

P

PAMP Pathogen-associated molecular pattern

PBMC Peripheral blood mononuclear cell

PBS Phosphate buffer saline

PCR Polymerase chain reaction

pDC	Plasmacytoid dendritic cell
PFU	Plaque-forming units
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
Q	
qPCR	Quantitative polymerase chain reaction
R	
RIG-I	Retinoic acid-inducible gene 1
ROR γ T	Retinoic acid related orphan receptor gamma T
rpm	Revolutions per minute
RPMI-1640	Roswell park memorial institute medium
RSV	Respiratory syncytial virus
S	
ssRNA	Single stranded RNA
STAT	Signal transducer and activation of transcription
T	
Tcm	Central memory T cell
TCR	T cell receptor
Tem	Effector memory T cell
Tfh	T follicular helper cell
Th1	T helper 1
Th17	T helper 17
Th2	T helper 2
TLR	Toll-like receptor
TNF- α	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor receptor
Treg	Regulatory T cell
U	
URTI	Upper respiratory tract infection
UV-RSV	UV inactivated respiratory syncytial virus

1. Introduction

1.1 Respiratory Syncytial virus

1.1.1 Epidemiology and clinical manifestation

Respiratory syncytial virus (RSV) is a ubiquitous pathogen and a major cause of lower respiratory tract infections in infants, the elderly and immunosuppressed individuals. Recent analysis of the global burden of acute lower respiratory tract infections (LRTI) caused by RSV in young children have estimated 34 million cases annually in children under 5 years of age (Nair et al., 2010). RSV accounts for 22% of all acute LRTIs with 3.4 million requiring hospital admission and 66,000 – 199,000 deaths. In addition, the incidence was more than twice as high in developing compared with developed countries. RSV infection is also recognised as a significant problem in certain adult populations; including the elderly, persons with cardiopulmonary diseases, and immunocompromised hosts. RSV infection in these individuals commonly leads to hospitalisation for cardiac failure (5.4%), pneumonia (10.6%), and exacerbation of chronic obstructive pulmonary disease (11.4%) (Falsey et al., 2005; Velling et al., 2014).

All children are infected with RSV at least once during the first 2 years of life and about half of these experience two infections (Glezen et al., 1986). While most infected children undergo a self-limiting upper respiratory tract infection (URTI), some develop bronchiolitis which may also lead to recurrent wheeze in later life (Schauer et al., 2002; Sigurs et al., 2000; Wu and Hartert, 2011). Typically, RSV in healthy adults induces mild URTI with symptoms of a cold with sore throat and rhinorrhoea (DeVincenzo et al., 2010). In experimental human adult infections, RSV shedding begins a day before the onset of symptoms, and can be detected for up to 7 days in nasal washes (DeVincenzo et al., 2010). In more severe cases where hospitalisation is required, LRTIs such as bronchiolitis and viral pneumonia may be driven as a result of inflammatory cell influx into the lung, increased mucus production and the cytopathic effects of the virus on airway epithelial cells (Habibi and Openshaw, 2012). Risk factors for developing a severe RSV infection includes preterm birth, chronic lung disease, immunodeficiencies and polymorphism in some innate immune genes (i.e. TLR4)

(Borchers et al., 2013; Janssen et al., 2007; Miyairi and DeVincenzo, 2008). While there are two subtypes of RSV – A and B, which display major variations in only one cell surface glycoprotein G – whether virulence differs between the different subtypes is unclear (Sullender and Edwards, 1999). Furthermore, even those with healthy mature immune systems suffer recurrent RSV infections throughout life, implying that immunity is incomplete even in the absence of major antigenic variation.

1.1.2 RSV genome and structure

RSV is an enveloped, non-segmented negative-sense, ssRNA virus belonging to the *Paramyxoviridae* family. Its genome is made up of 10 genes that encode 11 proteins; non-structural proteins 1 and 2 (NS1 and NS2), nucleoprotein (N), phosphoprotein (P), matrix proteins M, M2.1 and M2.2, small hydrophobic protein (SH), attachment glycoprotein (G), fusion protein (F) and RNA-dependent RNA polymerase (L) (Figure 1.1.1) (Lay et al., 2013).

NS1 and NS2 are non-structural proteins that have immunosuppressive roles via the inhibition of STAT2 expression and type I interferon responsiveness (Borchers et al., 2013; Spann et al., 2004). The M protein lines the inner surface of the viral envelope, having a central role in budding and formation of infectious extracellular virions (Rodríguez et al., 2004). The N, P, M2.1, M2.2 and L proteins are associated with the ssRNA and have roles in encapsidation, RNA replication and transcription (Cheng et al., 2005; Collins and Melero, 2011).

There are 3 surface proteins on RSV, namely the G, F and SH proteins. The SH protein has been suggested to form ion channels which enhance membrane permeability in the host cell and increasing RSV infectivity (Gan et al., 2012). G is an attachment protein that mediates virus binding and is important for efficient replication *in vivo* (Teng and Collins, 2002). Grouping of RSV strains is entirely based on variation in sequence of G. It is heavily glycosylated and bears a CX3C fractalkine-like motif, which is thought to modulate inflammatory responses, while the secreted form of G protein may act as a decoy for antibody (Bukreyev et al., 2008). Unlike G, the F protein is well conserved and responsible

for fusion of the viral and cellular membranes (McLellan et al., 2013a). Proteolytic cleavage of F leads to a metastable, prefusion form of the protein. On virus binding, triggering of prefusion F results in a conformational change yielding a thermodynamically stable postfusion bundle which drives viral and host membrane fusion, thus allowing viral entry (McLellan et al., 2013b).

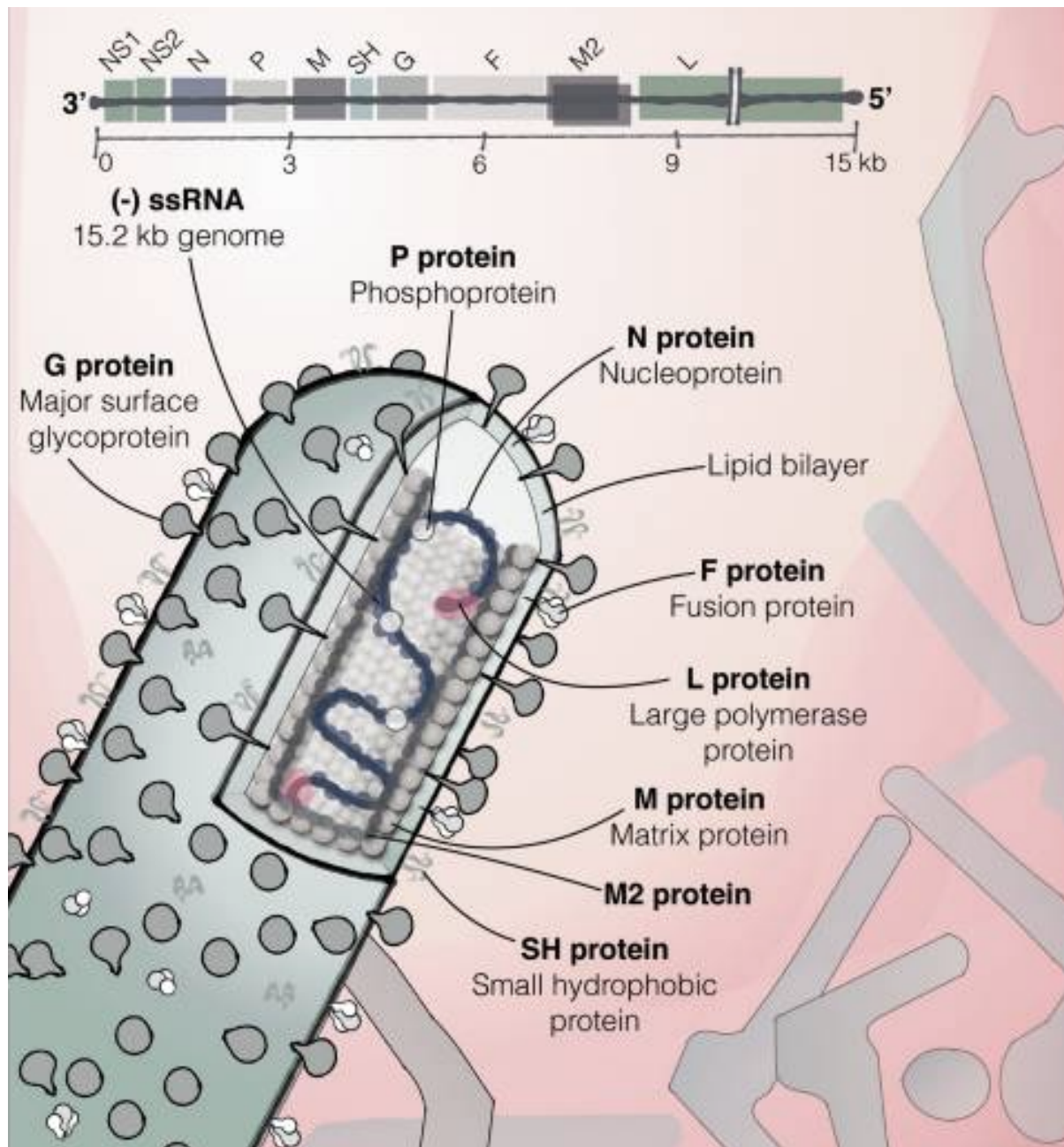


Figure 1.1.1 The structure of RSV. The 15.2 kb, negative sense, single stranded RNA RSV genome is made up of 10 genes that encode 11 proteins. Diagram illustrates a filamentous virus particle which bud from the infected cell (Lambert et al., 2014).

1.1.3 Intervention strategies

To date, there is no specific treatment for RSV infection. The only antiviral approved for use is ribavirin, a broad-spectrum antiviral occasionally used for severe RSV cases in children and immunocompromised patients (Casey et al., 2013; Marcelin et al., 2014). Its mechanism of action involves disruption of the viral RNA replication but has poor efficacy against RSV and extensive side effects so is seldom used (Leyssen et al., 2005). However, several candidate antivirals are in clinical trials, including the RSV fusion inhibitor GS-5806. This was recently evaluated for its antiviral activity in an experimental human challenge model and was shown to be safe with no adverse events in a phase I trial (DeVincenzo et al., 2014). This also showed that the GS-5806 treated group had lower cumulative viral load, symptom scores and total mucus weight compared to the placebo group.

The only licensed RSV-specific intervention is Palivizumab (MedImmune), a humanised mouse anti-F protein monoclonal IgG1 antibody against a postfusion F epitope (Bjornson et al., 2015; 1998). Although it is effective in reducing hospitalisation of premature infants by up to 50%, it is expensive and needs to be given as intramuscular injections every month during the RSV season. It's therefore of limited use in global RSV prevention and used only for prophylaxis in high-risk infants in high income countries.

The development of new candidate vaccines has been slow since the disastrous outcome of the formalin-inactivated RSV (FI-RSV) vaccine trial in late 1960s and there is still no licensed RSV vaccine. Lot-100 was an alum precipitated formalin-inactivated RSV, which was believed to have maintained viral protein structure for antibody recognition and in preclinical models prevented viral replication and disease (Kim et al., 1969). Results were initially promising following vaccination; 43% of the infants displayed at least four-fold rise in serum neutralising antibody titre and 91% had at least four-fold rise in complement fixing antibody. However, upon subsequent natural RSV infection, 80% of the vaccinees required hospitalisation for pneumonia and bronchiolitis compared to 5% in the control group inoculated with a parainfluenza vaccine. There were two fatal cases and the post-mortem analysis showed extensive peribronchiolar inflammation with influx of neutrophils and

eosinophils that caused obstruction of the small airways (Kim et al., 1969). Later studies in mouse models suggested that FI-RSV led not only to disruption of B cell epitopes and promotion of non-neutralising antibodies but also to CD4+ T cell responses biased towards Th2 with production of IL-4, IL-5 and IL-13 alongside decreases in Th1 cytokines upon challenge with wild-type RSV (Waris et al., 1996).

In the past decade, a number of RSV vaccines have been developed and tested in clinical trials. Recent advances in understanding of RSV F protein structure and the global burden of disease have boosted the field and there are now more than 50 candidates in development. These include live-attenuated, particle-based, subunit and gene-based vectors and have been reviewed extensively elsewhere (Guvenel et al., 2014).

1.2 Immune responses to RSV

Immune responses to RSV can be divided into 3 parts. In the first hours of infection, an early innate immune response is triggered, where the virus is detected by airway epithelial cells, alveolar macrophages and dendritic cells and the release of pro-inflammatory chemokines and cytokines from these cells attract other cells of the innate and adaptive response to the lungs. This is followed by the influx of inflammatory cells including neutrophils, natural killer (NK) cells and monocytes into the site of infection. Finally, antigen-specific T and B cells are recruited as part of the adaptive immune response; CD8+ T cells mediate cytotoxic function to kill virus-infected cells; B cells produce antibodies against the virus; and T helper cells coordinate their activity.

1.2.1 Innate immune responses

1.2.1.1 Airway epithelial cells

The airway epithelium is made up of ciliated cells and secretory goblet cells that act as the first line of defence against respiratory viruses through providing a physical barrier and mucociliary clearance (Vareille et al., 2011). While the exact mechanism of invasion is

unclear, RSV has been found to target ciliated airway epithelial cells, triggering acute pulmonary inflammation characterised by early infiltration of neutrophils and macrophages to the lungs (Welliver et al., 2007). Recently, an *ex vivo* model of RSV infection was developed using well-differentiated primary paediatric bronchial epithelial cells (WD-PBECs) (Villanave et al., 2012). These cultures were shown to resemble RSV infection in infant lungs and showed restriction of virus entry to the apical surface with occasional syncytium formation, goblet cell hyperplasia and mucus hypersecretion. Furthermore, there was secretion of pro-inflammatory chemokines CXCL10, CCL5, IL-6, and CXCL8. Other studies have highlighted the roles of the surface proteins G and F for viral entry *in vitro*. In immortalised cell lines, G protein has been shown to mediate attachment through the binding of its heparin binding domain to the cell-surface heparan sulfate (Hallak et al., 2007). However, expression of heparan sulfate is limited to immortalised cells and may misrepresent the human airway epithelium *in vivo*. Instead, primary, well differentiated human airway epithelial cell (HAE) cultures more closely resemble the function of the human airway epithelium and lack heparan sulfate expression (Pickles et al., 1998). Using HAE cultures, Johnson and colleagues suggested that CX3CR1 may be the receptor for G protein as blocking of CX3CR1 or mutations in the G protein CX3C motif resulted in reduced RSV infection *in vitro* (Johnson et al., 2015). RSV can infect HAE cells in the absence of G, albeit poorly, suggesting that RSV F protein also has attachment activity (Kwilas et al., 2009). The main function of F however is to promote fusion of the viral and host cell membranes and RSV lacking F protein cannot infect cells (Batonick et al., 2008). Conformational change from the prefusion to postfusion form results in refolding of the heptad repeat domains into a hairpin 6-helix bundle that promotes fusion (Zhao et al., 2000). In addition, nucleolin has been proposed as a potential cellular receptor for RSV (Tayyari et al., 2011). As nucleolin is expressed at the apical side of airway epithelial cells, it may have a role in viral entry by interacting with the RSV F protein. Therefore, Lay and colleagues proposed a model of RSV binding and entry in airway epithelial cells; RSV G protein binds cell-surface glycosaminoglycans (or CX3CR1 as more recently shown) which aids the binding of F protein (possibly interacting with nucleolin) and embedding in cholesterol-lipid microdomains on the apical cell surface. These interactions trigger the cytoskeleton reorganisation that allows the delivery of RSV contents into the cytoplasm (Lay et al., 2013).

1.2.1.2 Induction of type I interferons

Pattern recognition receptors (PRRs) are germ-line encoded innate immune receptors that recognise distinct evolutionarily conserved structures on pathogens, termed pathogen-associated molecular patterns (PAMPs) (Mogensen, 2009). There are several types of PRRs that differ by the location of expression the ligands recognised. RSV is primarily recognised by Toll-like receptors (TLRs), retinoic acid-inducible gene 1 (RIG-I)-like receptors and nucleotide-binding oligomerisation domain (NOD)-like receptors. A summary of PRRs that are involved in recognition of RSV is shown in Table 1.2.1.

PRR	Location	Ligand	Reference
TLR3	Endosome	dsRNA	Rudd et al., 2005
TLR4	Cell surface	RSV F protein	Kurt-Jones et al., 2000
TLR7/8	Endosome	ssRNA	Lindell et al., 2009
RIG-I	Cytoplasm	dsRNA	Loo et al., 2008
MDA5	Cytoplasm	dsRNA	Loo et al., 2008
NLRP3	Cytoplasm	RSV SH protein	Triantafilou et al., 2013
NOD2	Cytoplasm	ssRNA	Vissers et al., 2012

Table 1.2.1 RSV recognition by different PRRs.

In the context of viral infections, PRR signalling induces interferons (IFNs) which elicit the transcription of interferon-stimulated genes (ISGs) whose protein products exhibit antiviral activity. IFNs are soluble mediators that provide antiviral immunity by restricting viral growth and spread (Durbin et al., 2000). There are 3 types of IFNs that are grouped according to the receptors they use. Type I IFNs are a family of 30 subtypes in humans consisting of 13 IFN- α s, a single IFN- β , IFN- ω , IFN- κ , IFN- ϵ and 13 pseudogenes (Hardy et al., 2004). These are produced by most cell types and utilise the type I IFN α/β receptor (IFNAR) (Theofilopoulos et al., 2005). The type II IFN group consists only of IFN- γ that is mainly produced by the Th1 subset of CD4⁺ T cells, CD8⁺ T cells and NK cells and which signals through the IFN- γ receptor (IFNGR) (Platanias, 2005). Finally, type III IFNs are made up of three gene products of IFN- λ (IFN- λ 1, IFN- λ 2, IFN- λ 3) (Ank et al., 2006). Even though type III IFNs signal through a different heterodimeric receptor complex, they activate the same intracellular pathways as type I IFNs and therefore share some biological activities (Donnelly and Kutenko, 2010).

RSV infection *in vitro* using cell lines and *in vivo* using mouse models have been used to characterise the induction of the type I IFN response (Demoor et al., 2012; Goritzka et al., 2014; Jewell et al., 2007). The mechanism of this induction is thought to be dependent on recognition of dsRNA products of RSV by RIG-I which leads to the activation of IRF3 and NF- κ B. IRF3 is constitutively expressed in most cell types and induces the expression of IFN- α and IFN- β which initiate the first wave of the type I IFN response (Solis et al., 2006). In parallel, NF- κ B induces expression of proinflammatory cytokines and chemokines including TNF- α , IL-1 α and IL-1 β (Rubio et al., 2013). The binding of type I IFNs to the alpha/beta interferon receptor (IFNAR) activates downstream complexes that result in transcription of ISGs that limit early viral replication and direct immune responses (Bitko et al., 2007; Haller et al., 2006; Jin et al., 2014). The importance of type I IFNs in RSV infection was demonstrated in IFNAR knockout mice which showed increased viral loads and weight loss with significantly reduced levels of proinflammatory cytokines and chemokines (Goritzka et al., 2014).

ISGs induced by type I IFNs have many different roles in antiviral immunity. They contribute to the control of viral infections by upregulation of soluble mediators that enable cell-to-cell communication whereas negative regulators help resolve the IFN-induced state and facilitate return to cellular homeostasis. ISGs may intervene at every stage of the viral life cycle, during attachment, fusion, replication, translation, assembly and budding (Schneider et al., 2014). These include IFN-inducible transmembrane (IFITM) proteins that can intervene with viral entry through inhibition of viruses from traversing the lipid bilayer and accessing the cytoplasm (Diamond and Farzan, 2013). In RSV infection, IFITM proteins were shown to potentially interfere with virus entry and knocking down the endogenous and IFN-induced expression of IFITM proteins facilitated RSV infection (Zhang et al., 2015). In contrast, viperin in RSV infected cells has been associated with the inhibition of virus filament formation and reduced cell-to-cell virus transmission (Jumat et al., 2015).

RSV has evolved mechanisms to evade the highly antiviral type I IFN responses by the host. NS1 and NS2 proteins are rapidly synthesised and have the capability to inhibit the induction of type I IFNs. NS1 can suppress IFN synthesis by binding to IRF3 (Ling et al., 2009; Ren et al., 2011). On the other hand, NS2 induces the degradation of STAT2 as well as binding to RIG-I

that prevents its interaction with mitochondrial antiviral signalling (MAVS) which is important for the activation of NF- κ B. (Ling et al., 2009; Swedan et al., 2011). Furthermore, infection of human epithelial cells and macrophages with NS1 and NS2 deficient RSV results in significantly higher levels of type I IFN production compared to wild-type, with these deletion mutant unable to replicate *in vivo* demonstrating the importance of evasion of the early antiviral response for successful infection.

1.2.1.3 Natural killer cells

Natural killer (NK) cells are large granular lymphocytes that are among the next players in early antiviral immunity. They are activated in response to type I IFNs and have the ability to interfere with virus propagation by killing infected host cells in the early stages of infection as well as providing IFN- γ for the modulation of cells of the adaptive immunity (Jost and Altfeld, 2013). In the context of RSV infection, the role of NK cells has been controversial as they have been shown to both contribute to early viral defence and cause immunopathology. Clinical studies on severely RSV-infected infants showed a failure in generation of a robust NK cell response (De Weerd et al., 1998; Welliver et al., 2007). In mouse models of primary RSV infection, the frequency of NK cells peaked on day 4 and were the major source of IFN- γ production (Hussell and Openshaw, 1998). IFN- γ producing NK cells may be critical for the development of an effector Th1 and cytotoxic CD8⁺ T cell response (Mailliard et al., 2003) and their depletion during primary RSV infection of mice has been shown to promote Th2-type responses with increased viral load and pathology (Kaiko et al., 2010). On the other hand, Li and colleagues shown that RSV infection of BALB/c mice resulted in accumulation and activation of IFN- γ producing NK cells that induced lung damage while their depletion reduced the immunopathology (Li et al., 2012a). Furthermore, RSV sensitisation of neonatal mice followed by reinfection in adulthood resulted in enhanced weight loss accompanied by the influx of NK cells which was attenuated upon the depletion of NK cells (Harker et al., 2014).

NK cells might also promote later antiviral CD4⁺ T cell immunity by releasing cytokines and regulating the activity of antigen presenting cells (APCs). IFN- γ and TNF- α production by NK

cells promote T cell priming and differentiation of a Th1 phenotype (Vivier et al., 2008). Furthermore, production of proinflammatory chemokines CCL3, CCL4 and CCL5 aid the recruitment of antiviral T cells to the site of infection (Schuster et al., 2016). In addition, NK cells have been shown to promote Th1 differentiation *in vitro* by inducing the upregulation of MHC class II expression, costimulatory molecules CD80/86 and increased production of IL-12 by dendritic cells (DCs) (Piccioli et al., 2002). Similarly, NK cells can promote maturation of DCs *in vivo* which enhances their ability to prime CD4⁺ T cells (Mandaric et al., 2012).

1.2.1.4 Dendritic cells

Dendritic cells are professional APCs that play an important role in priming of the adaptive immune response. At steady state, mouse lung DCs can be subdivided into three distinct subsets based on the expression of cell surface markers and distribution in the lung (Neyt and Lambrecht, 2013). CD11c^{hi}CD103⁺ conventional DCs (cDCs) are found on the basal epithelial cells whereas the CD11c^{hi}CD11b⁺ cDCs are located in the lamina propria. Immature cDCs are capable of sampling the airway for antigens. Antigen encounter activates PRRs and triggers the maturation of the immature cDCs, which results in the activation of the antigen-processing machinery, expression of the costimulatory molecules CD80/CD86 and the upregulation of CCR7 that facilitates migration to the lymph nodes where they present endogenous and exogenous antigens to CD8⁺ and CD4⁺ T cells respectively (Mellman, 2013). In addition, CD11c^{dim}CD11b⁻ plasmacytoid DCs (pDCs) are found in the lung parenchyma, where gas exchange occurs, and are specialised in production of type I IFN (Kim et al., 2014a). Under pro-inflammatory conditions, such as during respiratory viral infection, monocyte-derived CD11b⁺ DCs (moDCs) are recruited to the lungs and where they may differentiate into specialised antigen presenting cells. Although these cells resemble the phenotype of cDCs, they can be discriminated by the expression of the marker Ly6C.

The role of DCs in RSV infection has been studied *in vitro* and in mouse models of disease. *In vivo*, the number of DCs starts to increase in the murine lung on day 6 after infection, and they persist for several weeks. These DCs have increased expression of costimulatory molecules in the lungs (Beyer et al., 2004) and RSV is capable of infecting both mouse and

human DCs *in vitro* (Munir et al., 2008; Le Nouën et al., 2009). Thus, DCs can in principle present antigen to both CD4⁺ and CD8⁺ T cells via MHC class I and II. The role of DCs in human RSV infection is less understood. In hospitalised RSV-infected children, DCs were found to mobilise to the nasal mucosa and the increase in the number of pDCs in the nasal compartment correlated with viral load (Gill et al., 2005). However, although infected DCs can mature, they have been shown to have reduced ability in stimulating naïve T cells. The exact mechanism for this is not clear, but it has been suggested that RSV N protein causes impairment of the immunological synapse with CD4⁺ T cells, which prevents their activation (González et al., 2008). *In vitro*, infection of moDCs with RSV led to impaired CD4⁺ T cell proliferation and reduced cytokine production (de Graaff et al., 2005). Given the important role of DCs in antigen presentation and activation of RSV-specific CD4⁺ T cells, it might be hypothesised that immunomodulation by RSV through inhibiting immune synapse formation can influence not only CD4⁺ T cell differentiation and function, but also downstream impairment of antibody and CD8⁺ T cell responses.

1.2.2 Adaptive immune response to RSV

Mediators produced by innate immune cells (e.g. type I IFNs) promote the recruitment and environment for activation of adaptive immune cells while DCs activated by the inflammatory milieu carry viral antigen to the lymph nodes to present to T cells and B cells. Once primed, CD4⁺ T cells migrate to the site of inflammation and release further mediators, coordinating the cytotoxic CD8⁺ T cell and antibody producing B cell responses (Chiu and Openshaw, 2015; Collins and Melero, 2011). A summary diagram of the adaptive immune responses to respiratory viral infections is shown (Figure 1.1.2.).

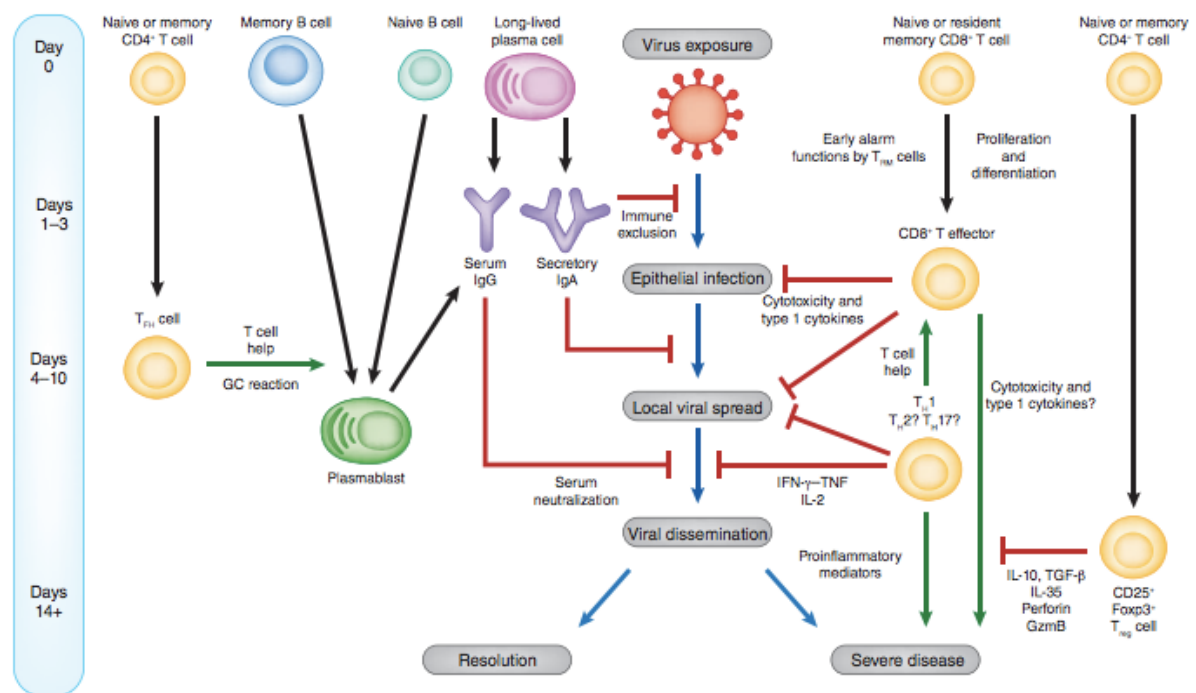


Figure 1.1.2. T and B cell responses to respiratory viral infections. Cellular and humoral immunity work together at different stages to limit disease (Chiu and Openshaw, 2015).

1.2.2.1 CD8+ T cell responses

CD8+ T cells are believed to play an important role in clearance of RSV from the lungs. Early studies showed that depletion of CD8+ T cells results in reduced viral clearance (Graham et al., 1991a). At the peak of infection, large numbers of epitope-specific CD8+ T cells were found to infiltrate the lungs (Chang and Braciale, 2002) and adoptive transfer of memory T cells isolated from previously infected animals into irradiated mice cleared the persistent infection (Cannon et al., 1988). CD8+ T cells have also been implicated in viral clearance in human studies. Children with severe combined immunodeficiency disorder (SCID) lack T cells development and have prolonged severe infections (Pachlopnik Schmid et al., 2014). In RSV-infected children, antigen-specific CD8+ T cells accumulate in the airways and PBMCs with an activated phenotype (Heidema et al., 2007). Using the experimental challenge model, our group has shown that CD8+ T cells expand with RSV infection, reaching peak frequencies in the blood at day 10 and BAL at day 28 (Jozwik et al., 2015). RSV-specific CD8+ T cells in BAL showed phenotypes consistent with advanced differentiation with downregulation of

costimulatory molecules and cytotoxicity molecules. More importantly, increased frequencies of antigen-specific CD8⁺ T cells in the airways prior to infection correlated with reduced disease severity, which supports the hypothesis that these cells have a direct role in clearance of RSV (Jozwik et al., 2015).

Primary CD8⁺ T cell responses have been shown to be partially independent of CD4⁺ T cells as they can undergo activation with inflammatory signals as a result of the infection. This phenomenon was demonstrated in *Listeria monocytogenes* infection of wild type and CD4⁺ T cell deficient mice where the primary CD8⁺ T cell response was equally able to clear infection (Sun and Bevan, 2003). However, while memory CD8⁺ T cells from wild type mice could respond rapidly upon rechallenge, those generated in the absence of CD4⁺ T cell help were defective in their recall response. Similar findings were reported for CD8⁺ T cell responses to LCMV infection (Janssen et al., 2003; Shedlock and Shen, 2003). Moving forward, Sun and colleagues found that CD4⁺ T cells were not essential for the induction of primary CD8⁺ T cell responses but the number of memory CD8⁺ T cells decreased over time in CD4⁺ T cell deficient mice, highlighting their importance for maintenance of long-lived memory CD8⁺ T cells (Sun et al., 2004). It is unclear whether a defective CD4⁺ T cell response could explain why CD8⁺ T cell mediated-protection against RSV is short-lived but improved knowledge of CD4⁺ T cell help might be useful in designing vaccines that induce more durable protection.

1.2.2.2 B cells and humoral immune responses

Antibodies are essential in preventing virus infection, through their neutralisation activity, ability to fix complement and antibody-mediated cellular cytotoxicity. Activation of B cells occurs following conformational antigen recognition and, with the help of T follicular helper (Tfh) cells in germinal centres, they differentiate into antibody-secreting plasma cells and memory B cells (Chiu and Openshaw, 2015). In RSV, antibodies against both G and F proteins are generated. However, since F is the most antigenically stable, the induction of F-specific neutralising antibodies is believed to result in broader protection against multiple strains. The protective effect of F-specific antibodies has been demonstrated by transplacental

maternal antibodies and the prophylactic monoclonal antibody Palivizumab. Even though RSV has a relatively well-conserved structure, reinfection is common throughout life and serum antibody levels are short-lived (Falsey and Walsh, 2006; Habibi et al., 2015). Previous experimental challenge studies showed that serum IgA and IgG neutralising antibodies might be associated with partial protection from RSV infection (Hall et al., 1991; Lee et al., 2004). However in our recent studies, the correlation between serum neutralising antibody titres and protection from experimental RSV infection was poor (Habibi et al., 2015). Instead, nasal IgA levels were found to significantly correlate with protection from subsequent challenge with RSV, although few individuals possessed the higher levels of mucosal IgA necessary for reliable protection (Habibi et al., 2015). Conversely, poorly neutralising antibodies have been shown to form immune complexes that caused immunopathology in FI-RSV vaccinated mice (Polack et al., 2002). These findings suggest that high levels of high affinity antibody are required for reliable protection which requires help from T follicular helper cells (discussed in detail later).

1.3 Importance of CD4+ T helper cells

T helper cells are a subset of $\alpha\beta$ T-cells that express the CD4 protein on their surface. CD4+ T cells play a central role in coordinating the different arms of the immune system to shape an effective response against a pathogen. They do so through their capacity to help B cells make antibodies, to induce antimicrobial mechanisms in macrophages, to recruit neutrophils, basophils, eosinophils to site of inflammation, and through activation of CD8+ T cells (Novy et al., 2007; Zhu and Paul, 2008). In addition, CD4+ T cells have been found to exhibit cytolytic activity in several acute and chronic viral infections including influenza and HIV (Nemes et al., 2010; Wilkinson et al., 2012). In HIV-infected patients, the inability of CD4+ T cells to mount an effective immune response increases susceptibility to infection (Pollock et al., 2015).

1.3.1 Differentiation and functionality of CD4+ T cell subsets

Beginning with the work of Mosmann and colleagues in the 1980s, CD4+ T cell clones were initially subdivided into 2 groups as IFN- γ or IL-4 cytokine producers (Mosmann et al., 1986). Similarly, Killar and colleagues reported subsets of CD4+ T cell clones based on their cytokine production nowadays referred to as Th1 and Th2 cells (Killar et al., 1987). Following on from these findings and with advances in molecular biology techniques, additional subsets of CD4+ T cells with distinct functionality have been found. Sakaguchi and colleagues discovered the regulatory potential of CD4+CD25+ T cells following findings that showed the transfer of CD25 depleted CD4+ T cells into athymic mice induced autoimmune disease (Sakaguchi et al., 1995). Regulatory T cells (Tregs) were later shown to be under the control of the transcriptional regulator FoxP3 (Hori et al., 2003). Autoimmune diseases such as multiple sclerosis and rheumatoid arthritis were initially attributed solely to the action of the Th1 subset until the discovery of IL-23 which was shown to inhibit the development of IFN- γ producing cells while expanding a 'pathogenic' CD4+ T cell population (Th17 subset) that produce IL-17A, IL-17F and IL-6 (Langrish et al., 2005; Park et al., 2005). Finally, CXCR5 expressing memory CD4+ T cells in the tonsils were described as a distinct subset that potently induce antibody production during co-culture with B cells, and were referred to as follicular B helper T cells (Tfh) (Schaerli et al., 2000). As a result of these findings, 5 main subsets of CD4+ T cells have been described by their distinct functionality and signature transcription factors (Figure 1.1.3).

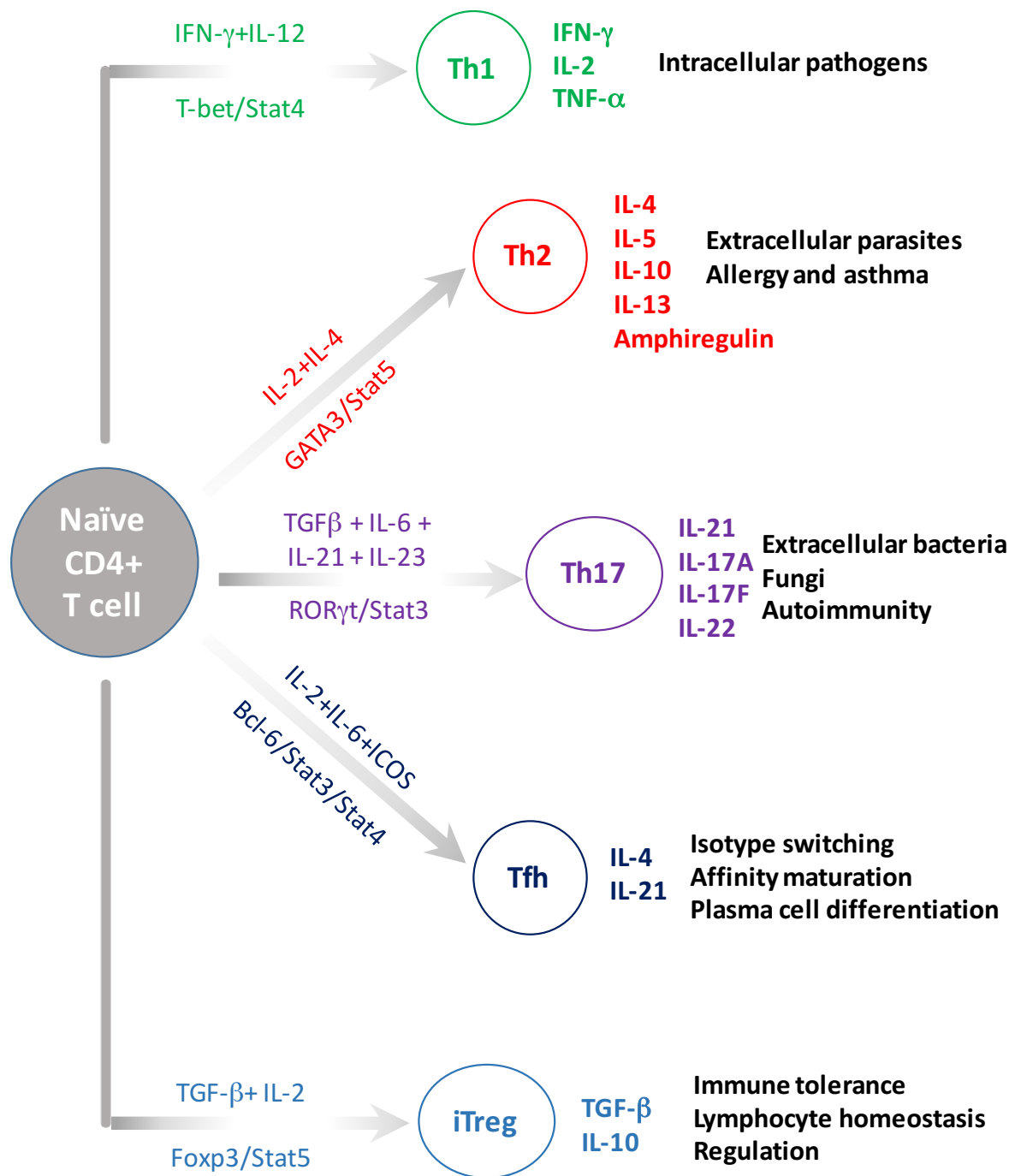


Figure 1.1.3 Summary diagram of T helper cell subset differentiation, effector cytokines and primary roles in immune responses.

1.3.1.1 T helper 1 cells

Th1 cells mediate immune responses against intracellular pathogens, particularly viruses and bacteria (Mosmann and Coffman, 1989; Del Prete, 1992). Their importance has been demonstrated in animal models and human viral infections; in LCMV infection, presence of virus-specific CD4⁺ T cells producing IFN- γ correlated with virus control (Gamadia et al., 2003). In influenza infection, Th1 clones were shown to be cytolytic *in vitro* and protective against lethal challenge *in vivo* (Graham et al., 1994). In human experimental infection, pre-existing influenza-specific CD4⁺ T cells that were capable of producing IFN- γ correlated with protection (Wilkinson et al., 2012).

Th1 differentiation requires IL-12 and IFN- γ which are critical cytokines in initiating the downstream signalling cascade (Smeltz et al., 2002). The major source of IL-12 is APCs that are activated through TLR stimulation (Ito et al., 2002; Thoma-Uszynski et al., 2000; Iwasaki and Medzhitov, 2004). In addition to acting on CD4⁺ T cells, IL-12 activates NK cells to produce IFN- γ (Arase et al., 1996; Ye et al., 1995). IFN- γ activates STAT1 signalling in the responding CD4⁺ T cells which results in induction of T-box transcription factor (T-bet), the master transcription factor of the Th1 subset (Afkarian et al., 2002; Lugo-Villarino et al., 2003). In the absence of IFN- γ , T-bet expression is diminished which prevents differentiation to the Th1 lineage (Szabo et al., 2000). T-bet thus induces further IFN- γ production which acts in a positive feedback loop to amplify T-bet expression (Afkarian et al., 2002). While IL-12R β 1 is constitutively expressed by naïve CD4⁺ T cells, T-bet upregulates the expression of IL-12R β 2 and this selects differentiating Th1 cells in the context of abundant IL-12 produced by the APCs (Afkarian et al., 2002; Kano et al., 2008). At later stages of Th1 differentiation, IL-18R α expression is upregulated through the action of IL12/STAT4 pathway (Luckheeram et al., 2012). IL-12 and IL-18 can induce further IFN- γ production by Th1 cells in the absence of T cell receptor (TCR) stimulation, which enhances the Th1 response (Zhu et al., 2010). In addition, T-bet coordinates with Runt-related transcription factors to suppress the development of other T helper cell lineages. Alongside T-bet, Runx3 is required for silencing of the genes that encode IL-4 and GATA3 to suppress Th2 whereas Runx1 is essential for

interfering with RoR γ T expression to block Th17 development. (Djuretic et al., 2007; Kohu et al., 2009; Lazarevic et al., 2011).

The resultant signature cytokines of the Th1 lineage are IFN- γ , IL-2 and TNF- α (Luckheeram et al., 2012). IL-2 acts on CD8+ T cells and promotes their acquisition of a cytolytic phenotype (Gattinoni et al., 2005). In tuberculosis, IFN- γ and TNF- α production by antigen-specific Th1 cells were shown to activate macrophages (Egen et al., 2011). Following influenza vaccination of murine models, protective immunity was shown to be dependent on CD4+ T cells *in vivo* through IFN- γ , IL-2 (Sun et al., 2011). In humans influenza challenge, antigen-specific CD4+ T cells producing IFN- γ correlated with protection (Wilkinson et al., 2012). These findings have therefore demonstrated the antibacterial and antiviral functions of Th1 effector cells.

1.3.1.2 T helper 2 cells

Th2 cells mediate immune responses to extracellular parasites and are responsible for the induction of allergic diseases and certain forms of asthma (Del Prete, 1992; Sokol et al., 2009). The differentiation of Th2 lineage requires IL-2, IL-4 and the master transcription factor GATA-binding protein (GATA3) (Le Gros et al., 1990; Zhu et al., 2001; Cote-Sierra et al., 2004). In addition, STAT5 and STAT6 are integrated at various stages of differentiation and it has been shown that the commitment to Th2 is impaired in the absence of these signal transducers and GATA3 (Usui et al., 2003; Cote-Sierra et al., 2004; Stritesky et al., 2011; Zhu et al., 2004).

TCR signalling plays an important role in early IL-4 production by naïve CD4+ T cells *in vitro*. Stimulation of naïve CD4+ T cells from TCR transgenic mice with low concentrations of peptide results in the induction of IL-2 mediated STAT5 signalling and low levels of endogenous IL-4 (Yamane et al., 2005). IL-4 acts on the STAT6 pathway to upregulate the expression of GATA3 which can then induce its own expression (Ouyang et al., 2000). In addition, the IL-4/STAT6 pathway induces the expression of a transcriptional repressor, growth factor independent-1 (Gfi-1) which strikingly enhances Th2 cell expansion by

selecting GATA3^{High} cells (Zhu et al., 2006a). Gfi-1 deficient mice show significantly reduced expansion of the Th2 cells (Zhu et al., 2006a). Furthermore, GATA3 inhibits the Th1 differentiation pathway by interacting with T-bet and downregulating STAT4 but must integrate with STAT5 to complete the differentiation into Th2 lineage (Zhu et al., 2001, 2006b). Binding of STAT5 and GATA3 together at the IL-4 locus induces the production of IL-4 (Zhu et al., 2006b).

Th2 cells have the capacity to produce multiple cytokines such as IL-4, IL-5, IL-10, IL-13 and amphiregulin (Zhu and Paul, 2008). In addition to its role as a positive feedback cytokine for Th2 differentiation, IL-4 promotes the differentiation of activated B cells into plasma cells (Hikida et al., 1999; Kobayashi et al., 2002; Mandler et al., 1993). In addition, IL-4 induces class switching to IgE by B cells, which can cross-link the FcεRI on basophils and mast cells to degranulate mediators such as histamine (Hikida et al., 1999; Mandler et al., 1993; Zhu and Paul, 2008). The role of other effector cytokines produced by Th2 cells include recruitment of eosinophils by IL-5 (Coffman et al., 1989), suppression of Th1 proliferation and dendritic cells by IL-10 (Taylor et al., 2006), induction of airway hypersensitivity by IL-13 (Taube et al., 2004) and proliferation of epithelial cells by amphiregulin (Johnson et al., 1993).

In acute respiratory infections, virus induced Th2 immune responses have been associated with immunopathology. Vaccination with formalin-inactivated RSV vaccine induced a Th2-biased response and upon viral challenge, this led to exacerbated pulmonary disease (Kim et al., 1969). In mouse models influenza infection, adoptive transfer of influenza-specific Th2 cells were shown to be associated with delayed viral clearance and the Th2 cytokines IL-4 and IL-5 were implicated in causing immunopathology in the lungs (Graham et al., 1994).

1.3.1.3 T helper 17 cells

Th17 cells mediate immune responses against extracellular bacteria and fungi (Weaver et al., 2006) but also contribute to generation of organ-specific autoimmune diseases (Luckheeram et al., 2012). Although they were only defined as a distinct CD4⁺ T cell lineage

in the last decade, their roles in controlling gram-negative bacteria in mouse models have been previously described as IL-17 producing CD4⁺ T cells (Ye et al., 2001).

Differentiation of naïve CD4⁺ T cells into the Th17 lineage requires the cytokines IL-6, IL-21, IL-23, TGF- β and the master transcription factor RoR γ T. The differentiation pathway of Th17 and Tregs are related; high concentration of TGF- β can induce signalling pathways for upregulation of FoxP3, the master regulator of Tregs, whereas low concentration of TGF- β in the presence of IL-6 leads to production of IL-21, upregulation of IL-23R and activation of RoR γ T (Ivanov et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006; Yang et al., 2008; Zhou et al., 2008). IL-21 signalling activates STAT3 which regulates RoR γ T as STAT3 deficiency enhances T-bet and FoxP3 expression initiating differentiation into Th1 and Treg lineages, respectively (Yang et al., 2007). The amplification of RoR γ T does not require IL-17, which is the signature cytokine of Th17 cells. Instead, TGF- β and IL-21 act synergistically in an IL-6 independent manner to amplify RoR γ T expression hence promoting Th17 differentiation (Nurieva et al., 2007). Finally, differentiating Th17 cells are responsive to IL-23 produced by APCs through the upregulation of IL-23R. The binding of IL-23 to its receptor is the final stage of Th17 differentiation and ensures expansion and maintenance of Th17 cells (Langrish et al., 2005).

The major effector cytokines of Th17 cells are IL-17A and IL-17F which act on a common receptor, IL-17RA expressed in multiple tissues including hematopoietic tissue and lungs (Gaffen, 2009; Luckheeram et al., 2012). Although antigen-specific Th17 cells confer mucosal immunity against bacterial infections such as *P. aeruginosa* and *S. pneumonia* (Moffitt et al., 2011; Priebe et al., 2008), they are associated with lung immunopathology in the context of respiratory viral infections. In mouse models of influenza infection, IL-17 in the absence of IFN- γ results in worse symptoms and longer viral shedding (Brown et al., 2012). In contrast, IL-17RA knockout mice have a less severe infection characterised by reductions in weight loss and neutrophil migration (Crowe et al., 2009). The exact role of Th17 in RSV infection is controversial and will be discussed in detail later.

1.3.1.4 Follicular helper T cells

The major role of Tfh cells are to help with generation of isotype switching and affinity maturation of antibodies produced by B cells, therefore they have a protective role against pathogens. In LCMV infection, defects in Tfh frequencies are associated with failure to control LCMV (Harker et al., 2011). Similarly in HIV infected patients, Tfh help to B cells is impaired, which makes it even more challenging to generate neutralising antibodies against HIV (Cubas et al., 2013). They can also be induced in response to influenza infection, but whether they were required for viral clearance in that study was not investigated (Boyden et al., 2012).

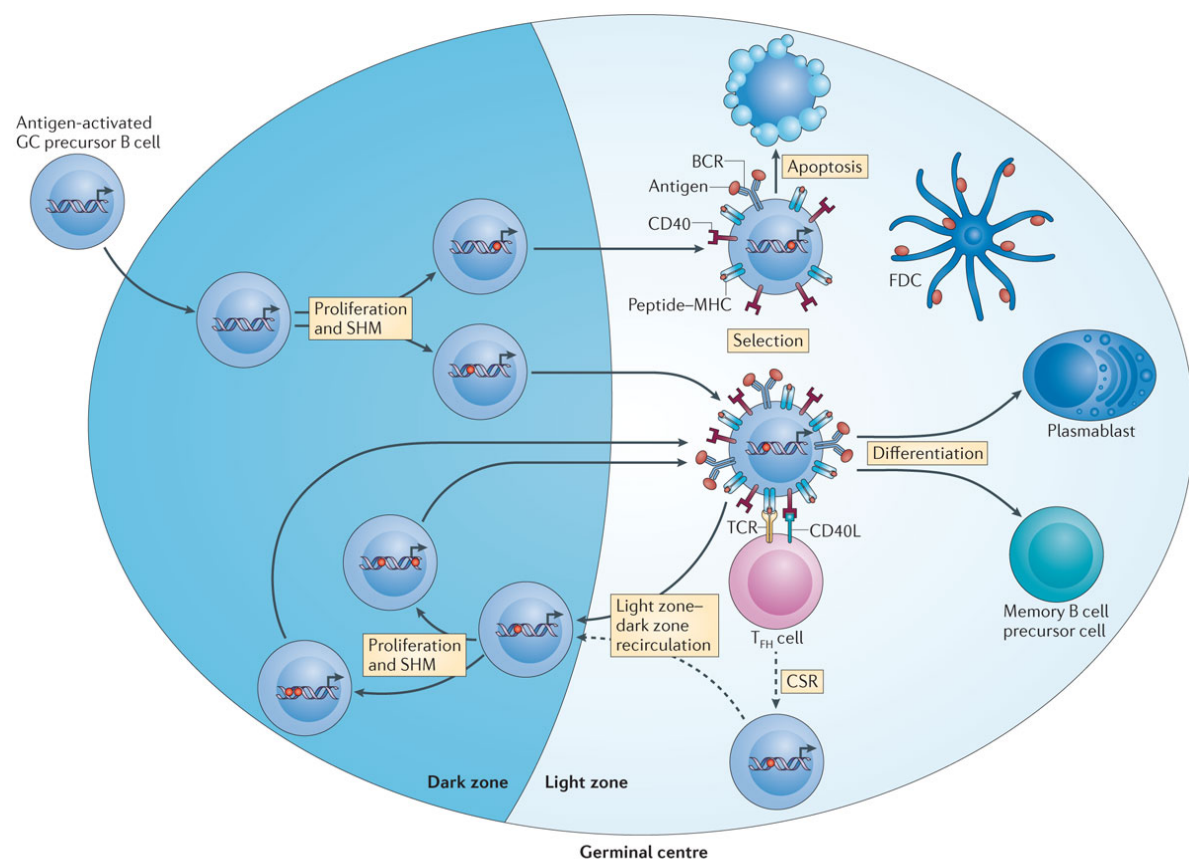
Unlike the other T helper cell subsets, Tfh cell differentiation is a multifactorial process and several Tfh differentiation pathways exist (Crotty, 2014). Canonical Tfh cell differentiation starts with upregulation of CXCR5, which leads to the migration of early Tfh cell to the border of B cell follicles (Haynes et al., 2007). The early differentiation to Tfh cells in mice is regulated by IL-2, IL-6, inducible costimulator (ICOS) and TCR signal strength (Tubo et al., 2013). IL-6 signalling induces Bcl-6 expression which is the master transcription factor of Tfh cells (Nurieva et al., 2009). As mentioned in the previous section, IL-6 collaborates with TGF- β for Th17 differentiation, which suggests the possibility of plasticity between Tfh and Th17 lineages. In contrast, IL-6 and ICOS act synergistically for Tfh differentiation (Weber et al., 2015). In mouse models, IL-6 driven STAT3 and in humans, STAT3 and STAT4 are involved in upregulation of CXCR5 expression (Ma et al., 2012; Schmitt et al., 2013; Suto et al., 2008). In addition, IL-2 is a negative regulator of Tfh differentiation and inhibits Tfh differentiation in a STAT5 dependent manner (Johnston et al., 2012). Recently, Tregs were found to overcome this inhibitory effect by controlling IL-2 availability and promoting influenza-specific Tfh responses (León et al., 2014). These highlight multiple regulatory mechanisms of Tfh differentiation even at its early stages. The second stage takes place in the interfollicular zone where the differentiating Tfh cells interact with B cells (Crotty, 2014). This engages ICOS on the surface of the Tfh cells with the ICOSL on B cells, which also act as APCs to enhance cell division of Tfh cells (Choi et al., 2013a, 2013b). Tfh cells complete their differentiation in the GCs through the upregulation of programmed death-1 (PD1), signalling lymphocyte

activation molecule (SLAM) and SLAM-associated protein (SAP) (Crotty, 2014). At this stage, fully differentiated Tfh cells can be identified as CXCR5+ICOS+PD1+Bcl-6+SAP+ and produce IL-4 and IL-21 for delivering help B cells (Crotty, 2014; Liang et al., 2012).

Humoral immunity depends on formation of GCs which are transient structures that form within lymphoid organs in response to T cell dependent antigen (De Silva and Klein, 2015). These are sites where B cells undergo the process of somatic hypermutation, class switching and memory B cell formation that mediate and sustain protection against invading pathogens (Figure 1.1.4.). Our understanding of the GC dynamics and the role of Tfh cells is restricted to mouse models due to limitations in sampling lymphoid tissue in humans. The initiation of GC formation has been visualised using intravital microscopy (Kerfoot et al., 2011; Kitano et al., 2011). These studies showed that the activation of cognate B and T cells promote the formation of long-lasting interactions in the interfollicular regions. This is accompanied by the full activation of B cells and the acquisition of the Tfh phenotype by T cells, characterised by the upregulation of CXCR5 and PD-1 as described earlier. This is followed by the migration of the B cells into the centre of the follicle where they begin to proliferate through interactions with follicular DCs (FDCs). This promotes the establishment of mature GCs, which consist of the dark zone with B cell blasts and light zone that contains Tfh cells and FDCs (De Silva and Klein, 2015). Somatic hypermutation takes place in the dark zone and is the process by which the immunoglobulin variable region of the B cells is modified by the activation-induced cytidine deaminase (AID) enzyme (Laskov et al., 2011). Positive selection of high-affinity B cells was shown to be driven by competition for antigen presented on FDCs in the light zone (MacLennan, 1994) and the survival of selected B cells was promoted by Tfh cells through the production of IL-21 (Linterman et al., 2010). However, a more dominant role for Tfh cells in the selection process has been suggested. Using a method for *in situ* labelling of GC B cells with photoactivatable green fluorescent protein (PA-GFP), Victora and colleagues found that GC B cells with high affinity for antigen were associated with greater antigen capture that led to higher density of MHC class II/peptide complexes on the surface (Victora et al., 2010). This facilitated interactions with Tfh cells to a larger extent and drove positive selection. In support of this, *in vivo* imaging of Tfh and B cell interactions showed that patrolling Tfh cells formed longer lasting contacts with B cells

that presented the highest density of MHC class II/peptide complexes (Shulman et al., 2014). These Tfh cells had increased levels of intracellular free calcium which was associated with the coexpression of IL-4 and IL-21, which aids the survival of high affinity B cells.

To date, the role of Tfh cells in the context of RSV infections has not been studied. As discussed in section 1.2.2.2, serum antibody levels against RSV are short-lived and this may be a result of an impaired Tfh response to human RSV infection. Addressing this directly is currently not possible given the limitations in sampling of human lymphoid tissues, but studying of Tfh cells in blood may provide useful insights to their role in human RSV infection.



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Figure 1.1.4 Events that take place in the germinal centres. Antigen activated B cells interact with FDCs and Tfh cells that initiate the formation of a mature GC. B cells undergo proliferation and somatic hypermutation in the dark zone. Following their migration to the light zone, B cells with high-affinities against their antigen are positively selected for survival which is promoted by the help of Tfh cells. Those B cells can then differentiate into plasmablasts or memory B cells (De Silva and Klein, 2015).

1.3.1.5 Regulatory T cells

Tregs are key players in maintenance of immunologic tolerance and regulation of immune responses following the clearance of pathogens to prevent immunopathology (Sakaguchi et al., 1995). As dramatically demonstrated in scurfy mice and IPEX patients (immunodeficiency polyendocrinopathy enteropathy X-linked syndrome), lack of detectable Tregs due to mutations in the *FoxP3* gene, usually results in death during infancy or early childhood (Brunkow et al., 2001; Myers et al., 2006).

There are two phenotypically and functionally distinct subsets of Tregs, natural Tregs (nTregs) and induced Tregs (iTregs) which collectively make up 5-10% of total peripheral CD4⁺ T cells in mice and humans (Shalev et al., 2011). nTregs originate in the thymus as CD4⁺CD25⁺FoxP3⁺ following high-avidity interactions with self peptide/MHC complexes and expression of the transcription factor Helios distinguishes them from iTregs (Thornton et al., 2010). In the absence of proinflammatory cytokines (i.e. IL-6), IL-2 and TGF- β can induce FoxP3 expression in CD4⁺CD25⁻ cells to form iTregs (Bettelli et al., 2006). TGF- β signalling activates Smad3 while TCR signalling activates NFAT and the collaboration of these downstream proteins bind the *FoxP3* enhancer region and promote FoxP3 expression (Tone et al., 2008). In addition, IL-2 acts through STAT5 for persistence of FoxP3 as well as the survival of the Tregs (Davidson et al., 2007). It is important to highlight that iTregs can lose their regulatory potential; while the FoxP3 promoter of nTregs is fully demethylated, TGF- β induced Tregs in the periphery have only a partially demethylated FoxP3 promoter (Floess et al., 2007). *In vitro*, iTregs can have downregulation of FoxP3 and may produce IFN- γ or IL-17 (Ziegler and Buckner, 2009).

The main function of Tregs is appreciated as limiting excessive inflammation in autoimmunity as well as viral and bacterial infections (Long and Buckner, 2011; Prabhakara et al., 2011; Veiga-Parga et al., 2013). Following influenza infection, there is a robust induction of Tregs in mice (Betts et al., 2012). In lymphocyte depleted mouse models, adoptive transfer of polyclonal Tregs (CD4⁺CD25⁺FoxP3⁺) increases the survival rate after influenza infection (Antunes and Kassiotis, 2010). Moreover, influenza-specific Treg clones from human donors

that display influenza-specific immunity displayed suppressive capacity *in vitro* even after 2 years, through the production of IL-10 (Piersma et al., 2010). These examples highlight the importance of Tregs in dampening down the activated immune responses to prevent immunopathology.

1.3.1.6 Plasticity of CD4+ T cells

Initially, differentiation of naïve CD4+ T cells into a distinct lineage was thought to be stable with a fixed fate as Mosmann and Coffman described T-bet or GATA3 expressing cells as selective producers of IFN- γ or IL-4, respectively (Mosmann and Coffman, 1989). However, following the discovery of additional Th lineages, it is now clear that many 'signature' cytokines can be broadly expressed by other lineages and there is plasticity between the different subsets (Nakayamada et al., 2012).

Genetic modifications reprogram differentiated Th lineages; conditional deletion of GATA3 in from Th2 cells abolish their capacity to produce IL-4 and IL-5, and induces Th1 differentiation even in the absence of IL-12 and IFN- γ (Zhu et al., 2004). Similarly, decreased FoxP3 expression in T cells leads to induction of Th2 cells, even in conditions that would favour Th1 polarisation (Wan and Flavell, 2007). Modifications such as these are unlikely to occur in a healthy immune system, but nonetheless CD4+ T cells may show plasticity in response to certain physiological stimuli. Culture of Th2 or Tregs with IFN- γ and IL-12 can induce these cells to produce the Th1 cytokine IFN- γ (Coomes et al., 2015; Wei et al., 2009). In the absence of TGF- β , IL-17 production is suppressed by both IL-12 and IL-23, and these cells acquire the capacity to produce IFN- γ in a T-bet/STAT4 dependent manner (Lee et al., 2009). Intriguingly, LCMV infection can reprogram fully differentiated Th2 cells, in a type I IFN dependent manner, to adopt a 'Th2+1' phenotype characterised by the expression of GATA-3, T-bet and production of IL-4 and IFN- γ (Hegazy et al., 2010). Tfh cells can be regarded as the most flexible subset. *In vitro* differentiated and *in vivo* generated Tfh cells can acquire Th1, Th2 or Th17 phenotypes in response to appropriate polarising conditions. Likewise, these cells can be reprogrammed to obtain Tfh cell characteristic following B cell contact and exposure to IL-6 and IL-21 (Lu et al., 2011). The plasticity between the different

Th subsets is summarised in Figure 1.1.5. Collectively, Th subsets may appear to be phenotypically distinct but under certain conditions, their plasticity in re-differentiating to other phenotypes should not be ignored.

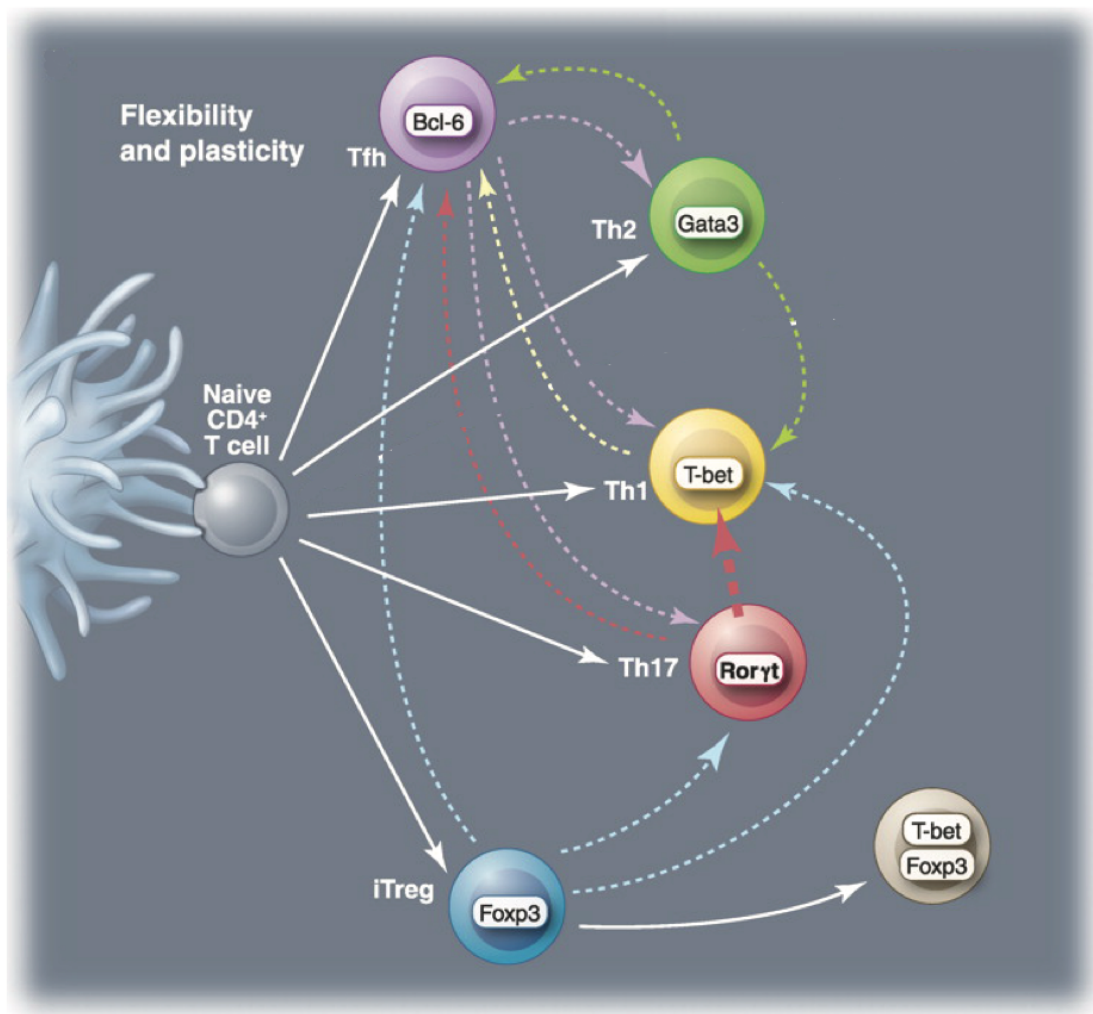


Figure 1.1.5 Summary diagram illustrating the plasticity of differentiated CD4⁺ T cells. CD4⁺ T cell subsets can be distinguished by the expression of their signature transcription factors. CD4⁺ T cells can change their profile of cytokine production as illustrated with dotted lines. (O'Shea and Paul, 2010).

1.4 CD4+ T cell responses to RSV

Our knowledge about the role of CD4+ T cells against RSV infection mainly comes from mouse models and much less is known about these responses in human RSV infection. The Th1 cell response has been reported to be beneficial; mice primed with recombinant vaccinia virus expressing the F protein resulted in a strong Th1 and CD8+ T cell response that resulted in higher IL-2 production and increased cytolytic ability (Alwan and Openshaw, 1993). Yamada and colleagues investigated the gene expression profiles of Th1 or Th2 primed human airway epithelial cells following RSV infection. Microarray analysis of these cells showed that viral replication was diminished in Th1-primed but not in Th2-primed cells (Yamada et al., 2011). In infants, RSV infection can induce mucus production and airway hyperreactivity, which are symptoms associated with Th2-biased immune responses (Christiaansen et al., 2014). This may be explained by a predisposition of the infant immune system toward the Th2 subset compared to adults; cord blood CD4+ T cells express higher levels of IL-4R following *in vitro* stimulation with RSV compared to PBMCs from adults (You et al., 2013). In addition, it has been shown that neonatal DCs produce lower levels of IL-12 compared to adults which results in less IFN- γ production by CD4+ T cells (Taylor and Bryson, 1985). However, an imbalance in the Th1/Th2 response may be associated specifically with RSV-induced disease. Clinical studies on nasal washes from infected infants showed that even though levels of IFN- γ was higher compared to IL-4, an increased ratio of IL-4 to IFN- γ was associated with more severe disease (Garofalo et al., 2001; Legg et al., 2003). *In vitro* studies on PBMCs of severely ill infants showed similar findings where significant decreases in IL-12 and IFN- γ levels were observed with an increased IL-4/IFN- γ ratio compared to control infants (Pinto et al., 2006; Román et al., 1997). While these findings suggest that Th2 biased responses may be found in more severe disease, these are based on detection in limited sampling of the upper respiratory tract. Although several Th2 cytokines have been detected in the nasal washes (IL-4, IL-5 and IL-13), only IL-4 was measurable in the lower respiratory tract (sampled using tracheal aspirates) and the contribution of Th2 cells to disease severity in infants remains unclear (Garofalo et al., 2001).

Emerging evidence on Th17 cells in RSV infection suggest these cells may also play a critical role in inducing more severe disease. In RSV-infected infants, high levels of IL-17 have been detected in the tracheal aspirates but not in the nasal washes (Mukherjee et al., 2011). Bystrom and colleagues found that IL-17 enhanced IL-13 production that skewed the Th1/Th2 balance towards a Th2 biased response. In animal models, an inadequate Th1 response was shown to enhance Th17-mediated responses which was detrimental during RSV co-infection in experimental asthma (Bystrom et al., 2013). Neutralisation of IL-17 following RSV infection in mice resulted in reduced MUC5A mRNA expression and histology suggesting their role in inducing mucus production and airway hyperreactivity (Mukherjee et al., 2011). Moreover, IL-17 but not Th2 cytokines was found to stimulate the MUC5B mRNA expression by both well differentiated primary normal human bronchial epithelial cells and a human bronchial epithelial cell line (Fujisawa et al., 2011). Conversely, Faber and colleagues reported potentially beneficial effects of IL-17 during convalescence from primary RSV bronchiolitis. They measured IL-17 levels in nasopharyngeal aspirates from non-ventilated and ventilated children at admission and discharge. IL-17 concentrations were significantly higher only in children with non-ventilated RSV disease at discharge (Faber et al., 2012). Following these findings, it might be speculated that airway IL-17 facilitates resolution during convalescence. Therefore, the exact role of IL-17 in the pathogenesis of RSV needs further study.

Regulation of T cell responses is essential for a balanced antiviral response without causing tissue damage. Following RSV infection in mice, the number of Tregs identified through the co-expression of CD25 and FoxP3 has been found to increase in lung, BAL, mediastinal lymph nodes and spleen (Fulton et al., 2010; Lee et al., 2010). Depletion of Tregs resulted in enhanced disease characterised by higher weight loss and increased mucus production, delayed viral clearance, delayed recruitment of CD8⁺ T cells and greater inflammatory cell migration (i.e. NK cells, eosinophils and neutrophils) to the lungs. There are several mechanism of regulation by Tregs; (i) inhibition of pro-inflammatory cytokines and chemokines as well as regulation of the number of IFN- γ producing cells in a granzyme B dependent manner (Loebbermann et al., 2012a), (ii) via upregulation of CTLA-4 that interacts with the costimulatory molecules CD80/86 to inhibit DC maturation (Fulton et al., 2010) and

(iii) dampening of the immune responses by the production of IL-10 during resolution from RSV (Loebbermann et al., 2012b). While Tregs appear to be key during the convalescence from RSV infection in mice, their role in human RSV infection is yet to be elucidated.

Human Tfh responses to respiratory viral infections as a whole are still poorly understood, due to limitations in sampling lymphoid structures. To date, there is no literature on the role of these cells in human RSV infection. Work from PBMCs of influenza vaccinated adults has shown the increase in the frequency of Tfh-like cells, characterised as CD4+CXCR5+CXCR3+ICOS+, induced by influenza vaccination and which is associated with vaccine-specific antibody production, but much work remains to be done to establish the mechanisms by which CD4+ T cell help of B cell response occurs in humans (Bentebibel et al., 2013).

1.5 CD4+ T cell activation

Activation of naïve CD4+ T cells is essential for clonal expansion and differentiation into effector cells. This process requires three distinct signals; (i) interaction of the TCR with the cognate antigen, (ii) costimulation through interactions with molecules expressed on the surface of APCs and (iii) a third signal commonly provided by pro-inflammatory cytokines (Curtsinger and Mescher, 2010).

1.5.1 TCR signalling

TCR signalling upon recognition of antigenic peptides on MHC serves as signal 1 for activation of a T cell. T cells interact with antigen through the variable TCR α and β chains, which are incapable of initiating signalling pathways due to their short cytoplasmic tails (Wucherpfennig et al., 2010). Instead, conformational changes following recognition of a cognate MHC-peptide complex by TCR $\alpha\beta$ trigger changes in associated CD3 subunits (δ -, γ -, ϵ - and ζ - chains) that contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic domains (Kane et al., 2000; Alarcón et al., 2003). Constitutively active Src

family member leukocyte-specific tyrosine kinases (Lck) associate with the CD4 co-receptor, which recruits them to TCR complexes for phosphorylation of ITAMs (Nika et al., 2010). As a result, activation of the numerous downstream signalling events required for cytoskeletal remodelling and signal transduction to the nucleus occurs, activating the transcription factors activator protein (AP1), nuclear factor of activated T cells (NFAT) and nuclear factor κ B (NF- κ B) (Nika et al., 2010; Pollizzi and Powell, 2014; Thaker et al., 2015). These act together to promote the transcription of the IL-2 gene and the expressed protein is released to the extracellular milieu to act in an autocrine manner. The cascade of complex signalling events downstream of TCR activation was recently reviewed and will therefore not be discussed here in detail (van der Merwe and Dushek, 2011; Oeste et al., 2016; van Panhuys, 2016).

1.5.2 Costimulation

TCR signalling is an integral part of T cell activation, but is insufficient in the absence of additional signals and alone results in anergy (Frauwrith et al., 2002; Mueller et al., 1989). Signal 2 is mediated through costimulation which has been shown to amplify TCR signalling to induce proliferation and IL-2 production (Jenkins et al., 1991; June et al., 1987). While naïve T cells require costimulation to become fully activated, signal 2 is also important in effector T cells for optimal cytokine production (Dubey et al., 1996). There are 2 major groups of costimulatory receptors; immunoglobulin superfamily (IgSF) and tumour necrosis factor receptor (TNFR) superfamily (Chen and Flies, 2013).

The IgSF consists of several co-stimulatory families including the well-studied CD28. Early studies demonstrated that T cells isolated from CD28 deficient mice showed reduced proliferation in response to peptide stimulation (Shahinian et al., 1993). CD28 interacts with members of the B7 family which includes CD80/86. In contrast to activation of ITAMs by TCR triggering, signal transduction of CD28 is mediated through phosphorylation of tyrosines (independent of ITAMs) and proline-rich motifs in its cytoplasmic tail (Holdorf et al., 1999). However, like ITAMs, tyrosine residues on the intracellular domain of CD28 are phosphorylated by Src family protein Lck and bound by phosphatidylinositol 3-kinase (PI3K)

(Céfaï et al., 1998). Downstream CD28 signalling can induce priming, cell growth and survival of CD4⁺ cells. CD28 costimulation of T cells isolated from human PBMCs was found to increase their glycolytic rate through enhancing the expression of glucose transporters, glucose uptake and glycolysis (Frauwirth et al., 2002). This increase in cell metabolism through CD28 was found to be dependent on PI3K signalling. CD28 signalling also increases proliferation of CD4⁺ T cells by reducing the number of TCRs that have to be triggered as well as facilitating activation by low affinity peptides (Bachmann et al., 1997). In addition, CD28 signalling was shown to specifically regulate the stability of mRNAs for IL-2, IFN- γ and TNF- α by preventing their degradation (Lindstein et al., 1989) through activation of the NF- κ B pathway (Kalli et al., 1998). Finally, CD28 signalling through PI3K has been shown to generate pro-survival signals that prevent T cell apoptosis. CD28 prevents apoptosis via death receptors by decreasing FasL expression and interfering with the formation of the death-inducing signalling complex (Jones et al., 2002; Sperling et al., 1996) as well as by inducing the expression of anti-apoptotic proteins Bcl-2 and Bcl-X_L (Boise et al., 1995; Burr et al., 2001).

TNFR superfamily members, such as CD27, are also involved in providing costimulation for T cells and can have a positive impact on priming, cell growth, T helper cell differentiation, effector function and cell survival (Nolte et al., 2009). CD27 is expressed on the cell surface as a dimer and its intracellular tail couples to TNFR-associated factor 2 (TRAF2) and TRAF5 that have been shown to be necessary for CD27-mediated costimulation in knock out experiments (Akiba et al., 1998; Nakano et al., 1999). Interaction with CD70, the only known ligand for CD27 (Welten et al., 2013), induces ubiquitination of TRAF2 and TRAF5 which results in the activation of the NF- κ B signalling pathway (Ramakrishnan et al., 2004). Consequently, NF- κ B triggers the upregulation of IFN- γ and T-bet expression (Xiao et al., 2008), favouring the development of a Th1 phenotype as well as enhancing cell survival through the induction of Bcl-X_L (van Oosterwijk et al., 2007). However, CD27 knock out studies in mouse models showed that naïve CD4⁺ T cells can be generated and maintained independently of CD27 (Hendriks et al., 2000). Nevertheless, primary CD4⁺ and CD8⁺ T cell responses to influenza infection were found to be impaired in CD27^{-/-} mice. Furthermore, the absence of CD27 signalling was associated with delayed response kinetics and reduction

in the number of antigen-specific CD8⁺ T cells suggesting the requirement of CD27 for generation of T cell memory.

1.5.3 Signal 3

Pro-inflammatory cytokines provide a third signal for optimal differentiation and clonal expansion of naïve T cells (Curtsinger et al., 1999). This was initially based on findings that showed minimal CD4⁺ T cell response to vaccination in the absence of adjuvants, while the differentiation of antigen-specific cells was considerably enhanced with co-administration of IL-1 (Pape et al., 1997). IL-1 acts directly on antigen-specific naïve or memory CD4⁺ T cells, independently of CD28, and inhibition of the IL-1 signalling can result in up to 50% reduction in response to antigen (Ben-Sasson et al., 2009). IL-12 and type I IFNs can also provide signal 3 for CD8⁺ T cell expansion and differentiation (Curtsinger et al., 2005). In addition to their antiviral role in innate immunity, type I IFN signalling have been shown to regulate effector and memory CD4⁺ T cells. As discussed earlier, development of a Th1 phenotype requires IL-12 signalling through STAT4 and the expression of the transcription factor T-bet. Type I IFN signalling in the absence of IL-12 has also been shown to induce the phosphorylation of STAT4 in mice and human cells (Berenson et al., 2004; Farrar et al., 2000). However, *in vitro*, STAT4 phosphorylation through type I IFN signalling in the absence of IL-12 was short-lived and not enough for commitment to a Th1 phenotype (Ramos et al., 2007). Nevertheless, *in vivo* studies using IL-12 deficient mice did show generation of Th1 cells against murine hepatitis virus infection (Havenar-Daughton et al., 2006). Furthermore, IFN α and IL-12 can work synergistically to enhance a subset of IL-2 secreting cells that resemble the phenotype and function of central memory CD4⁺ T cells (Davis et al., 2008). These cells were shown to be capable of generating IFN- γ secreting effector cells upon secondary infection. Finally, type I IFN production promotes antiviral Th1 responses through negative regulation of Th2 and Th17 differentiation. In human cells, type I IFNs inhibited the ability of IL-4 to induce GATA3, therefore Th2 differentiation (Huber et al., 2010). Similarly, IL-17 mRNA and protein expression was significantly suppressed by IFN α treatment of PBMCs *in vitro* (Moschen et al., 2008). Collectively, these findings highlight the importance of type I IFNs in promoting an antiviral Th1 response. Recurrent infection with RSV in the absence of substantial antigenic

variation is common and immunomodulation of type I IFN responses by NS1/NS2 proteins have been shown. In this context, defective type I IFN responses may be associated with an impaired T cell response due to suboptimal signal 3 that might contribute to short lived memory responses against RSV infection. It is also worth highlighting that the order in which signals 1, 2 and 3 occur may be critical; for example, both mouse and human CD4⁺ T cells can transiently become anergic if they are exposed to signal 3 (IL-1 signalling) in the absence of signal 1 (TCR engagement) (Sckisel et al., 2015). The induction of this anergic state is dependent on the expression of suppressor of cytokine signalling-3 (SOCS3) and prevents mounting of CD4⁺ T cell responses both *in vivo* and *in vitro* (Sckisel et al., 2015).

1.5.4 Influence of signals 1 and 2 on differentiation of CD4⁺ T cell

Differentiation of CD4⁺ T cells into different Th cell subsets can be influenced by signal 1 and signal 2. It is well established from *in vitro* studies that a “strong” TCR signal promotes Th1 differentiation whereas a “weak” TCR signal can lead to Th2 differentiation (Constant et al., 1995; Hosken et al., 1995). The strength of the TCR signal is dependent on the combination of a number of factors including the affinity of binding between the peptide/MHC complex and the specific TCR, and the quantity of peptide/MHC complex present on the surface of APCs (Hosken et al., 1995). *In vivo*, the strength and duration of TCR signalling has therefore been shown to influence Th cell differentiation. van Panhuys and colleagues developed a model system that enabled manipulation of the cytokine milieu by varying the adjuvant exposure and the strength of antigen stimulation by modulating MHC class II/peptide display encountered by the responding CD4⁺ T cells. They found that high doses of antigen induced Th1 differentiation even under Th2-polarising conditions and conversely, low doses of antigen regardless of the polarising conditions induce Th2 differentiation (van Panhuys et al., 2014). Furthermore, TCR signalling can also induces IL-2R expression in a signal strength-dependent manner (van Panhuys, 2016). If the induction of IL-2R expression is accompanied by a strong TCR signal, differentiation of Th1 subset is promoted. Conversely, weak TCR signalling inhibits responsiveness of the cell to IL-2 and favours the development of Th17 and iTregs in a STAT5 dependent manner (Gomez-Rodriguez et al., 2014; Gottschalk et al., 2010). TCR signalling has also been associated with regulation of T cell memory development. This

was illustrated in a mouse model with point mutations in the transmembrane of the TCR β chain that resulted in partial TCR signalling with no modifications to primary effector T cell responses (Teixeiro et al., 2009). Upon *Listeria* infection, the mutant cells were impaired in generation of memory T cells which was shown to be a result of defective of NF- κ B signalling pathway. These results highlight the requirement of TCR-dependent NF- κ B signalling for the generation of memory T cells.

Costimulatory signals also play a role in Th cell development and effector function. CD28 stimulation of CD4⁺ T cells *in vitro* promotes the production of both Th1 and Th2 cytokines, but longer periods of CD28 ligation preferentially enhance the production of Th2 cytokines while diminishing Th1 responses (King et al., 1995). The CD28 dependent response for induction of IL-5 persists following the administration of anti-IL-4 antibodies, suggesting that this process is IL-4 independent (King et al., 1995). *In vivo* studies partially support this, as Th1 biased responses to LCMV infection remain intact in CD28-deficient mouse models as long as there is persistent TCR signalling through prolonged viral replication (Kündig et al., 1996). In contrast, Th1 responses in other settings have been shown to be highly CD28 dependent (Palmer et al., 1996) and Th2 responses can still be mounted in the absence of costimulation in certain models (Harris et al., 1999). Furthermore, *in vivo* mouse studies investigating the role of CD28 signalling in memory immune responses have shown the requirement of costimulation for generation of memory T cells (Borowski et al., 2007). Influenza-specific T cells were generated by infection of wild type C57Bl/6 mice and adoptively transferred to CD80/86^{-/-} recipients. CD80/86^{-/-} mice were challenged with influenza and compared to the wildtype group, there was a 9-fold reduction in the absolute number of influenza-specific T cells at the peak of T cell response.

ICOS is another member of the immunoglobulin superfamily that acts through the same signalling pathway as CD28 (Rudd et al., 2009). Unlike CD28, ICOS is not constitutively expressed and is induced following TCR/CD28 signalling (Kaminski et al., 2009) after which it has been shown to regulate the polarisation of Th1, Th2 and Th17 subsets (Salama et al., 2003). In particular, ICOS is important for induction of CD4⁺ memory subset differentiation, as ICOS deficient patients show impairment in their memory T cell compartment and

consequently immunodeficiency (Takahashi et al., 2009). ICOS has an important role in driving Th2 responses with ICOS ligation *in vitro* increasing the production of Th2 cytokines by enhancing IL-4R signalling (Watanabe et al., 2005). Similarly, prolonged expression of ICOS is critical for differentiation and expansion of Th17 cells as costimulation through CD28 has only a short-term influence on sustainability of Th17 cells (Paulos et al., 2010). The role of ICOS signalling for Th1 cells is more complex as it can augment or dampen Th1 responses depending on the pathogen. For example, ICOS-deficient mice produce a strong Th1 response in *M. Tuberculosis* infection, characterised by an increase in the number of IFN- γ producing CD4⁺ T cells (Nouailles et al., 2011). Conversely, ICOS-deficient mice are unable to mount a Th1 response and are unable to resolve *Salmonella enterica* infection (Vidric et al., 2006). Finally, ICOS has a direct impact on induction of Tfh cells by promoting the expression of CXCR5 during GC responses and its interaction with ICOSL on B cells is essential for maintenance of Tfh:B cell contact within the GCs (Akiba et al., 2005; Liu et al., 2015).

Collectively, activation of CD4⁺ T cells relies on three signals that are mediated through the TCR, costimulatory molecules and cytokines such as IL-1. These signals are essential for regulating the differentiation of the various Th subsets as well as development of T cell memory.

1.6 Class-II antigen processing and presentation

1.6.1 Antigen uptake

CD4⁺ T cells recognise MHC class II-restricted antigens presented by APCs and this interaction is essential for their activation and differentiation (Doyle and Strominger, 1987). MHC class II molecules are primarily expressed by professional APCs such as DCs, macrophages and B cells, and APCs use various processes to capture foreign antigen for processing. Of these, phagocytosis is a fundamentally important mechanism of antigen uptake by DCs and macrophages *in vivo* and involves direct recognition using phagocytic receptors on the surface (Stuart and Ezekowitz, 2005). Macropinocytosis is another major pathway utilised by DCs and macrophages, which results in non-specific internalisation of

extracellular molecules (West et al., 2004; Lim and Gleeson, 2011). B cells have low macropinocytic activity compared to DCs as demonstrated by significantly lower pinocytic rates *in vitro* (Sallusto et al., 1995). Instead, B cells preferentially undertake receptor-mediated endocytosis and uses antigen binding on surface receptors, such as mannose receptor and DEC205, to mediate internalisation of antigen (Platt et al., 2010). Both DCs and B cells express receptors that enhance their capacity for endocytosis and it has been recently shown that binding of DEC205-targeted antigen by B cells amplifies antigen-specific CD4⁺ T cells *in vivo* (Leung et al., 2013). In addition, B cells can use the B cell receptor (BCR) for antigen uptake and triggering of BCR signalling induces endocytosis and recruitment of MHC class II containing vesicles towards the late endosomal HLA-DM⁺ antigen processing compartments (Roche and Furuta, 2015). Early studies showed that B cells with antigen-specific BCRs are more efficient (up to 1,000 fold) in processing and presentation to CD4⁺ T cells when compared to irrelevant antigens (Rock et al., 1984).

1.6.2 MHC class II structure and assembly

MHC class II genes are encoded by three pairs of polymorphic genes, HLA –DR, –DP and –DQ and are expressed as non-covalent heterodimers on macrophages, dendritic cells and B cells (Trowsdale et al., 1991). The use of molecular typing methods for defining an individuals HLA haplotype, which involves amplification of DNA with primers targeted against the different HLA-class II antigens, has been well characterised (Doherty et al., 1992). HLA class II haplotypes of individuals have been used in numerous studies to address a spectrum of scientific questions. Certain haplotypes have been associated with autoimmunity (Gough and Simmonds, 2007) as well as susceptibility to and severity of infectious diseases including HIV, hepatitis B, tuberculosis, malaria and leprosy (Blackwell et al., 2009). Furthermore, HLA class II restriction has been important for epitope mapping of pathogens, including influenza. These advances subsequently led to construction of class II tetramers, which have provided important insights into role of CD4⁺ T cells during infection (Danke and Kwok, 2003; Novak et al., 1999).

HLA-DR is best characterised structurally and functionally. Most variability comes from the beta chain of the heterodimer (DRB) with over 700 known alleles, whereas there are only 3 DRA variants (van Lith et al., 2010). Conversely, 17 DPA1 and 86 DPB1 allelic forms exist in the global population, although 50% of the European population express DPA1*0103/DPB1*0401 and DPA1*0103/DPB1*0402, which differ by only three amino acids (Sidney et al., 2010). Finally, 33 DQA1 and 78 DQB1 alleles have been characterised in human populations (Klitz et al., 2003).

MHC class II molecule assembly takes place in the endoplasmic reticulum (ER) where the MHC class II α and β subunits associate in a process facilitated by a chaperone called the invariant chain (Ii) (Romagnoli and Germain, 1994). This protein is required for the folding of MHC class II $\alpha\beta$ chains and blocking of the peptide binding groove from loading with peptides at early stages of biosynthesis as demonstrated in Ii deficient cells and mouse models (Roche and Cresswell, 1990; Bikoff et al., 1993; Busch et al., 1996). Furthermore, Ii contains a dileucine motif in the N-terminal cytoplasmic domain that directs the MHC class II to the late endosomal compartment (Pond et al., 1995; Zhong et al., 1997). The 3 different class II alleles have different affinities for Ii chain, and in the absence of this interaction MHC class II molecules fail to reach the cell surface (van Lith et al., 2010). The Ii-bound HLA traffics to mildly acidic, lysosome-like organelles referred to as MHC II compartments (MIICs) or late endosomes (Peters et al., 1991). Upon arrival, the class II associated Ii is degraded, leaving behind a short fragment called the class II-associated invariant chain peptide (CLIP) that remains bound to the peptide binding groove of the class II complex in a manner similar to antigenic peptides (Ghosh et al., 1995). Using X-ray crystallography, Ghosh and colleagues showed that the binding of CLIP to HLA-DR3 was very similar to that in which antigenic peptides bind but later studies have found that some class II molecules (i.e HLA-DR4) interact to a lesser extent with CLIP and are associated with autoimmunity (Ghosh et al., 1995; Patil et al., 2001).

Chaperone proteins HLA-DM and HLA-DO are residents of late endosomes that form complexes with the class II/CLIP and induce conformational changes which result in the release of CLIP to vacate the binding groove for loading of antigenic peptide (Kropshofer et

al., 1998; Morris et al., 1994). HLA-DO stabilises and preserves the chaperone activity of HLA-DM at low pH which catalyses the removal of CLIP and facilitates loading of high-affinity antigenic peptide onto the class II molecule (Denzin and Cresswell, 1995; Douek and Altmann, 1997; Kropshofer et al., 1995). Even though HLA-DM can carry out its function alone, HLA-DO-DM complexes are superior suggesting that HLA-DO acts as a co-chaperone protein to HLA-DM (Kropshofer et al., 1998). These processes have been summarised in Figure 1.1.6

1.6.3 Antigen processing and loading in the endocytic pathway

Following internalisation, antigens enter low pH microenvironments (i.e early endosome, lysosome) that favour protein denaturation (Blum et al., 2013). The main proteases found in these organelles are cysteine proteases (cathepsins S, L, B, C and H) and aspartyl proteases (cathepsins D and E) (Bühling et al., 2004; Costantino et al., 2008). In addition, γ -interferon induced lysosomal thiol reductase (GILT) is recognised as an additional lysosomal processing enzyme. Even though it lacks proteolytic activity, it catalyses the processing of proteins with internal disulphide bonds for presentation to antigen-specific CD4⁺ T cells (Maric et al., 2001). HLA-DO-DM complexes have the ability to initiate the dissociation of low-affinity peptide (such as CLIP) and this provides the opportunity for high-affinity peptides to associate on the peptide binding groove (Sloan et al., 1995; Weber et al., 1996). These processes have been summarised in Figure 1.1.6.

1.6.4 Trafficking of peptide/MHC class II complex

Following complete degradation of the li chain, the MHC-li complex dissociates into $\alpha\beta$ heterodimers and depending on whether they have been successfully loaded with a high affinity peptide, they are sorted into lysosomes for degradation or transported to the cell surface (Théry et al., 1998; Wubbolts et al., 1996). MHC class II molecules on immature DCs are rapidly internalised and recycled, with a half-life of 10 hours. Following DC maturation, inflammatory stimuli induce an increase in MHC class II synthesis while their half-life increases to over 100 hours (Cella et al., 1997). The major mechanism by which their

downregulation is controlled is through the ubiquitin E3 ligase membrane-associated RING-CH I (MARCH I) that acts on the β chain of the class II molecule (De Gassart et al., 2008). The expression of MARCH I increases with DC maturation as well as IL-10 activity, leading to downregulation of the expression of MHC class II (Shin et al., 2006; Thibodeau et al., 2008).

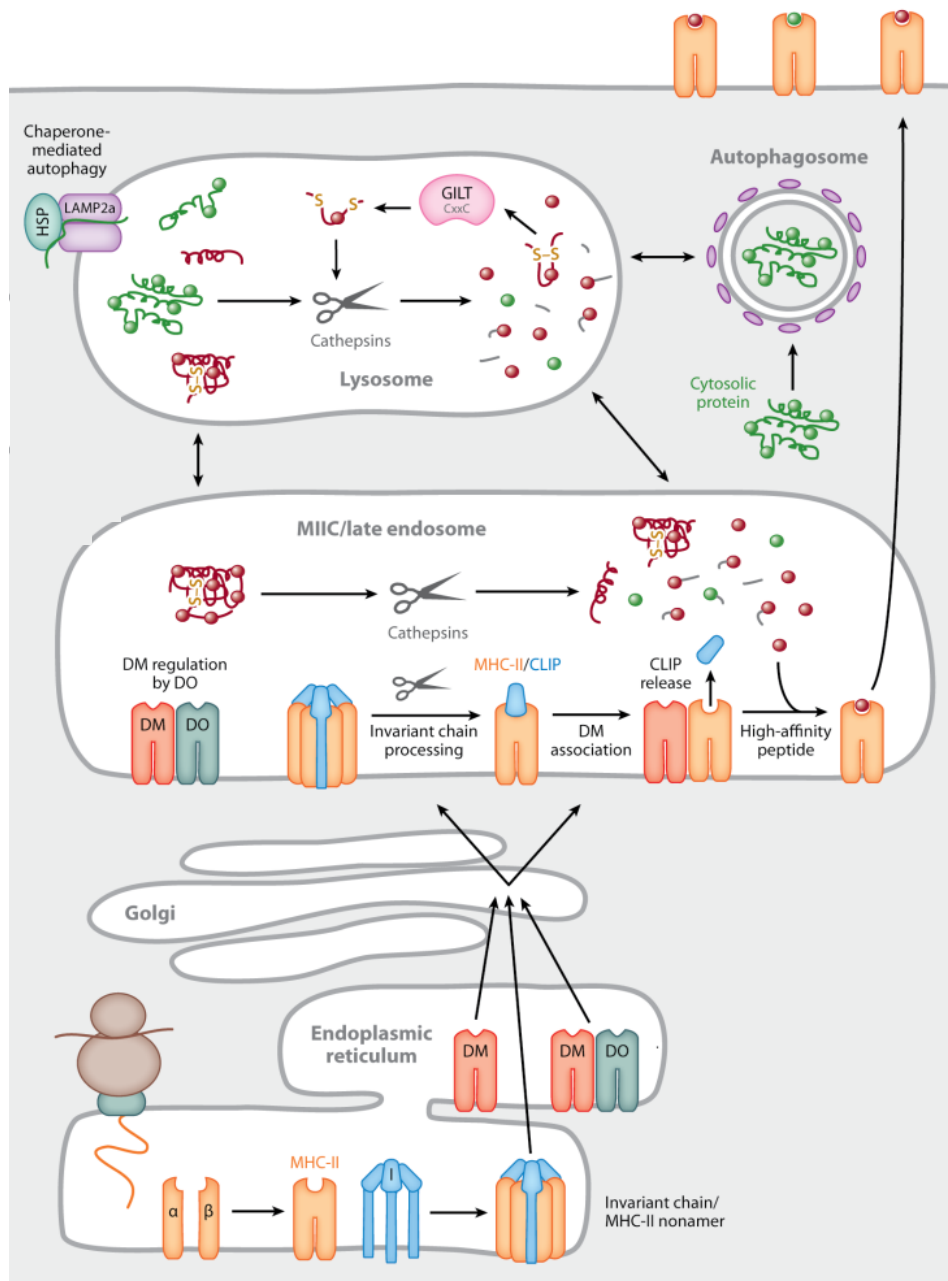


Figure 1.1.6 Summary diagram illustrating the endocytic class II presentation pathway. The MHC class II α and β chains associate and form a stable complex with the invariant chain. These then transit to endosomes where proteolysis of the invariant chain yields the residual CLIP fragment on the peptide binding groove. HLA-DM and DO facilitate the dissociation of CLIP that allow binding of high-affinity peptides. The loaded MHC class II/peptide complexes are then transported to the cell surface for presentation to CD4+ T cells (Blum et al., 2013).

1.7 Human challenge studies

1.7.1 Background

Deliberate challenge of humans goes back to 1796, when Edward Jenner deliberately exposed James Phipps to smallpox and demonstrated that previous cowpox variolation protected from disease. This was an important breakthrough and critical for the subsequent development of vaccination. More recently, a range of studies using various bacterial or viral pathogens have been carried out to enhance understanding of pathogenesis or evaluation of vaccine candidates (Levine et al., 1977, 1988; McMichael et al., 1981; Tacket et al., 1999; Wilkinson et al., 2012). The majority of these have been designed as proof of concept studies, to assess potential vaccine candidates and therapeutic interventions at an early stage. Experimental challenge models allow greater standardisation through inoculation with a desired dose of pathogen to subjects that have well-defined characteristics, including age and comorbidity. More recent advances in technology and logistics have meant that not only blood but also nasal and lower airway samples can be collected intensively at different stages of disease, making it possible to study symptoms, dynamics of transmission and kinetics of immune responses in detail (Pollard et al., 2012). However, despite having these advantages over natural infection studies, extrapolating into high risk populations can be difficult. With ethical considerations in mind, eligible subjects must be healthy and expected to undergo mild-to-moderate symptoms only.

1.7.2 RSV challenge studies

The first experimental challenge of humans with RSV was conducted by Kravetz and colleagues in 1961, where 20 healthy adults were infected with the RSV long strain (Kravetz et al., 1961). Since then, there have been several other studies using wild-type or attenuated strains (Lee et al., 2004; Pringle et al., 1993; Watt et al., 1990; Wright et al., 1971). These studies mainly addressed the different routes of infection, optimal dose, and the duration of antibody responses. In 2006, an RSV A strain (Memphis 37) was isolated from a child with bronchiolitis and produced to good manufacturing practice (GMP) standards. The first

published study of human experimental challenge with this virus was by Zaas and colleagues in 2009 who proposed nasal IL-6, IL-8 and MIP1 α as biomarkers of clinical disease with elevated levels of these pro-inflammatory mediators positively correlating with cumulative viral load (Zaas et al., 2009). Furthermore, DeVincenzo and colleagues implied that disease is driven by viral load, rather than a delayed or aberrant host immune response as seen in animal models of RSV infection (DeVincenzo et al., 2010). However, these RSV challenge studies were not designed to investigate in detail the host immune response during the course of the infection. In summer 2011, Openshaw and colleagues commenced a programme of RSV challenge studies in humans using the RSV M37 strain the main objective of which was to investigate the antiviral adaptive immune response in detail. The advantages of using this model, especially the longitudinal sampling through the course of the infection will help understanding the kinetics, phenotype and functionality of CD4⁺ T cells in detail.

1.8 Hypothesis

The RSV-specific CD4⁺ T cell response is essential for the generation of high affinity antibodies and persistent adaptive immune memory. RSV-specific B cell responses correlate with the induction of RSV-specific Tfh-like cells, detectable in the peripheral blood. In healthy adults, effective control of RSV disease relies on an anti-viral Th1 response and the induction of Th2 or Th17 cytokine-producing cells does not occur in mild upper respiratory tract disease.

1.9 Aims of the project

This study aims to characterise the kinetics, phenotype, specificity and functionality of CD4⁺ T cell responses in healthy adults experimentally challenged with RSV. Kinetics and functionality of CD4⁺ T cells during RSV infection will be studied using longitudinal sampling. In order to further investigate CD4⁺ T cells at an antigen- and epitope-specific level, we aim to determine the breadth and specificities of the CD4⁺ T cell response to RSV. Having identified immunodominant public epitopes in RSV, we will use class II tetramers to investigate the kinetics, memory, homing and costimulatory profile of RSV-specific CD4⁺ T cells in order to further understand their role in the generation of protective immunity.

2. Materials and Methods

2.1 Reagents

2.1.1 Commercial reagents

Reagent	Company
BD Cytofix™ BD Compbeads	BD Bioscience (Oxford, UK)
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid L-glutamine (200 mM) Roswell Park Memorial Institute (RPMI) medium Live-Dead fixable blue	Gibco (Paisley, UK)
Phosphate buffered saline (PBS) Dulbecco's Modified Eagle Medium (DMEM) Penicillin-streptomycin (P/S) (100X)	PAA (New Jersey, USA)
Bovine serum albumin (BSA) Ethylenediaminetetraacetic acid (EDTA) 0.4% Trypan Blue solution Tween20	Sigma-Aldrich (Dorset, UK)
Fetal calf serum (FCS)	BioScience (Paisley, UK)
Anti-human IFN- γ coating antibody Anti-IFN γ -biotin detection antibody	Mabtech (Mariemont, USA)
96 well Elispot plates	Millipore (Stockholm, Sweden)

2.1.2 Laboratory prepared reagents

	Composition	Concentration
ACK Buffer	Distilled water KHCO ₃ Na ₂ EDTA NH ₄ Cl	0.15mM 1mM 0.1mM
Complete RPMI	RPMI Heat-inactivated FCS L-glutamine Pencilin-Streptomycin	1x 10% 2mM 100U/ml
Serum-free DMEM	DMEM L-glutamine Pencilin-Streptomycin	1x 2mM 100U/ml
Staining buffer	FCS PBS	2% 1x
Washing buffer	PBS Tween20	1x 0.05%

2.1.3 Equipment & Software

Name	Supplier
BD Fortessa cell analyser	BD Bioscience, Oxford, UK
Automated ELISpot plate reader	ABcam, Cambridge, UK
FACSDiva	BD Bioscience, Oxford, UK
GraphPad Prism v6.0	GraphPad Software, CA, USA
Flowjo v9.5	Tree Star, Oregon, USA
Nanodrop 1000 Spectrophotometer	Thermo Scientific, DE, USA
7500 Fast Real-Time PCR system	Applied Biosystems, Paisley, UK
Gene Amp PCR System 9700	Applied Biosystems, Paisley, UK
Heraeus Megafuge 40R	Thermo Scientific, DE, USA
Sonicator	Scientific laboratory supplies, Nottingham, UK

2.1.4 Antibodies

Specificity	Fluorochrome	Clone	Vendor
Bcl-6	PerCPCy5.5	7D1	eBiosciences, Hatfield, UK
CCR5	BV421	2D7	BD Pharmingen Oxford, UK
CCR6	PE	R6H1	eBiosciences, Hatfield, UK
CCR7	BB515	3D12	BD Pharmingen Oxford, UK
CD107a	V450	H4A3	BD Pharmingen Oxford, UK
CD14	PE	61D3	eBiosciences, Hatfield, UK
CD27	APC-R700	M-T271	BD Pharmingen Oxford, UK
CD28	BV650	CD28.2	BD Pharmingen Oxford, UK
CD3	eFluor 450	OKT3	eBiosciences, Hatfield, UK
CD3	PE-CF594	UCHT1	BD Pharmingen Oxford, UK
CD38	BV510	HIT2	BD Pharmingen Oxford, UK
CD4	APCeFluor780	OKT4	eBiosciences, Hatfield, UK
CD45RA	PerCPCy5.5	HI100	Biolegend, Cambridge, UK
CD62L	PE	DREG-56	BD Pharmingen Oxford, UK
CD8	PerCPCy5.5	RPA-T8	eBiosciences, Hatfield, UK
CD8	V450	OKT8 (OKT-8)	eBiosciences, Hatfield, UK
CXCR3	PerCP-Cy5.5	G025H7	Biolegend, Cambridge, UK
CXCR5	APC	MU5UBEE	eBiosciences, Hatfield, UK
FITC	ICOS	ISA-3	eBiosciences, Hatfield, UK
IFN- γ	BV650	4S.B3	Biolegend, Cambridge, UK
IFN- γ	APC	B27	BD Pharmingen Oxford, UK
IL-17A	V450	N49-653	BD Pharmingen Oxford, UK
IL-17A	APC	eBio64DEC17	eBiosciences, Hatfield, UK
IL-17F	AFluor488	M5E2	Biolegend, Cambridge, UK
IL-17F	PECy7	SHLR17	eBiosciences, Hatfield, UK
IL-2	FITC	5344.111	BD Pharmingen Oxford, UK
IL-2	BV 510	MQ1-17H12	Biolegend, Cambridge, UK
IL-21	PE	eBio3A3-N2	eBiosciences, Hatfield, UK
IL-21R	PE	17A12	BD Pharmingen Oxford, UK
IL-4	FITC	MP4-25D2	Biolegend, Cambridge, UK
IL-4	PE-Cy7	8D4-8	eBiosciences, Hatfield, UK
IL-5	PE	HP6017	Biolegend, Cambridge, UK
IL-5	APC	TRFK5	Biolegend, Cambridge, UK
PD-1	PECy7	eBioJ105	eBiosciences, Hatfield, UK
TNF- α	PEcy7	M5E2	BD Pharmingen Oxford, UK

2.2 Study design of experimental RSV infection

2.2.1 Screening

As part of the recruitment process, volunteers attended a screening visit where they were seen by the study doctor (Dr Christopher Chiu or Dr Maximilian Habibi) for a brief interview to determine their suitability for enrolment. Questions were asked about their current and previous health, lifestyle, and social and domestic circumstances. A physical examination and bedside observations (temperature, pulse, blood pressure, and oxygen saturation) were performed and blood was obtained full blood count, electrolytes, renal function, liver function, coagulation, C-reactive protein, immunoglobulin subsets, lymphocyte subsets, and blood borne virus serology (HIV, hepatitis B, hepatitis C) measurements. A urine test for drugs of abuse was performed for all subjects and women of childbearing potential underwent a pregnancy test.

2.2.2 Study eligibility

2.2.2.1 Inclusion criteria

Healthy persons aged 18 to 55 years who were able to give informed consent were deemed eligible for the study.

2.2.2.2 Exclusion criteria

Any significant medical or psychological condition or medication deemed by the study doctor to make the participant unsuitable resulted in exclusion.

Specifically, volunteers with any of the following conditions were excluded:

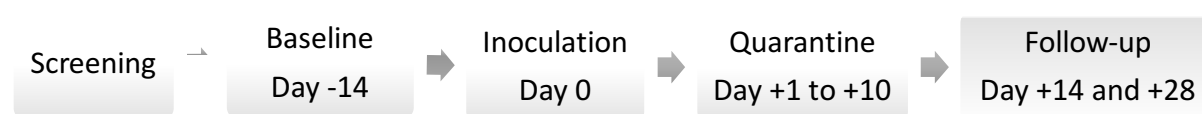
- Chronic respiratory disease (asthma, COPD, rhinitis, sinusitis) in adulthood
- Inhaled bronchodilator or steroid use within the last 12 months

- Use of any medication or other product (prescription or over-the-counter) for symptoms of rhinitis or nasal congestion within the last 6 months
- Acute upper respiratory infection (URTI or sinusitis) in the past 6 weeks
- Smoking in the past 6 months OR > 5 pack-year lifetime history
- Subjects with allergic symptoms present at baseline
- Those in close domestic contact (i.e. sharing a household with, caring for, or daily face to face contact) with children under 3 years, the elderly (> 65 years), immunosuppressed persons, or those with chronic respiratory disease
- Subjects with known or suspected immune deficiency
- Receipt of systemic glucocorticoids (in a dose $\geq$ 5 mg prednisone daily or equivalent) within one month, or any other cytotoxic or immunosuppressive drug within 6 months prior to challenge
- Known IgA deficiency, immotile cilia syndrome, or Kartagener's syndrome
- History of frequent nose bleeds
- Pregnant or breastfeeding women
- Positive urine drug screen

2.2.3 Study timetable and sampling

Eligible subjects were invited for a baseline visit fourteen days (day -14) prior to viral challenge in order to obtain samples at healthy state (Table 2.2.1). They were then inoculated (day 0) with 10^4 plaque-forming units (PFU) of RSV M37 in 1mL of DMEM/10% sucrose by intranasal drops and were quarantined for eleven nights (day 0 to +10) at facilities provided by the study. Ten days post inoculation subjects were allowed to return home, as by this point no further live virus could be detected in any individual. They returned for follow up visits at days +14 and +28 for sampling in the convalescence period. At all time-points, subjects were asked to fill in a self-reported symptom diary (Supplementary Figure S1) cards based on the Jackson score, a symptom scoring system developed to differentiate adults with viral upper respiratory tract infections from other conditions (Jackson et al., 1958). This was modified by addition of lower respiratory tract and systemic symptoms due to the longer incubation period and protracted course of RSV infection. Upper respiratory symptoms were

assessed by: sneezing, sore throat, nasal discharge, nasal obstruction, hoarseness and watery eyes. Lower respiratory tract symptoms were assessed by: cough, difficulty breathing, chest discomfort and wheezy chest. Systemic symptoms were assessed by: headache, fatigue, fever, diarrhoea, feeling/being sick and muscle aches. These were graded 0 – 3 where 0 = absent; 1 = mild – does not affect normal daily activities; 2 = moderate – limitation of normal daily activities; 3 = severe – prevents some normal daily activities. Subjects were asked to fill in their symptom diaries before any sampling (i.e. bronchoscopy) to avoid recording of symptoms due to other factors. In addition, subjects were sampled daily from the upper airway with nasal washes. They were less frequently sampled for serum and peripheral blood for isolation of PBMCs. In the 2011 and 2012 cohorts, lower airway samples were collected at baseline at days 7 and 28. However, the preliminary results from this showed that peak of T cell responses was at a later stage, therefore the bronchoalveolar lavage (BAL) sampling time-point was rescheduled from day 7 to day 10 for the later cohorts.



Day	-14	0	1	2	3	4	5	6	7	8	9	10	14	28
Inoculation		X												
Symptoms diary	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Nasal washes	X	X	X	X	X	X	X	X	X	X	X	X	X	X
PBMCs	X	X			X				X			X	X	X
BAL	X											X		X
Serum	X				X				X			X		X

Table 2.2.1 A summary of the study scheduling (top) and sampling strategy (bottom).

Subjects were sampled by clinicians and research nurses of the study.

2.3 Laboratory techniques and assays

2.3.1 Plaque Assay

Vero cells (2×10^4 /well) were seeded into a flat-bottomed 96 well plate in DMEM + 10% HI FBS and incubated overnight at 37 °C. The following day, each well was washed with serum free media before 50 µl of virus stock was added. Virus stock was added in triplicate at the dilutions indicated started at 1:10 and incubated at 37°C for 2 hours. Each well was then supplemented with 150 µl DMEM + 10% HI-FCS and incubated overnight at 37°C. The media in each well was then discarded and cells were washed with PBS before being fixed in methanol with 2% H₂O₂ for 20 minutes. Cells were then washed twice with 200 µl PBS containing 1% BSA (PBS/BSA) and incubated overnight in 200 µl staining buffer. Each well was then incubated with 100 µl anti-RSV antibody diluted 1:500 in PBS/BSA and subsequently washed twice with 200 µl PBS/BSA. 100 µl extravidin peroxidase diluted 1:500 in PBS/BSA was added and cells were incubated for 30 minutes at room temperature. Cells were washed twice in PBS/BSA before incubation in 50 µl DAB for 20 minutes at room temperature in the dark. The reaction was stopped by washing the cells in PBS and plaques were counted. Plaque forming units were calculated as following number of plaques x dilution x 20.

2.3.2 Production of RSV stock

1×10^7 Vero cells were seeded in 25 ml DMEM complete media in a 175 cm² flask and incubated overnight at 37°C until approximately 70% confluency. DMEM was removed from the monolayer of cells which were then washed with 10 ml serum free DMEM. The monolayer cells were then infected with RSV virus at a concentration of 0.1 PFY/ml and incubated for 2 hours at 37°C. Following this, the flasks were supplemented with 26 ml of complete DMEM and further incubated for 24 hours. FCS in the media was reduced to 2% and cells were incubated for a further 24 hours at 37°C. When approximately 50% of cells had detached, cells were harvested and a rubber policeman was used to detach the remained adhered cells. Cells were then sonicated in a water bath sonicator for 20 seconds

before centrifugation at 1500 rpm for 5 minutes. The supernatant containing RSV virus was then aliquoted and snap-frozen in liquid nitrogen for further application.

2.3.3 Determination of viral load in nasal wash

2.3.3.1 Viral RNA extraction

Total RNA in 250 µl of thawed nasal wash samples were extracted using the QIAamp Viral Mini kit (Qiagen). For each sample, 10 µl of carrier RNA and 1 ml of lysis buffer AVL were added in 15 ml canonical tubes. The mixture was pulse vortexed for 15 seconds and incubated for 10 minutes at room temperature. 1 ml of 96% ethanol was added to the tubes and pulse vortexed for 15 seconds. Next, samples were treated with kit supplied buffers as per the manufacturer's instructions. To prevent DNA contamination, samples were treated with DNase digest using the Qiagen DNase kit. Extracted RNA was eluted in 60 µl of nuclease free water and stored in a cryotube at -80°C.

2.3.3.2 cDNA synthesis

Extracted RNA was converted to cDNA using the Omniscript RT kit (Qiagen). Reverse transcription was primed using random hexamer primers (Qiagen). A master mix consisting of dNTP, primers, RNA inhibitor and omniscript reverse transcriptase was added to the extracted RNA sample. Reaction conditions were 37°C for 60 minutes for reverse transcription followed by 95°C for 5 minutes to inactivate reverse transcriptase on the GeneAmp 9600 PCR cycler.

2.3.3.3 Real-time PCR (RT-PCR)

For quantification of RSV N gene copy number, a plasmid DNA standard was made by Maximilian Habibi from a mock infected nasal lavage sample (unpublished data). Briefly, an N gene PCR product was made by real-time PCR and purified using the instructions of the QIAquick PCR Purification Kit (Qiagen). The purified product was then ligated into a plasmid

vector and transformed into *E. coli* using the TOPO TA Cloning Kit (Invitrogen) kit according to manufacturer's instructions. *E. coli* contained an ampicillin resistant gene, which allowed the selection of single colonies containing the N gene PCR product using LB agar plates with ampicillin. A plasmid stock from a single colony was produced using QIAfilter Plasmid Maxi Kit (Qiagen) according to manufacturer's instructions. The presence of N gene product was confirmed by real-time PCR and the prep was stored at a concentration of 10^{10} copies per μ l at -80°C .

Quantification of RSV N gene was performed using real-time TaqMan PCR using the primers and probes described in (Perkins et al., 2005). A master mix containing 6.25 μ l of TaqMan Universal Master Mix II, 1.125 μ l each of forward and reverse primer, 0.25 μ l of probe and 1.25 μ l of nuclease-free water was mixed with 2.5 μ l of cDNA. Cycling conditions were as follows: 50°C for 2 minutes (Uracil DNA-glycosylase incubation to eliminate carryover PCR products), 95°C for 10 minutes (polymerase activation) followed by 40 cycles of 95°C for 15 seconds (denaturing) and 60°C for 1 minute (annealing and extension). A linear fit on the standard curve allowed estimation of the copy number therefore RSV viral load.

2.3.4 BAL and whole blood sample processing

BAL sample collection from study participants was obtained using 120 ml of saline delivered via a fiberoptic bronchoscope. The cells were filtered through a 70 μ M strainer to obtain a single cells suspension. Following a wash step, cells were resuspended in ACK buffer to lyse red blood cells and incubated for 2 minutes. The cells were then centrifuged at 1500 rpm for 5 minutes and resuspended in staining buffer before being counted using a haemocytometer. Trypan blue was used to assess cell viability.

Whole blood samples was obtained from study participants and a 300 μ l aliquot was lysed by incubation with 2 mls of 1X FACS/Lyse Buffer for 10 minutes at room temperature. 250ul of PBS was added to wash the cells which were the centrifuged at 1500 rpm for 5 minutes before resuspension in staining buffer for further applications. The remaining whole blood

sample was used for PBMC isolation, which was performed using density gradient separation.

In order to isolate PBMCs, 10 ml of blood was diluted with 10 ml sterile PBS before layering onto 15ml of Ficoll. The sample was then centrifuged for 30 minutes at a speed of 400 x g at 20°C in a swinging bucket rotor without acceleration or brake. Cells at the interface, which includes lymphocytes, monocytes and thrombocytes, were collected using a sterile pipette and transferred to a 50 ml conical tube and washed with 10 ml staining buffer. Cells were then resuspended in an appropriate volume of staining buffer before being counted using a haemocytometer. The isolated PBMCs were frozen for future use by resuspension in FSC containing 20% DMSO, followed by storage at -80°C for a minimum of 24 hours and then transferred to liquid nitrogen for long term storage.

2.3.5 Processing of tonsils

Tonsils were obtained from children undergoing routine tonsillectomy and processed as previously described (Johnston et al., 2009a). Tonsils were cut into 10mm tissue fragments before being placed into a stainless steel sieve within a large petri dish containing 2 ml of isotonic saline. A single cell suspension was acquired by forcing the tonsil tissue through the sieve using a 5 ml syringe plunger three times with isotonic saline. The cell solution was then filtered through a 70 µm cell strainer to ensure a single cell suspension was obtained. Following this, the lymphocyte population was isolated using density gradient separation and resuspended in an appropriate volume of complete R10 media for further applications, or resuspended in DMSO and stored in liquid nitrogen for future use.

2.3.6 Thawing of cryopreserved cells

Samples were removed from liquid nitrogen and placed onto dry ice until thawing in a water bath at 37°C. The contents of the vial were transferred to a 15 ml conical tube and 10 ml warm complete RPMI media was added drop by drop. The cells were then washed twice with warm complete RPMI media and cell viability was determined using Trypan blue dye

exclusion. The cells were then resuspended at a desired concentration for use. All washing steps were done by centrifugation of the samples at 1500rpm for 5 minutes.

2.3.7 Antibody staining for flow cytometry analysis

2.3.7.1 Surface staining

Following BAL or PBMC isolation, cells were washed with 500 µl of staining buffer and cell pellets were stained with antibodies directed against surface markers of interest for 15 minutes in the dark at 4°C. 500 µl of staining buffer was added to wash away any unbound antibody, centrifuges at 1500 rpm for 5 minutes and the supernatant was then discarded. Cells were then resuspended in fresh staining buffer for flow cytometric analysis.

2.3.7.2 Intracellular cytokine staining

For analysis of intracellular cytokines, cells isolated from BAL or PBMC samples were resuspended in 100 µl of complete R10 media. Stimulation with PMA (25 ng/ml) and ionomycin (1000 ng/ml) or SEB (3 µg/ml) at 37°C for 6 hours was utilised as a positive control for these experiments. For stimulations with RSV, virus was added to cells at various MOI and incubated for 24 hours. Brefeldin A (1 µg/ml) was added 4 hours prior to the end of the 24 hours incubation, after which cells were washed with staining buffer and stained with antibodies against surface markers of interest (as described above). Cells were then washed with staining buffer and incubated with 150 µl of Fix/Perm Buffer for 30 minutes and then washed with 200 µl Perm/Wash buffer. Monoclonal antibodies against cytokines of interest were added and cells were incubated in the dark for approximately 45 minutes. The stained samples were then washed twice for Perm/Wash buffer before being acquired on a flow cytometer (Fortessa, BD Biosciences). All washing steps were done by centrifugation of the samples at 1500rpm for 5 minutes.

2.3.7.3 MHC-class II tetramer staining

MHC-class II tetramers were synthesised and gifted by the NIH tetramer core facility (Atlanta, GA, USA). Prior to tetramer staining, cells were treated with protein kinase inhibitor, dasatinib (Axon Medchem, Reston, VA, USA) for 30 minutes at 37°C at a final concentration of 50nM. Cells were washed and stained with class II tetramer of interest and its relevant negative control, a tetramer specific against the CLIP peptide. Each class II tetramer had specific optimum staining conditions in terms of concentration, temperature and incubation period (Table 2.3.1). Following class II tetramer staining, cells were stained for surface and intracellular markers as described in previous sections.

Specificity	Class-II allele	Concentration	Temperature	Time
HA-PRY	DRB1*01:01	2µg/ml	4°C	20 minutes
F-EFY	DRB1*04:01	2µg/ml	4°C	30 minutes
F-EFY	DRB1*11:01	2µg/ml	4°C	30 minutes
G-DDF	DPB1*04:01	2µg/ml	37°C	30 minutes

Table 2.3.1 The optimum staining conditions with different class II tetramer reagents.

2.3.8 CFSE labelling of PBMCs

To assess proliferation of T cells in response to RSV peptides of interest, frozen PBMC samples were thawed and washed as described previously. Cells were resuspended at a concentration of 10^7 cells/ml in serum-free media and transferred to a 15 ml canonical tube. 0.5 µl of 5mM stock of CFSE in 110 µl of PBS was added to the cells (final CFSE concentration 2.5 µM) which were then vortexed quickly for uniform mixing of the solutions. The cells were incubated for 7 minutes at 37°C and washed 3 times with complete RPMI medium. Cells were then counted and resuspended at a desired concentration for T cell culture assays. All washing steps were done by centrifugation of the samples at 1500rpm for 5 minutes.

2.3.9 Pooling of RSV peptides

Peptides corresponding to the RSV genome were generated by designing 15 amino acid sequences, each overlapping by 10 amino acids. The peptides were synthesised by Mimotopes, Minneapolis, MN and were supplied as lyophilised reagents with an average mass of 1.7 mg. Stocks of each peptide was generated by solubilising in an appropriate volume of DMSO to obtain a stock solution of 50 mg/ml. If peptides were not fully dissolved in DMSO, they were placed on a heat block for a few seconds to aid solubilisation. Peptides were then pooled in order to identify candidate epitopes that were able to stimulate T cell responses (Table 2.3.2). The final concentration of each peptide in the pool was 1.470 µg/µl. Aliquots of the pooled peptides was made prior to storage in DMSO at -20°C, as freeze/thawing of peptides was not recommended by the manufacturers.

	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16	V17	V18	V19	V20	V21	V22	V23	V24	V25	V26	V27	V28	V29	V30
H1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
H2	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
H3	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
H4	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
H5	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150
H6	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
H7	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210
H8	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240
H9	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270
H10	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300
H11	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330
H12	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360
H13	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390
H14	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420
H15	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450
H16	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480
H17	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510
H18	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533	534	535	536	537	538	539	540
H19	541	542	543	544	545	546	547	548	549	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570
H20	571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	591	592	593	594	595	596	597	598	599	600
H21	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629	630
H22	631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	650	651	652	653	654	655	656	657	658	659	660
H23	661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684	685	686	687	688	689	690
H24	691	692	693	694	695	696	697	698	699	700	701	702	703	704	705	706	707	708	709	710	711	712	713	714	715	716	717	718	719	720
H25	721	722	723	724	725	726	727	728	729	730	731	732	733	734	735	736	737	738	739	740	741	742	743	744	745	746	747	748	749	750
H26	751	752	753	754	755	756	757	758	759	760	761	762	763	764	765	766	767	768	769	770	771	772	773	774	775	776	777	778	779	780
H27	781	782	783	784	785	786	787	788	789	790	791	792	793	794	795	796	797	798	799	800	801	802	803	804	805	806	807	808	809	810
H28	811	812	813	814	815	816	817	818	819	820	821	822	823	824	825	826	827	828	829	830	831	832	833	834	835	836	837	838	839	840
H29	841	842	843	844	845	846	847	848	849	850	851	852	853	854	855	856	857	858	859	860	861	862	863	864	865	866	867	868	869	870
H30	871	872	873	874	875	876	877	878	879	880	881	882	883	884	885	886	887	888	889	890	891									

Table 2.3.2 The matrix illustrates the peptide pooling strategy. Final concentration of each individual peptide was 1.470 µg/µl. DMSO was added for pools H30, V22-V30 to have an equal concentration of peptides in all pools.

2.3.10 Enzyme linked immunosorbent spot (ELISpot)

30 µl of 70% methanol was added to each well of 96-well PVDF-backed Elispot plates to pre-wet the wells, and were subsequently washed three times with 150 µl of sterile distilled water per well. 50 µl of anti-human IFN-γ capture antibody (1 µg/ml) was added to each well and the plates were incubated at 4 °C overnight. The wells were then washed three times with 125 µl PBS and subsequently blocked with 125 µl of complete R10 media for 1 hour at 37°C. Following this, 2×10^5 PBMCs in 50 µl of media were added to each well along with 50 µl of peptides (5 µg/ml) and then incubated for a further 24 hours at 37°C. Wells were then washed four times with 200 µl of PBS containing 0.05% Tween-20, followed by 2 washes with 200 µl of PBS. 50 µl of anti-IFN-γ-biotin detection antibody diluted in PBS with 0.05% BSA (1 µg/ml) was then added to each well and the plate was incubated for 2 hours at room temperature. Plates were then washed four times with PBS/Tween and twice with PBS before being developed with BCIP/NBTPLUS for 15 minutes following the manufacturer's instructions. Each well was then washed with tap water to stop the reaction and plates were then allowed to dry before spots were counted.

2.3.11 Magnetic activated cell sorting (MACS)

Freshly isolated or thawed PBMCs were obtained in a single cell suspension and washed using MACS buffer. The cell pellet (containing up to 10^7 cells) was resuspended in 80 µl of MACS buffer and 20 µl of CD8 MicroBeads (Miltenyi Biotec, UK). Cells were incubated for 15 minutes at 4°C for labelling. At the end of the incubation, cells were washed with 1 ml MACS buffer and resuspended in 500 µl of MACS buffer. The cell suspension was then applied onto LD columns (Miltenyi Biotec, UK) for depletion of the CD8+ T cells using magnetic separation. A small aliquot of the eluted sample was stained with anti-CD3, CD4 and CD8 antibodies for purity check using flow cytometry. Purity of CD8+ cell depletion was at least 95% for all samples. All washing steps were done by centrifugation of the samples at 1500rpm for 5 minutes.

2.3.12 CD4+ T cell culture assay

Thawed PBMCs (CFSE labelled for proliferation assay) were resuspended at a concentration of 1×10^6 /ml and 1 ml was transferred to a 24-well plate. The peptide of interest was added to complete RPMI medium at a concentration of 20 μ g/ml and 1 ml of this was transferred to the 24-well plate containing PBMCs (final peptide concentration 10 μ g/ml). As the peptides were dissolved in DMSO, DMSO containing complete RPMI medium was used as a negative control. The culture was supplemented with human recombinant IL-7 (R&D Systems, Minneapolis, MN, USA) at a final concentration of 25 ng/ml at days 0, 7 and 10 and with human recombinant IL-2 (Thermo Fisher, Waltham, MA, USA) at a final concentration of 50 IU/ml at days 3, 7 and 10. Cell cultures were incubated at 37°C for 7 days for proliferation assay and 14 days for T cell expansion. At the end of the culture period, cells were stained for flow cytometry analysis as described previously. All washing steps were done by centrifugation of the samples at 1500rpm for 5 minutes.

2.3.13 *In vitro* peptide binding affinity

Measurement of *in vitro* peptide binding affinity was done in collaboration with John Sidney and Alessandro Sette (La Jolla institute, California, USA). Briefly, each peptide of interest was incubated at a range of doses with an MHC mix (purified MHC, protease inhibitors and radiolabelled peptides) for 2 – 4 days at 37°C. MHC capture using monoclonal antibodies was used to calculate the percent inhibition through determination of the amount of bound labelled peptide. This was then converted to IC₅₀ values which represent the concentration of an inhibitor where the binding is reduced by half, therefore a lower IC₅₀ indicates higher binding affinity.

2.3.14 HLA typing

HLA typing of the class II genes (DR, DP and DQ) was performed to intermediate resolution by Anthony Nolan typing services (London, United Kingdom). Different HLA typing methods were recently described in detail (Dunckley, 2012). Briefly, genomic DNA was extracted from

whole blood samples and amplified by PCR using allele specific forward and reverse primers. This was followed by sequencing reactions for individual genes using sequencing primers. The allele assignment can be determined by analysis of the sequence data using Sanger sequencing-based typing software (i.e. SBTengine).

2.3.15 Modular IMMune *In vitro* Construct (MIMIC)

Collagen I (Sigma-Aldrich, Dorset, UK) at a final concentration 1mg/ml was prepared on ice by the addition of NaOH (1:40), PBS (1:10) and antigen of interest (1:10). 100 µl of collagen/antigen mix was added to flat bottom 96 well plates and incubated at 37°C for 60 minutes for the collagen to set. The collagen layer was rinsed 3 times with 200 µl of PBS. Lyophilised fibronectin (Sigma-Aldrich, Dorset, UK) was dissolved in distilled water at a final concentration of 500 µg/ml and 50 µl of this fibronectin was added onto the collagen layer and incubated at room temperature for 30 minutes. The collagen layer was rinsed 3 times with 200 µl of PBS. Human Umbilical Vein Endothelial Cells (Lonza, Basel, Switzerland) were thawed and resuspended at a concentration of 1.5×10^5 cells/ml and 200 µl of this suspension was added onto the collagen layer and incubated at 37°C for 4 days.

At day 5, PBMCs were thawed and a fraction of cells were surface stained with anti-CD14 as pre-migration sample. 200 µl of 5×10^5 PBMCs was added onto the collagen layer and incubated at 37°C for 2 hours to allow the migration of monocytes. The non-migrating cells were collected from the collagen layer and rinsed twice with complete RPMI medium. A fraction of these cells were surface stained with anti-CD14 and a decrease in the frequency of CD14 expression compared to the matched pre-migration samples suggest that monocyte migration was successful. The non-migrating cells were collected and re-incubated in fresh complete RPMI medium for additional 3 days to allow transmigration and differentiation.

At day 7, the reverse migrated cells are collected. In order to collect the cells within the collagen layer, wells were treated with 2mg/ml of Collagenase D in PBS containing 1M EDTA for at least 60 minutes at 37°C. The digested collagen was collected and washed with PBS

containing 1M EDTA by centrifugation at 1500rpm for 5 minutes. Cells containing antigen-loaded APCs were used for culture with autologous PBMCs.

2.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism. Two-group comparisons were performed using two-tailed Student's t-test; Mann-Whitney test was used for unpaired and Wilcoxon matched-pairs signed rank test was used for paired groups. Comparison between more than two groups were by one-way ANOVA. For all tests, a value of $p < 0.05$ was considered significant. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, NS: not significant.

3. Results

3.1 Clinical responses of adults experimentally infected with RSV

3.1.1 Introduction

Experimental challenge of humans with RSV have been conducted since the early 1960s, which addressed the different routes of infection, optimal dose, and the duration of antibody responses (discussed in Section 1.7.2). However, these studies were not designed to investigate in detail the cellular immune responses during the course of the infection. Despite several decades of research into the immunology of RSV infection, there remains limited data on the role of CD4⁺ T cells in humans, which are currently limited to analyses of blood and upper airway samples obtained opportunistically from naturally infected patients (Breindahl et al., 2012; Legg et al., 2003; Stoppelenburg et al., 2014; Tabarani et al., 2013). We therefore conducted an experimental human RSV infection study to collect longitudinal samples and relevant clinical information necessary to investigate in detail the CD4⁺ T cell response to RSV infection in adults. The study was approved by the UK National Ethics Service London—Fulham (study numbers 10/H0711/94 and 11/LO/1826). Written informed consent was obtained from all volunteers.

3.1.2 Viral load peaks at 8 days post-infection

RNA extracted from daily nasal lavage samples was used for assessment of viral load in the upper respiratory tract by quantitative PCR for RSV N gene. Virus was detectable at day 1 post inoculation in most subjects, which was likely to have been due to the remains of inoculum in the nose. The mean onset of viral shedding was at day 4 ($1.13 \log_{10}$ copies per ml $\pm$ s.e.m. 0.204) and ranged from day 3 to day 5. The increase in viral load continued and peaked at day 8 with a mean of $2.76 \log_{10}$ copies per ml ($\pm$ s.e.m. 0.322; Figure 3.1.1a) before rapidly decreasing. In most individuals, no virus was detectable at day 14 and virus was undetectable in all subjects by day 28.

3.1.3 Experimental human RSV infection induces primarily upper respiratory symptoms

In summer 2012 and 2013, a total of 49 healthy adult subjects were challenged with RSV. Of these, 26 (53%) were deemed infected as RSV was detectable on at least two consecutive days between day 2 and day 10 post-inoculation by quantitative PCR. The other 23 subjects remained uninfected, despite being challenged with the same inoculum. Within the infected subjects, 17 (65%) developed cold-like symptoms which was defined by two out of the following three conditions having been fulfilled: nasal discharge lasting ≥ 3 days, subjective feeling of cold reported or cumulative 14-day symptom score ≥ 14 using a modified Jackson score (Jozwik et al., 2016). The remaining infected subjects were minimally or asymptomatic. Subject characteristics including age, gender, and ethnicity had no significant effect on infection rates or development of symptoms. These results are summarised in Table 3.1. 1

Subjects completed symptom diaries that evaluated upper and lower respiratory tract and systemic symptoms throughout the study. There was wide variation in the reported symptoms, we found a number of uninfected subjects reported symptoms in the early days of the quarantine where the highest total symptom score was 15 at day 3 (Figure 3.1.1b).

	Uninfected (PCR-)		Infected (PCR+)		P
Number of subjects	23	47%	26	53%	Ns
Median age (range)	20.5	18-50	23	18-39	Ns
Gender (M:F)		15:11		15:08	Ns
Ethnicity					Ns
White	17	74%	22	85%	
Black	4	17%	1	4%	
Chinese	0	0%	2	8%	
Mixed	2	9%	1	4%	

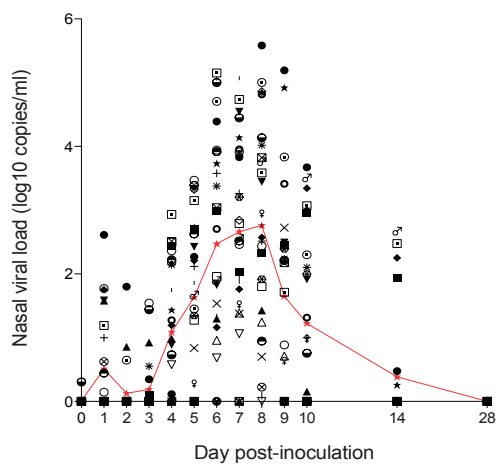
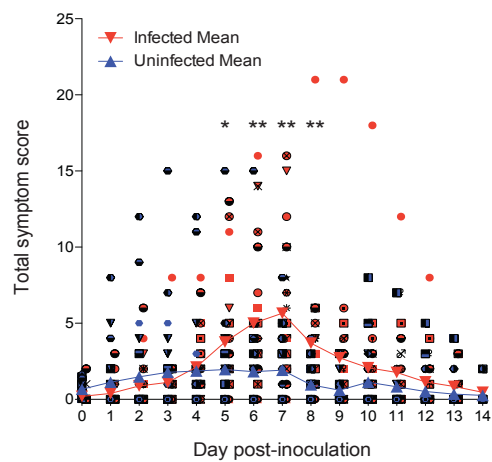
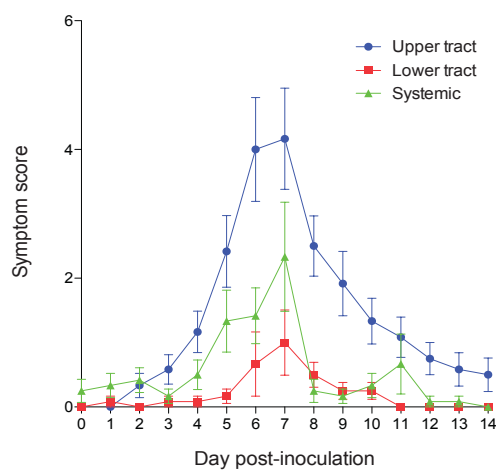
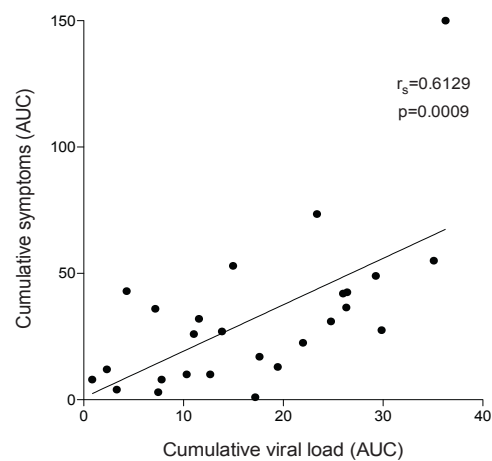
Table 3.1.1 Study subject's demographical characteristics and outcome of challenge.

Mann-Whitney- Wilcoxon test and p-values for differences in frequencies of the outcomes are shown (ns = $p > 0.05$)

Nevertheless, the cumulative scores were significantly different starting at day 5 between the infected and uninfected subjects ($p=0.0114$), reaching a peak at day 7 ($p=0.006$). One infected subject had a different pattern for onset of symptoms; a maximum symptom score of 20 peaked at day 8 remained higher than the rest of the infected subjects. Overall, the symptoms recorded were mild with a mean of 5.65 ($\pm$ s.e.m. 1.01) out of a maximum of 48 (Figure 3.1.1b). These were mainly reported as upper respiratory symptoms, and cases of lower respiratory or systemic symptoms were unusual (Figure 3.1.1c).

3.1.4 Symptoms correlate with viral load in the nasal lavage

In keeping with previous studies, symptoms started around the same time as initial viral detection, peaked around day 7 and fell as viral load started to decline. This translated to a significant positive correlation between cumulative total symptom score and cumulative viral load (as trapezoidal AUC for both parameters) in infected subjects ($r_s= 0.6129$, $p= 0.0009$; figure 3.1.1d).

a**b****c****d**

3.1.5 There is no association between infection rate, viral load and symptoms and HLA haplotype

Human HLA class II glycoproteins DP, DQ and DR are expressed by antigen presenting cells and essential in presenting peptide antigens for the initiation of CD4⁺ T cell responses (Schwartz, 1985). Typing of HLA class II alleles of the study subjects was conducted by Anthony Nolan (London, UK) by Luminex xMAP Sequence Specific Oligonucleotide Probe testing in order to identify any biases towards particular haplotypes in our cohort as well as for later investigation of the restriction of CD4⁺ T cell epitopes identified within the RSV proteome. The class II HLA types of the study participants are summarised in Table 3.1.2 The majority of the experimental data presented in this thesis was from the 2013 cohort and stored cells from previous cohorts were used in epitope mapping experiments discussed later. All subjects were typed for the HLA-DRB1 locus with HLA-DP and –DQ loci typed for a subset. Overall, the predominant HLA-DRB1 alleles in the study cohort were *15:01 (n=25, 18%), *07:01 (n=22, 16%), *03:01 and *04:01 (n=9, 4%; Figure 3.1.2a). In terms of the HLA-DPB1 locus, most prevalent alleles were *04:01 (n=20, 34%), *04:02 (n=11, 19%) and *02:01 (n=9, 16%; Figure 3.1.2b). These allelic frequencies are similar to those reported in the literature for the British Caucasoid population (Doherty et al., 1992; González-Galarza et al.,

Figure 3.1.1 Inoculation of healthy adults with RSV M37 causes an acute upper airway infection that correlates with symptoms. 49 subject were inoculated with an infection rate of 56%. (a) RSV N gene in nasal lavage was used to quantify the viral load in the upper respiratory tract. Individual data points and means are shown. (b) Subjects were asked to fill in a symptoms diary daily throughout the study. Means for infected (red) and uninfected (blue) individuals are shown. Infected individuals were defined by RSV detected from nasal lavage on at least 2 consecutive days after day +3 by qPCR of nasal lavage. (c) Reported symptoms were categorized as originating from the upper respiratory tract (blue), lower respiratory tract (red) or as systemic (green). Data are shown as mean $\pm$ s.e.m. (d) Correlation between cumulative symptom scores and cumulative viral load were calculated using trapezoidal area under the curve (AUC). Mann-Whitney was used for statistical testing. (* = $p < 0.05$, ** = $p < 0.01$).

2015). There was no evidence of infection outcome being biased towards a single haplotype, as there was a mixture of infected and uninfected subjects within the most frequently occurring DRB1 and DPB1 alleles (Figure 3.1.2a, b).

Subject ID	HLA-DR β 1		HLA-DP α 1		HLA-DP β 1		HLA-DQ α 1		HLA-DQ β 1		Outcome
MH 002	*15:01	*04:04			*04:01						Uninfected
MH 003	*12:02				*04:02	*13:01					Infected
MH 007	*13:02	*07:01			*02:01	*04:01					Infected
MH 008	*11:01	*11:04			*04:02	*104:01					Infected
MH 010	*01:01	*15:01			*04:01						Infected
MH 011	*13:02	*07:01			*04:02	*17:01					Infected
MH 013	*03:01	*04:01			*01:01	*04:02					Uninfected
MH 014	*15:01	*03:01			*04:01						Infected
MH 015	*15:01	*07:01									Uninfected
MH 017	*01:01	*11:01			*03:01	*10:01					Infected
MH 022	*03:01	*04:07			*02:01	*03:01					Uninfected
MH 023	*15:01	*07:01									Infected
MH 024	*15:01	*03:01									Uninfected
MH 025	*14:01	*07:01									Uninfected
MH 026	*15:01	*15:02									Infected
MH 031	*01:01	*15:01									Uninfected
MH 033	*04:01	*07:01									Uninfected
MH 034	*11:01	*07:01									Infected
MH 036	*04:04	*08:02			*04:02	*104:01					Infected
MH 037	*07:01	*08:01			*04:01						Infected
MH 040	*08:01	*10:01									Infected
MH 041	*04:01	*14:01									Infected
MH 043	*15:01	*12:01			*04:01						Infected
MH 047	*15:01	*11:01			*02:01	*04:01					Infected
MH 049	*04:01	*07:01			*01:01	*04:01					Uninfected
RF 006	*01:01	*04:04									Infected
RF 012	*15:01	*10:01			*02:01	*04:01					Infected
RF 013	*15:01	*04:01			*04:02	*11:01					Uninfected

Subject ID	HLA-DR β 1		HLA-DP α 1		HLA-DP β 1		HLA-DQ α 1		HLA-DQ β 1		Outcome
RF 014	*15:01	*07:01			*01:01	*04:01					Infected
RF 016	*01:02	*03:01			*04:01						Infected
RF 017	*03:01	*07:01			*04:01						Infected
RF 024	*11:02	*08:04			*02:01	*04:01					Uninfected
RF 025	*15:01	*04:08			*02:01	*04:01					Uninfected
RF 026	*15:01	*04:03			*04:01	*17:01					Uninfected
RF 033	*13:01	*07:01			*02:01	*04:02					Infected
RF 034	*14:04	*09:01									Infected
RF 036	*04:01	*10:01	*01:03		*02:01	*04:01	*01:01	*03:01	*05:01	*03:01	Infected
RF 037	*11:04	*08:01									Uninfected
RF 038	*11:04	*07:01	*01:03	*02:01	*04:01	*11:01	*02:01	*05:05	*02:02	*03:01	Infected
RF 040	*15:01	*04:04	*01:03		*02:01	*04:02	*01:02	*03:01	*06:02	*03:02	Infected
RF 041	*03:01	*13:01									Uninfected
RF 042	*07:01	*09:01	*01:03	*02:01	*03:02	*03:03	*02:01	*03:01	*03:02	*03:03	Infected
RF 044	*04:07	*11:01									Uninfected
RF 045	*15:01	*03:01									Uninfected
RF 047	*04:01	*07:01									Infected
RF 058	*15:01	*13:03									Uninfected
RF 059	*15:01	*13:01									Uninfected
RF 061	*01:01	*15:01									Infected
RF 062	*11:01	*07:01	*01:03	*02:01	*03:01	*17:01	*02:01	*05:05	*03:03	*03:01	Infected
RF 064	*12:01	*07:01	*01:03		*04:01		*02:01	*05:05	*03:03	*03:01	Infected
RF 065	*13:01	*13:02									Uninfected
RF 067	*15:01	*03:01	*01:03		*04:01	*04:02	*02:01	*05:01	*06:02	*02:01	Infected

Table 3.1.2 HLA haplotypes of the study subjects. All subjects were HLA-typed for HLA-DRB1. A subset of the infected subjects was typed for HLA-DP and HLA-DQ loci.

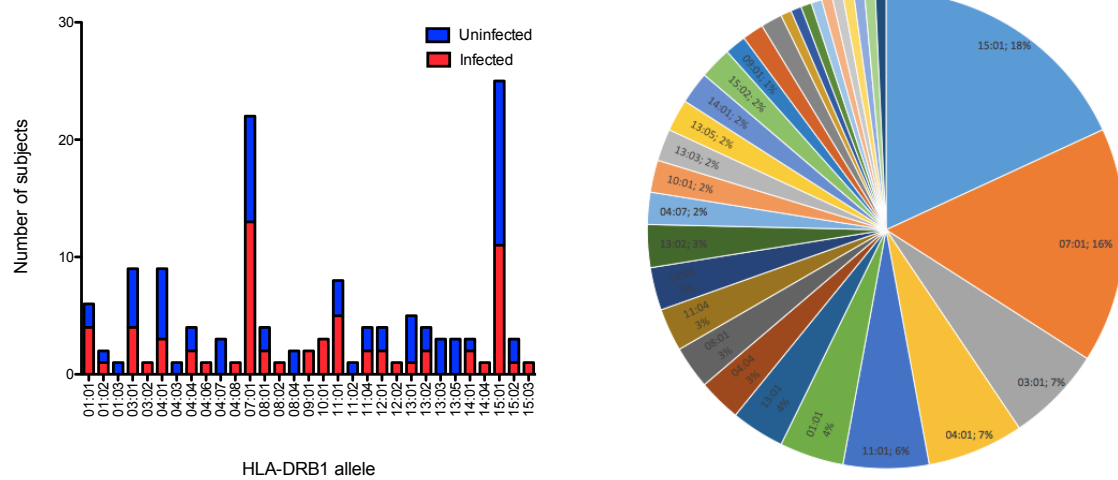
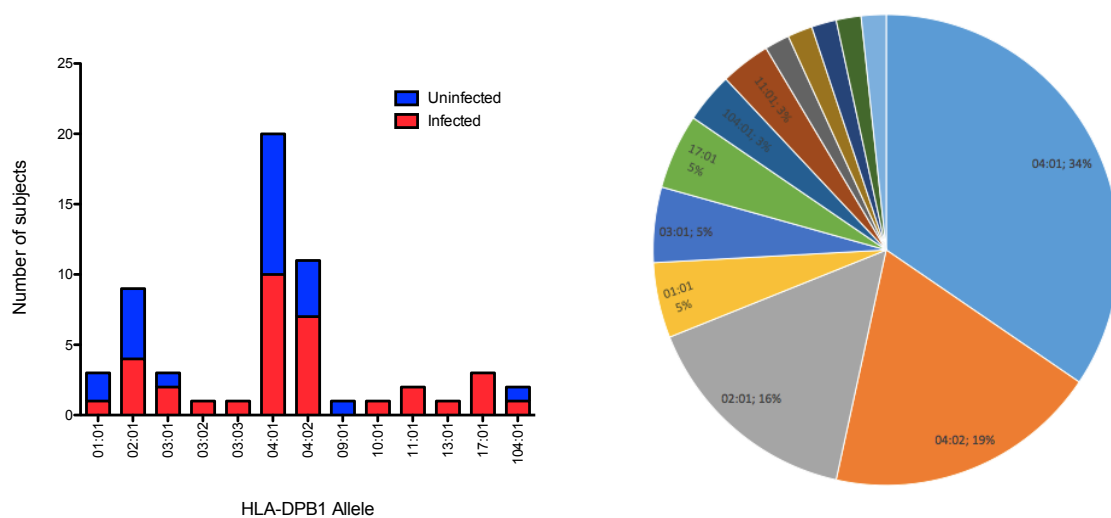
a**b**

Figure 3.1.2 Susceptibility to infection is not associated with specific HLA alleles. Histograms (left) show the number of subjects with (A) HLA-DRB1 or (B) HLA-DP1 alleles that were infected (red) or uninfected (blue) and pie-charts (right) show the allelic frequency of (A) HLA-DRB1 and (B) HLA-DPB1 alleles of the study subjects.

3.1.6 Discussion

The mechanisms underlying RSV-induced airway inflammation in humans are not known. Studies in naturally infected infants have shown that viral load detected in nasal aspirates correlates with disease severity, patients with respiratory failure having higher viral loads compared to those who do not require ventilation (Buckingham et al., 2000). This is supported by DeVincenzo and colleagues, who conducted an experimental challenge study where healthy adults pre-selected for having low serum neutralizing antibody titres were inoculated with increasing quantities of RSV (DeVincenzo et al., 2010). This resulted in a range of frequencies of subjects successfully infected (from 71.4% to 85.7%) with an average of 77%. Increasing the size of RSV inoculum did not lead to increased infection rate in this study. All infected subjects manifested mild clinical symptoms of disease and symptom severity correlated with viral load. This study was not designed to study cellular immune responses and the effects on T cell responses of inoculation with increasing doses of RSV is still unknown.

In contrast to the study conducted by DeVincenzo and colleagues, we achieved a 56% infection rate and classified individuals into three groups according to clinical outcome; uninfected, infected but asymptomatic and infected with cold-like symptoms. This reduced infection rate could be explained by the different screening criteria: in this study, we did not select volunteers on the basis of pre-existing serum neutralizing antibody levels so that the enrolled cohort would be more representative of an unselected population, where there was likely to be a broader range of infection histories and RSV-specific responses.

Although immune correlates of protection from human RSV infection are poorly understood, previous work in this model has highlighted two immunological factors that may explain why some people remain uninfected. Firstly, our group showed that RSV-specific mucosal IgA (but not serum IgG) titres were predictive of susceptibility to RSV infection (Habibi et al., 2015). Moreover, nasal, but not plasma or BAL, LL-37 levels were shown to be higher at baseline in subjects who remained uninfected (Currie et al., 2016). However, titres of both were also highly variable amongst the uninfected subjects, and the large overlap with levels seen in those who were subsequently infected suggest that neither mechanisms is overridingly

correlated with protection. It is known that RSV can re-infect throughout life even in the absence of antigenic variation (Hall et al., 1991). Nevertheless, differences in previous infection history are likely to have an impact on an individual's susceptibility to reinfection. In mouse models, early re-challenge (2 months after primary infection) with RSV was cleared rapidly from the nose and the lungs whereas late re-challenge (16-21 months) caused infection in 87% of the animals (Graham et al., 1991b). In humans, work from our group has also implied that antigen-specific CD8⁺ T resident memory cells contribute to decreased disease severity but that frequencies decrease significantly in the lungs after RSV infection, possibly as a regulatory mechanism to reduce immunopathology (Jozwik et al., 2015). Differences in the rate of waning of RSV-specific immune memory therefore probably contributes to variation in susceptibility.

In addition, other factors that have yet to be addressed including inherited characteristics and innate immunity may contribute to why some subjects remained uninfected. For example, differential responses to the surface G and F proteins of RSV, which have previously shown to have immunomodulatory mechanism, may play a role. The G protein bears a CX3C fractalkine-like motif which is thought to modulate inflammatory responses and the secreted form of G protein may act as a decoy for antibody (Tripp et al., 2001). TLR4 recognises the F protein and is important for initiating an innate immune response against RSV. Polymorphisms in TLR4 have been speculated to influence the genetic predisposition to RSV infection (Puthothu et al., 2006). Therefore, the variability in host response may also be due to a variety of factors that influence the susceptibility of individual to RSV-induced immunomodulatory mechanisms.

A proportion of subjects who were infected remained asymptomatic. We hypothesise that this was the result of certain individuals being able to better control viral replication, as we found higher viral load correlating with more severe symptoms. This relationship was also reported in a previous experimental challenge with RSV M37 (DeVincenzo et al., 2010). However, there are caveats to making this interpretation; viral load does correlate with symptoms but is confounded by the fact that it is measured in the nose, where more severe symptoms may enhance viral shedding. They may therefore not be genuinely independent variables. In addition, quantification of symptoms is difficult, as the self-reported symptoms diary method is subjective, making the results highly variable. One infected subject reported

a late onset of symptoms, but this did not seem to be driven by viral load as peak viral load for this individual was at day 7. Moreover, subjective scoring of symptoms probably explains why the uninfected subjects, who had no detectable viral shedding, also reported some symptoms. This was found mainly during the first 5 days following inoculation and it is possible that subjects were influenced by the act of inoculation itself. These symptoms do not occur in uninfected individuals in studies where subjects are individually housed rather than in groups.

Study subjects were not pre-screened for their class II alleles and the HLA haplotypes of our study cohort were heterogeneous with allelic frequencies comparable to published reports (Doherty et al., 1992; González-Galarza et al., 2015). Unsurprisingly, we found no HLA class II association with susceptibility to RSV infection although HLA haplotyping was incomplete and sample size was too small to reliably identify HLA associations. HLA class I and II are linked with susceptibility to a number of autoimmune diseases, including rheumatoid arthritis, type I diabetes and coeliac disease (Mangalam et al., 2013). Autoimmunity is predominantly caused by poorly regulated auto-reactive T and B cells with some evidence showing contributions from the components of innate immunity (Tsai and Santamaria, 2013). In contrast, factors involved in susceptibility and severity of viral infections have added complexity related to pathogen characteristics and host-pathogen interactions. HLA associations have only been made in some chronic viral infections, such as HIV, hepatitis B and C, where effective host responses against viruses that breach the early innate defences rely heavily on HLA-restricted T cell responses (Blackwell et al., 2009). Despite 70% similarity between the three different HLA class II genes (DP, DQ and DR), the polymorphism in the sequences of the peptide binding groove offers a wide range of peptide-binding affinities (van Lith et al., 2010; Wills and Green, 1995).

In conclusion, we inoculated a cohort of healthy adults with a 56% infection rate that was in line with what might be expected from a cohort that was not pre-selected for low pre-existing antibody titres. The study was safe, with no unexpected adverse effects throughout. We found that overall viral load correlated with symptoms in infected subjects and which mainly caused upper respiratory tract symptoms. There were no significant differences in subject demographics and the HLA class II haplotypes of the enrolled participants were in line with reported frequencies in the population. Thus the study population and clinical

outcomes make this an appropriate model to further investigate CD4+ T cells in relation to infection and HLA class II-restricted responses against RSV.

3.2 Kinetics, phenotype and functionality of CD4+ T cells in human RSV infection

3.2.1 Introduction

Our knowledge of CD4+ T cell responses in human RSV infection has primarily derived from paediatric studies where upper and lower respiratory tract samples from hospitalised children have been used to analyse the levels of cytokines ascribed to helper T cell subsets. These reports have shown increases in amount of pro-inflammatory cytokines IFN- γ , IL-4, IL-5, IL-13 and IL-17 but the contribution of these to disease severity in RSV-infected infants remains controversial (Breindahl et al., 2012; Garofalo et al., 2001; Legg et al., 2003; Stoppelenburg et al., 2014; Tabarani et al., 2013).

In the mouse model of RSV infection, CD4+ T cells have been shown to reduce viral shedding but their depletion also reduces weight loss suggesting a causal role in immunopathology (Graham et al., 1991b). Suggested mechanisms have included Th17-mediated inflammation, with IL-17 having been shown to be induced with RSV infection, enhancing local inflammation and delaying viral clearance (Mukherjee et al., 2011). Conversely, regulatory T cells have been reported to proliferate and accumulate in the lungs following RSV infection (Fulton et al., 2010). Their depletion results in enhanced viral clearance, but also immunopathology as a result of increased numbers of CD8+ T cells (Lee et al., 2010). Thus, CD4+ T cells can contribute both positively and negatively to the outcome of RSV infection.

To date, there have been no reports providing systematic analysis of the overall CD4+ T cell response in human RSV infection. Using the experimental challenge model, longitudinal analysis of these cells has the potential to overcome the limitations of natural infection studies and to investigate their phenotype and function in more detail. Studies of this kind have been particularly informative in the context of influenza infection (McMichael et al., 1983; Wilkinson et al., 2012). Using experimental human challenge with both H3N2 or H1N1

influenza strains, Wilkinson and colleagues found that pre-existing CD4⁺ T cells in peripheral blood correlated with lower viral shedding and fewer symptoms. They also showed a burst in CD4⁺ T cell activation at day 7, which was directed against conserved internal proteins. Moreover, they showed cytolytic capacity of influenza-specific CD4⁺ T cell lines that may have directly limited viral replication (Wilkinson et al., 2012). An earlier human experimental challenge study, McMichael and colleagues measured cytotoxic T cell activity following inoculation with influenza A/Munich/1/79 strain. This study showed that cross-reactive cytotoxic T cells were played a part in resolution of influenza infection (McMichael et al., 1983). Collectively, these results suggest that T cell-mediated protection against influenza infection are not limited to a single cell type and is multifactorial.

3.2.2 Short-lived polyclonal CD4⁺ T cell activation peaks at day 10 in peripheral blood

CD4⁺ T cells have been shown to respond to experimental challenge with influenza virus and yellow fever vaccination by an increase in the expression of proliferation and activation markers (Blom et al., 2013; Wilkinson et al., 2012). In the context of human RSV infection, the kinetics of the CD4⁺ T cell response has not been described. To address this, CD4⁺ T cells in whole blood samples from forty-nine challenged subjects at baseline, during acute infection and convalescence were stained for CD38 and Ki67, markers of activation and proliferation, respectively (Figure 3.2.1a).

CD4⁺ T cells from challenged but uninfected subjects showed variable CD38 and Ki67 expression, showing no significant increase in the frequency of the activated phenotype throughout the study period (Figure 3.2.1b, d). In infected subjects, there was an increase in the median frequency of activated CD4⁺ T cells 7 days post-infection (median of 0.808%, IQR 0.61 – 1.11) that peaked at day 10 (median of 1.33%, IQR 1.08 – 1.87), and rapidly returned to baseline levels during the convalescence period (median 0.67%, IQR 0.449 – 0.757; Figure 3.2.1c, d). The peak frequency of CD38⁺ Ki67⁺ CD4⁺ T cells was therefore significantly increased in the infected subjects compared to baseline ($p < 0.0001$; Figure 3.2.1d). The peak frequency of activated CD4⁺ T cells was also significantly different ($p = 0.008$) between the infected (mean 2.04% $\pm$ s.e.m. 0.295) and uninfected subjects (mean 1.34% $\pm$ s.e.m. 0.144; Figure 3.2.1e).

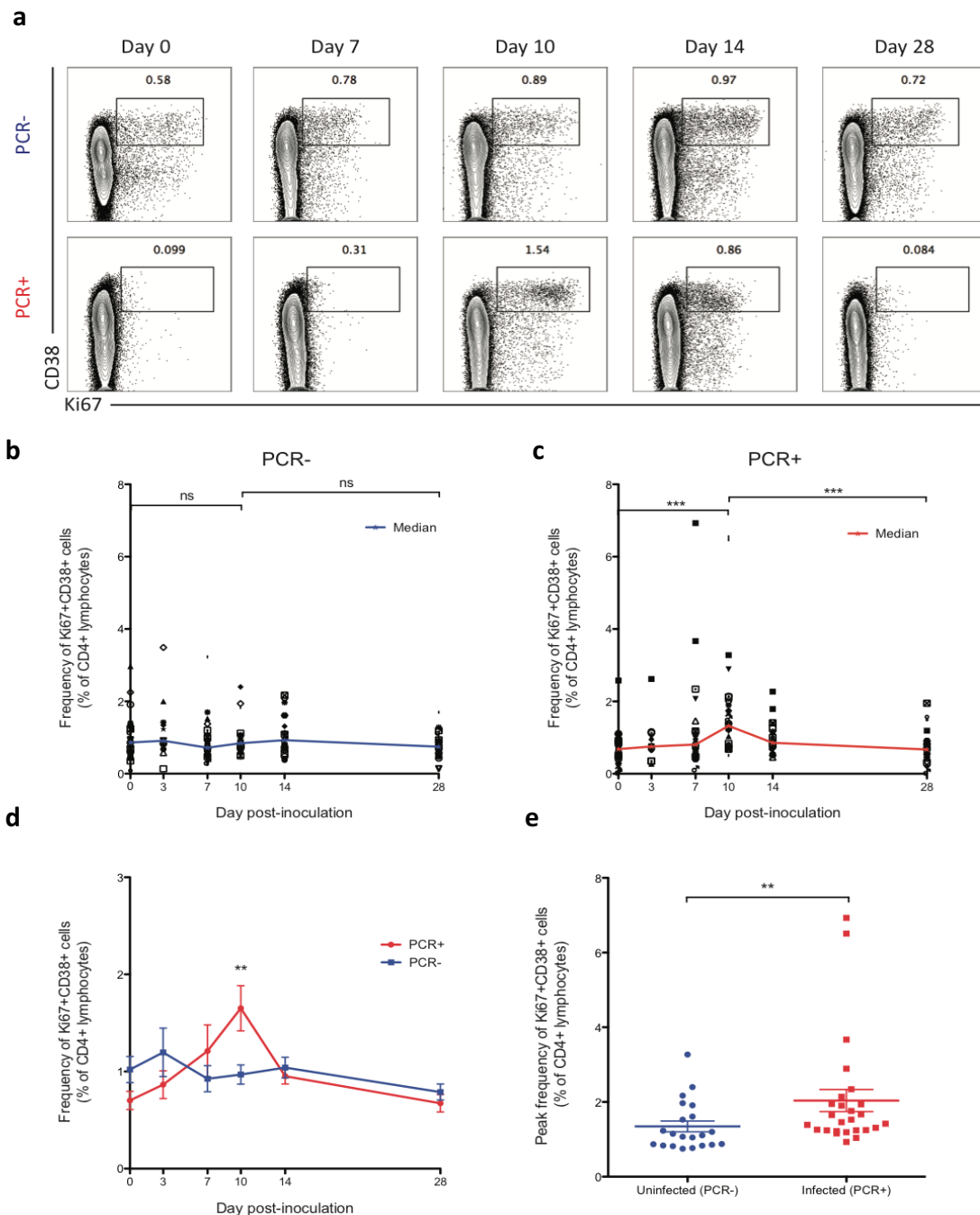


Figure 3.2.1 The activated polyclonal CD4+ T cell burst peaks around day 10 in blood. Whole blood samples were stained with anti-CD3, CD4, CD8, CD38 and Ki67, and analysed by flow cytometry. (a) Plots are gated on CD3+CD4+CD8- lymphocytes. Numbers represent the frequency of Ki67+CD38+ cells as a percentage of CD4+ T cells. Representative subject are shown from uninfected (PCR-) and infected (PCR+) groups. (b) Frequency of Ki67+CD38+ cells amongst the PCR- subjects. (c) Frequency of Ki67+CD38+ cells amongst the PCR+ subjects. (d) Mean $\pm$ SEM of Ki-67+CD38+ CD4+ T cell frequencies in the blood of PCR- and PCR+ subjects are shown. The P-value for a two-tailed Wilcoxon matched pairs test is shown (**p<0.01 and ***p<0.001; ns = not significant). (e) Peak frequencies of Ki-67+CD38+ cells as a proportion of CD4+ T cells are shown. Mean $\pm$ SEM are shown with the P-value for a two-tailed Mann–Whitney test (**p<0.01). Data was acquired by Agnieszka Jozwik.

3.2.3 The activated polyclonal CD4+ T cell burst peaks around day 10 in BAL

The kinetics of CD4+ T cells in the airway have not been studied in human respiratory infections. However, Zhao and colleagues found that in two adults with severe influenza infection, approximately 8% of CD4+ T cells in BAL upregulated the expression of the activation markers CD38 and HLA-DR (Zhao et al., 2012). In addition, Jambo and colleagues reported a higher percentage of antigen-specific CD4+ T cells against influenza virus could be detected in BAL compared to blood in HIV-uninfected adults (Jambo et al., 2011). We used the approach described in the previous section to analyse the induction of activated CD4+ T cells in BAL samples of twenty challenged subjects of whom ten developed PCR+ infection. Bronchoscopies were performed at baseline, day 7 (2012 cohort) or day 10 (2013 cohort) and day 28 post infection. These results followed the same trend as their counterparts in blood; peak Ki67+CD38+ frequency was at day 10 in the infected subjects (median of 4.05%, IQR 1.55 – 6.59) whilst the uninfected subjects showed no change throughout the course of the infection (Figure 3.2.2a). There was high variability between individuals in both groups and the increase at day 10 was not statistically significant compared to baseline ($p=0.2188$) or uninfected subjects ($p=0.3939$; Figure 3.2.2b, c). The wide spread of the data and relatively small sample size could explain the absence of statistical significance. Nevertheless, there was a strong trend towards an increase in the median of Ki67+CD38+ CD4+ cells at days 7 and 10 in the infected individuals compared to those that remained uninfected (Figure 3.2.2d). Importantly, we did find more activated CD4+ T cells in BAL than blood following infection, with the average peak frequency of these cells in BAL (mean 4.73% $\pm$ s.e.m. 1.97) significantly higher when compared to matched peripheral blood samples (mean 1.17% $\pm$ s.e.m. 0.137; $p=0.0273$).

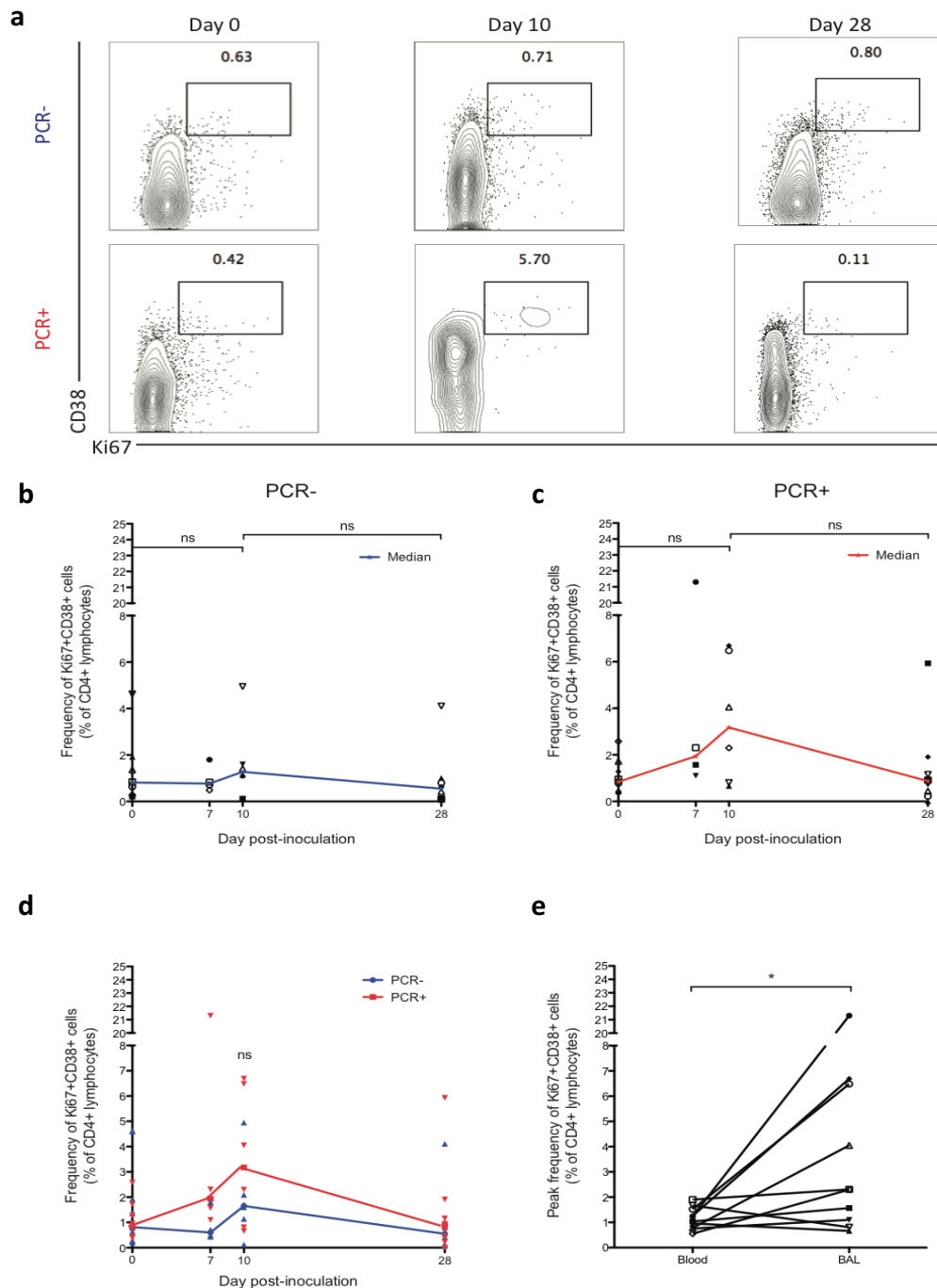


Figure 3.2.2 CD4+ T cells in the BAL of infected subjects are highly activated during acute RSV infection. Broncho-alveolar lavage samples were stained with anti-CD3, CD4, CD8, CD38 and Ki67, and analysed by flow cytometry. (a) Plots are gated on CD3+CD4+CD8- lymphocytes. Representative subjects are shown from challenged but uninfected (PCR-) and infected (PCR+) groups. (b) Frequency of Ki67+CD38+ cells amongst the PCR- subjects. (c) Frequency of Ki67+CD38+ cells amongst the PCR+ subjects. (d) Median of Ki-67+CD38+ CD4+ T cell frequencies in the BAL of PCR- and PCR+ subjects are shown. (e) Frequencies of Ki-67+CD38+ cells, as a proportion of CD4+ T cells at day 10 post inoculation in matched blood and BAL samples. The P-value for a two-tailed Wilcoxon matched pairs test is shown (* $p < 0.05$; ns = not significant). Data was acquired by Agnieszka Jozwik.

3.2.4 Polyclonal CD4⁺ T cells in blood and BAL display distinct costimulatory, memory and homing receptor profiles

There is limited data characterising the phenotype of CD4⁺ T cells in human RSV infection. de Bree and colleagues used RSV antigens (lysates from infected cell cultures) to stimulate PBMCs from healthy adults and identified RSV-specific cells with a memory phenotype and moderate to high CD27 and CCR7 expression (de Bree et al., 2007). In the context of influenza virus, *ex vivo* staining of peripheral blood from healthy adults with MHC class II tetramers showed CD4⁺ T cells to be of a predominantly central memory phenotype, with high expression of both costimulatory molecules CD27 and CD28 (Lucas et al., 2004).

To characterise CD4⁺ T cells during RSV infection, whole blood (n=14) and BAL (n=5) samples from PCR⁺ subjects were stained for markers associated with costimulation (Figure 3.2.3a), memory subsets (Figure 3.2.3b) and homing (Figure 3.2.3c). These showed distinct differences in expression profiles between blood and BAL. In blood, at least 95% of the CD4⁺ T cells co-expressed the costimulatory molecules CD27 and CD28, whereas in BAL CD27 expression on average was around 35% (Figure 3.2.3a).

T cells can be characterised into four sub-populations using the markers CD45RA and CCR7; naïve T cells are CD45RA⁺CCR7⁺, central memory T (T_{cm}) cells are CD45RA⁺CCR7⁺, effector memory T (T_{em}) cells are CD45RA⁺CCR7⁻ and terminally differentiated effector memory (T_{emra}) cells are CD45RA⁺CCR7⁻ ((Sallusto et al., 1999)). Using the combination of these markers we found three populations in blood; at baseline, mean 54.41% $\pm$ s.e.m. 2.71 were naïve, mean 19.94% $\pm$ s.e.m. 1.48 were T_{em} with the remaining exhibiting the T_{cm} phenotype (Figure 3.2.3b). However, in BAL we found the majority of CD4⁺ T cells displaying the T_{em} phenotype, with a small population of T_{cm} cells and none naïve (Figure 3.2.3b).

Finally, the majority of CD4⁺ cells in blood expressed the lymphoid homing marker CD62L which is required for recirculation and entry to lymphoid tissue (Hengel et al., 2003), while, in BAL, this was expressed in only 10% of CD4⁺ T cells (Figure 3.2.3c). In contrast, low frequencies of CCR5-expressing CD4⁺ T cells (mean 3.93% $\pm$ s.e.m. 0.667) were found in blood with higher frequency of cells expressing this chemokine receptor in BAL (mean 10.8% $\pm$ s.e.m. 3.93). Despite failing to reach statistical significance (p=0.0625), there was a trend

towards an increase throughout the infection, reaching the peak frequency at day 28 (figure 3.2.3c).

Notably, the phenotypes in both blood and BAL compartments were stable throughout the course of infection, with no change in the frequency of expression for the majority of phenotypic markers (except CCR5 in BAL). Since these results were derived from a CD4⁺ T cell population of mixed clonality and specificity, it is possible that changes in the phenotype of antigen-specific cells may have been swamped by resting cells of unrelated specificities, which in the context of RSV infection would not be expected to respond. This highlights the need for tools (such as MHC class II tetramers) that can allow tracking of RSV-specific cells *ex vivo*.

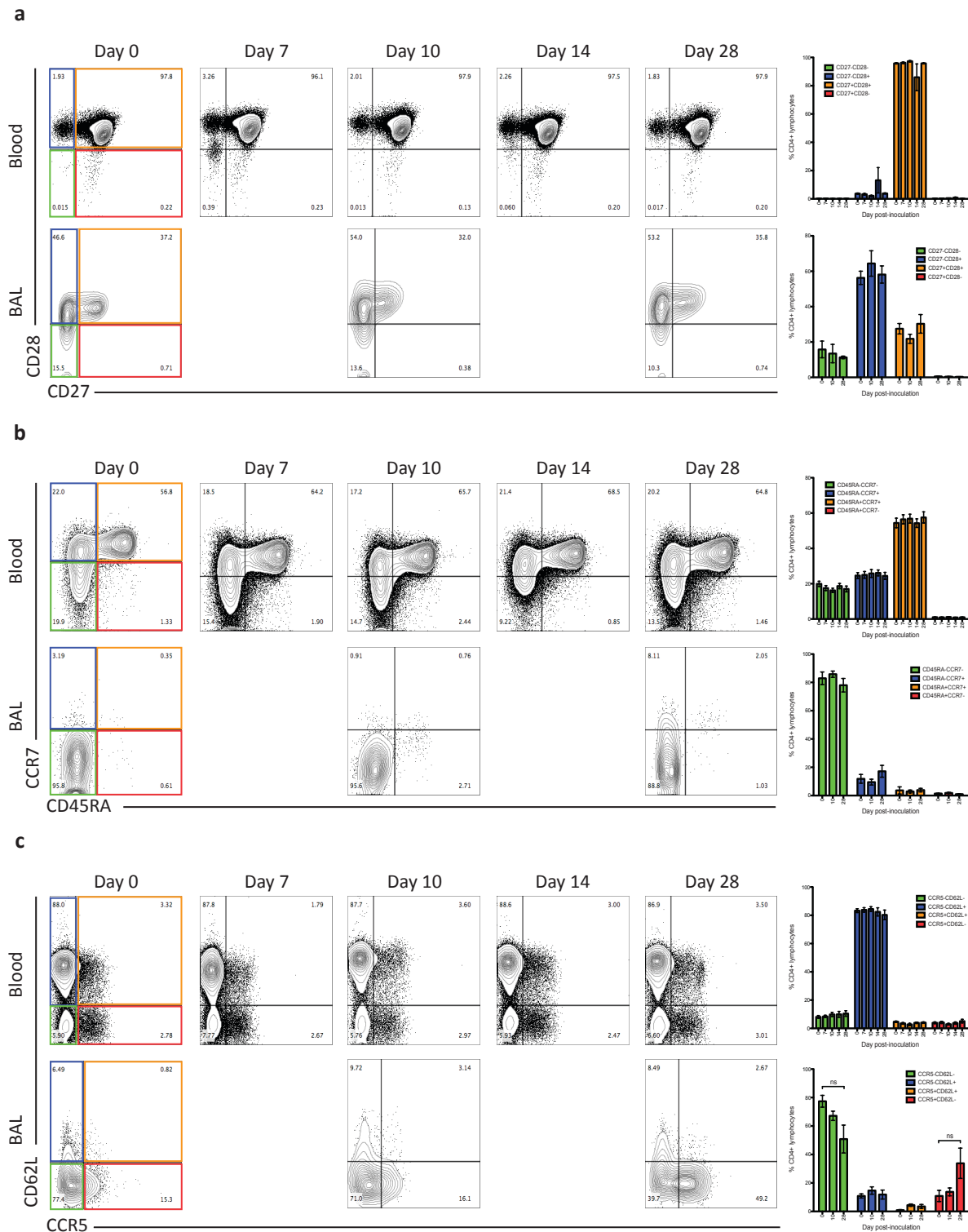


Figure 3.2.3 Differential expression of costimulatory, memory and homing markers by CD4⁺ T cells between blood and BAL samples. Whole blood and broncho-alveolar lavage samples were stained with anti-CD3, CD4, CD8, CD27, CD28, CD45RA, CCR7, CCR5 and CD62L, and analysed by flow cytometry. All plots are gated on CD3⁺CD4⁺CD8⁻ lymphocytes. A representative PCR⁺ subject is shown for the expression of (a) costimulatory molecules CD27 and CD28, (b) markers of memory subsets CD45RA and CCR7 and (c) homing molecules CCR5 and CD62L throughout the infection. Two-tailed Wilcoxon matched pairs test was used for statistical testing (ns = not significant).

3.2.5 CD4+ T cells minimally express markers of cytotoxicity during RSV infection

CD4+ T cells have been shown in some settings to express cytotoxicity molecules and may contribute to direct killing of infected cells in chronic (Norris *et al.*, 2004; Zaunders *et al.*, 2004) or acute (Wilkinson *et al.*, 2012) viral infections. To investigate this in the context of human RSV infection, whole blood (n=14) and BAL (n=5) samples from PCR+ subjects were stained with granzyme B and perforin, which are markers associated with killing of target cells. Expression profiles in both blood and BAL samples were similar, with low frequencies of granzyme B+ perforin+ CD4+ T cells (figure 3.2.4a). Over the course of infection, there was no change in the mean frequency of cytotoxic molecules in blood ($p=0.3054$; figure 3.2.4b) and BAL ($p=0.8125$; figure 3.2.4c).

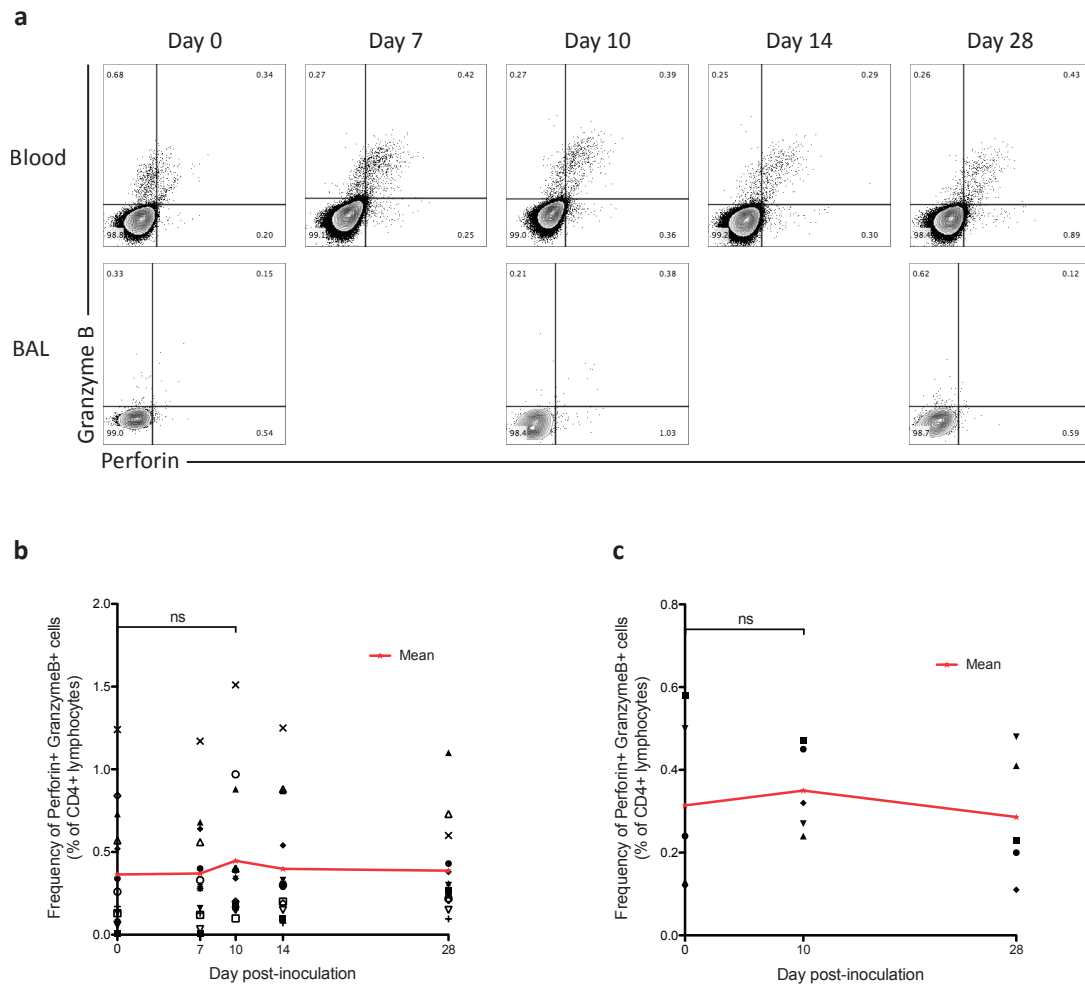


Figure 3.2.4 CD4+ T cells show no change in cytolytic markers following infection. Blood and broncho-alveolar lavage samples were stained with anti-CD3, CD4, CD8, granzyme B and perforin, and analysed by flow cytometry. (a) Plots are gated on CD3+CD4+CD8- lymphocytes. Results from a representative PCR+ subject is shown for expression of granzyme B and perforin in whole blood and BAL samples. Frequency of granzyme B+ perforin+ cells amongst the PCR+ subjects in whole blood (b) and in BAL (c). Two-tailed Wilcoxon matched pairs test was used for statistical testing (ns = not significant).

3.2.6 RSV-specific CD4+ T cells predominantly express type 1 cytokines, with no evidence for Th2 or Th17 cytokine production

CD4+ T cell subsets can be distinguished by expression of unique transcription factors and cytokine production profiles (Zhu et al., 2010). To optimise staining panels for Th1, Th2 and Th17 cytokines, healthy adult PBMCs were stimulated with PMA and ionomycin, which acts on the protein kinase C pathway resulting in stimulation of all T cells regardless to their antigen specificity. Using this method, we established an antibody panel that defined the Th1 subset by production of IL-2, IFN γ and TNF- α (Figure 3.2.5a), Th2 subset by IL-4 and IL-5, and Th17 subset by IL-17A and IL-17F (Figure 3.2.5b). Previous studies had indicated an optimal duration of stimulation with live viruses. We further optimised the stimulation period and MOI for RSV M37 by stimulating PBMCs from infected individuals at day 10 post-infection with MOIs ranging from 0.01 to 6 and stimulation periods of 24 and 48 hours. From the cytokines we have studied, higher MOI only resulted in an increased frequency of IFN- γ and TNF- α . Furthermore, longer incubation of PBMCs with higher MOI resulted in detection of fewer antigen-specific responses (Figure 3.2.5c).

We used these optimised conditions to stimulate PBMCs from challenged subjects at day 10 with RSV at MOI 6 for 24 hours. Flow cytometry analysis showed that RSV-specific CD4+ T cells were mainly producers of Th1 cytokines with no increase in Th2 or Th17 cytokines examined (Figure 3.2.6a). In particular, RSV-specific CD4+ T cells were double-producers of IFN- γ /TNF- α (mean 0.6% $\pm$ s.e.m. 0.182; Figure 3.2.6b).

In order to validate these findings, we used a similar stimulation strategy to quantify IFN γ producing cells using IFN γ ELISpot. While PCR- subjects showed no changes longitudinally (figure 3.2.7a), we found RSV-specific T cells from PCR+ subjects had significantly higher frequencies of IFN γ producing cells at day 10 compared to baseline and convalescence (figure 3.2.7b). In order to determine whether CD4+ T cells contributed to this response, we depleted CD8+ T cells prior to RSV stimulation, by labelling CD8+ T cells with microbeads and separating them using MACS columns. The total number of cells pre- and post-depletion was used to normalise (Section 2.3.11) for variability in the proportion of CD8+ T cells in individual

samples. RSV stimulation of CD8 depleted PBMCs resulted in a reduction IFN γ producing cells (mean 62.9% $\pm$ s.e.m. 6.38; Figure 3.2.7c).

There is evidence from other infections that CD4 $^{+}$ T cells are protective and we therefore tested whether there was a correlation between baseline IFN- γ producing RSV-specific T cell frequencies and subsequent viral load. However, there was no significant difference in IFN- γ producing T cell frequencies at baseline between PCR $^{+}$ and PCR $^{-}$ subjects (Figure 3.2.7d). Furthermore, the frequency of RSV-specific memory T cells that produced IFN γ did not correlate with cumulative viral load in PCR $^{+}$ subjects (Figure 3.2.7e). Thus, in this model, there was no evidence of an association between RSV-specific memory CD4 $^{+}$ T cells and disease prevention or amelioration.

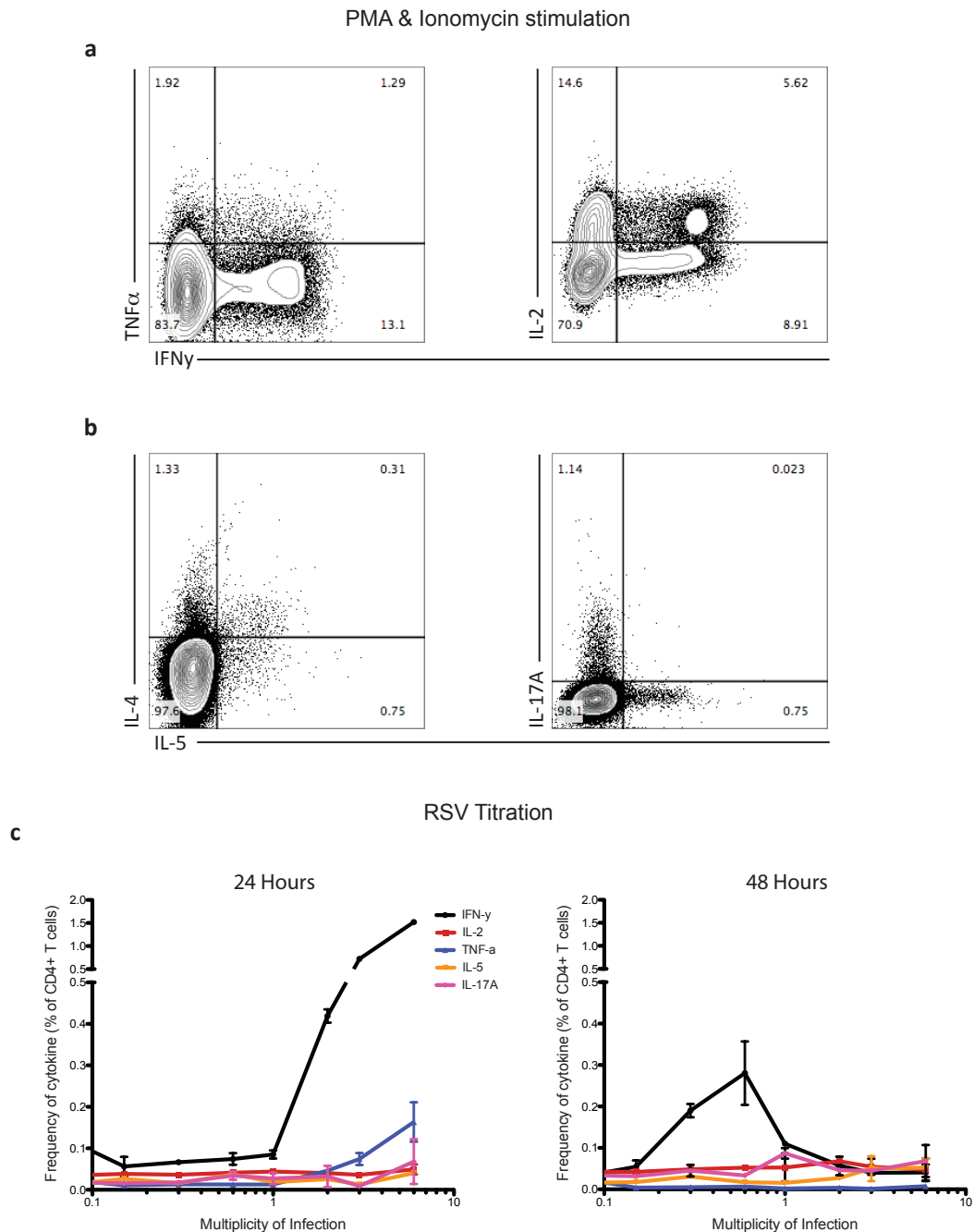


Figure 3.2.5 Optimisation of intracellular cytokine staining panels and titration of RSV. 1×10^6 healthy adult PBMCs were stimulated with PMA (25ng/ml) and ionomycin (1 μ g/ml) for 6 hours. Brefeldin A (10 μ g/ml) was added for the last 4 hours of the culture. Cells were then stained with anti-CD3, CD4, CD8, IL-2, IL-4, IL-5, IL-17A, IFN γ , TNF α and fixable viability dye, and analysed by flow cytometry. All plots are gated on live CD3⁺CD4⁺CD8⁻ lymphocytes. (a) Plots showing expression of IL-2, IFN γ and TNF α , cytokines associated with Th1 subset. (b) Plots showing expression of IL-4, IL-5 and IL-17A, cytokines associated with Th2 and Th17 subsets, respectively. (c) 2×10^5 healthy adult PBMCs were stimulated with vero-grown RSV at varying multiplicity of infection for 24 or 48 hours with brefeldin A (10 μ g/ml) added in the last 4 hours of the culture.

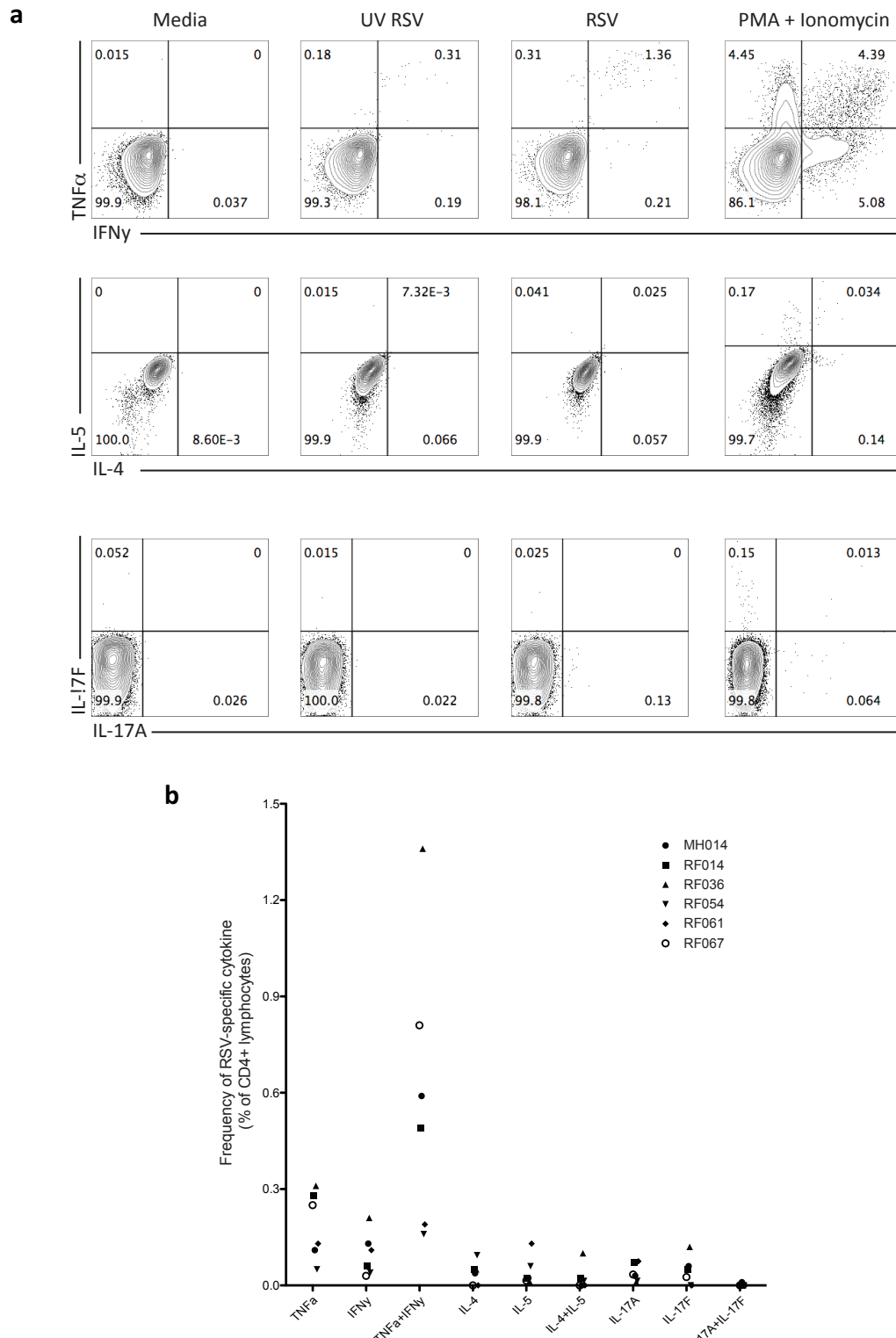


Figure 3.2.6 RSV stimulation induces only Th1 cytokines in CD4⁺ T cells. PBMCs from the infected subjects at day 10 were cultured *in vitro* with RSV (MOI 6) for 24 hours with Brefeldin A (10 μ g/ml) added 4 hours before staining with fixable viability dye, anti-CD3, CD4, CD8; and IFN γ IL-2 and TNF- α ; or IL-4, IL-5, IL-17A and IL-17F. (a) Representative FACS plots showing frequencies of cytokines associated with Th1 (top panel), Th2 (middle panel) and Th17 (lower panel) subsets. (b) Expression of T helper cell subset cytokines in blood of RSV stimulated PCR⁺ subjects.

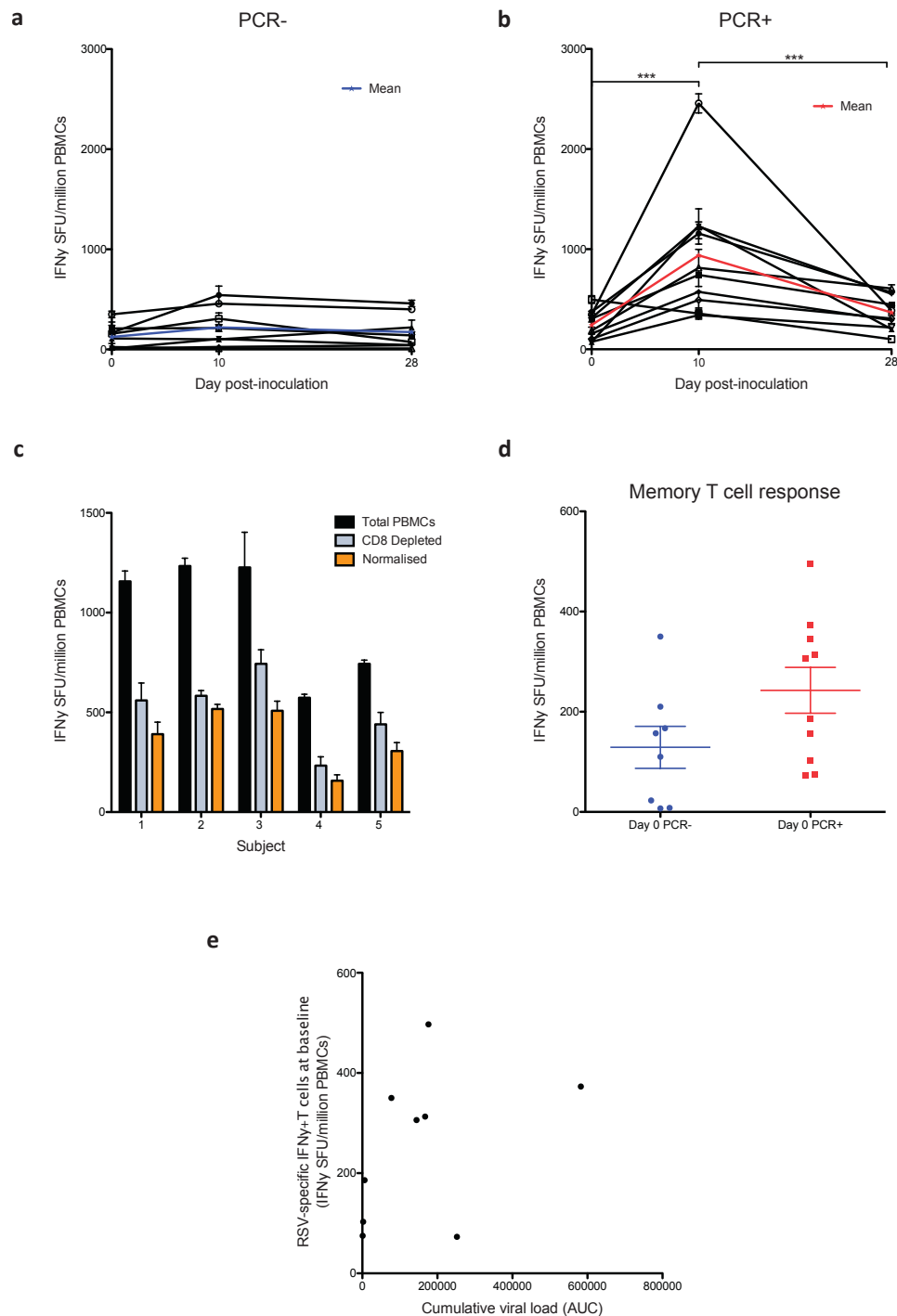


Figure 3.2.7 IFN- γ expressing CD4+ T cells of PCR+ subjects pre-infection are not associated with decreased viral load. PBMCs (2×10^5) from the RSV challenged subjects were cultured *in vitro* with RSV (MOI 6) for 20 hours IFN- γ ELISpot assay. Graphs show IFN γ production by total T cells following stimulation of PBMCs from (a) PCR- and (b) PCR+ subjects. (c) Of five infected subjects, CD8+ T cells were depleted using MACS depletion (>95% purity) and prior to RSV stimulation for IFN γ ELISpot assay. (d) IFN γ producing memory T cells following RSV stimulation of day 0 samples. (e) Correlation between baseline RSV-specific IFN γ producing T cells and cumulative viral load.

3.2.7 Discussion

Little direct evidence is available characterising CD4⁺ T cells themselves in the context of RSV infection. Instead, understanding of the role of CD4⁺ T cells in the context of human RSV infection is primarily restricted to studies using nasal washes and tracheal aspirate samples from severely infected children (Breindahl et al., 2012; Garofalo et al., 2001; Legg et al., 2003; Stoppelenburg et al., 2014; Tabarani et al., 2013). These have shown production of T helper-associated cytokines, which are hypothesised to contribute to both protection and pathogenesis. Taking advantage of the detailed and longitudinal sampling possible in the human experimental challenge model, we aimed to elucidate the features of the polyclonal and RSV-specific CD4⁺ T cell response to infection through the course of RSV infection.

CD4⁺ T cells underwent polyclonal activation and proliferation that peaked at day 10 before returning rapidly to baseline frequencies in both blood and BAL. This trend in CD4⁺ T cell kinetics in peripheral blood has been similarly observed following trivalent inactivated influenza vaccination (Li et al., 2012b) and other acute respiratory infections including hantavirus (Lindgren et al., 2011) and influenza (Wilkinson et al., 2012). We have also previously shown CD8⁺ T cells that follow this pattern of short-lived expansion, although the mean peak frequency of CD38 and Ki67 expression is significantly higher compared to CD4⁺ T cells (Jozwik et al., 2015). To date, there are no reports investigating the CD4⁺ T cell phenotype in serial bronchial samples following human RSV infection. Our results showed that a large proportion of CD4⁺ T cells in BAL upregulated expression of activation and proliferation markers in infected subjects. While this finding was not statistically significant (probably due to a relatively small sample size), the peak frequency of activated and proliferating CD4⁺ T cells was significantly higher than in matched peripheral blood samples. As this was only observed in infected subjects, we conclude that this expansion was in response to RSV infection. However, the absence of tools in assessing the antigen-specificity of CD4⁺ T cells in human RSV infected limited us in characterising this in more detail.

Memory T cells have been grouped into four phenotypically distinct populations using the markers CD45RA and CCR7 in humans (Sallusto et al., 1999). Compared to their naïve counterparts, memory CD4⁺ T cells can respond to infectious challenge more rapidly at lower

antigen doses and requiring less co-stimulation (McKinstry et al., 2010). In general, Tem cells can display immediate effector function at inflamed peripheral tissues, whereas Tcm cells mainly home to secondary lymphoid organs with reduced effector function but bearing the capacity to proliferate and differentiate into effector cells following antigenic stimulation (Sallusto et al., 2004). However, these cells are relatively heterogenous in their differentiation and their function may be altered under different experimental models (Sallusto et al., 2004). In addition, the expression of various adhesion molecules and chemokine receptors are important for the trafficking patterns of these cells, and can result in localisation to sites of previous infection (Sallusto et al., 1999). Blood CD4⁺ T cells were found in three phenotypically distinct populations. Majority were naïve cells and these circulate through lymphoid organs where they may encounter antigen and upregulate effector functions (Cose, 2007). Moreover, similar frequencies of blood CD4⁺ T cells displayed a Tcm and Tem phenotype. In contrast, majority of the CD4⁺ T cells in BAL were Tem. Tem cells have been described to have the ability of rapid effector responses upon antigenic recall (Verhoeven et al., 2008). In neither compartment were CD4⁺ cells of the Temra phenotype, which is mainly associated with CD8⁺ T cells with cytolytic ability and production of IFN- γ and TNF- α (Hamann *et al.*, 1997). These observations have been made in other systems; the Tem cells were abundant in the lungs and proliferated in response to influenza virus stimulation (Purwar et al., 2011). Moreover, our results showed differential expression of markers associated with costimulation, memory and homing when comparing peripheral blood and BAL. In the periphery, the vast majority of CD4⁺ T cells expressed CD27 and CD28 which identifies them as relatively undifferentiated cells (Koch et al., 2008). This is not surprising as the majority of the circulating cells were of a naïve phenotype. Furthermore, the expression of these two receptors are required for T cell activation, providing costimulatory signals to naïve CD4⁺ T cells and enhancing IL-2 production (Jenkins et al., 1991; de Jong et al., 1991). In contrast, the largest population of cells in BAL consisted of differentiating CD4⁺ T cells at intermediate stages, which lose the expression of CD27, and subsequently CD28 at a later phase (Proserpio et al., 2016). This profile is typically seen in effector memory CD4⁺ T cells and may allow immediate effector function upon antigen encounter (Koch et al., 2008). Finally, CD62L expression may suggest capability of a T cell to migrate to secondary lymphoid organs (Catron et al., 2006). CCR5 expression on the other hand is associated with responding to inflammatory chemokines, which allows migration to

site of inflammation (Zhang et al., 2010). Given that most CD4⁺ T cells are naïve in the periphery, the CD62L⁺CCR5⁻ phenotype implies that these cells have the capacity to home to lymphoid tissue where they may encounter antigen. In comparison, CD62L expression in BAL was reduced as most cells in this compartment possess an effector memory phenotype. In mouse models of influenza infection, lung resident memory CD4⁺ T cells were found enhance viral clearance and survival (Teijaro et al., 2011). Conversely, memory CD4⁺ T cells isolated from the spleen failed to mediate protection to influenza infection, despite their migration to multiple tissues including the lung. Moreover, in mice deficient of CD8⁺ T cells and B cells, lung resident memory CD4⁺ T cells were found to eliminate low doses of influenza via a perforin-dependent pathway (McKinstry et al., 2012). Collectively, these findings highlight tissue compartmentalisation of memory CD4⁺ T cells as a major factor for immune-mediated protection. Our results also shown a trend in increasing CCR5 expression through to day 28. CCR5 contributes to migration of T cells to sites of inflammation and has previously been shown to be important in recruitment of CD8⁺ T cells to the lung during influenza infection (Kohlmeier et al., 2008). Therefore, CCR5 expression by lung CD4⁺ T cells may be used for recruitment of other cells to respond to RSV infection.

Although it is not generally recognised as a major function of virus-specific CD4⁺ T cells, there is evidence of CD4⁺ T cell-mediated cytotoxicity following acute and chronic infections, including influenza A, dengue, cytomegalovirus and HIV (Appay et al., 2002; Weiskopf et al., 2015; Wilkinson et al., 2012; Zaunders et al., 2004). In these studies, CD4⁺ T cells have been shown to upregulate perforin, granzyme B and CD107a, while downregulating the costimulatory molecules CD27 and CD28, typical features of cytolytic effector cells. We therefore investigated whether CD4⁺ T cells in response to RSV expressed any cytolytic markers. However, no changes in the expression of perforin and granzyme B were detectable in either blood and BAL CD4⁺ T cells during RSV infection, suggesting that these cells contribute little to direct cytotoxicity in this context.

Our results up to this point indicated that CD4⁺ T cells respond to RSV infection but the contribution of individual T helper subsets was unclear. Our knowledge of these subsets in the context of human RSV infection comes from paediatric studies and their contribution to disease severity remains controversial. In some studies, the ratio of IL-4 to IFN γ has been

shown to be increased with severe disease (Legg et al., 2003) and higher levels of IFN γ compared to IL-4 in nasal washes of infected infants have been associated with decreased severity (Garofalo et al., 2001). Conversely, Tabarani and colleagues reported only minimal levels of Th2 cytokines in RSV-infected infants suggesting they are not directly associated with immunopathology (Tabarani et al., 2013). On the other hand, IL-17 has been shown to be increased in tracheal aspirates (but not nasal washes) of RSV-infected infants that required mechanical ventilation compared with controls (Mukherjee et al., 2011). However, in a different study, IL-17 levels were found to peak during convalescence in non-ventilated patients, which might suggest an association with recovery (Faber et al., 2012). Taken together, the contribution of helper T cells to disease severity in RSV-infected infants remains unclear.

In RSV-infected adults, we found IFN- γ and TNF- α production with little detection of Th2 or Th17 cytokines. One disadvantage of relying on flow cytometry when working with low numbers of antigen-specific cells is that the frequency of cytokine-producing cells may not reach the limit of detection. To confirm our findings, we used IFN γ ELISpot assays to enumerate IFN γ producing cells following stimulation with RSV *in vitro*. As expected, PCR-subjects showed no increase in RSV-specific T cells through the different stages of infection, but we found a significant increase in IFN γ producing cells at day 10 of infected subjects. The main disadvantage of using IFN γ ELISpot over flow cytometry was the inability to distinguish between CD4+ and CD8+ T cells. To address this, we depleted CD8+ T cells from total PBMC populations using magnetic-activated cell sorting with at least 95% purity. Following stimulation of this fraction, IFN γ producing cells were still detected and therefore most likely represented RSV-specific CD4+ T cells. Collectively, these results suggest that the antiviral response in healthy adults is strongly Th1 biased.

Effective antiviral responses have commonly been shown to be mediated by the Th1 subset in various viral infections (Graham et al., 1994; Iijima et al., 2008). The minimal contribution of Th2 and Th17 cytokines in infected adults highlights the differences between adult and infant responses. The foetal immune system is heavily Th2 biased; *in vitro* stimulation of neonatal cord blood with PRR agonists revealed low levels of IFN- γ and TNF- α and high levels

of Th2-type cytokines (Angelone et al., 2006). Presumably, this Th2-bias prevent the pro-inflammatory Th1-mediated immune rejection of the foetus (Philbin and Levy, 2009). A number of mechanisms may account for the differential responses in infants; neonatal dendritic cells produce less IL-12, which is required for the development of the Th1 subset (Goriely et al., 2001). More recently, CD4⁺ T cells from cord blood have been shown to express higher levels of IL-4 receptor following RSV stimulation *in vitro* when compared to PBMCs from adults (You et al., 2013). The bias towards the Th17 cytokine production might also be explained by immaturity of the infant immune system. Black and colleagues found that infants express higher levels of IL-23R, ROR γ T and STAT3 which inversely relate to developmental age (Black et al., 2012). Moreover, acute RSV infection of infants causes increased production of Th17 polarizing cytokines by dendritic cells compared to healthy controls (Stoppelenburg et al., 2014). Together these features of the immature immune system in infants predispose the cellular responses toward Th2 and Th17 subsets. Together, these may explain why data from naturally infected infants are different from experimentally challenged healthy adults.

In human influenza infection, pre-existing CD4⁺ against internal proteins has been associated with lower viral titre and less severe symptoms on subsequent infection (Wilkinson et al., 2012). In the context of natural infection, Sridhar and colleagues also found that in the absence of cross-reactive neutralizing antibodies, CD8⁺ T cells against conserved viral epitopes correlated with protection against influenza (Sridhar et al., 2013). We therefore tested whether IFN- γ producing RSV-specific memory T cells in peripheral blood at baseline would correlate with protection from disease. There was a large overlap in frequencies of RSV-specific memory T cells in PBMCs of both infected and uninfected groups and the difference was not statistically significant. Furthermore, these RSV-specific memory T cells in peripheral blood did not correlate with cumulative viral load from RSV-infected subjects. One might argue that the small sample size prevent the finding of a statistically significant association, if it truly exists. However, it is more likely that the RSV-specific CD4⁺ T cells in peripheral blood are a surrogate marker of their counterparts in the lungs, and are therefore not directly involved in protection.

In conclusion, we characterised the expansion of CD4⁺ T cells in response to RSV infection, as shown by increased expression of activation and proliferation markers. However, these cells showed no evidence of cytotoxicity and, despite having distinct phenotypic profiles between blood and BAL, the expression of costimulatory, memory and homing markers showed no change through the course of infection. We showed that RSV-specific CD4⁺ T cell responses in healthy adults were restricted to the IFN γ producing Th1 subset. Although these showed no correlation with protection against RSV, overall CD4⁺ T cell responses were relatively modest and therefore more sensitive methods for tracking of RSV-specific CD4⁺ T cells are probably required to demonstrate their role.

3.3 Identification of candidate CD4+ T cell epitopes against RSV M37

3.3.1 Introduction

Epitopes recognised by CD4+ T cells are important targets for generating effective immunity against infectious diseases. In both mouse models (Hervas-Stubbs et al., 2006) and humans (Goletti et al., 2006), epitope-specific CD4+ T cells against *Mycobacterium tuberculosis* have been observed to correlate with protection. Moreover, immunization with a single CD4+ T cell epitope can induce vigorous CD4+ T cell responses and provide protection against rotavirus (McNeal et al., 2007) and salmonella (Kurtz et al., 2014). The development of future vaccines may therefore include epitopes with the aim of inducing robust CD4+ T cell responses. In addition, T cell epitopes can be used in the generation of tools (i.e. MHC class II tetramers) for better characterisation of antigen-specific cell-mediated immunity, which may provide further insights regarding correlates and mechanisms of protection (Jozwik et al., 2015).

T cell epitope mapping to identify novel peptide epitopes can be done using predictions *in silico* and/or experimental systems *in vitro*. Predictive methods have been developed for human HLA class II alleles which use algorithms to test the potential capacity of a given peptide sequence to bind the HLA of interest. There are a number of methods that are publicly available for quantitative class II epitope prediction; ARB (Bui et al., 2005), SVRMHC (Wan et al., 2006), MHCpred (Guan et al., 2003), NetMHCII (Nielsen et al., 2007) and SYFPEITHI (Rammensee et al., 1999). This approach provides candidate epitopes with theoretical binding affinity and has advantages in terms of determining the HLA restriction of a given sequence. However, there are limitations of using these algorithms especially when predicting class II binding. MHC class II proteins consist of α and β chains, which are highly polymorphic with both participating in peptide binding (Lundegaard et al., 2012). Wang and colleagues compared 10,000 MHC-peptide binding affinities, 29 peptide/MHC crystal structures and 664 experimentally tested peptides for CD4+ T cell responses and developed the consensus method (Wang et al., 2008). This combines the top three predictive methods for each MHC class II molecule and only achieved 76% accuracy against all HLA-DR molecules.

Purely experimental approaches for T cell epitope mapping rely on testing of synthetic peptides with a given length and overlapping sequences that span the candidate proteins. Peptide matrices can be generated such in a way that peptide pools derived from each column and row in a matrix of peptides are used for screening. This specific arrangement, such that each peptide is in only one row-pool and one column-pool, allows for efficient identification of responses at the intersection of positive pools. In this way, non-stimulatory peptides can be eliminated (Fiore-Gartland et al., 2016). This primary screening can then be followed by deconvolution for testing of individual epitope candidates. The screening using *in vitro* assays can be done by several different methods (Pira et al., 2010): lymphoproliferation, flow cytometry analysis of activation markers, intracellular cytokine staining, sandwich-ELISA or IFN- γ ELISpot. While these approaches have been extensively used to characterise MHC class I epitopes, the relatively low frequency of even immunodominant epitope-specific memory CD4⁺ T cells in human blood has represented a limitation in the detection of these cells *in vitro* using any of these strategies.

To date, comprehensive epitope mapping has not been undertaken to investigate human T cell responses against the RSV proteome. Nevertheless, following *in silico* predictions and focused investigation of specific RSV proteins using *in vitro* culture, a number of CD4⁺ and CD8⁺ T cell epitopes in human RSV have previously been reported. Class I-restricted epitopes have exclusively been found in the internal proteins of the virus within N, M, NS1 and NS2 (Goulder et al., 2000; Heidema et al., 2004; Jozwik et al., 2015). Rock and colleagues also used *in silico* prediction and *in vitro* binding assays to report 220 peptides from RSV or MPV restricted to MHC class I alleles (Rock et al., 2011). The major limitation of this study was that peptides were not tested using a cellular assay and it is likely that most of these peptides would not be recognised by naturally-generated T cells. CD4⁺ T cell epitopes have been studied to a lesser extent. van Bleek and colleagues designed overlapping peptides from the F protein of the RSV A2 strain and found 31 antigenic peptides recognised by CD4⁺ T cells in blood samples of healthy adults (van Bleek et al., 2003). In addition, de Waal and colleagues generated T cell clones from healthy adults and found a CD4⁺ T cell epitope within the central conserved region of the RSV G protein (de Waal et al., 2004). The major limitation of these studies were their biased nature as a result of the requirement to expand low level responses in the samples of healthy donors. In addition, the HLA restrictions of these epitopes were

inferred after testing of only a small number of individuals and could therefore not be confidently extrapolated to a general population.

3.3.2 RSV M37 strain is genotypically well conserved with other RSV strains

To construct a peptide library that spans the whole RSV proteome, we obtained the RSV M37 genomic sequence from Meridian Life Science (Memphis, Tennessee) and translated it with BlastX (NCBI). The nucleotide sequence was translated into polypeptides using all six possible open reading frames and each peptide sequence was then run against known RSV proteins in the reference database (NCBI accession number NC_001781.1). The hits were then filtered by setting an E value of $\leq 1e-6$ to ensure the smallest probability of finding a matching sequence by chance. With the exception of the G protein, each full length homologous protein was found in the M37 strain. G was split between two results, which was remedied by taking the results for the first half and filling the remaining half by manually examining the open reading frame prediction in that area until the stop codon. Finally, all predicted proteins were manually validated by comparison to the RefSeq entries (NCBI).

We used the multiple sequence alignment tool ClustalW to investigate the homology of the translated amino acid sequences with other reported RSV strains. Results are shown for the G and F proteins in Figure 3.3.1 and for the nine additional proteins in appendix (Supplementary Figure S2). There was close alignment of RSV M37 to both RSV A and B subtypes and sequences were well conserved for majority of the proteins, except G and M2.2 (Table 3.3.1). It is important to highlight that the sequence diversity in the G protein was primarily due to the variable regions at the N and C termini. In contrast, the central conserved region (amino acid positions 148 – 198) was at least 95% conserved with all RSV strains tested (Kim et al., 2014b).

	NS1	NS2	N	P	M	SH	G	F	M2.1	M2.2	L
RSVA A2	99.3	96.8	99.5	99.6	100	100	84.9	96.9	98.5	88.6	98.8
RSVB B1	87.1	89.5	95.4	91.3	91.8	72.3	50.8	90.8	93.3	62.2	92.0

Table 3.3.1 Percentage of RSV M37 sequence conservation with RSV A2 and B1 strains.

Table was generated using the sequence conservation data obtained from alignment with M37 stain using ClustalW software. Accession numbers used in the alignment are; RSVA A2 – M74568.1 and RSVB strain B1 – AF012354.1.

a

RSVM37	1	MELPILKTNAITTLAAVTLCPASSONITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
RSVA2	1	MELLILKANAITTILTAVTFCFASGONITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
RSVB	1	MELLIHRLSAIFLTLAINALYLTSSONITEEFYQSTCSAVSRGYFSALRTGWYTSVITIE
RSVM37	61	LSNIKENKCNGTDAKVKLIKQELDKYKNAVTELQLLMQSTPAANNRRARRELPRFMNYTLN
RSVA2	61	LSNIKENKCNGTDAKVKLIKQELDKYKNAVTELQLLMQSTPTNNRRARRELPRFMNYTLN
RSVB	61	LSNIKEKCNGTDTKVKLIKQELDKYKNAVTELQLLMQNTPAANNRRAREAPQYMNITIN
RSVM37	121	NTKNNVNTLSKKRRRRFLGFLLGVGSAIASGIAVSKVLHLEGEVKNKIKNALLSTNKAVVS
RSVA2	121	NAKKTNVNTLSKKRRRRFLGFLLGVGSAIASGVAVSKVLHLEGEVKNKIKSALLSTNKAVVS
RSVB	121	TTKNLVNVSISKKRRRRFLGFLLGVGSAIASGIAVSKVLHLEGEVKNKIKNALLSTNKAVVS
RSVM37	181	LSNGVSVLTSKVLDLKNYIDKQLLPVKNQSCSISNIETVIEFQOKNNRLLLEITREFSVN
RSVA2	181	LSNGVSVLTSKVLDLKNYIDKQLLPVKNQSCSISNIETVIEFQOKNNRLLLEITREFSVN
RSVB	181	LSNGVSVLTSKVLDLKNYINNQLLPVKNQSCRISNIETVIEFQOKNSRLLLEINREFSVN
RSVM37	241	AGVTPPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYV
RSVA2	241	AGVTPPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYV
RSVB	241	AGVTPPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYV
RSVM37	301	VQLPLYGVIDTPCWKLHSTPLCTTNTKEGSNICLRTDRGWYCDNAGSVSFFPQAETCKV
RSVA2	301	VQLPLYGVIDTPCWKLHSTPLCTTNTKEGSNICLRTDRGWYCDNAGSVSFFPQAETCKV
RSVB	301	VQLPLYGVIDTPCWKLHSTPLCTTNTKEGSNICLRTDRGWYCDNAGSVSFFPQAETCKV
RSVM37	361	QSNRVFCDTMNSLTLPSEVNLCNIDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT
RSVA2	361	QSNRVFCDTMNSLTLPSEINLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT
RSVB	361	QSNRVFCDTMNSLTLPSEVSLCNTDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKT
RSVM37	421	KCTASNKNRGIKTFSGNCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDP
RSVA2	421	KCTASNKNRGIKTFSGNCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDP
RSVB	421	KCTASNKNRGIKTFSGNCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDP
RSVM37	481	LVFPSDEFDASISQVNEKINQSLAFIRKSDLEHNVNKGSTTNIMITTIIIVIIIVILL
RSVA2	481	LVFPSDEFDASISQVNEKINQSLAFIRKSDLEHNVNKGSTTNIMITTIIIVIIIVILLS
RSVB	481	LVFPSDEFDASISQVNEKINQSLAFIRKSDLEHNVNKGSTTNIMITTIIIVIIIVILLS
RSVM37	541	LIAVGLFLYCKARSTPVTLSKDQLSGINNIAFSN
RSVA2	541	LIAVGLLLYCKARSTPVTLSKDQLSGINNIAFSN
RSVB	541	LIAVGLLLYCKARSTPVTLSKDQLSGINNIAFSN

b

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RSVM37   1  MSKTKDQRTAKTLEKTWDTLNHLLFISSCLYKLNKLSIAQITLSILAMIISTSLIIVAI
RSVA2    1  MSKNKDQRTAKTLEKTWDTLNHLLFISSCLYKLNKLSIAQITLSILAMIISTSLIAAII
RSVB     1  MSKHKNQRTARTLEKTWDTLNHLIVISSCLYKLNKLSIAQIALSVLAMIISTSLIAAII

RSVM37   61  FIASANNKVTLLTTAI IQDATSQIKNTTPTYLTONPOLGISFFNLSGTISQTAILALTP
RSVA2    61  FIASANHKVTPPTTAI IQDATSQIKNTTPTYLTONPOLGISFNPSEITSQITLILASTTP
RSVB     61  FIISANHKVTLTTVTVQTIKNHTEKNITTYLTQVPPERVSSSKOPTTSPIHNTSATSP

RSVM37   121 SVESILQSTTVKTKNTTTTQIQPSKPTTKQRQNKPPNKPNDDFHFEVFNFPVPCSI CSNNP
RSVA2    121 GVKSTLQSTTVKTKNTTTTQTQPSKPTTKQRQNKPPSKPNNDFFHFEVFNFPVPCSI CSNNP
RSVB     121 NTKSETHHTTAQTGRTTSTQTQNKPS TKPR LKNPPKPKDDHFEVFNFPVPCSI CGNNQ

RSVM37   181 TCWAICKRIPSKKPGKKTTTKPTKKPTIKTT-KKDLKPQTKPKAPTTKPTDKPTINIT
RSVA2    181 TCWAICKRIPNKKPGKKTTTKPTKKPTLKT-KKDPKPQTKSKVPTTKPTEPTINTT
RSVB     181 LCKSICKTIPSNKPKKKPTIKPTNKPTTKTTNKRDPKTPAKTTKETTTNPTKKPTLTTT

RSVM37   240 KPNIRTTLLTNS TTGNPEHTSQEETLHSTSSEGNTPSPSQLYTTSEYLSQPPSPSNITD-Q
RSVA2    240 KTNIIITLLTNS TTGNPELTSQMETFHTSTSSEGNTPSPSQVSTTSEYPSQPSPPNTPR-Q
RSVB     241 ERDTS TSQSTVLDTTTLEHTIQQOSLHSTTPENTPNSTQTPASE-PSTSNSTQNTQSHA

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Figure 3.3.1 Alignment of the translated amino acid sequence for RSV M37 G and F proteins with their counterparts in RSV A2 and B strains. Multiple sequence alignment tool ClustalW (version 1.83) was used to align the (a) F and (b) G protein sequences of RSV M37 against RSV A2 (accession number M74568.1) and B1 accession number NC_001781.1. Black box indicates positions of a fully conserved residue; grey box indicates conservation between groups of strongly similar properties – scoring > 0.5 in the Gonnet PAM 250 matrix; white box indicates conservation between groups of weakly similar properties – scoring ≤ 0.5 in the Gonnet PAM 250 matrix.

3.3.3 Stimulation of PBMCs with overlapping peptides show inter-individual variability in size and breadth of the T cell response to RSV

We constructed a peptide library that consisted of 891 15-mer peptides overlapping by 10 amino acids and that spanned the entire RSV M37 proteome. The peptides were pooled according to a matrix into 30 vertical (V1 – V30) and 30 horizontal (H1 – H30) pools, as shown in Table 3.3.2 Each PBMC sample was assayed for peptide-specific IFN- γ producing T cells using every horizontal and vertical pool by ELISpot. Peptides found at the intersections of pools that induced IFN- γ producing T cells were considered candidate epitopes and were further tested in deconvolution experiments.

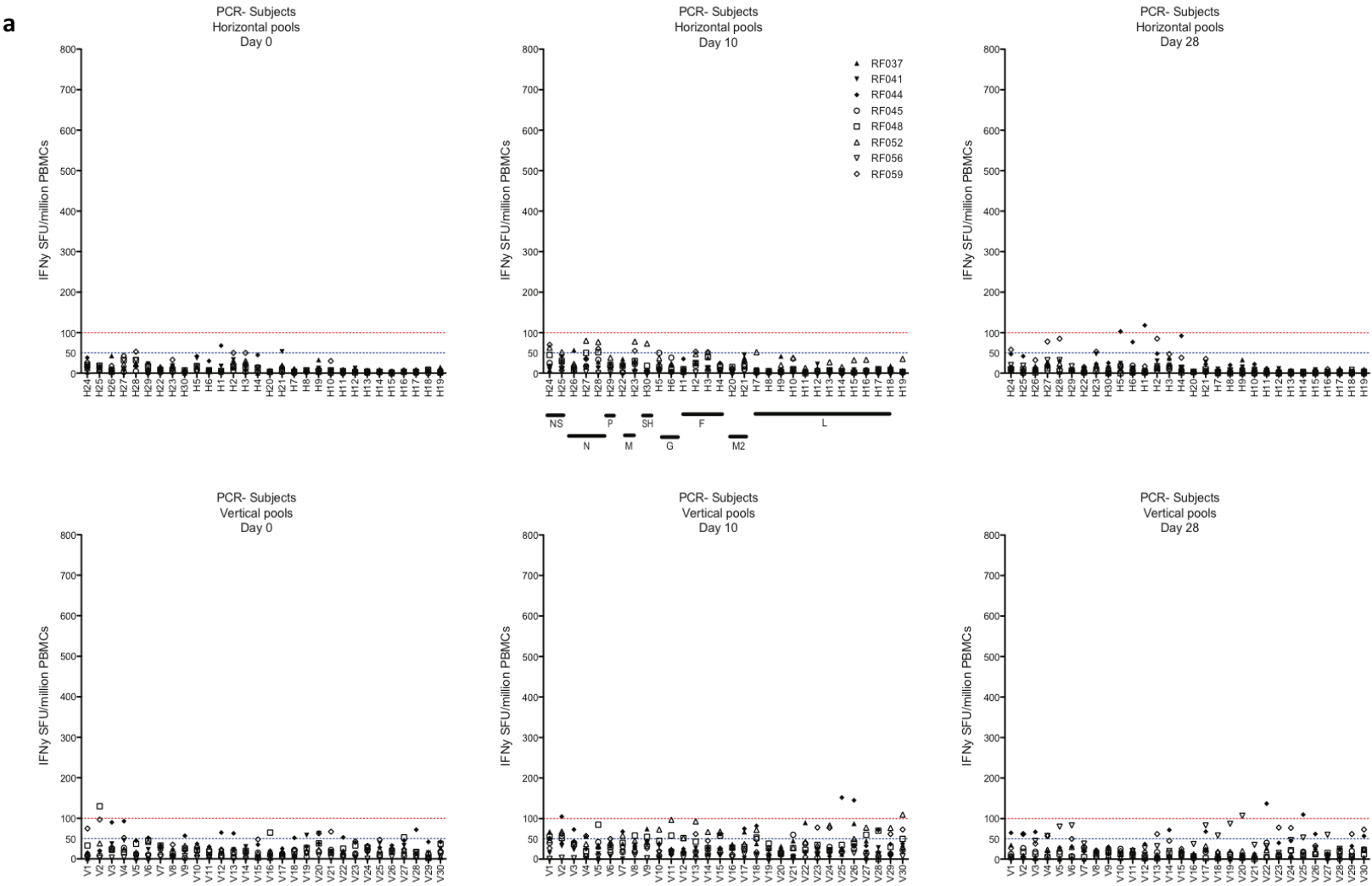
	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16	V17	V18	V19	V20	V21	V22	V23	V24	V25	V26	V27	V28	V29	V30
H1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
H2	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
H3	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
H4	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
H5	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150
H6	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
H7	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210
H8	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240
H9	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270
H10	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300
H11	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330
H12	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360
H13	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390
H14	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420
H15	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450
H16	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480
H17	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510
H18	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533	534	535	536	537	538	539	540
H19	541	542	543	544	545	546	547	548	549	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570
H20	571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	591	592	593	594	595	596	597	598	599	600
H21	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629	630
H22	631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	650	651	652	653	654	655	656	657	658	659	660
H23	661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684	685	686	687	688	689	690
H24	691	692	693	694	695	696	697	698	699	700	701	702	703	704	705	706	707	708	709	710	711	712	713	714	715	716	717	718	719	720
H25	721	722	723	724	725	726	727	728	729	730	731	732	733	734	735	736	737	738	739	740	741	742	743	744	745	746	747	748	749	750
H26	751	752	753	754	755	756	757	758	759	760	761	762	763	764	765	766	767	768	769	770	771	772	773	774	775	776	777	778	779	780
H27	781	782	783	784	785	786	787	788	789	790	791	792	793	794	795	796	797	798	799	800	801	802	803	804	805	806	807	808	809	810
H28	811	812	813	814	815	816	817	818	819	820	821	822	823	824	825	826	827	828	829	830	831	832	833	834	835	836	837	838	839	840
H29	841	842	843	844	845	846	847	848	849	850	851	852	853	854	855	856	857	858	859	860	861	862	863	864	865	866	867	868	869	870
H30	871	872	873	874	875	876	877	878	879	880	881	882	883	884	885	886	887	888	889	890	891									

Table 3.3.2 Epitope mapping with a 30x30 peptide matrix. 891 15-mer peptides overlapping by 10 amino acids were arranged into 30 vertical (V1 – V30) and 30 horizontal (H1 – H30) pools for testing on IFN- γ ELISpot. Final concentration of each peptide was 5 μ g/ml. For example, if V7 – V9 and H12 – H14 pools were stimulatory (highlighted in Table 3.3.2) all peptides in the intersection (337, 338, 339, 367, 368, 369, 397, 398, 399) could be antigenic. However, the same result would be obtained if only peptides 337, 368 and 399 were antigenic. Therefore, this would require an additional deconvolution step in which individual epitope candidates would be tested. Originating proteins of the peptides are; 1 – 113 from F, 114 – 171 from G, 172 – 603 from L, 604 – 657 from M2, 658 – 707 from M, 708 – 756 from NS1/2, 757 – 833 from N, 834 – 880 from P and 881 – 891 from SH. Amino acid sequences of all 891 peptides are listed in Supplementary Figure S3.

Freshly isolated PBMCs from study subjects in the 2013 cohort (n=8 uninfected, n=10 infected) at days 0, 10 and 28 were stimulated with the peptide pools for detection of IFN- γ producing cells using IFN- γ ELISpot. The threshold for a positive response was set to 50 spots/million PBMCs which has been validated to distinguish true responses in a number of independent studies, discussed further in the next section (Dubey et al., 2007; Gill et al., 2010; Schmitt et al., 1997). In individuals who remained uninfected, responses to peptide pools quantified by frequency of IFN- γ producing cells were minimal and remained below the threshold throughout the course of the infection (Figure 3.3.2a). On the other hand, responses to peptide pools in infected subjects was similar to the trend found with whole RSV stimulation, where number of IFN- γ producing cells peaked at day 10 before returning to baseline levels by day 28 (Figure 3.3.2b). Graphs showing responses for every individual can be found in the appendix (Supplementary Figure S4). The order in which the horizontal pools were arranged was based on sequential peptides from originating proteins. Therefore, we used the IFN- γ ELISpot responses to horizontal pools to estimate the magnitude of T cell responses against individual RSV proteins. This showed substantial inter-individual variability in the size and specificities of the responses, ranging from subject RF061, who had a total of 685 spots/million PBMCs, to subject RF054 with a total of 4891 spots/million PBMCs (Figure 3.3.3a). In order to estimate the magnitude of T cell responses to each protein, we normalised the data to protein length to avoid bias towards larger proteins. We found the highest responses in all subjects against the internal proteins NS1/2 (median = 397 spots per 10^6 PBMCs, range: 166-681 spots per 10^6 PBMCs), M (median = 392 spots per 10^6 , range: 45-787 spots 10^6 PBMCs), N (median = 318 spots per 10^6 , range: 82-1589 spots per 10^6 PBMCs) as well as surface protein F (median = 539 spots per 10^6 , range: 93-1460 spots per 10^6 PBMCs) which were dominant compared to rest of the viral proteins. To a lesser extent, all subjects responded to P (median = 119 spots per 10^6 , range: 27-341 spots per 10^6 PBMCs) and G (median = 166 spots per 10^6 , range: 33 – 812 spots per 10^6 PBMCs; Figure 3.3.4b). Responses to the remaining proteins were less common; 7/10 individuals had responses to L (median = 238 spots per 10^6 , range: 0 – 692 spots per 10^6 PBMCs) and M2 (median = 89 spots per 10^6 , range: 0 – 170 spots per 10^6 PBMCs), and 5/10 individuals responded to SH protein (median = 20 spots per 10^6 , range: 0 – 152 spots per 10^6 PBMCs). However, since each peptide pool

was made up of 30 peptides, whether the responses within each protein were due to a single epitope or a combination of many was not possible to determine at this stage.

Theoretically, there should be a relationship between IFN- γ responses to whole virus stimulation and to peptide pools, as these are derived from the viral proteome. As expected, the sum of IFN- γ producing T cells in response to peptide pools at day 10 significantly correlated with those in response to whole RSV stimulation ($r_s = 0.6727$, $p = 0.0390$; Figure 3.3.4a). However, once again we found no correlation between pre-existing RSV-specific T cells and reduced disease severity in our model (see discussion in section 3.2.6). In addition, we tested whether viral load might drive T cell responses by analysing the relationship between viral load and number of IFN-producing cells at day 10 post infection. However, we found no correlation between viral load and the sum of IFN- γ producing cells to all peptide pools ($r_s = 0.2000$, $p = 0.5837$; Figure 3.3.5b), to peptides from surface proteins ($r_s = 0.0303$, $p = 0.9460$; Figure 3.3.4c) or to peptides from internal proteins ($r_s = 0.1273$, $p = 0.7330$; Figure 3.3.4d).



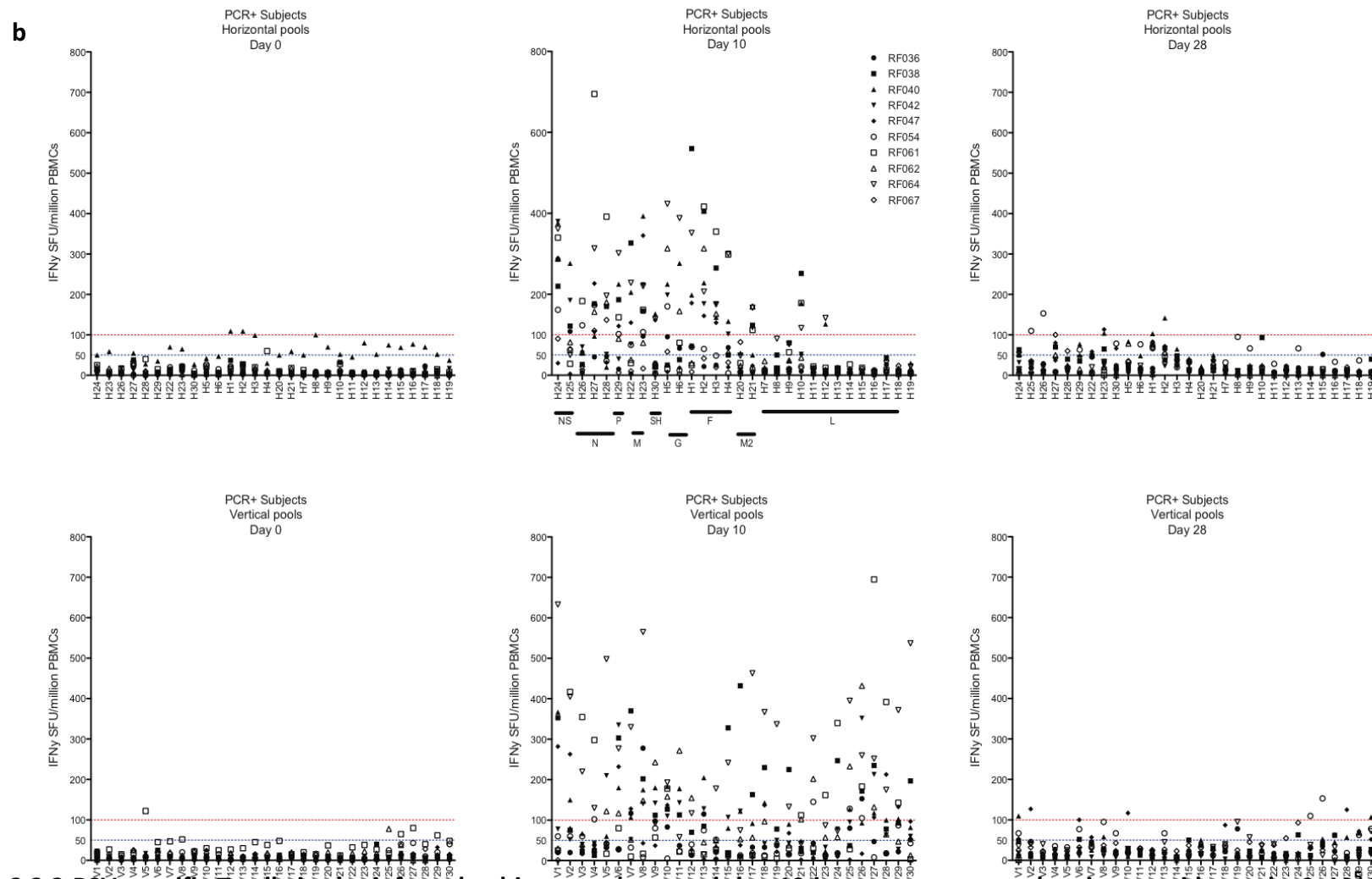


Figure 3.3.2 RSV-specific T cells in the infected subjects peak around day 10 in response to overlapping peptide pools. 2×10^6 PBMCs were cultured with overlapping peptide pools at days 0, 10 and 28. Graphs show the mean IFN- γ spot forming cells responsive to peptide pools in (a) uninfected and (b) infected subjects. Horizontal pools were organised in the order of appearance in the genomic sequence. All pools were tested in triplicate and the threshold for a positive response was ≥ 50 spots/million PBMCs.

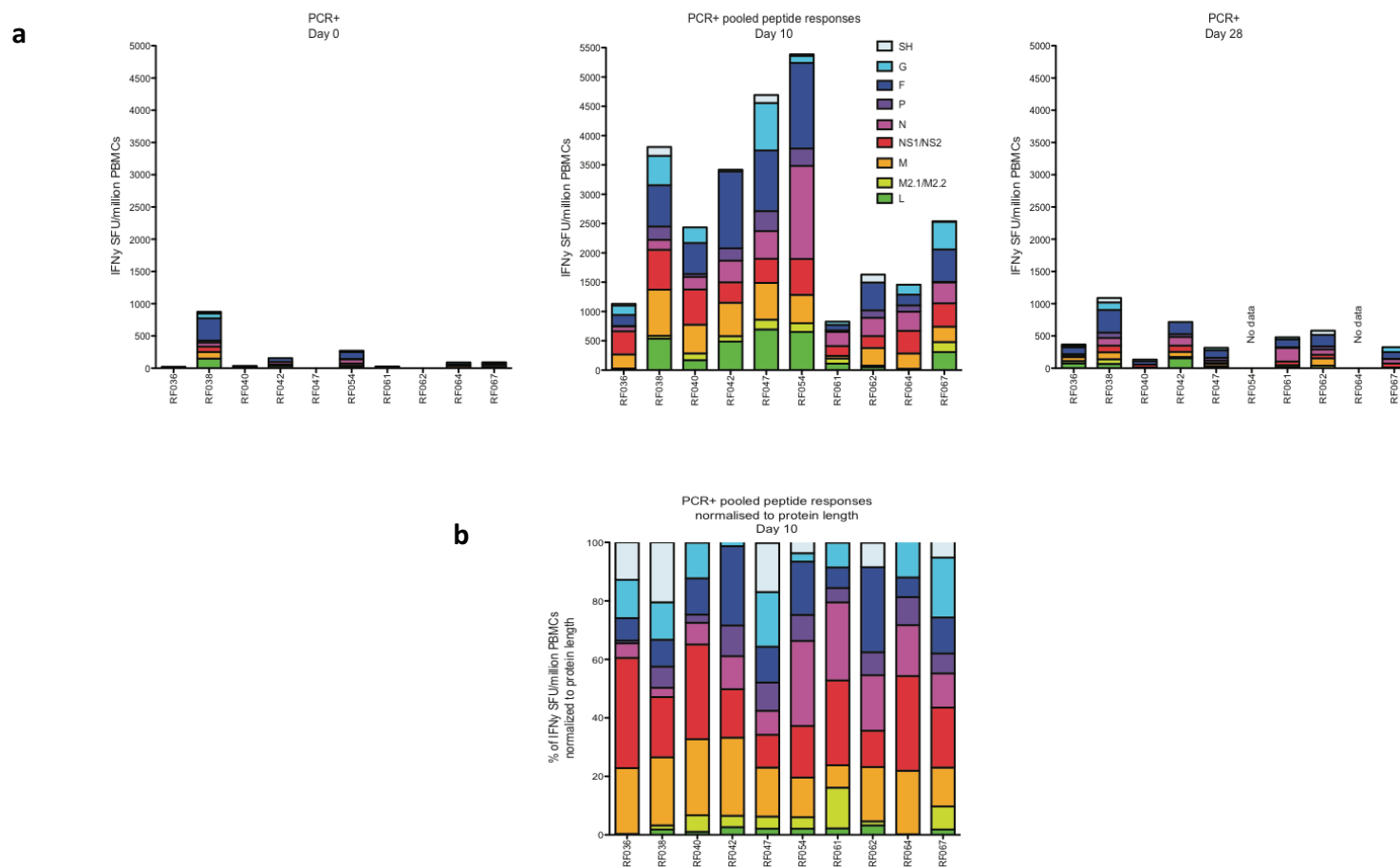


Figure 3.3.3 T cell responses to proteins within the RSV genome are mostly against NS1/2, M, F and G. The data represent the sum of IFN- γ spots detected upon peptide stimulations according to their originating proteins. (A) The graphs show T cell responses against each protein within the RSV M37 genome throughout the course of infection for the infected subjects. (B) The proportion of IFN- γ producing cells from day 10 PBMCs of infected subjects, normalised to the protein lengths. Threshold for a positive response was >25 spots/million PBMCs.

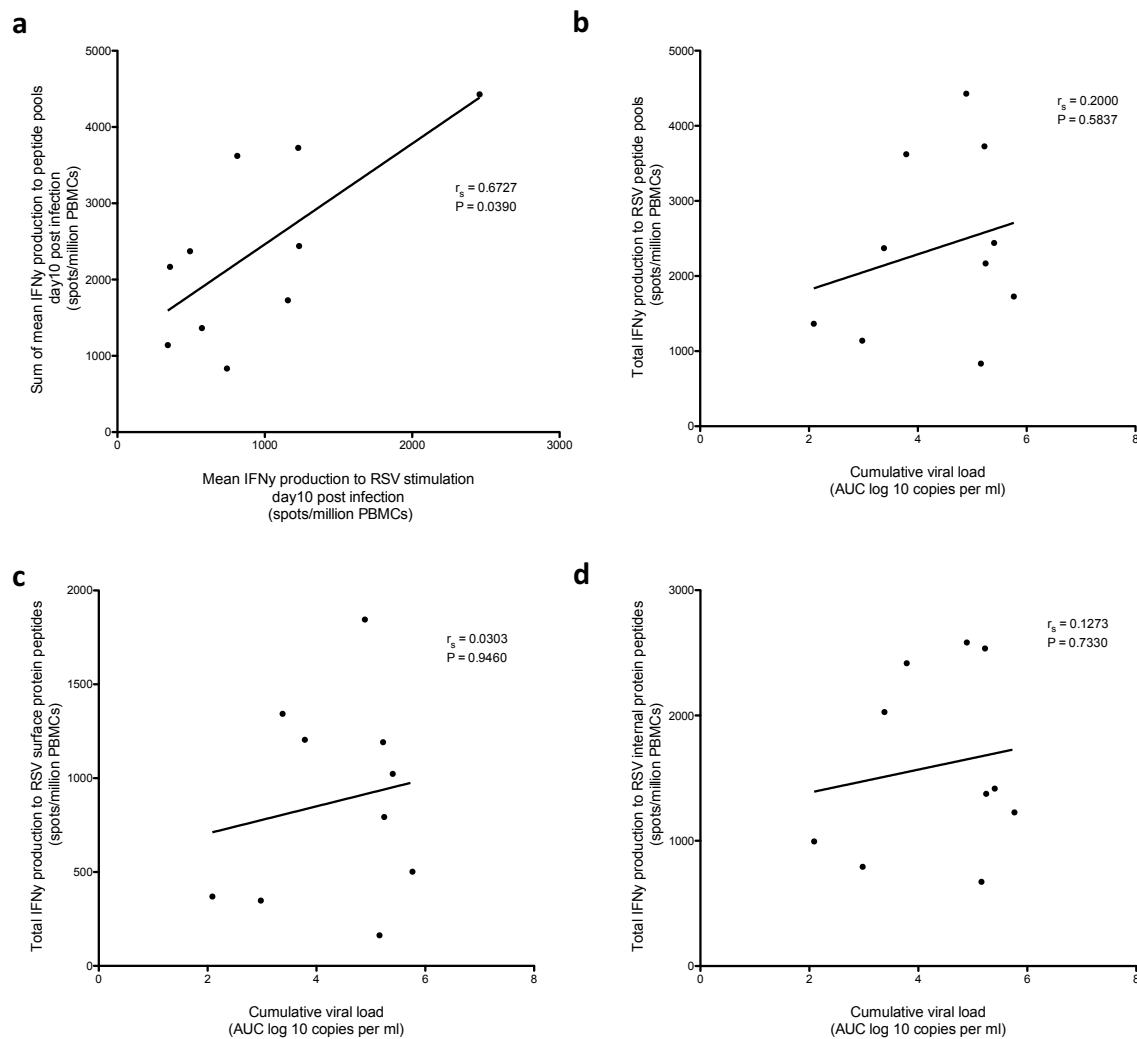


Figure 3.3.4 IFN- γ production in response to peptide pools correlate with responses to RSV stimulation, but not with viral load. Non-linear regression was used for infected subjects from the 2013 cohort ($n=10$) to assess the association between (a) IFN- γ production to peptide pool stimulation and responses to whole virus or (b) disease severity in infected individuals as measured by nasal viral load (trapezoidal area under the curve (AUC)). In addition, total IFN- γ response to (c) surface proteins and (d) internal proteins was examined for their relationship with nasal viral load. Spearman's rank correlation coefficient (r_s) and P values are shown.

3.3.4 Epitope mapping with pooled peptides indicates both CD4- and CD8-restricted T cell responses to RSV

From the time-points we investigated, it was apparent that the number of RSV-specific IFN- γ producing T cells was highest at day 10. Samples at this time-point were therefore further analysed to investigate the range of immunodominant T cell epitopes in RSV. In this study, the threshold for positive responses was therefore selected according to empirical criteria at ≥ 50 spots per 10^6 cells and also a statistical value of $p < 0.05$ by the DFR method.

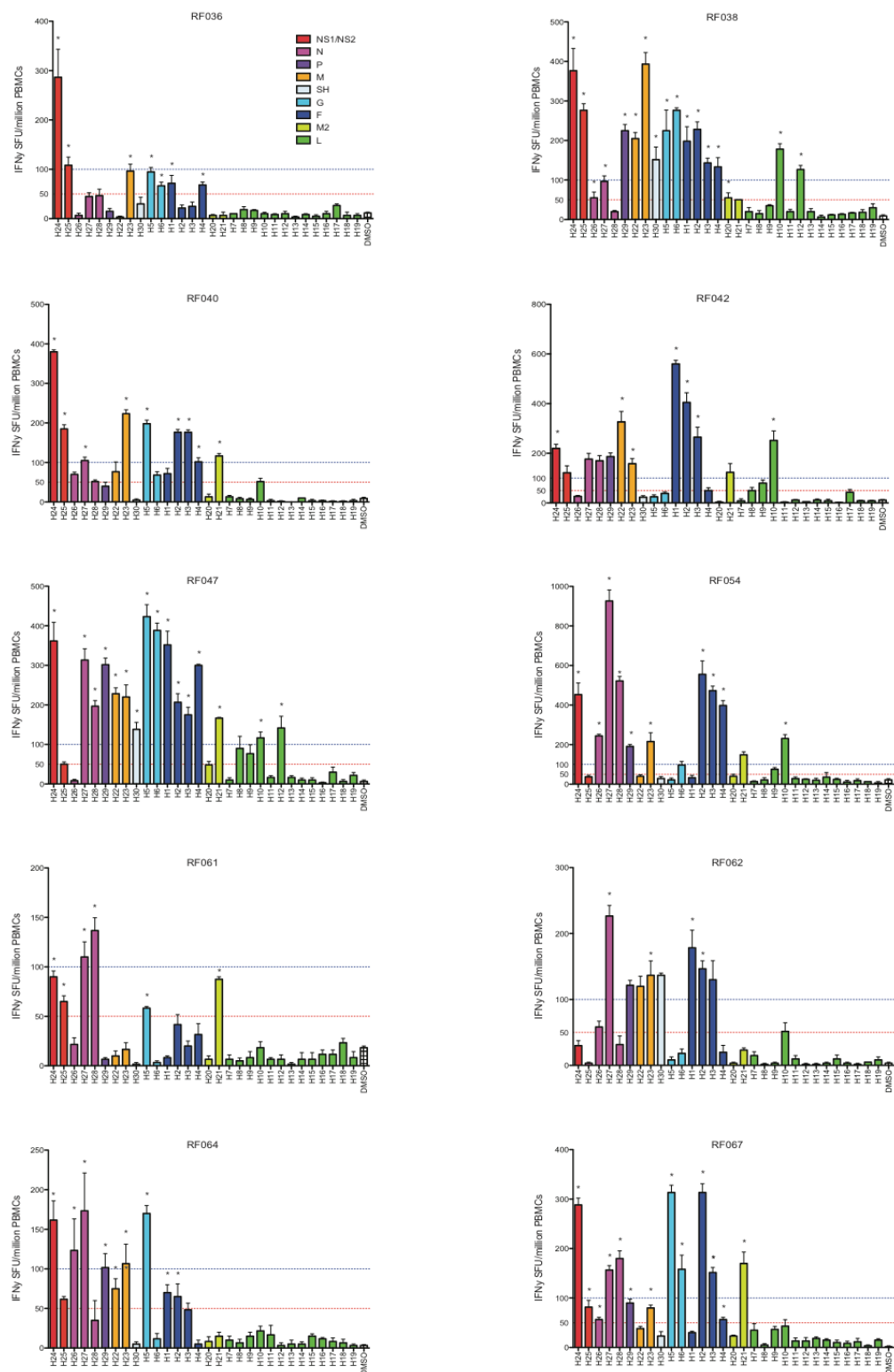
Despite some overlap in responses to peptide pools that originated from NS1/2, M, G and F proteins, it was clear that the size and breadth of responses varied greatly between individuals (Figure 3.3.5a, b). For example, for subject RF061, 6 horizontal and 6 vertical pools gave positive responses whereas subject RF038 had 17 horizontal and 22 vertical pools above the threshold (Table 3.3.3). To test every potential peptide using this threshold for some subjects was not feasible as the number of PBMCs isolated at day 10 post inoculation was a limiting factor.

The strategy so far involved stimulation of total PBMCs with 15-mer peptides meaning that both CD4+ and CD8+ T cells could contribute to IFN- γ production. Where number of isolated PBMCs permitted, we proceeded with a CD8+ T cell depletion step to identify the peptide pools primarily stimulating CD4+ T cell responses. In principle, if stimulation of CD8+ T cell-depleted PBMCs with previously positive peptide pools resulted in the loss of IFN- γ production, this would indicate that the peptides therein stimulating cytokine production were restricted to CD8+ T cells. Conversely, if there were no change in the number of IFN- γ spots after depletion, the stimulating peptides would most likely be recognised by CD4+ T cells.

CD8+ T cells were labelled with micro-beads and depleted by positive selection using MACS columns (see Materials and Methods) from 5 infected subjects to at least 98% purity (Supplementary Figure S5). These cells were then stimulated with peptide pools that were previously found to stimulate positive responses. In comparison with total PBMCs, CD8+ T

cell depletion resulted in either a) loss of IFN- γ production, b) a reduction in the number IFN- γ spots or c) no change (Figure 3.3.6). For instance, CD8+ T cell depletion for subject RF054 shown a remarkable decrease in number of IFN- γ spots for peptide pools H27 (mean 930 to 23 spots/million cells), V26 (mean 712 to 30 spots/million cells) and V27 (mean 745 to 67 spots/million cells). These were therefore likely to have contained epitopes recognised by CD8+ T cells that led to demonstrable IFN- γ production in stimulated total PBMCs. In other examples, depletion of CD8+ T cells from PBMCs in subject RF064 resulted in a decrease in IFN- γ production that nevertheless remained above threshold for peptide pools H23 (mean 106 to 60 spots/million cells) and V26 (mean 153 to 53 spots/million cells). This suggested that multiple epitopes stimulating both CD4+ and CD8+ T cells might have been in those pools. Finally, CD8+ T cell depletion did not alter the number of IFN- γ spots in response to some peptide pools (i.e. subject RF062, pools H22 and V28) which suggested CD4+ T cell-restriction of stimulating peptides therein.

a



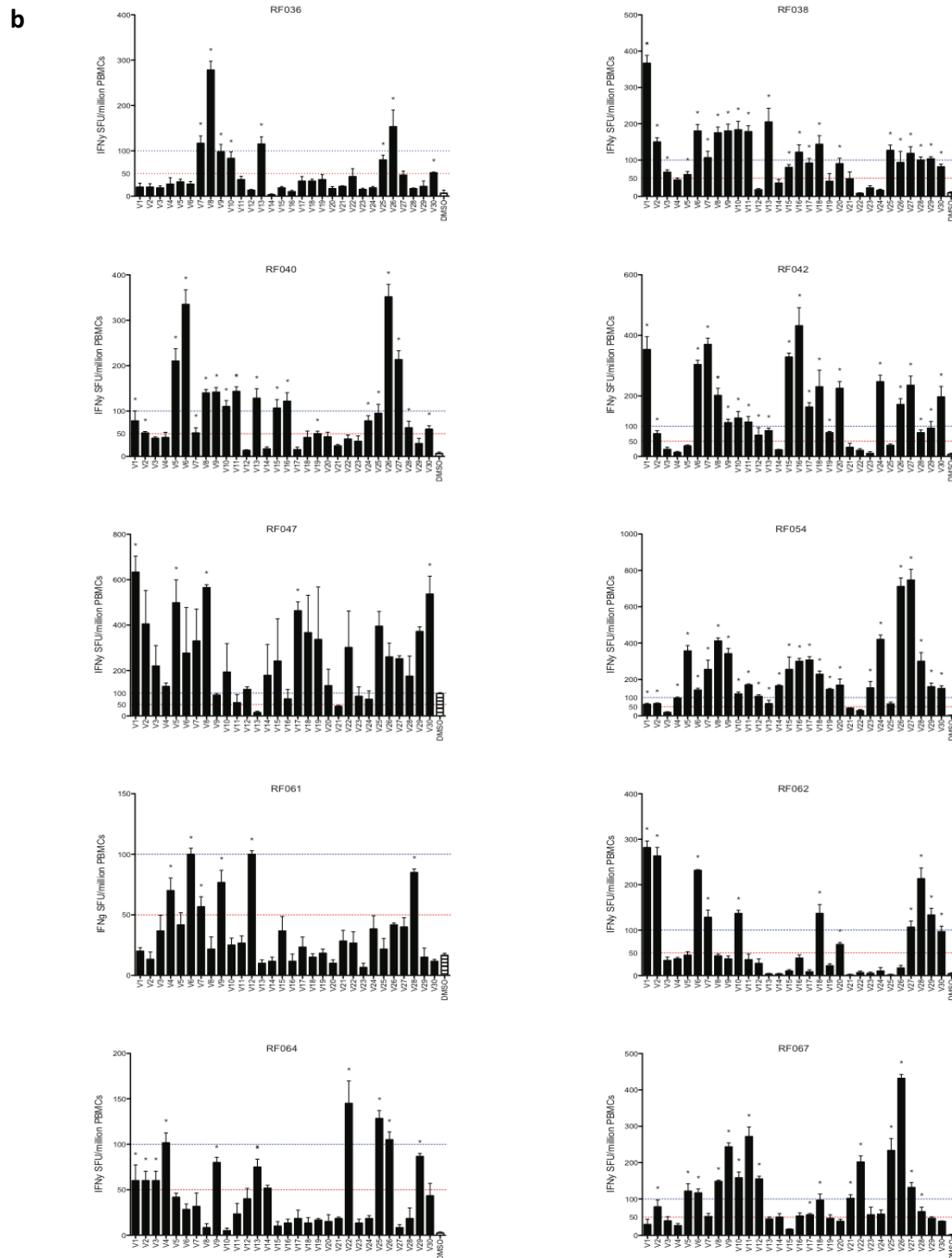


Figure 3.3.5 Day 10 responses to overlapping peptide pools show inter-individual variability in size and breadth of the response. 2×10^5 PBMCs from infected subjects ($n=10$) were cultured for 20 hours with overlapping peptide pools at day 10. Graphs show the mean $\pm$ s.e.m. IFN- γ spot forming cells responsive to peptide pools in (a) horizontal and (b) vertical pools of infected subjects. All pools were tested in triplicate. DMSO stimulation was used as a negative control. Threshold for a positive hit was ≥ 50 spots/million PBMCs and statistical analysis was carried out using the DFR method described by Moodie *et al.* where * = $p < 0.05$

	H24	H25	H26	H27	H28	H29	H22	H23	H30	H5	H6	H1	H2	H3	H4	H20	H21	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	Total
RF036	*	*						*		*	*	*			*																7
RF038	*	*	*	*		*	*	*	*	*	*	*	*	*	*	*					*		*								17
RF040	*	*		*				*		*			*	*	*		*														9
RF042	*						*	*				*	*	*							*										7
RF047	*			*	*	*	*	*	*	*	*	*	*	*	*		*				*		*								17
RF054	*		*	*	*	*		*					*	*	*						*										10
RF061	*	*		*	*					*							*														6
RF062				*				*				*	*																		4
RF064	*		*	*		*	*	*		*		*	*																		9
RF067	*	*	*	*	*	*		*		*	*		*	*	*																12
Total	9	5	4	8	4	5	4	9	2	7	4	6	8	6	6	1	3				4		2								

	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16	V17	V18	V19	V20	V21	V22	V23	V24	V25	V26	V27	V28	V29	V30	Total	
RF036							*	*	*	*			*												*	*			*		8	
RF038	*	*	*		*	*	*	*	*	*	*		*		*	*	*	*		*					*	*	*	*	*	*	*	22
RF040	*	*			*	*	*	*	*	*	*		*		*	*			*					*	*	*	*	*	*	*	19	
RF042	*	*				*	*	*	*	*	*	*	*		*	*	*	*	*	*	*			*		*	*	*	*	*	*	22
RF047	*				*			*									*												*		5	
RF054	*	*		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		*	*		*	*	*	*	*	*	26
RF061				*		*	*		*			*																*			6	
RF062	*	*				*	*			*								*		*						*	*	*	*	*	11	
RF064	*	*	*	*					*				*									*			*	*			*		10	
RF067		*			*	*		*	*	*	*	*					*	*			*	*			*	*	*	*			16	
Total	7	7	2	3	5	7	7	7	8	7	5	4	6	1	4	4	5	5	3	4	1	2	1	3	5	7	6	7	5	7		

Table 3.3.3 Summary of IFN- γ responses to horizontal (upper panel) and vertical pools (lower panel). * indicates responses ≥ 50 spots per 10^6 cells and a statistical value of $p < 0.05$ by DFR method. Total number of positive pools for each subject and total positive responses in each pool are also shown. Table created with results presented on Figure 3.3.4.

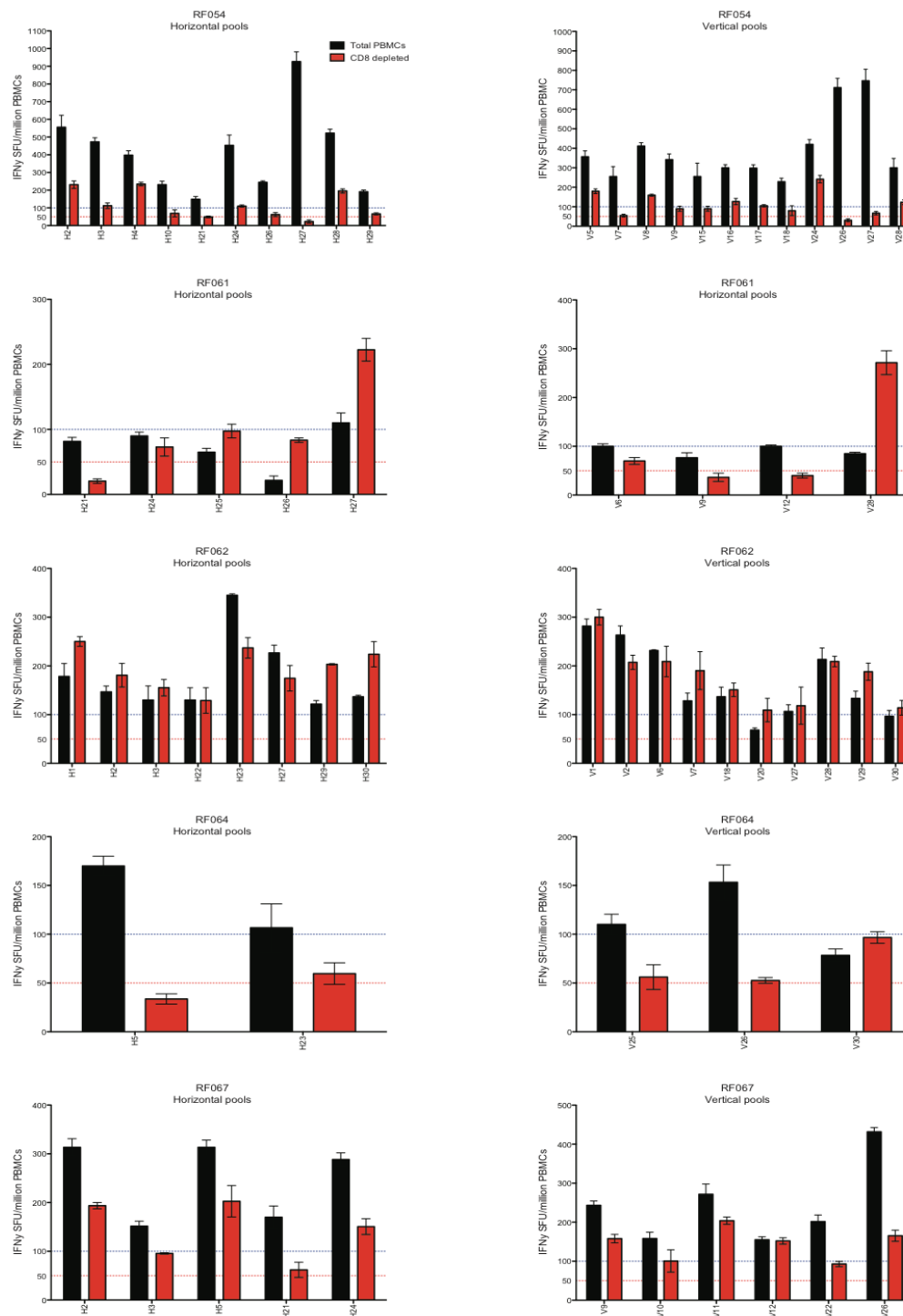


Figure 3.3.6 Depletion of CD8+ T cells reveals likely CD4+ restricted responses with pooled peptides. CD8+ T cells labelled with micro-beads were depleted manually using MACS columns from day 10 PBMCs of 5 infected subjects to at least 98% purity (Supplementary Figure 40). 2×10^5 depleted PBMCs were cultured with positive peptide pools for 20 hours. Graphs show the mean $\pm$ s.e.m. IFN- γ spot forming cells responsive to horizontal (left) and vertical (right) peptide pools in total PBMCs (black bars) or CD8+ T cell depleted samples (red bars). All pools were tested in triplicate.

3.3.5 Immunodominant RSV-specific T cell epitopes identified in internal and external proteins

There was a large number of positive peptide pools and the number of PBMCs required for the next set of experiments was a limiting factor. Therefore, we set a threshold of 100 sfu/million PBMCs for positive responses to focus on deconvolution of stimulating pools that resulted in the highest frequency of IFN- γ spots, which should therefore have contained the most immunodominant epitopes. PBMCs from 8 of 10 infected subjects were used to test individual peptides found in F, G, M, N and NS1/2 proteins (Figure 3.3.7). As we showed previously, the size of the response to a single peptide was highly variable between individuals. The number of peptides tested for each individual as well as those that stimulated at least 20 IFN γ producing cells per 10^6 PBMCs but were not tested are summarised in Table 3.3.4.

In order to identify common responses that might be applicable on a population level, we looked for peptides that stimulated IFN- γ producing cells with over a 2-fold increase compared to negative control in at least 3 subjects. In M protein, we found responses to peptides 698, 699 and 703 in 5 subjects (RF036, RF038, RF042, RF047 and RF064). Peptides 698 and 699 shared 10 amino acids and positive hits to both of these indicated that T cells recognized the overlapping region. This phenomenon was also observed within the G protein; peptides 145 and 146 were stimulatory for 4 subjects (RF038, RF047, RF064 and RF067). Finally, we found peptides 7 (F protein) and 808 (N protein) to be stimulatory in 3 subjects; RF036, RF042, RF062 and RF042, RF061, RF062 respectively. These findings are summarized in Table 3.3.5.

Although deconvolution experiments were carried out using total PBMCs and it was not possible using IFN- γ ELISpots to distinguish which T cell subset responded to these immunodominant epitopes, several publications indicated that the epitopes identified from within the internal proteins were CD8 $^{+}$ T cell-restricted. Of these, peptides 698 (M – LLV) and 703 (M – LGA) had been used to construct effective MHC class I tetramers (Heidema et al., 2004; Jozwik et al., 2015). In addition, peptide 808 (N – VML) was predicted as a CD8 $^{+}$ T

cell epitope *in silico* (Kim et al., 2014b). In contrast, candidate epitopes identified within the surface proteins had been suggested as potentially MHC class II-restricted in independent publications (van Bleek et al., 2003; de Waal et al., 2004), supporting the further investigation of these peptides from F (F-EFY) and G protein (G-DDF) as CD4+ T cell epitopes.

Subject ID	Number of peptides tested	20-49 spots per 10 ⁶ PBMCs	50-99 spots per 10 ⁶ PBMCs	>100 spots per 10 ⁶ PBMCs
RF036	40	2	2	1
RF038	6	1	0	0
RF042	27	1	0	0
RF047	54	4	2	0
RF061	42	7	2	2
RF062	11	4	0	0
RF064	19	9	3	0
RF067	30	7	1	0

Table 3.3.4 Summary of total number of individual peptides tested for each subject.

Peptide #	Number of hits (n = 8)	Peptide sequence	Originating protein
7	3	EFYQSTCSAVSKGYL	F
146	4	DDFHFEVFNFPVCSI	G
698	5	LLVITVTDNKGAFKY	M
703	5	LGAYLEKESIYYVTT	M
808	3	VMLRWGVLAKSVKNI	N

Table 3.3.5 Summary of immunodominant RSV-specific T cell epitopes and sequences.

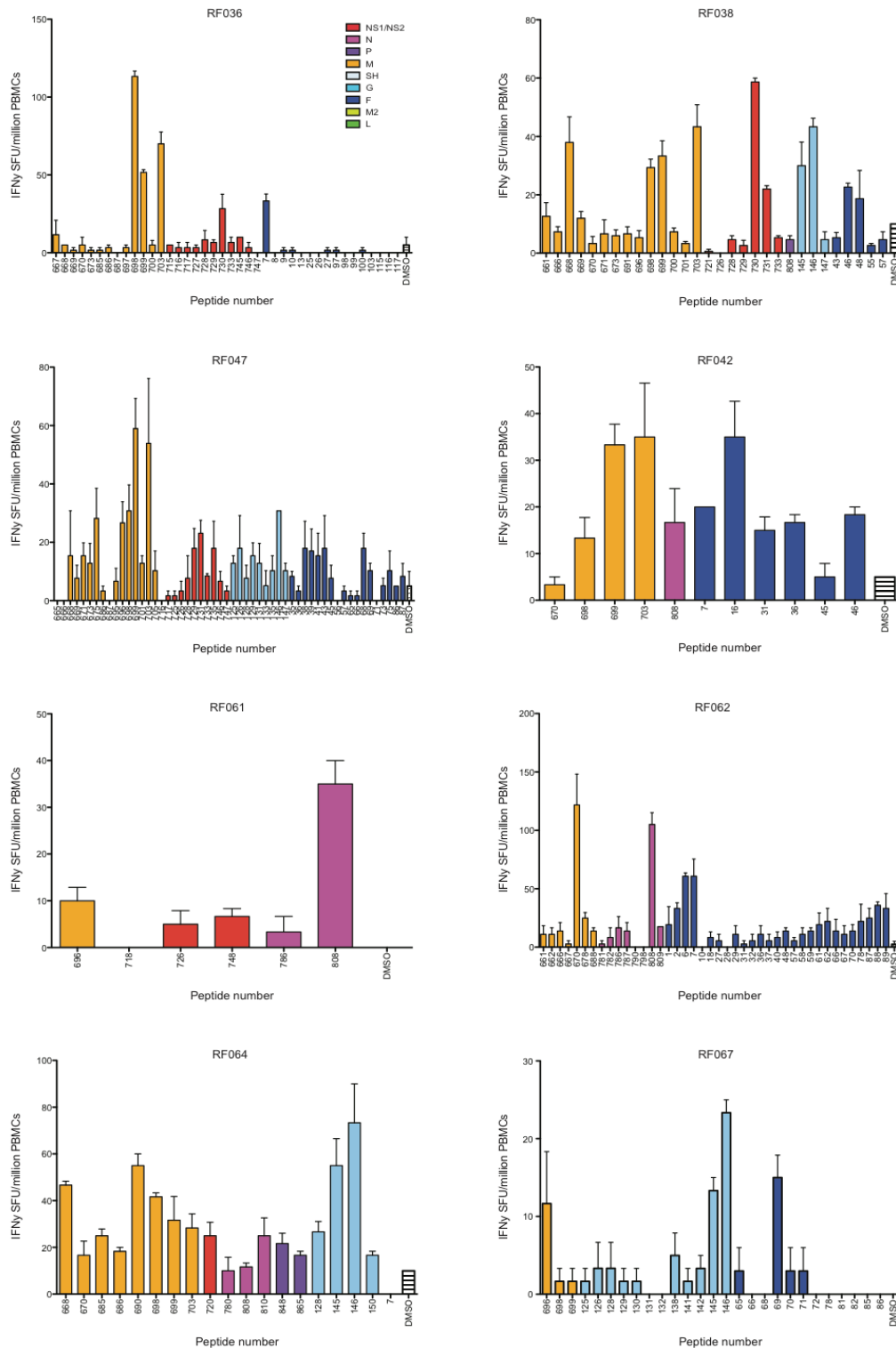


Figure 3.3.7 T cell responses to 5 highly immunodominant epitopes within F, G, M and N proteins. 2×10^5 PBMCs from day 10 samples of 8 infected subjects were cultured with candidate T cell epitopes identified in previous experiments. Graphs show the mean $\pm$ s.e.m. IFN- γ spot forming cells to individually tested peptides. All pools were tested in triplicate. DMSO stimulation was used as a negative control.

3.3.6 Discussion

CD4+ and CD8+ T cells have been shown in mouse models of RSV infection to mediate viral clearance with depletion of these cells resulting in prolonged viral shedding (Graham et al., 1991b). However, this also reduced severity of disease with the lack of T cells associated with less severe weight loss during primary infection (Graham et al., 1991b). Conversely, in severely ill children, antigen-specific CD8+ T cells have been proposed to mediate viral clearance without enhancing severity of disease (Heidema et al., 2007). Meanwhile, the frequency of activated T cells in blood was higher in RSV-infected adults who required hospitalisation compared to less ill outpatients (Walsh et al., 2013). Therefore, the role of T cells in human RSV pathogenesis remains controversial.

CD4+ T cell-polarising cytokines in human RSV infection have been extensively studied in clinical samples from hospitalised individuals (Christiaansen et al., 2014). However, the breadth of the T cell response and antigen-specificity of CD4+ T cells have not been systematically investigated. To address these using the experimental challenge model, we first translated the genomic sequence of RSV M37 using *in silico* methods. Alignment of this sequence against known RSV A2 and B strain sequences in GenBank showed a high level of conservation for most proteins, confirming the accuracy of the predicted protein sequences via their relationship with proteins from several other RSV strains. Recently, Kim and colleagues also compared the sequence of RSV M37 to other published sequences and found at least 85% similarity to other RSV A strains (Kim et al., 2014b). The least homology was found in the G protein due to the variable regions found at the N- and C- termini. Nevertheless, the central cysteine loop region was relatively well conserved. In addition, it is important to appreciate the high level of similarity between the RSV M37 and recently circulating strains of RSV as well as laboratory-adapted strains (i.e. RSVA A2), which supports the applicability of these experimental findings with the M37 strain to contemporary natural infection.

We used the translated sequence of RSV M37 to construct an overlapping peptide library spanning the entire viral genome. The peptide binding grooves of MHC class I and class II typically accommodate 9-mers and 15-mers, respectively (van Bleek and Nathenson, 1991;

Rudensky AY et al., 1991). However, testing two different libraries of variable peptide lengths would be impractical due to time, costs and limitations in samples. Peptide sets of 15 amino acids in length that overlap by 10 amino acids have been shown to be a good compromise for epitope mapping of CD4⁺ and CD8⁺ T cells in previous studies (Betts et al., 2001; Kiecker et al., 2004; McDermott et al., 2014). Betts and colleagues compared CD4⁺ and CD8⁺ T cell responses to different HIV Gag peptide mixtures and found that the highest frequency of IFN- γ was produced against 15-mers compared to 11-mers and 20-mers (Betts et al., 2001). Based on published data and practical considerations, the peptide library for RSV M37 was synthesised as 15-mers that overlap by 10 amino acids which resulted in a total of 891 peptides that were pooled using a 30x30 matrix. Epitope mapping using these peptide pools could have been assayed using IFN- γ ELISpot or flow cytometry. These methods were previously compared and produced results that statistically correlated for enumeration of antigen-specific T cells (Karlsson et al., 2003). As whole-RSV stimulation resulted only in production of IFN- γ from the panel of cytokines investigated, multi-parameter analysis by flow cytometry would have been unlikely to provide any advantage over IFN- γ ELISpot in terms of additional functional data. In addition, ELISpot has an advantage when used for detection of cells that are of low frequency due to the sensitivity of the technique with a lower limit detection of at least 25 IFN- γ producing T cells per 10⁶ PBMCs (Schmittl et al., 1997).

Two approaches were combined in determination of a cut-off value for positive responses. In the published literature, an empirical approach is commonly used, which sets a threshold value at some point in the difference between the mean number of spots in the experimental wells and the negative-control, and/or an x-fold change between the means of the antigen-stimulated and negative-control wells (Moodie et al., 2010). Although these methods are somewhat arbitrary, it has been used in a number of studies, including vaccine trials, in establishing thresholds for a positive response (Dubey et al., 2007; Gill et al., 2010; Schmittl et al., 1997). Dubey and colleagues tested HIV gag, nef and pol peptides in PBMCs from 20 seronegative individuals to produce a model with antigen/mock ratio that defined positivity as ≥ 55 spots per 10⁶ cells and ≥ 4 -fold increase compared to negative control (Dubey et al., 2007). They then validated this using the same strategy but with samples from 6 HIV-infected

individuals and all responses fell in the positive region. The second approach uses statistical testing, developed by Moodie and colleagues, designed to compare antigen-stimulated and negative-control replicates (Moodie et al., 2006). This distribution-free resampling (DFR) method is a nonparametric test that compares background and experimental wells using a Westfall-Young permutation with a false-positive rate of 5% (Westfall and Troendle, 2008).

To date, there have been no studies that have investigated human T cell responses to peptides spanning the entire RSV proteomic sequence. Wilkinson and colleagues used a similar strategy to characterise responses to influenza H1N1 and H3N2 proteins using PBMCs from experimentally challenged subjects at baseline, day 7 and 28 (Wilkinson et al., 2012). In mice, CD4⁺ T cells isolated from the spleen after primary infection were tested against overlapping peptides that originated from all 11 proteins of RSV (McDermott et al., 2014). As a result, broad specificity of T cells was reported with CD4⁺ T cells recognising 6 of the 11 RSV-derived proteins (G, F, N, P, M2 and L), demonstrating some overlap with our own human data but with notable differences in terms of responses to NS1/2, M and L. Stimulation of PBMCs from experimentally challenged subjects with RSV-derived peptide pools showed limited memory T cell responses at baseline, with 9 out of 10 subjects having less than 200 spot-forming cells per million PBMCs responding to RSV in total. This finding was similar to the proportion of pre-existing T cells against influenza H3N2 (Wilkinson et al., 2012). However, there is a suggestion that influenza specific responses might be more focused on individual proteins in contrast to RSV where multiple proteins were recognised at baseline. The kinetics of the IFN- γ producing T cell response to RSV peptides correlated with the activation and proliferation phenotype of CD4⁺ T cells (see section 3.3.3) and there was expansion of T cells at day 10 in all infected subjects. This was similar to influenza H3N2 infection, where the breadth and the proportion of T cells responding to influenza peptides increased in PBMCs of infected subjects and T cell responses were detected against all H3N2 proteins (Wilkinson et al., 2012). However, there was substantial variability between individuals in terms of the size and the breadth of response. As T cell responses were highest at day 10 of the infected subjects, we concentrated on this time point for further deconvolution experiments. Organisation of the horizontal peptide pools in order of the genomic sequence provided more clues to why there might be limited T cell responses against M2 and L proteins. For RSV, the gene order and polar gradient of sequential

transcription are thought to be major factors in transcriptional control of viral mRNA synthesised during infection. Krempl and colleagues shown that the promoter-proximal genes NS1 and NS2 mRNA are more than two-fold more abundant compared to the N-P-M-G-F-M2 block of genes (Krempl et al., 2002). This may be one of the factors explaining the low level of responses to the L protein, as it is the most distant gene from the promoter. This was demonstrated in HIV as CD8⁺ T cell responses against the early proteins, Tat, Rev and Nef have been found to be better targets for inhibition of viral replication than the late expressed structural proteins Gag, Pol and Env (van Baalen et al., 2002; Loffredo et al., 2005). In addition, not all translated proteins will be packaged into virions, and it could be speculated that the early viral proteins will be more abundant, which increases the amount of antigen presentation and likelihood recognition by T cells. On the other hand, limited responses against SH and M2 proteins may be due to their sizes (64 and 194 amino acids in length, respectively) (Collins et al., 1990; Cuesta et al., 2000). The relatively small sizes of these proteins may mean a lower chance of containing peptide sequences that bind HLA. By day 28, most RSV-specific responses had contracted to baseline levels. A similar trend was also observed in influenza, where immunodominant responses were still detectable at very low levels at day 28 whereas most newly generated responses against other proteins had diminished (Wilkinson et al., 2012). Collectively, these could explain the responses seen against RSV proteins with, in general, the highest IFN- γ responses against early proteins NS1/2, N, P, M, G and F with much lower frequencies directed towards the later proteins M2 and L.

Peptide pools that induced IFN- γ production were used in the 30x30 matrix to identify potential T cell epitopes. This resulted in a large number of peptides to be deconvoluted, and with limited number of stored PBMCs, only the most immunodominant responses could be tested. These were peptides that originated from the internal proteins NS1/2, M, N and from the surface proteins F and G. To identify epitopes that would be recognised most commonly at a population level, we selected stimulatory peptides that were recognised by at least 3 infected subjects. This resulted in identification of 5 immunodominant T cell epitopes. However, their CD4⁺ or CD8⁺ restriction remained ambiguous. It is generally believed that cellular immune responses offer broad cross-protection through recognition of conserved internal viral proteins. There have been a number of publications that provide evidence of

this in the context of influenza infection (Machkovech et al., 2015; Sridhar et al., 2013; Testa et al., 2012). Indeed, the T cell responses we found against the RSV internal proteins were against highly conserved sequences described or predicted to be immunodominant CD8+ T cell epitopes (Heidema et al., 2004; Jozwik et al., 2015; Kim et al., 2014b). CD4+ T cells typically, but not exclusively, recognise antigens derived from surface proteins, in order to help B cells to produce antibody responses. Antibodies recognise their epitopes in three-dimensional structures rather than linear sequences and therefore mainly respond to surface antigens (McLellan et al., 2013a). Given the fact that CD4+ T cells and B cells form cognate interactions in order to allow bidirectional signalling; it might not be surprising that CD4+ T cell epitopes commonly originate from surface proteins (Crotty, 2014). In influenza, CD4+ T cell epitopes have been reported against the surface HA protein (Babon et al., 2012; Danke and Kwok, 2003), which are thought to specifically help production of anti-HA antibodies (DiPiazza et al., 2016).

A few focused studies have been undertaken previously to identify epitopes in the F and G proteins of human RSV (van Bleek et al., 2003; de Waal et al., 2004). However, there were several limitations to these studies, including need to expand T cells *in vitro*, the small sample sizes and limited determination of the HLA restriction (discussed in more detail in the next chapter). Nevertheless, these data predicted that F-EFY and G-DDF might stimulate CD4+ T cells of infected subjects. We also found responses to other novel peptides in the F protein, these were not common to a number of subjects, and could represent subdominant epitopes. Furthermore, whilst the immunodominant responses may be ascribed to public T cell responses, the less common responses may be due to private T cell repertoires. Public TCRs are present and recognise specific epitopes in the majority of individuals of the same haplotype whereas private TCRs allow immune responses to specific epitopes that are rarely observed in multiple individuals (Venturi et al., 2008). In this regard, it is possible that responses observed in single individuals may represent a private T cell response. This highlights the importance of testing candidate epitopes in a number of individuals to distinguish between public and private T cell responses, which has been a major limitation of previous studies that characterised RSV specific CD4+ T cell epitopes. Furthermore, many other peptide pools induced IFN- γ responses, mostly towards the internal proteins. Although these were not deconvoluted due to limited number of PBMCs from the infected subjects,

they are likely to contribute to the variability of immune responses seen between individuals exposed to RSV. In future studies, next generation sequencing of TCR repertoires in RSV might elucidate the relative contributions of public and private T cell responses.

In conclusion, we have shown for the first time the degree of variability in breadth and the size of human RSV-specific T cell responses. These responses were highest at day 10 post-infection, and responses were predominantly directed towards proteins at the start of the viral genome. We confirmed several immunodominant CD8⁺ T cell epitopes found within the internal proteins M and N, and identified immunodominant novel candidate CD4⁺ T cell epitopes in the surface proteins F and G.

3.4 Immunodominant CD4+ T cell epitopes in human RSV

3.4.1 Introduction

Peptides are known to be promiscuous in their binding to class II molecules, which means a single immunogenic peptide is capable of binding multiple HLA types (Southwood et al., 1998). While this may be advantageous in eliciting T cell responses using vaccines even in genetically heterogeneous human populations, it does make defining the HLA restriction of class II epitopes more challenging (Greenbaum et al., 2011). To some extent, the likelihood of an individual epitope binding to related class II molecules can be predicted. HLA–DR and –DP molecules can be grouped into supertypes based on certain motifs at the peptide binding groove (Greenbaum et al., 2011; Nielsen et al., 2008; Sidney et al., 2010; Southwood et al., 1998). Peptides bound to HLA-DRB1 molecules *01:01, *04:01 and *07:01 have been found to bear a motif characterised by a large aromatic or hydrophobic residue in position 1 and a small, uncharged residue in position 6 of the peptide binding groove (Southwood et al., 1998). Therefore, peptides binding these residues were shown to be cross reactive for DRB1 molecules *09:01, *13:02, *15:01 and to a lesser extent for *04:05, *08:02 and *11:01. For HLA-DP molecules, a common motif has been characterised with a phenylalanine at position 1, aromatic or hydrophobic residues in position 6 and a leucine at position 9 of the peptide binding groove (Andreatta and Nielsen, 2012). Peptides bearing this motif have been reported to be cross-reactive for DPA1*02:01/DPB1*01:01, DPA1*02:01/DPB1*05:01 and DPA1*03:01-DPB1*04:02.

Prediction of class II restrictions of epitopes therefore has variable success depending on the alleles of interest. In addition, degenerate HLA–DR and –DP binding peptides are relatively common in view of these well characterised binding motifs. Therefore, it is important to validate *in silico* prediction data using *in vitro* assays that confirm the functionality of CD4+ T cells in response to candidate epitopes.

3.4.2 Immunodominant epitopes in F and G proteins are restricted to CD4+ T cells

Previous publications have indicated that several immunodominant epitopes we found within the internal proteins were stimulating CD8+ T cells, whereas the epitopes within F and G might be restricted to CD4+ T cells. These studies, conducted using PBMCs from healthy adults, resulted in a number of potential epitopes within each protein (van Bleek et al., 2003; de Waal et al., 2004).

To confirm that the epitopes we identified were specific to CD4+ T cells, we depleted CD8+ T cells as described previously to at least 98% purity (Figure 3.4.1a). These cells were then cultured with the peptides F-EFY or G-DDF using IFN- γ ELISpots, comparing with responses to DMSO as negative control (Figure 3.4.1b,d). Using PBMCs from 3 infected subjects each, we showed that both F-EFY and G-DDF induced significantly higher frequencies of IFN- γ spots than negative controls (Figure 3.4.1c,e). Furthermore, the number of IFN- γ spots formed by CD8-depleted cells were not reduced compared with total PBMCs when stimulated, strengthening the conclusion that these epitopes were recognized by CD4+ T cells.

In order to confirm our findings from these IFN- γ ELISpot assays and to assess the functionality of CD4+ T cells in response to the F-EFY and G-DDF epitopes, we stimulated day 10 PBMCs from infected individuals with peptides of interest for 6 hours and analysed cytokine production by ICS and FACS (Figure 3.4.2). Although the frequencies were lower, we found that stimulation with these peptides also induced IFN- γ production by RSV-specific CD4+ T cells. However, staining of stimulated cells for other Th1 cytokines to assess the polyfunctionality of the antigen-specific cells revealed no IL-2 (Figure 3.4.2) or TNF- α production (results not shown).

In conclusion, our results show using two independent methods that F-EFY and G-DDF are immunodominant epitopes recognised by CD4+ T cells, with the HLA class II restriction of these epitopes yet to be determined.

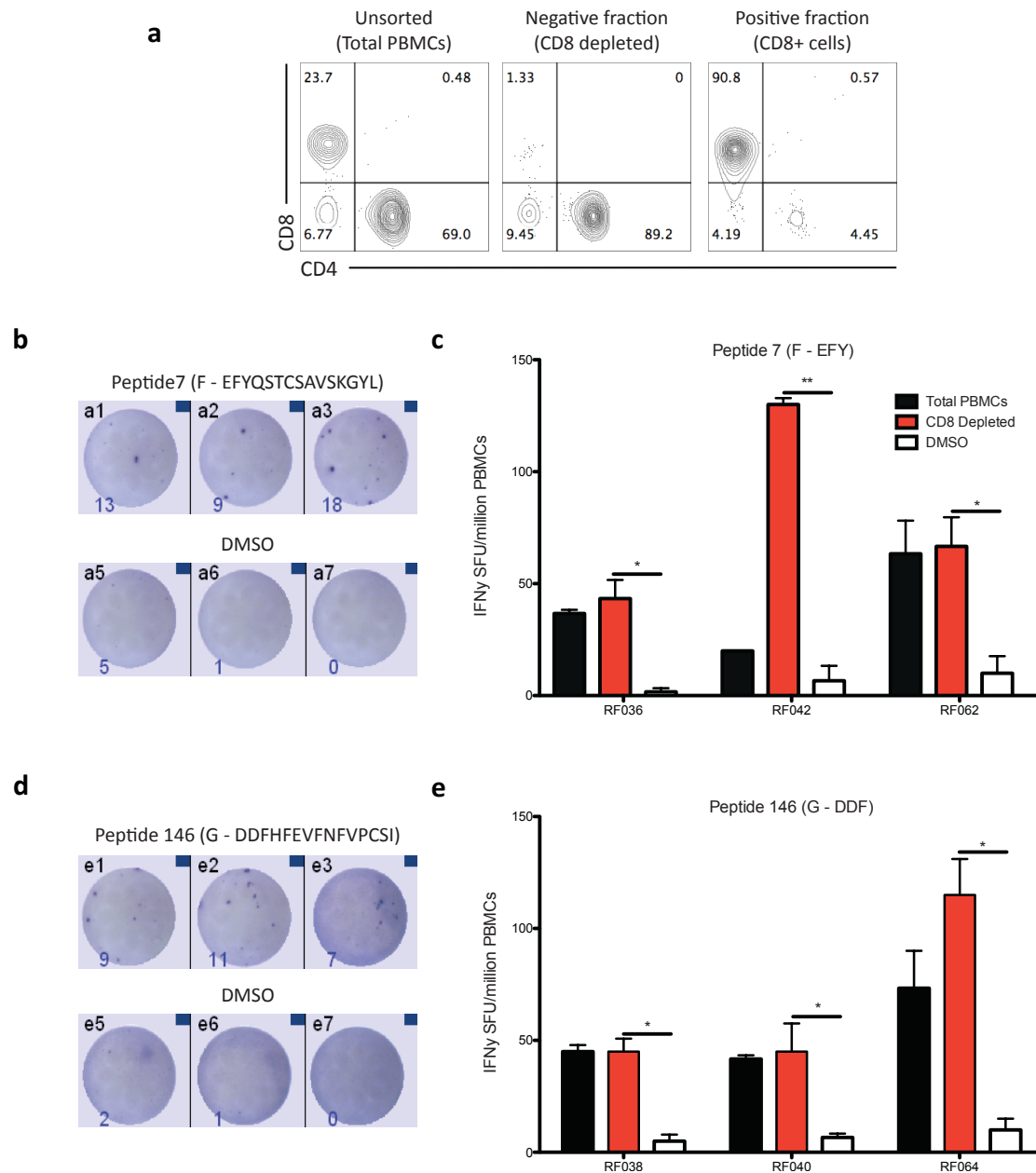


Figure 3.4.1 Peptides F-EFY and G-DDF are immunodominant CD4+ T cell epitopes. CD8+ T cells were depleted from PBMCs of infected subjects that previously showed positive IFN- γ ELISpot responses to F-EFY (n =3) and G-DDF (n=3). (a) Representative flow cytometry analysis gated on live CD3+ lymphocytes. (b,d) Representative IFN- γ ELISpot scans following culture of 2×10^5 CD8+ depleted cells with F-EFY or G-DDF. (c,e) Graphs show the mean $\pm$ s.e.m. IFN- γ spot forming cells in response to F-EFY and G-DDF by total PBMCs (black) and CD8-depleted PBMCs (red). All pools were tested in triplicate. DMSO stimulation was used as a negative control. P-values for the two-tailed Wilcoxon matched pairs test is shown (*p < 0.05; **p < 0.01).

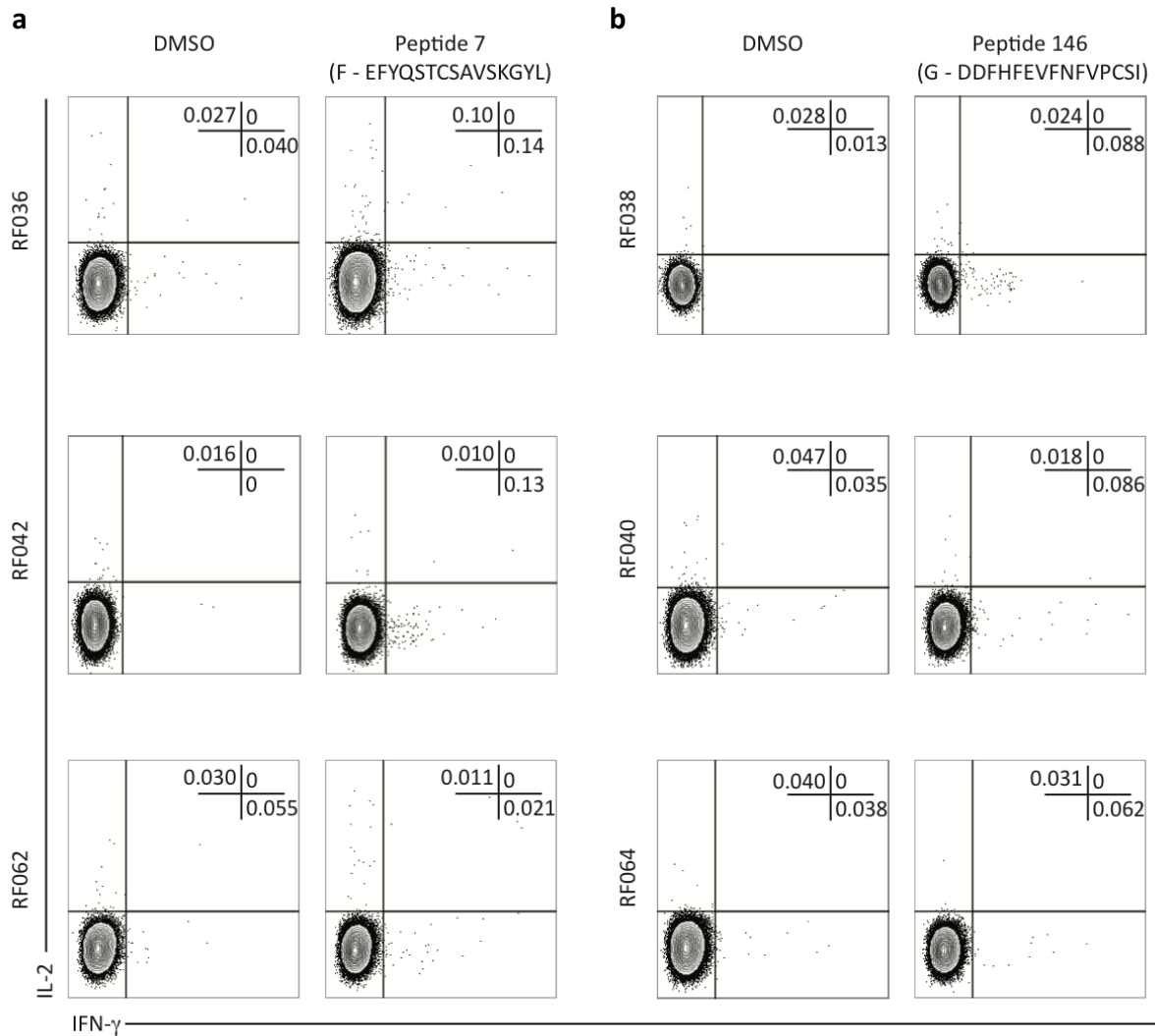


Figure 3.4.2 RSV-specific CD4⁺ T cells in blood show no polyfunctionality. PBMCs from infected subjects that were previously found to be responsive to F-EFY (n=3) or G-DDF (n=3) were stimulated with the peptide of interest (10 μ g/ml) for 6 hours. Brefeldin A (1 μ l/ml) was added in the last 4 hours of the culture. Cells were then stained with live/dead marker, and anti-CD3, CD4, CD8, IL-2, IFN- γ and TNF- α . Results show flow cytometry analyses gated on live CD3⁺ lymphocytes following stimulation with (A) F-EFY or (B) G-DDF.

3.4.3 F-EFY and G-DDF are predicted to be restricted to HLA-DR and HLA-DP, respectively

To determine the HLA class II restriction of the F-EFY and G-DDF epitopes, we initially used the IEDB MHC-II binding prediction tool, an *in silico* prediction model of peptide binding to class II molecules. This program uses the consensus method for prediction, which as described earlier has the highest success rate. For each peptide, a percentile rank was generated by comparing the peptide's score against the scores of five million random 15mers selected from the SWISSPROT database. It is suggested that class II consensus percentiles <20% can be considered binders, and <10% are very good binders (Chaves et al., 2012). The prediction was applied against all known human HLA class II alleles, and the top class II binding predictions for F-EFY and G-DDF are shown (Tables 3.4.1 and 3.4.2).

F-EFY was predicted to bind multiple DRB molecules with the lowest percentiles against *04:01, *09:01, *04:05, *11:01 and *04:04 (Table 41). This was in line with the HLA-DRB1 types of the subjects that produced IFN- γ in response to peptide stimulation; RF036 – DRB1*04:01, RF042 – DRB1*09:01 and RF062 – DRB1*11:01 (Section 3.1.5). On the other hand, the G-DDF epitope was predicted to bind DP molecules with the highest rank for DPA*01:03/DPB*02:01 and DPA*01/DPB*04:01 (Table 42). These alleles were also typed in the responding subjects; RF038 – DPA*01:03/DPB*04:01, RF040 – DPA*01:03/DPB*02:01 and RF064 – DPA*01:03/DPB*04:01.

HLA allele	Sequence	Method	Percentile rank
DRB1*04:01	EFYQSTCSAVSKGYL	Consensus	0.42
DRB1*09:01	EFYQSTCSAVSKGYL	Consensus	1.72
DRB1*04:05	EFYQSTCSAVSKGYL	Consensus	2.62
DRB1*11:01	EFYQSTCSAVSKGYL	Consensus	3.13
DRB1*04:04	EFYQSTCSAVSKGYL	Consensus	4.20
DRB1*08:02	EFYQSTCSAVSKGYL	Consensus	8.08
DRB1*07:01	EFYQSTCSAVSKGYL	Consensus	12.75
DRB1*01:01	EFYQSTCSAVSKGYL	Consensus	13.04
DRB1*03:01	EFYQSTCSAVSKGYL	Consensus	18.64

Table 3.4.1 Highest predicted HLA-class II binding affinity for F-EFY epitope. In silico analysis was carried out using the IEDB MHC-II binding predictions tool (Wang et al., 2010).

HLA allele	Sequence	Method	Percentile rank
DPA1*01:03/DPB1*02:01	DDFHFEVFNFPVCSI	Consensus	0.21
DPA1*01/DPB1*04:01	DDFHFEVFNFPVCSI	Consensus	0.55
DPA1*02:01/DPB1*01:01	DDFHFEVFNFPVCSI	Consensus	2.23
DQA1*01:01/DQB1*05:01	DDFHFEVFNFPVCSI	Consensus	3.32
DPA1*02:01/DPB1*05:01	DDFHFEVFNFPVCSI	Consensus	5.14

Table 3.4.2 Highest predicted HLA-class II binding affinity for G-DDF epitope. *In silico* analysis was carried out using the IEDB MHC-II binding predictions tool (Wang et al., 2010).

Although the MHC class II peptide binding groove can accommodate peptides between 13 and 25 amino acids, the core region is known to fit a 9mer that largely determines binding affinity and specificity (Jones et al., 2006). Therefore, we aimed to determine the predicted core 9mer region of the F-EFY and G-DDF epitopes using NetPANMHC3.1, an *in silico* tool trained on quantitative peptide binding data covering multiple MHC class II molecules (Andreatta et al., 2015). For F-EFY, the binding core sequence was predicted to be YQSTCSAVS with the highest affinity for DRB1*04:01 (61.79nM) followed by DRB1*09:01 (77.11nM) and DRB1*11:01 (373.1nM; Table 3.4.3). This predicted F-EFY as a strong binder to DRB1*04:01 and DRB1*09:01, but weak binder to DRB1*11:01 (Table 3.4.3). For G-DDF, the binding core sequence was predicted to be FHFVFNFPV with high predicted affinities to both DPA*01:03/DPB*02:01 (26.10nM) and DPA1*01:03/DPB1*04:01 (17.63nM). Based on these, both epitopes were predicted to be strong binders to the class II molecules of interest.

HLA allele	Sequence	Core	Affinity (nM)
DRB1*04:01	EFYQSTCSAVSKGYL	YQSTCSAVS	61.79
DRB1*09:01	EFYQSTCSAVSKGYL	YQSTCSAVS	77.11
DRB1*11:01	EFYQSTCSAVSKGYL	YQSTCSAVS	373.1
DPA1*01:03/DPB1*02:01	DDFHFEVFNFPVCSI	FHFVFNFPV	26.10
DPA1*01:03/DPB1*04:01	DDFHFEVFNFPVCSI	FHFVFNFPV	17.63

Table 3.4.3 Predicted peptide binding core sequences of the F-EFY and G-DDF to HLA-class II alleles of interest. *In silico* analysis was carried out using NetMHCIIpan 3.1 server (Andreatta et al., 2015).

3.4.4 Measured *in vitro* binding affinities confirm *in silico* predictions of HLA restriction

Following the results from *in silico* predictions, we measured the binding affinities of F-EFY and G-DDF to class II molecules of interest *in vitro* in collaboration with John Sidney and Alessandro Sette (La Jolla Institute of Allergy and Immunology, USA). An IC₅₀ value was calculated which represent the concentration of an inhibitor where the binding is reduced by half, therefore a lower IC₅₀ indicates higher binding affinity (Table 3.4.4). These results supported the *in silico* prediction data: F-EFY had highest binding affinity for DRB1*04:01 (IC₅₀ 12nM) followed by DRB1*09:01 (IC₅₀ 460nM) and lowest binding affinity was observed for DRB1*11:01 (IC₅₀ 681nM). In comparison, binding affinity of F-EFY was low for the DP alleles. Conversely, the measured binding affinities of G-DDF for DPB1*02:01 (IC₅₀ 13nM) and DPB1*04:01 (IC₅₀ 1.8nM) alleles were both high while the binding of this epitope to the tested DRB alleles was relatively weak (Table 3.4.4).

These results validated the *in silico* predicted HLA class II restriction of the F-EFY and G-DDF epitopes, which are restricted to DRB1*04:01, -09:01, 11:01 or DPB1*02:01,-04:01, respectively.

Sequence	DRB1*04:01	DRB1*09:01	DRB1*11:01	DPB1*02:01	DPB1*04:01
EFYQSTCSAVSKGYL	12	460	681	1122	2742
DDFHFEVFNFPVCSI	583	3411	9951	13	1.8

Table 3.4.4 *In vitro* measured peptide binding affinities of F-EFY and G-DDF to HLA-class II molecules of interest. Results show the calculated IC₅₀ values (nM). Data provided by Dr John Sidney and Dr Alessandro Sette, La Jolla Institute, USA.

3.4.5 F-EFY and G-DDF stimulate proliferation and cytokine production by CD4⁺ T cells in HLA-matched PBMCs

The functionality of antigen-specific T cells can be studied in a number of ways including carboxyfluorescein succinimidyl ester (CFSE) labelling of lymphocytes to track cell division that indicates proliferation (Lyons and Parish, 1994) and intracellular cytokine staining following stimulation with the peptide of interest (McDermott et al., 2014). We used both these methods to further validate our findings in HLA-matched subjects.

Initially, pre-infection PBMC samples from HLA matched subjects (RF036 – DRB1*04:01, RF062 – DRB1*11:01 and RF042 – DPB1*04:01) were labelled with CFSE and cultured with F-EFY or G-DDF for 7 days (Figure 3.4.3a). However, using peptide alone, we found little, if any, CFSE dilution in CD4⁺ T cells irrespective of subject or HLA type (Figure 3.4.3b). This was further confirmed following peptide re-stimulation and ICS of the cultured PBMCs with no IFN γ production by any CD4⁺ T cells (Figure 3.4.3c). In peripheral blood, the frequencies of antigen-specific CD4⁺ T cells in general are low and estimated to be in the range of 10^{-4} to 10^{-5} for resting memory cells (Braendstrup et al., 2014). This low frequency of antigen-specific precursors may explain why no proliferation or cytokine-production could be detected by CD4⁺ T cells. In order to enhance their proliferation, we then supplemented the cultures with recombinant IL-7 at the start and day 7, and with IL-2 at days 3 and 7 which has previously been shown to successfully augment antigen-specific T cell expansion (Figure 3.4.4a) (Fogg et al., 2013).

Following cytokine supplementation, CFSE dilution was apparent in all cultured PBMCs, even in the DMSO control groups and the non-CD4⁺ T cell population (Figures 3.4.4b, c, d), indicating non-specific proliferation of cells due to supplementation with IL-2 and IL-7 cytokines alone. Nevertheless, PBMCs cultured with the appropriate epitopes contained at least a two-fold higher frequency of cells that proliferated to the maximum number of divisions compared to negative controls (Figures 3.4.4b, c, d, left panels). This is more clearly seen when represented as overlaid histograms, suggesting that these peptides induced more proliferation when present in comparison to DMSO alone (Figures 3.4.4b, c, d, middle panels). This increase in CFSE^{lo} cells was statistically significant (Figure 3.4.4e, $p=0.0039$).

To further validate these results, PBMCs from the same subjects were cultured for 10 days with cytokines and peptide, then re-stimulated with the peptides of interest to measure the ability of CD4⁺ T cells to produce type I cytokines (Figures 3.4.4b, c, d, right panels). In most cases, there was an approximate 2-fold increase ($p=0.0029$, Figure 3.4.4f) in the frequency of IFN γ ⁺ CD4⁺ cells in response to culture with peptide compared to the negative controls. Similar to results from CFSE dilution, there were some IFN γ ⁺ cells (range: 0.13-0.98%) in the DMSO control, which might be explained by non-specific expansion of cells by the addition of IL-2 and IL-7 to the culture wells.

In conclusion, *in vitro* culture of HLA-matched PBMCs with F-EFY or G-DDF in the presence of IL-2 and IL-7 stimulated the proliferation and cytokine production of RSV-specific CD4⁺ T cells.

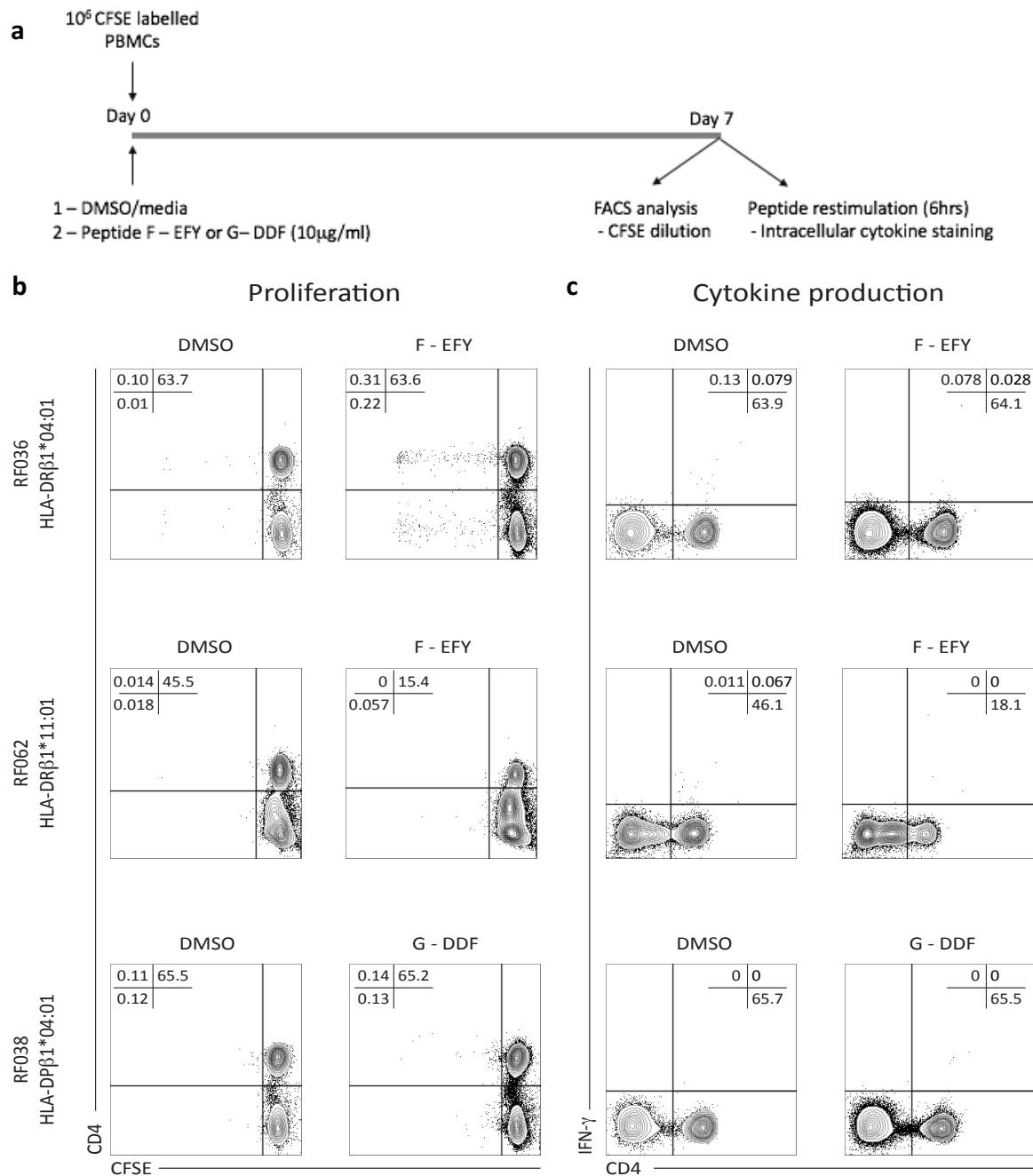
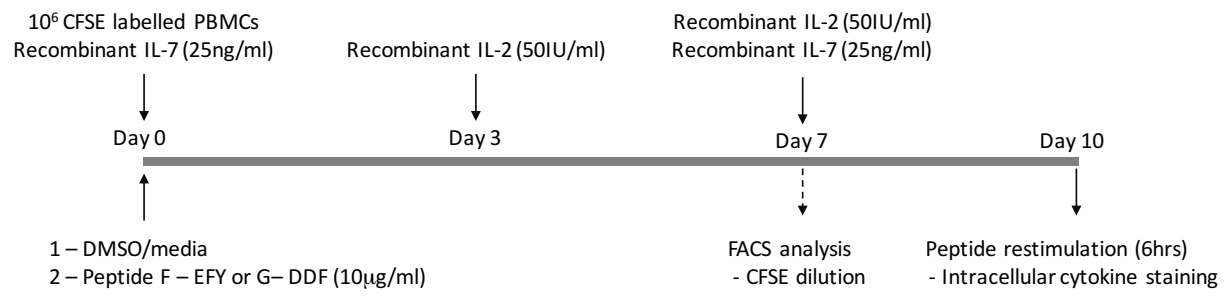
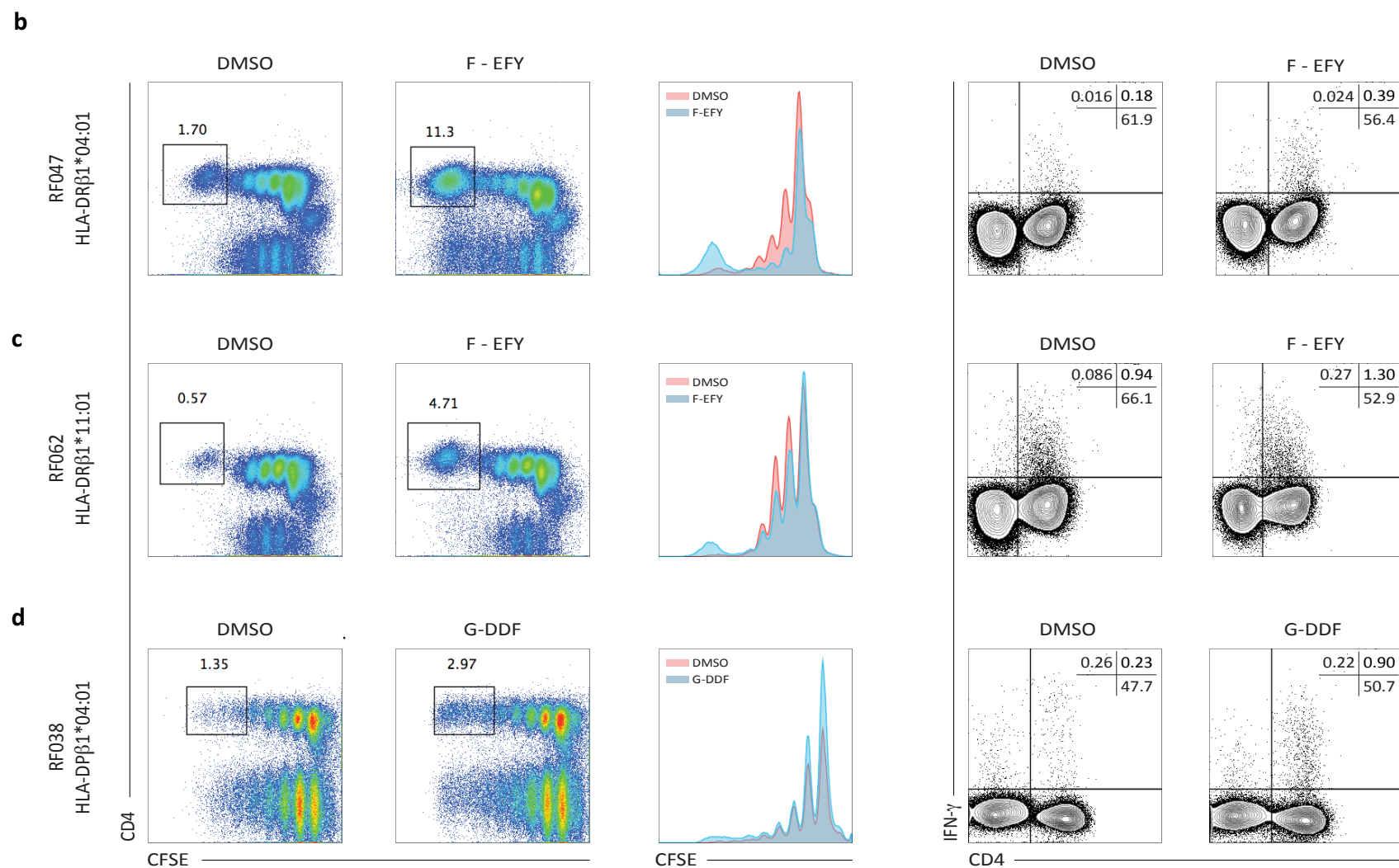


Figure 3.4.3 Stimulation of HLA-matched PBMCs with peptide alone does not induce detectable proliferation of CD4+ T cells. (a) 1x10⁶ CFSE labelled PBMCs from HLA-matched study subjects pre-infection were cultured with DMSO and 10µg/ml of F-EFY or G-DDF for 7 days at 37°C. At the end of the culture, cells were re-stimulated with the peptide of interest for 6 hours. Brefeldin A was added in the last 4 hours to inhibit intracellular transport. (b) Cells were surface stained with anti- CD3, CD4, CD8 and Live/Dead marker to assess proliferation of CD4+ T cells by CFSE dilution. (c) Re-stimulated cells were stained for intracellular markers with anti- IFN γ and IL-2 to assess cytokine production by CD4+ T cells. On all FACS plots, cells were gated on live CD3+ lymphocytes.

a



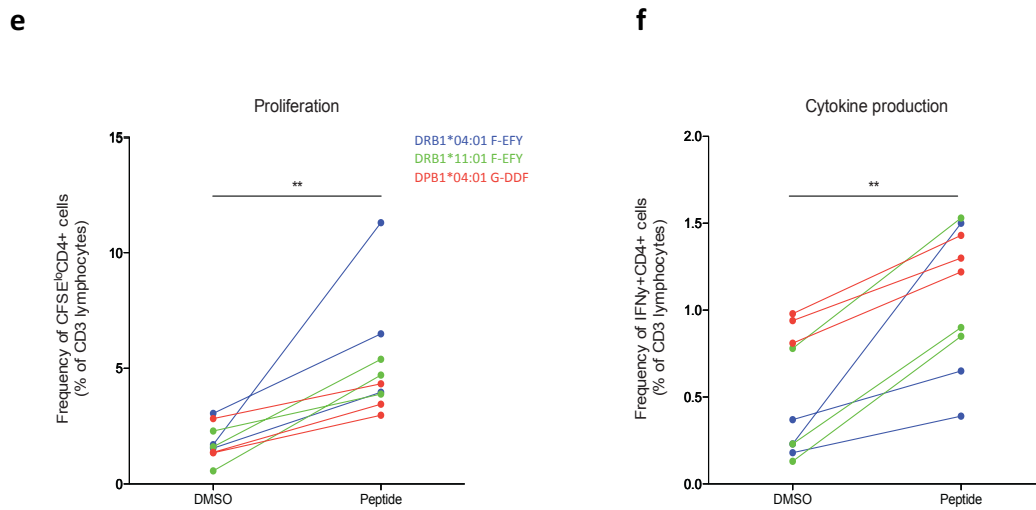


Figure 3.4.4 Culture of HLA-matched PBMCs with peptide with cytokine supplementation induces proliferation and cytokine production of CD4⁺ T cells. (a) 1×10^6 CFSE labelled baseline PBMCs from HLA-matched study subjects were cultured with DMSO and $10 \mu\text{g/ml}$ of F-EFY or G-DDF for 7 or 10 days at 37°C . The cultures were supplemented with recombinant IL-7 (25 ng/ml) at days 0, 7 and recombinant IL-2 (50 IU/ml) at days 3 and 7. At day 7, cells were surface stained with anti- CD3, CD4, CD8 and Live/Dead marker to assess proliferation of CD4⁺ T cells by CFSE dilution. Representative FACS plots show CFSE dilution on cells and IFN- γ ⁺ cells gated on live CD3⁺ lymphocytes for (b) DRB1*04:01, (c) DRB1*11:01 and (d) DPB1*04:01. Summary graphs show the change in frequency for (d) CFSE dilution and (e) IFN- γ between DMSO control and peptide stimulation. The p value for for a two-tailed Wilcoxon matched pairs test is shown (** P<0.01).

3.4.6 Discussion

Antigen-specific CD4⁺ T cells are important in mounting effective virus-specific host responses, as it was recently shown in influenza challenge studies (Wilkinson et al., 2012). However, previous attempts to define immunodominant epitopes in human RSV had major limitations, discussed further below (van Bleek et al., 2003; de Waal et al., 2004).

Previous reports and *in silico* peptide binding predictions had indicated F-EFY and G-DDF might be epitopes restricted to class II molecules. We confirmed this in two independent assays, IFN- γ ELISpot and intracellular cytokine staining for analysis by flow cytometry. Following the depletion of CD8⁺ T cells from PBMCs, the major producers of IFN- γ are CD4⁺ T cells and NK cells (Dercamp et al., 2002). Comparison of IFN- γ ELISpot results from peptide stimulated total or CD8 depleted PBMCs showed no change in the number of spots, indicating that the major cell population responding to F-EFY and G-DDF was not CD8⁺ T cells. IFN- γ producing cells are unlikely to be NK cells as they lack antigen-specific receptors and recognise virus-infected cells through activating or inhibitory receptors (Dercamp et al., 2002). Therefore, the vast majority of IFN- γ producing cells detected in this assay should be CD4⁺ T cells. We then used intracellular cytokine staining to validate our conclusions from IFN- γ ELISpot and to also assess whether the CD4⁺ T cells were multiple cytokine producers. This showed that CD4⁺ T cells produce IFN- γ in response to stimulation with F-EFY or G-DDF but not IL-2 or TNF- α . Conversely, influenza-specific CD4⁺ T cells have been shown to produce multiple cytokines in human PBMCs (Chen et al., 2014; Kannanganat et al., 2007). Multiple cytokine producing antiviral CD4⁺ T cells have been shown to be functionally superior to single cytokine producing cells in the context of vaccinia, influenza and CMV (Kannanganat et al., 2007). CD4⁺ T cells producing more than one Th1 cytokine (IFN- γ , TNF α and IL-2) were shown to have higher CD40L levels, which may provide better help to B cells and CD8⁺ T cells. Moreover, multiple cytokine producing CD4⁺ T cells could degranulate better than single producers, suggesting that they possess enhanced cytolytic potential. One potential explanation for the impaired polyfunctionality of CD4⁺ T cells may be the immunomodulatory mechanisms of RSV. NS1 and NS2 have both been shown to suppress production of type I interferons by epithelial cells (Spann et al., 2004) and this may decrease

Th1 cell polarisation (Brinkmann et al., 1993). Moreover, RSV can infect dendritic cells *in vitro* which has been shown to result in poor induction of T cell effector function (Guerrero-Plata et al., 2006). Compared to metapneumovirus, RSV infected dendritic cells led to impaired CD4+ T cell proliferation and reduced production of cytokines (Guerrero-Plata et al., 2006). Work from our group has shown limited polyfunctionality of RSV-specific CD8+ T cells in blood, but multiple cytokine production by influenza-specific CD8+ T cells from the same individuals (Jozwik et al., 2015). IL-2 production by CD4+ T cells act on CD8+ T cells to encourage improved cell division and survival (Whitmire, 2011). It might be speculated that restricted cytokine production by RSV-specific CD4+ T cells may therefore contribute to the limited functionality of blood CD8+ T cells in RSV compared influenza, although this needs to be addressed in future studies.

Although *in silico* prediction methods have limited accuracy, particularly for HLA class II-restricted epitopes, we found F-EFY to have high binding affinity predicted for HLA-DRB1*04:01, -09:01 and 11:01, HLAs for which average prediction accuracy has previously been shown at 69, 68 and 80%, respectively (Wang et al., 2008). These were in line with the HLA-types of the subjects in whom we detected F-EFY-specific responses. Interestingly, these HLA-types group into an HLA supertype family (discussed in section 3.6.1). We found that the predicted binding core sequence of F-EFY sequence bears the P1-P6 motif that is recognised by this supertype and may explain the promiscuity of this epitope. Thus, position 1 was occupied by a tyrosine (Y) whereas a serine (S) was found at position 6, which have properties that fitting the P1-P6 motif. Similarly, a common motif for HLA-DP had previously been characterised as containing a phenylalanine at position 1 and aromatic or hydrophobic residues in position 6 of the peptide binding groove (Andreatta and Nielsen, 2012). The predicted binding core of the G-DDF peptide showed phenylalanine (F) at both positions 1 and 6, which therefore has properties that fit the characteristics of the motif described previously. The open ended class II binding groove allows long peptides to extend beyond the binding region at both N- and C- terminus (Holland et al., 2013). Since these flanking residues have been shown to modulate T cell function (Larsen et al., 1996), a number of studies have investigated whether altering the flanking residues could improve TCR binding. Carson and colleagues found that removal of C-terminus flanking residues from the immunodominant epitope in hen egg lysosome significantly reduced the immunogenicity of

the epitope (Carson et al., 1997). Conversely, flanking regions with basic residues at the peptide C-terminus, or acidic residues in the N-terminus generated enhanced CD4⁺ T cell responses across different class II alleles and peptides (Holland et al., 2013). In this regard, substitution of a basic residue in the C-terminal flank of known immunodominant epitopes from hemagglutinin and myelin basic protein resulted in significantly increased CD4⁺ T cell responses (Cole et al., 2012; Godkin et al., 2001). More recently, substitution of tyrosine with arginine on the C-terminal flank of HA_{306–318} was accompanied by a 2-fold increase in peptide binding affinity, while having no impact to peptide specificity (Holland et al., 2015). These findings suggest that the manipulation of the C-terminal flanking regions of F-EFY and G-DDF may further enhance peptide binding affinity and CD4⁺ T cell responses.

Limitations of previous studies that attempted to identify potentially immunodominant epitopes have included the low abundance of antigen-specific cells and the small number of subjects tested. van Bleek and colleagues used overlapping peptide pools derived from F protein to induce IFN- γ production in healthy donor cells but mapped these in few individuals with a range of different HLA haplotypes (van Bleek et al., 2003). The study was designed to stimulate memory T cell responses from healthy uninfected subjects, which resulted in detection of very few IFN- γ producing cells and may have limited the analysis. We observed a similar result in our study from stimulation of PBMCs at day 0 for IFN- γ ELISpot assay, with the majority of the responses remaining below the threshold. In comparison, we found a striking increase in the overall number of IFN- γ producing T cells 10 days post infection (as shown in section 3.2.6). This highlights the technical limitations of identifying immunodominant T cell epitopes using healthy PBMCs. Moreover, van Bleek and colleagues tested samples from only 11 donors with the most common HLA-type being DRB1*04:01 expressed by only 4 subjects. Other DRB alleles such as DRB1*11:01, -13:01 and -15:01 were limited to single individuals. The main limitation of this is that observed responses could have been due to T cell clones with a private specificity and epitopes found in these subjects might not be immunodominant in a wider population (Venturi et al., 2008). On the other hand, de Waal and colleagues generated three T cell clones from different individuals and showed that one recognised the central conserved region of G, where the DDF epitope is found (de Waal et al., 2004). To validate their findings, they cultured PBMCs from healthy adults with

the 31-mer covering this region for 2.5 weeks, and measured CD69 expression. They found at least a 2-fold increase in the frequency of CD69 expressing CD4⁺ T cells in 7 of the 13 samples tested. The limitation of using this as a readout is that CD69 expression can be transient unless T cells are continuously exposed to antigen challenge (Werfel et al., 1997). Another limitation of this study was the prolonged culture with persistently infected B-lymphoblastic cell lines to expand RSV-specific cells. It is not possible to tease out whether presenting antigen in this way biases the response or is analogous to what occurs *in vivo*.

To confirm that the immunodominant epitopes F-EFY and G-DDF stimulate common T cell responses in memory T cells, we cultured baseline samples from multiple HLA-matched subjects with each peptide. Culturing with peptide alone was not enough to drive proliferation or cytokine production by CD4⁺ T cells, probably due to the low starting number of antigen-specific cells. To overcome this, we supplemented the culture with recombinant IL-2 and IL-7, which has been shown to improve *ex vivo* expansion of memory T cells (Besser et al., 2009; Fogg et al., 2013). Addition of recombinant cytokines resulted in proliferation of both CD4⁺ and CD8⁺ T cells even in the absence of peptide. Addition of these cytokines has previously been shown to act non-specifically to induce expansion of polyclonal T cells (Di Genova et al., 2010; Wong and Pamer, 2001). Nevertheless, using this method, we showed enhanced proliferation and cytokine production by CD4⁺ cells in response to peptide stimulation in at least 3 subjects for HLA-DRB1*04:01, HLA-DRB1*11:01 and HLA-DPB1*04:01. The peptide binding predictions and measured binding affinity suggested restriction of F-EFY to HLA-DRB1*09:01 as well, but due to the limited number of donors with this HLA allele we could not test this further.

In conclusion, by taking advantage of the expanded T cell populations in experimentally infected individuals, we found and validated immunodominant CD4⁺ T cell epitopes in human RSV *ex vivo*, thus avoiding the biases inherent in previous study designs. These originated from the surface F and G proteins. F-EFY was found to be restricted to HLA-DRB1*04:01, HLA-DRB1*09:01 and HLA-DRB1*11:01 whereas G-DDF was found to be restricted to DPA*01:03/DPB*04:01. The detailed analysis of HLA-restriction therefore allows the construction of class II tetramers, which will permit greater in-depth analysis of epitope-specific CD4⁺ T cells during the course of human RSV infection.

3.5 Blood T follicular helper cells in human RSV infection

3.5.1 Introduction

T cell help to B cells is an important aspect of adaptive immunity and essential for generation of high-affinity class-switched antibodies. T follicular helper (Tfh) cells in humans were described in 2000, following the finding that a large proportion of CD4⁺ T cells in tonsils had a unique phenotype and expressed high levels of CXCR5 (Breitfeld et al., 2000; Schaerli et al., 2000). For almost a decade, these cells were not acknowledged as a distinct lineage of CD4⁺ T cells, mainly because there was no specific transcription factor ascribed to this subtype. However, identification of Bcl-6 as a master regulator of Tfh differentiation, their requirement for helping B cells *in vivo* and the importance of IL-21 for their function established Tfh cells as a distinct CD4⁺ T cell subset (Yu et al., 2009; Johnston et al., 2009b; Linterman et al., 2010; Nurieva et al., 2009; Zotos et al., 2010). Tfh cells interact with B cells in germinal centres (GCs) which are transient structures that form in lymphoid organs during T cell dependent antibody responses. These are where B cell affinity maturation, class switching and plasma cell differentiation mainly occur (Crotty, 2011; MacLennan, 1994). Expression of CXCR5 by Tfh cells allows recognition of CXCL13, allowing their migration to follicles where T cell-B cell interactions takes place (Ansel et al., 1999). GC Tfh cells have upregulated expression of several surface proteins, including CD40L, ICOS and PD-1 which form direct physical contacts with B cells (Han et al., 1995; Rasheed et al., 2006; Nurieva et al., 2008). Tfh differentiation pathway, plasticity, role in germinal centre formation and function has been reviewed extensively (Crotty, 2011).

As Tfh cells are predominantly found in lymphoid tissues, human studies initially focused on tonsil samples. Recently, Tfh-like (also referred to as circulating Tfh) cells have been reported in human blood (Bentebibel et al., 2013; Morita et al., 2011; Pallikkuth et al., 2012). These studies used combinations of ICOS, PD-1 and CCR7 expression to define different subsets of Tfh-like cells in blood (Locci et al., 2013; Schmitt et al., 2014). ICOS⁺PD1⁺CCR7^{lo} cells were reported as activated, whereas ICOS⁺PD1⁺CCR7^{int} and ICOS⁺PD1⁺CCR7^{hi} cells were defined as quiescent cells (Locci et al., 2013; Schmitt et al., 2014). Differential expression of CXCR3

and CCR6 allowed further characterisation of these cells; Morita and colleagues have shown that CXCR3+CCR6⁻ cells display a Th1-like profile as they express T-bet and produce IFN- γ , while CXCR3-CCR6⁻ cells express GATA3 and produce IL-4, IL-5 and IL-13 thus resembling the Th2 subset. On the other hand, CXCR3-CCR6⁺ cells express RoR γ T and produce IL-17A and IL-22 which are typical markers of Th17 cells (Morita et al., 2011). Following on from these findings, Tfh-like cells have been grouped into Tfh1, Tfh2 and Tfh17 subsets (Schmitt et al., 2014).

Defining the functional subset of Tfh-like cells in blood that truly reflects GC Tfh cells has been controversial. Locci and colleagues found that CXCR5+PD1+CXCR3⁻ Tfh-like cells shared a transcriptional profile signature with their counterparts in the GCs, produced IL-4 and IL-21 upon PMA and ionomycin stimulation, and correlated with broadly neutralizing antibody responses (Locci et al., 2013). Conversely, Bentebibel and colleagues found that CXCR5+ICOS+CXCR3⁺ cells correlated with antibody responses to influenza vaccination (Bentebibel et al., 2013). Cells of this phenotype expressed IFN- γ , IL-2, IL-10 and IL-21 upon influenza antigen stimulation, and therefore a Tfh1 profile was ascribed to them. To confirm their functionality, CXCR5+ICOS+CXCR3⁺ cells in blood were sorted 7 days after vaccination. These were then cultured with memory B cells and induced their differentiation into plasma cells (Bentebibel et al., 2013).

To date, functional studies of human Tfh-like cells in blood have been limited to simple co-culture assays with sorted B cells. In collaboration with GSK Vaccines (Rixensart, Belgium) we attempted to optimise the 'MIMIC' (Modular Immune In vitro Construct) *in vitro* system to generate activated APCs that might more accurately model antigen presentation in tissue or lymph node. Well-established PBMC assays require artificially doses of peptides for pulsing of APCs and exogenous cytokine supplementation for expansion of lymphocytes (Moser et al., 2010). In addition, the number of APCs in PBMCs may be too few for efficient triggering of T cells. In contrast, MIMIC theoretically allows self-differentiation of monocytes into potent APCs to generate various cell-mediated and humoral immune responses in a physiologically relevant environment. MIMIC cultured cells can also be used at optimum DC:T cell ratios for generation of primary or recall antigen-specific CD4⁺ T cell responses. MIMIC was originally designed by Sanofi Pasteur VaxDesign (Florida, USA) and described as

a system that could better recapitulate a human immune response in a well-based format for rapid assessment of vaccine responses (Byers et al., 2009; Higbee et al., 2009).

To validate this approach, Byers and colleagues tested tetanus toxoid (TT)-specific antibody levels in 13 subjects before and after vaccination and found that the vaccination induced TT-specific IgG production. In parallel, they used MIMIC to culture pre-vaccination samples with the TT vaccine and found an increase in TT-specific IgG production similar to that measured post-vaccination (Byers et al., 2009). In a case study reported by Vaxdesign, MIMIC was also shown to be better at inducing proliferation of CD3⁺ T cells (~2 fold increase in frequency of CFSE^{lo} cells) in response to TT compared to *in vitro* culture of antigen with PBMCs (<http://www.vaxdesign.com/case-studies/lte-module-versus-pbmc-assay>).

The MIMIC system involves incubation of the antigen of interest within a 3D collagen matrix that is overlaid by a monolayer of human umbilical vein endothelial cells (HUVEC). Cultured endothelial cells produce chemokines at low levels which accumulate in the culture medium and in the underlying collagen matrix (Muller and Luscinskas, 2008; Muller and Weigl, 1992). As the culture medium is washed away, a chemotactic gradient is created which facilitates the migration of leukocytes to the collagen (Muller and Luscinskas, 2008). Therefore, application of donor PBMCs to the monolayer results in migration of monocytes through to collagen. Upon encountering the antigen, these monocytes take it up and mature into professional APCs. It was previously shown that CD14⁺ monocytes are maintained on collagen after 4 hours incubation but by 96 hours, CD14 gets downregulated while CD71 expression is upregulated which suggests that the differentiation of CD14⁺CD71⁻ monocytes into CD14⁻CD71⁺ macrophages. These cells then migrate back through the HUVEC layer as antigen-loaded APCs that can be isolated and used for downstream assays such as culturing with autologous T cells. This process is summarised (Figure 3.5.1) and has been discussed in detail elsewhere (Higbee et al., 2009).

To better model Tfh cell help in the context of acute infection, we therefore characterised the phenotype of Tfh-like cells in blood of our RSV-challenged cohort using markers previously reported in the literature and attempted to optimise MIMIC in-house as a more accurate model of the three-way interactions between APC, Tfh and B cell.

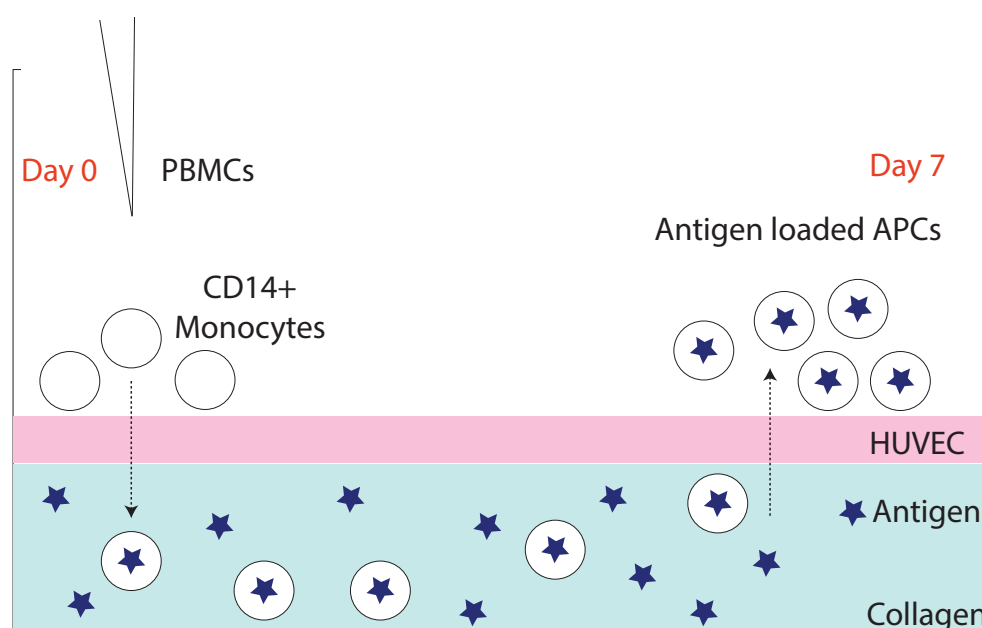


Figure 3.5.1 Diagram illustrating the principle of MIMIC system. PBMCs are loaded onto the HUVEC monolayer which results in migration of CD14+ monocytes towards the collagen matrix where they meet antigen. At the end of 7 days, antigen loaded APCs migrate back through the HUVEC and can be collected for use in functional assays.

3.5.2 Restricted cytokine production in tonsillar RSV-specific CD4+ T cells

Production of IL-21 (and IL-4 to some extent) by CD4+ T cells in tonsils has been shown to be essential in helping B cells produce antibody (Bentebibel et al., 2011; Bryant et al., 2007). However, influenza-specific CD4+ T cells have not been studied and there are no reports investigating the functionality of RSV-specific CD4+ T cells in human tonsil. We stimulated tonsillar cells from 3 donors using RSV to study the cytokine production profile of antigen-specific CD4+ T cells in the lymphoid tissue. Overall, we observed a low frequency of cytokine production in response to RSV stimulation (Figure 3.5.2a). Tonsillar RSV-specific cells produced no IL-4 and low levels of IFN- γ (mean 0.098% $\pm$ s.e.m. 0.037) and IL-21 (mean 0.21% $\pm$ s.e.m. 0.586; Figure 3.5.2b).

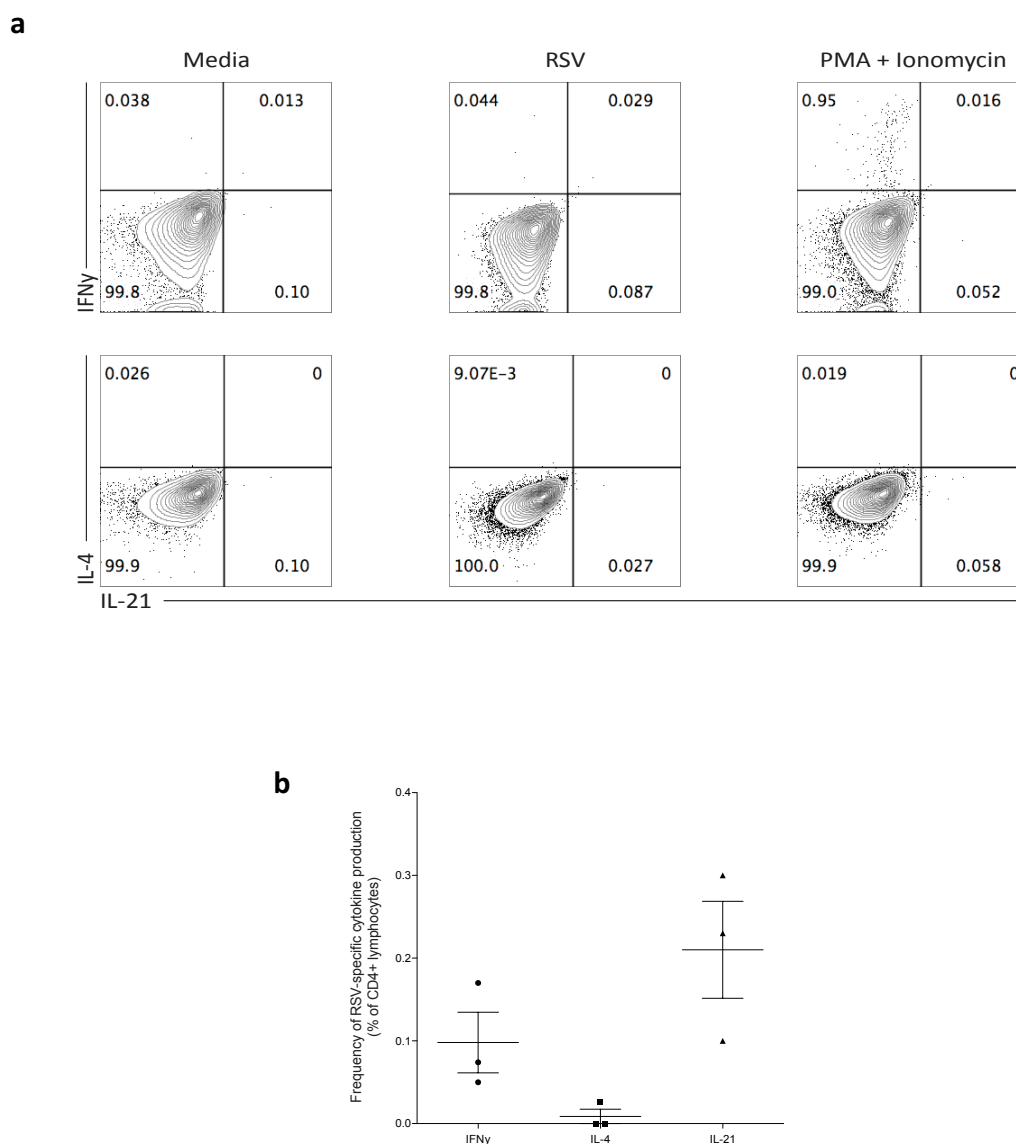


Figure 3.5.2 Low frequencies of cytokine-producing RSV-specific CD4⁺ T cells in tonsils. 1×10^6 tonsillar cells from 3 donors were stimulated *in vitro* with RSV (MOI 6) for 24 hours or PMA (25ng/ml) and ionomycin (1 μ g/ml) for 6 hours with Brefeldin A (10 μ g/ml) added 4 hours before staining with fixable viability dye, anti-CD3, CD4, CD8; and IFN γ , IL-4 and IL-21. (a) Representative FACS plots showing frequencies of cytokines IFN γ and IL-21 (top panel) and IL-4 and IL-21 (lower panel). (b) Summary of cytokine production by RSV-specific CD4⁺ T cells.

3.5.3 Phenotype of Tfh cells are different between PBMCs and tonsillar cells

Biological differences between blood and tonsillar Tfh cells have been described previously by comparison of cellular markers ascribed to the Tfh cell subset (Schmitt et al., 2014). To optimise the flow cytometry panel and to investigate the differences in Tfh phenotype between blood and lymphoid tissue, we collaborated with ENT surgeons at St Mary's Hospital (London, United Kingdom) to collect tonsils from children undergoing routine tonsillectomy. We stained PBMCs from healthy adults (n=3) and tonsillar cells isolated from healthy children (n=3) for markers associated with the Tfh phenotype; CXCR5, ICOS, PD-1 and Bcl-6 (Figure 3.5.3a). The phenotype of Tfh cells in PBMCs was similar to those in tonsils, but with lower frequencies of canonical marker expression. The frequency of CXCR5 expression in tonsillar CD4⁺ T cells (mean 32.4 % $\pm$ s.e.m. 0.867) was significantly higher ($p < 0.001$) than in blood (mean 5.04 % $\pm$ s.e.m. 0.514; Figure 3.5.3b). In tonsils, majority of CXCR5+CD4⁺ tonsillar cells co-expressed ICOS and PD-1 (mean 90.6 % $\pm$ s.e.m. 4.67; Figure 3.5.3b). In comparison, the frequency of ICOS+PD1⁺ cells within the CXCR5+CD4⁺ population in blood was significantly lower ($p = 0.002$), although detectable. Finally, we detected Bcl-6 expression by blood-Tfh cells (MFI 427) which was also lower ($p = 0.0041$) compared to tonsil-Tfh cells (MFI 659; Figure 3.5.3a).

In conclusion, Tfh-like cells make up a small proportion of CD4⁺ T cells in peripheral blood and are phenotypically distinct from tonsillar Tfh cells, with differential expression of canonical markers ICOS and PD-1. Furthermore, Bcl-6 expression in Tfh-like cells in blood reduced in comparison with tonsils. However, previous evidence of their capacity for B cell help implies that these subsets do have a functional relationship and the quantity and/or quality of these cells in peripheral blood may be reflective of the overall Tfh cell response to RSV infection.

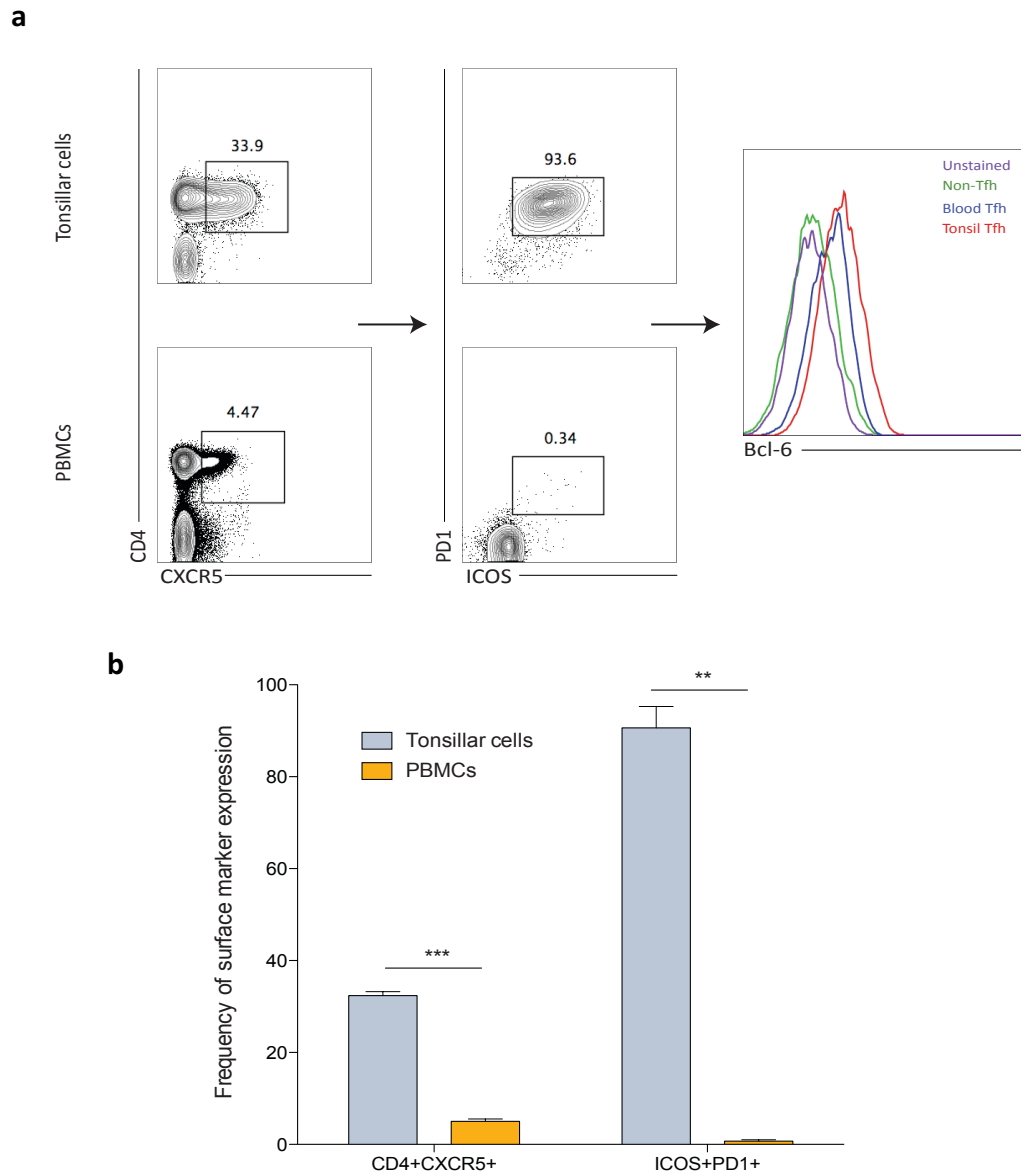


Figure 3.5.3 Tfh-like cells in peripheral blood share phenotypic characteristics with tonsillar Tfh cells. Healthy donor PBMCs (n=3) or tonsillar cells (n=3) were stained with anti-CD3, CD4, CD8, CXCR5, ICOS, PD1, Bcl-6 and LiveDead marker, and analysed by flow cytometry. (a) Plots from representative donors show the frequency of CXCR5+CD4+ cells as a percentage of live CD3+ lymphocytes (left panel), frequency of ICOS+PD-1+ cells as a percentage of CXCR5+CD4+ (middle panel) and histograms (right panel) show Bcl-6 expression within unstained (purple; MFI 122), CXCR5-CD4+ (green; MFI 169), blood CXCR5+ICOS+PD-1+CD4+ (blue; MFI 427) or tonsil CXCR5+ICOS+PD-1+CD4+ (red; MFI 659). (b) Mean±SEM of CXCR5+CD4+ and ICOS+PD1+ T cell frequencies in the blood and tonsil donors subjects are shown. The P-value for a two-tailed Wilcoxon matched pairs test is shown (**p<0.01 and ***p<0.001).

3.5.4 Tfh-like cells in PBMCs peak at day 10 in the infected subjects

We previously showed the activation and proliferation of polyclonal CD4⁺ T cells in response to RSV infection. To investigate whether Tfh-like cells undergo expansion during infection, we stained PBMCs from uninfected (n=10) and infected subjects (n=10) at days 0, 10 and 28 for phenotyping of Tfh-like cells (Figure 3.5.4). In the uninfected subjects, we found no change in the frequency of ICOS⁺PD1⁺ cells within CXCR5⁺CD4⁺ T cells throughout the course of infection (Figure 3.5.4b). In contrast, compared to baseline frequencies (mean 0.712% $\pm$ s.e.m. 0.167) there was a significant increase in ICOS⁺PD1⁺ cells at day 10 in infected subjects (mean 1.92% $\pm$ s.e.m. 0.258; $p=0.0013$), which returned to baseline levels during the convalescence period (mean 1.08% $\pm$ s.e.m. 0.207; $p=0.0207$; Figure 3.5.4c). At its peak, the mean frequency of ICOS⁺PD1⁺ cells in infected subjects was significantly higher compared to the uninfected ($p=0.0005$; Figure 3.5.4d).

Activated blood CD4⁺ T cells have been shown to upregulate the expression of ICOS (Tuettenberg et al., 2009) and PD-1 (Parry et al., 2005) and may not be sufficiently specific for identification of Tfh-like cells in peripheral blood. Bcl-6 expression in blood was reported as undetectable previously (Morita et al., 2011; Bentebibel et al., 2013; He et al., 2013) and we found low level of expression. In contrast, CXCR3 was reported to discriminate functional Tfh-like cells in blood (Bentebibel et al., 2013) and was used as a surrogate to Bcl-6. We therefore investigated the frequency of CXCR5⁺CXCR3⁺ICOS⁺ CD4⁺ T cells as they were previously shown to have the functional capacity to help B cells produce antibody (Bentebibel et al., 2013). The frequency of CXCR3⁺ICOS⁺ cells in uninfected subjects remained unchanged throughout the infection (Figure 3.5.5b). However, compared to baseline frequencies (mean 1.10% $\pm$ s.e.m. 0.13), there was a significant increase in CXCR3⁺ICOS⁺ expressing CXCR5⁺CD4⁺ T cells at day 10 (mean 3.55% $\pm$ s.e.m. 0.412; $p=0.002$) in infected subjects which returned to baseline levels by day 28 (mean 1.40% $\pm$ s.e.m. 0.209; $p=0.0005$; Figure 3.5.5c). The increase in the frequency of CXCR3⁺ICOS⁺ cells in infected subjects was significantly higher compared to those who remained uninfected ($p=0.0017$; Figure 3.5.5d).

Recent studies in the context of influenza vaccination have suggested that the frequency of Tfh-like cells acutely post-vaccination correlates with seroconversion and might be used as an early predictor of vaccine efficacy (Bentebibel et al., 2013). To investigate this, we investigated the correlation of Tfh-like cell frequencies with humoral markers of infection. There was no relationship between ICOS+PD1+ CXCR5+CD4+ T cells and number of antibody-secreting cells at day 10 in infected subjects ($r=-0.1333$, $p=0.7435$; Figure 3.5.6a) or the fold change in serum neutralising antibody titre ($r=0.2333$, $p=0.5517$; Figure 3.5.6b). However, there was a positive trend between the frequency of CXCR3+ICOS+ within the CXCR5+CD4+ cells and number of antibody secreting cells ($r=0.4500$, $p=0.2298$; Figure 3.5.6c) and fold change in serum neutralising antibody titre ($r=0.4333$, $p=0.2449$; Figure 3.5.6d) but neither relationship was statistically significant.

Thus, RSV infection induces the expansion of Tfh-like cells in peripheral blood and our data suggest that the frequency of CXCR5+ICOS+CXCR3+ CD4+ T cells acutely may correlate with the magnitude of the B cell and subsequent antibody response with increased sample size.

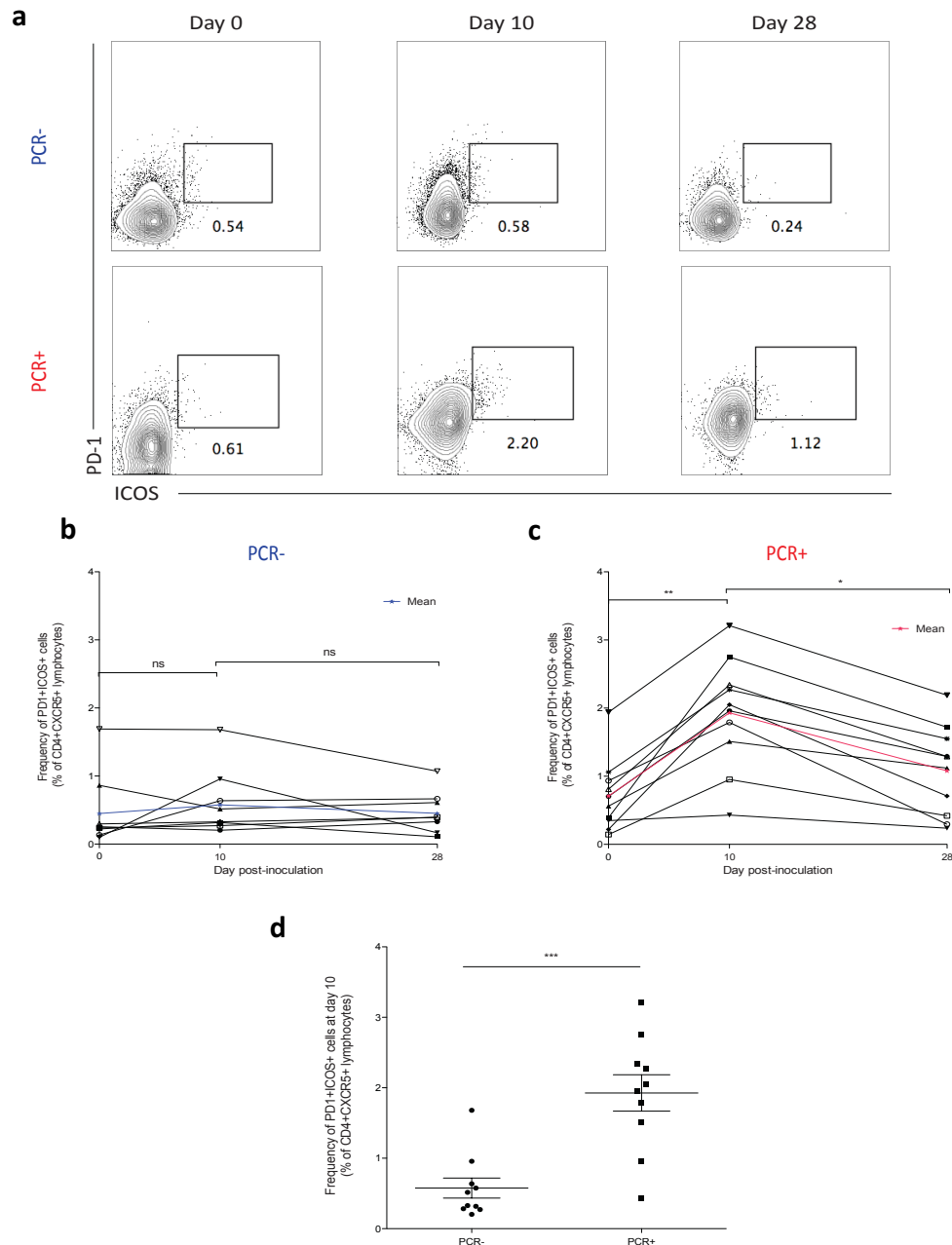


Figure 3.5.4 CXCR5+ICOS+PD1+ CD4+ T cells peak at day 10 in infected subjects. PBMCs from uninfected (n=10) and infected (n=10) subjects were stained with anti-CD3, CD4, CD8, CXCR5, ICOS, PD1 and LiveDead marker, and analysed by flow cytometry. (a) Numbers represent the frequency of ICOS+PD1+ cells as a percentage of CXCR5+CD4+ T cells. Representative subject are shown from uninfected (PCR-) and infected (PCR+) groups. (b) Frequency of ICOS+PD1+ cells amongst the PCR- subjects. (c) Frequency of ICOS+PD1+ cells amongst the PCR+ subjects. (d) Mean±SEM of ICOS+PD1+ CXCR5+CD4+ T cell frequencies in the blood of PCR- and PCR+ subjects at day 10 are shown. The p-value for a two-tailed Wilcoxon matched pairs test is shown (*p<0.05, **p<0.01 and ***p<0.001; ns = not significant).

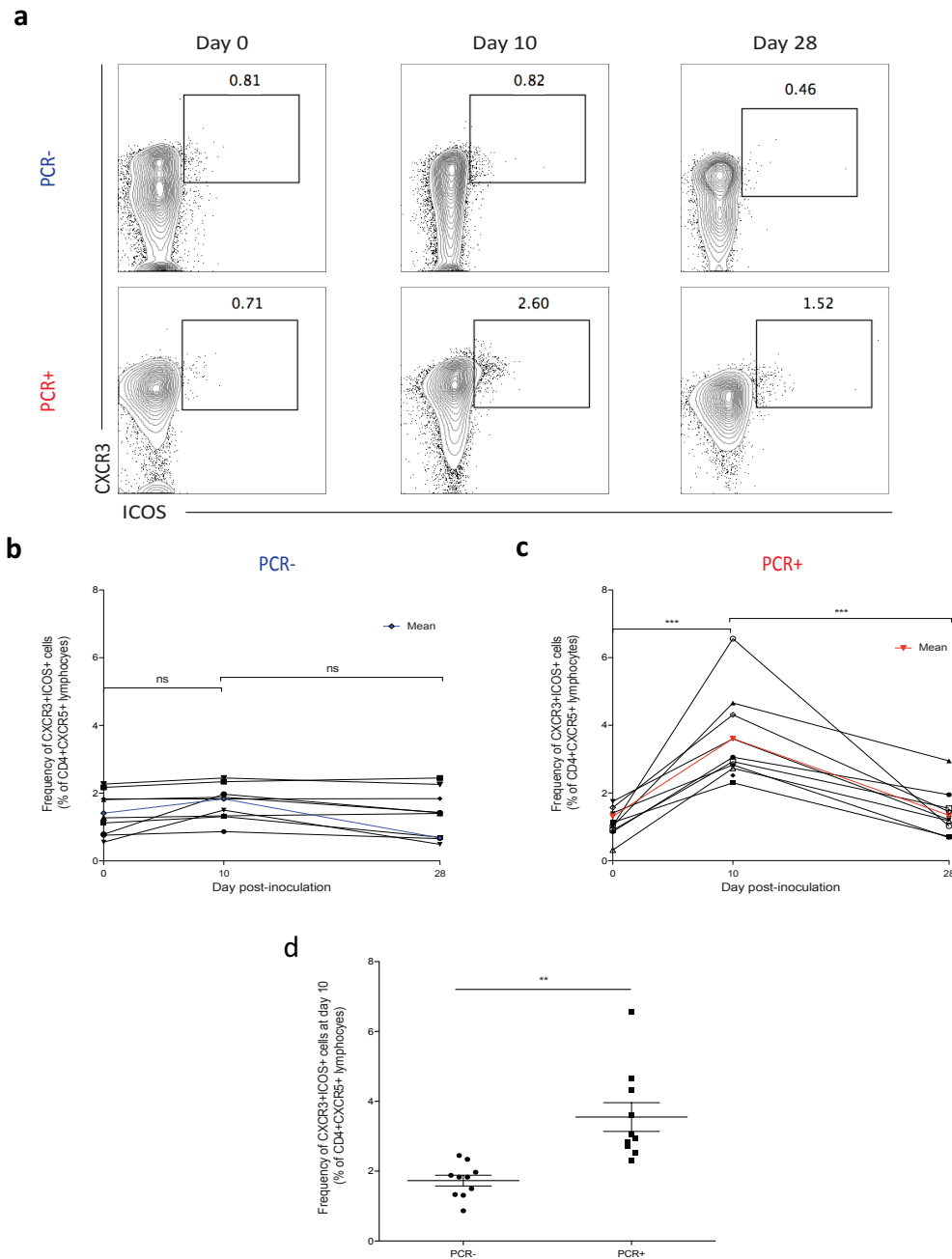


Figure 3.5.5 CXCR5+ICOS+CXCR3+ CD4+ T cells peak at day 10 in infected subjects. PBMCs from uninfected (n=10) and infected (n=10) subjects were stained with anti-CD3, CD4, CD8, CXCR5, CXCR3, ICOS and LiveDead marker, and analysed by flow cytometry. (a) Numbers represent the frequency of ICOS+CXCR3+ cells as a percentage of CXCR5+CD4+ T cells. Representative subject are shown from uninfected (PCR-) and infected (PCR+) groups. (b) Frequency of ICOS+CXCR3+ cells amongst the PCR- subjects. (c) Frequency of ICOS+CXCR3+ cells amongst the PCR+ subjects. (d) Mean $\pm$ SEM of ICOS+CXCR3+ CXCR5+CD4+ T cell frequencies in the blood of PCR- and PCR+ subjects at day 10 are shown. The p-value for a two-tailed Wilcoxon matched pairs test is shown (**p<0.01 and ***p<0.001; ns = not significant).

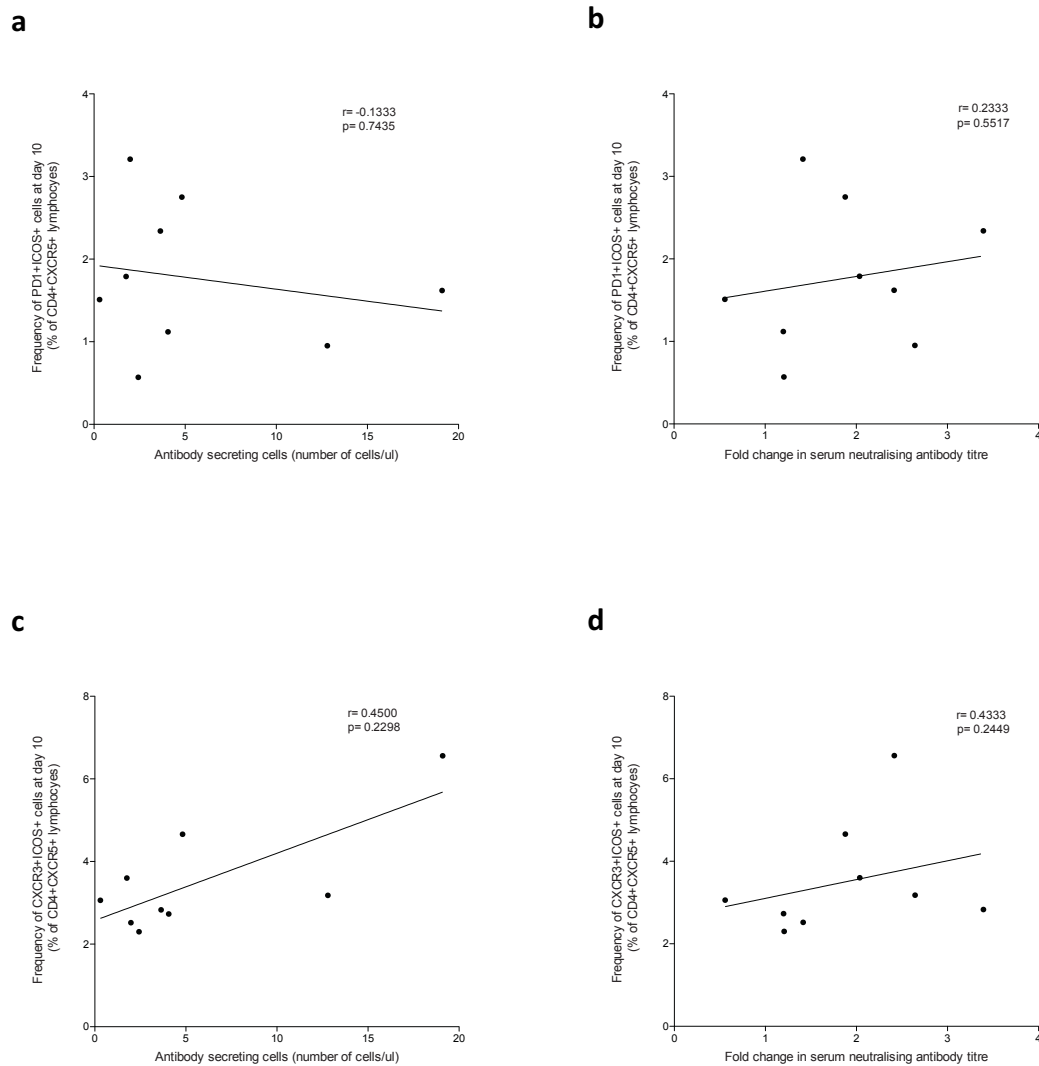


Figure 3.5.6 Peak frequency of Tfh-like cells in blood is not associated with plasmablasts or fold change in serum antibody. Non-linear regression was used to assess the association between peak ICOS+PD1+CXCR5+CD4+ Tfh-like cells and (a) number of antibody secreting cells, (b) fold change in serum neutralising antibody titre and between CXCR3+ICOS+CXCR5+CD4+ Tfh-like cells and (c) number of antibody secreting cells, (d) fold change in neutralising antibody titre. Spearman's rank correlation coefficient (r) and P values are shown.

3.5.5 Culture of cells using the MIMIC system results in non-specific proliferation

The exact combination of surface markers that describes Tfh-like cells in blood with potential to help antibody production remains controversial. Studies that have assessed functionality of different Tfh subsets have relied on sorting of PBMCs by FACS for simple co-culture with autologous B cells and may not accurately recapitulate the interactions between APC, Tfh cell and B cell *in vivo* (Bentebibel et al., 2011, 2013; Locci et al., 2013; Morita et al., 2011). We therefore investigated alternative methods that may provide a more physiological *in vitro* microenvironment and which could potentially generate Tfh cells with properties more similar to their counterparts in lymphoid organs. Although the MIMIC system was not commercially available, we collaborated with GSK Vaccines (Rixensart, Belgium) who had preliminary data on optimising this technique.

We initially followed the protocol optimised by GSK Vaccines and compared with conventional cultures of PBMCs with an antigen of interest. The MIMIC system consists two parts; the first part involves production of the 3D collagen matrix containing RSV antigen and formation of the HUVEC monolayer, after which the addition of PBMCs allows the migration of monocytes towards the collagen where they take up the antigen and differentiate into professional APCs (Figure 3.5.7a). In part 2, cells (containing antigen-loaded APCs) are collected back from the MIMIC system and cultured with autologous PBMCs with proliferation of CD4⁺ T cells assessed by CFSE dilution (Figure 3.5.7a). In parallel, we set up co-cultures where CFSE labelled PBMCs were cultured with media or RSV PreF for 7 days (Figure 3.5.7b).

Migration of the monocytes through the HUVEC is essential for these cells to become antigen-loaded APCs. To check this, a fraction of cells pre- and post-migration were analysed for the frequency of CD14-expressing monocytes (Figure 3.5.7c). In the pre-migration sample, 16% of all cells were CD14⁺, but following a 2-hour incubation, this frequency was reduced to 4.69% and 2.27% in wells containing media (no antigen control) and RSV preF, respectively. These results suggested that the monocytes had migrated through to the collagen matrix.

The simple culture of PBMCs and RSV preF resulted in proliferation of 0.41% of CD4+ T cells with no noticeable background staining (Figure 3.5.7d, top panel). On the other hand, although the culture of preF-loaded APCs with PBMCs resulted in significantly enhanced proliferation with 27.6% of the CD3+ cells having diluted CFSE staining, 12.9% of CD3+ cells in the negative control were also CFSE^{lo} (Figure 3.5.7d, lower panel).

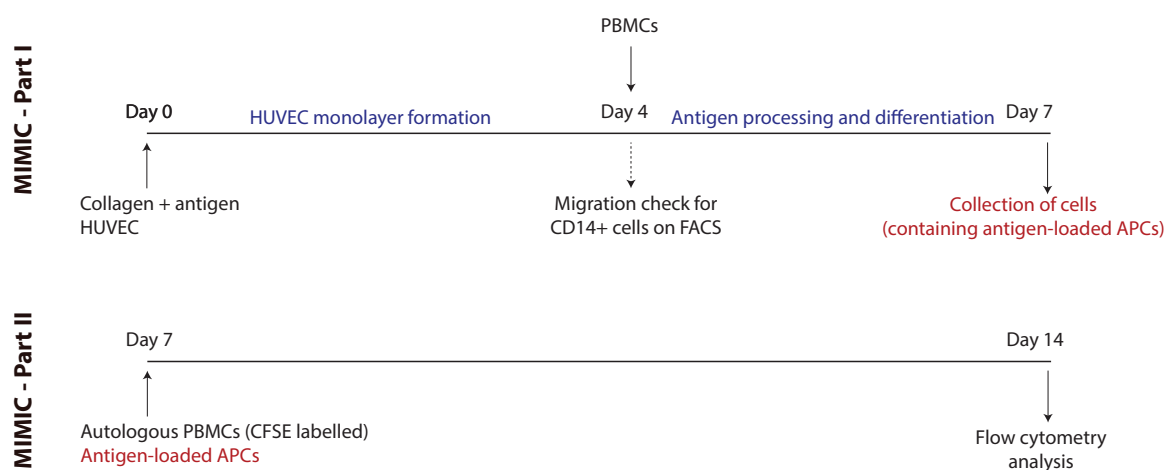
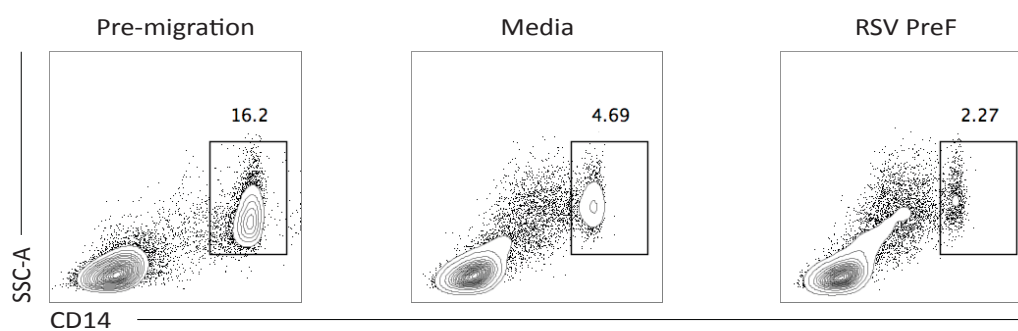
Despite the more than 2-fold difference in the frequency of CFSE^{lo}CD4+ cells, the high frequency of CFSE^{lo} T cells in the absence of antigen influences the sensitivity of the assay. We hypothesised, in view of the absence of a CFSE^{int} population in negative control wells, that these CFSE-unlabelled CD3+ cells were derived from the MIMIC-derived PBMC/APC fraction. In addition, we questioned whether proliferation to F-EFY would be higher as peptides can be presented to T cells without the need for antigen processing. To investigate this, we repeated the MIMIC experiment (as shown in Figure 3.5.7a). Once again, we were reassured that the cells were capable of migrating as there was a decrease in the frequency of CD14+ cells in the post-migration samples (Figure 3.5.8a). The cells that were collected back from MIMIC at day 7 for culturing with autologous PBMCs were then assessed for the frequency of CD3 expression. We found that CD3+ cells accounted for ~80% of the total population (Figure 3.5.8b). Following the culture of antigen-loaded APCs and autologous PBMCs, we found a similar result to the previous experiment, where 27.5% of cells in the negative control were CFSE^{lo} (Figure 3.5.8c). Although this frequency increased in response to antigen (28.4% with F-EFY and 33.1% with preF), the high-level of background staining made these results unreliable.

As the majority of cells collected from MIMIC were CD3+, it is likely that these cells contributed substantially to the CFSE^{lo} population without actual proliferation. To overcome this, a further MIMIC experiment was set up in which the cells collected back from the MIMIC system were also CFSE-labelled prior to culturing with PBMCs (Figure 3.5.9). This overcome the contribution of MIMIC-derived cells to the CFSE^{lo} population. However, despite this, non-specific background background proliferation was seen between 0.50% and 1.84%, which in some cases was higher than the antigen-specific response (Figure 3.5.9).

We previously used the expression of Ki67 and CD38 to identify proliferating and activated cells, respectively (Section 3.2.2). As an alternative to CFSE dilution, we investigated the expression of these markers by MIMIC cultured CD4⁺ T cells against F-EFY (Figure 3.5.10). Following culture, 19-24% of CD4⁺ T cells co-expressed Ki67 and CD38 indicating that they were activated. However, there was a substantial variability in the negative control with 5-17% of cells also identified as Ki67⁺CD38⁺, indicating that non-specific activation was occurring in the absence of antigen.

Finally, we investigated the functionality of MIMIC cultured antigen-specific CD4⁺ T cells by assessing cytokine production upon re-stimulation with F-EFY peptide (Figure 3.5.11). Overall, the frequencies of IFN- γ positive CD4⁺ T cells were low and TNF- α and IL-2 producers were not detectable. There was a large variability in the level of background and the increase in the frequency of IFN- γ +CD4⁺ cells was modest in response to culture with F-EFY and RSV preF. Collectively, the variability in results has dissuaded us from pursuing this technique.

In conclusion, although the MIMIC system was initially proposed as generating a more physiologically relevant *in vitro* culture system, we identified a number of issues in trying to optimise it. CFSE labelling of the cells collected back from MIMIC was crucial in overcoming the large non-proliferating CFSE^{lo} population artefact, but even then high level of non-specific background proliferation was observed. As an alternative, cells were stained at the end of the 14-day culture period with markers of activation and proliferation, but again the high variability in the background made the results unreliable. This was further reflected on investigation of the antigen-specific functionality of these cells. These results suggest that the MIMIC technique offers little benefit over conventional co-culture and is unsuitable for use, perhaps even with further extensive optimisation.

a**b****c**

d

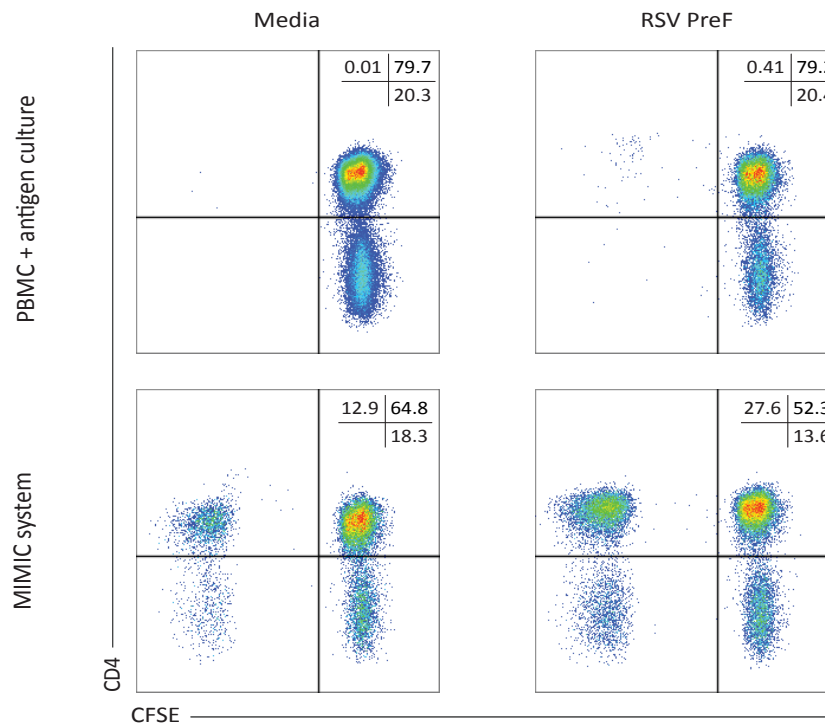


Figure 3.5.7 CD4⁺ T cell culture using MIMIC-derived APCs results in high background frequency of CFSE^{lo} cells. PBMCs from healthy donors (n=2) were cultured with media or RSV PreF (10µg/ml) for comparison of MIMIC and *in vitro* culture of PBMCs with antigen. (a) Diagram illustrates the steps of culture using MIMIC. (b) CFSE labelled PBMCs from the same donors were cultured with media or RSV PreF (10µg/ml) for 7 days and analysed using the same staining procedure. (c) At day 4 of the MIMIC culture, a fraction of PBMCs were stained with CD14 to assess the disappearance of monocytes. (d) Flow cytometry plots show CFSE dilution in CD4⁺ T cells using conventional culture (top panels) and the MIMIC system (lower panels).

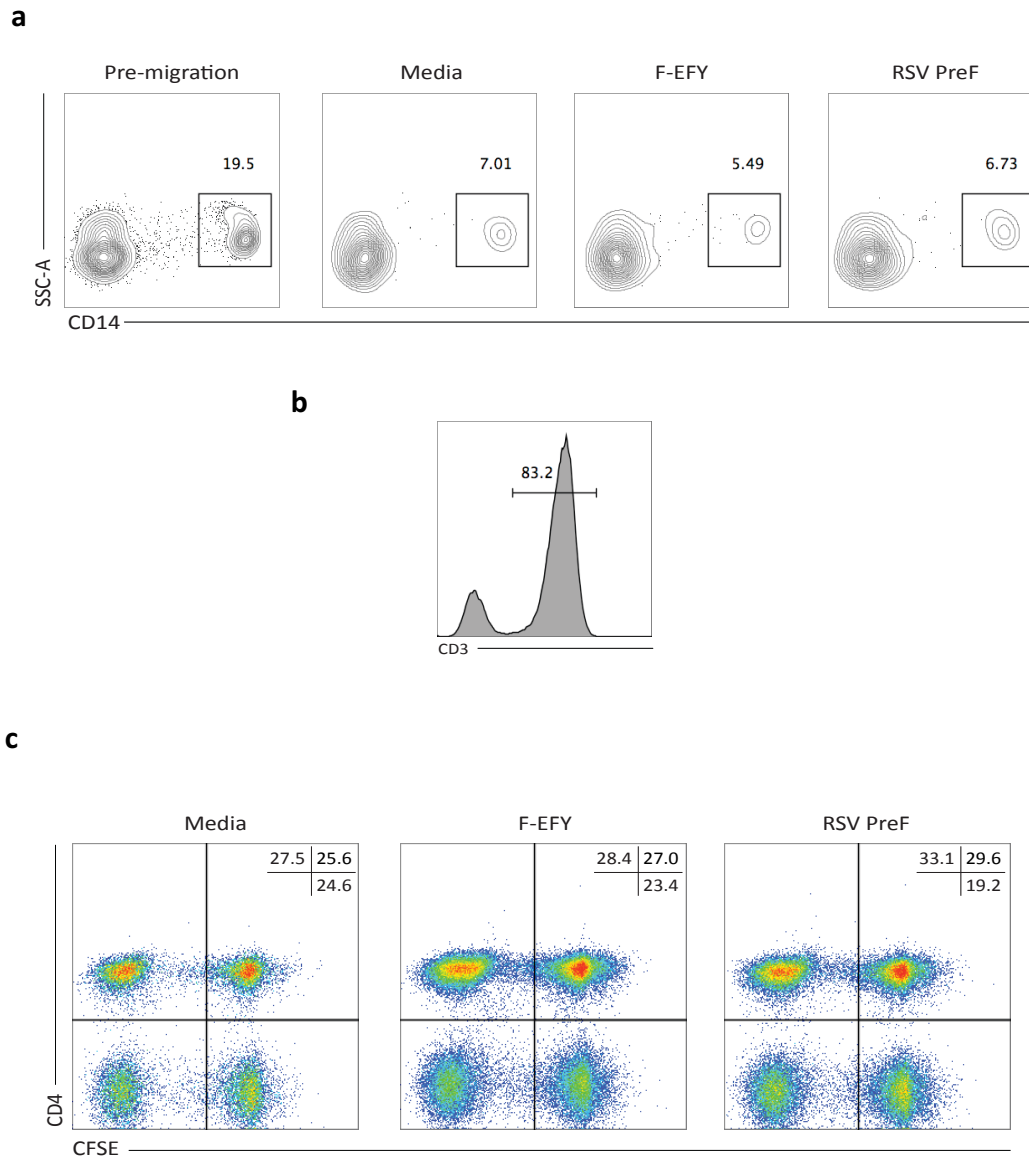


Figure 3.5.8 CFSE^{lo} cells resulting from the MIMIC system are predominantly unlabelled CD3⁺ collected along with antigen-loaded APCs. PBMCs from healthy donors (n=2) were used to set up MIMIC as described previously with F-EFY and RSV PreF. (a) Migration of cells through the HUVEC was evaluated by analysing the frequency of CD14⁺ cells pre- and post-migration. (b) At day 7, the cells collected back from MIMIC were labelled with anti-CD3. (c) At the end of the 7 day incubation of antigen-loaded APCs with autologous CFSE-labelled PBMCs, cells were stained with anti-CD3, CD4, CD8 and LiveDead marker. Representative plots are shown from one donor and gated on live CD3⁺ lymphocytes.

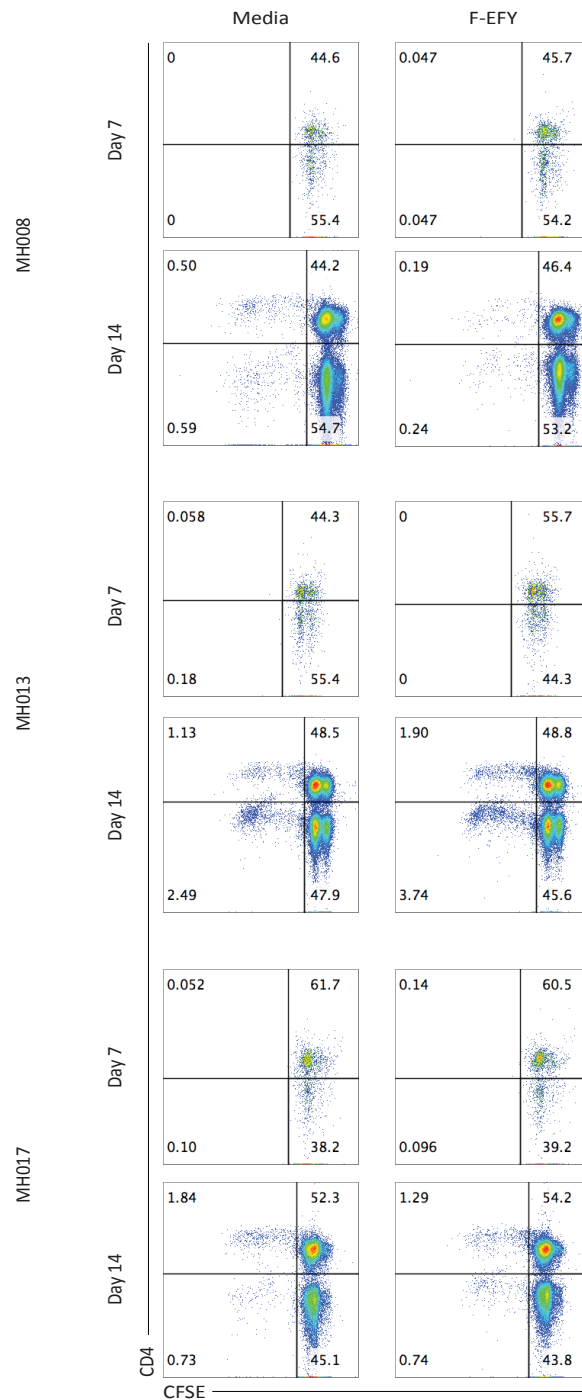


Figure 3.5.9 Non-specific proliferation of CD3+ T cells occurs following MIMIC culture. HLA-matched baseline PBMCs from study subjects (n=3) were used to set up MIMIC as described previously with F-EFY. Cells were stained with anti- CD3, CD4, CD8 and LiveDead marker prior to analysis. At day 7, the cells collected back from MIMIC were stained additionally stained with CFSE prior to culture with autologous PBMCs. At day 14, CFSE dilution by CD4+ T cells was investigated. Flow cytometry plots are gated on live CD3+ lymphocytes.

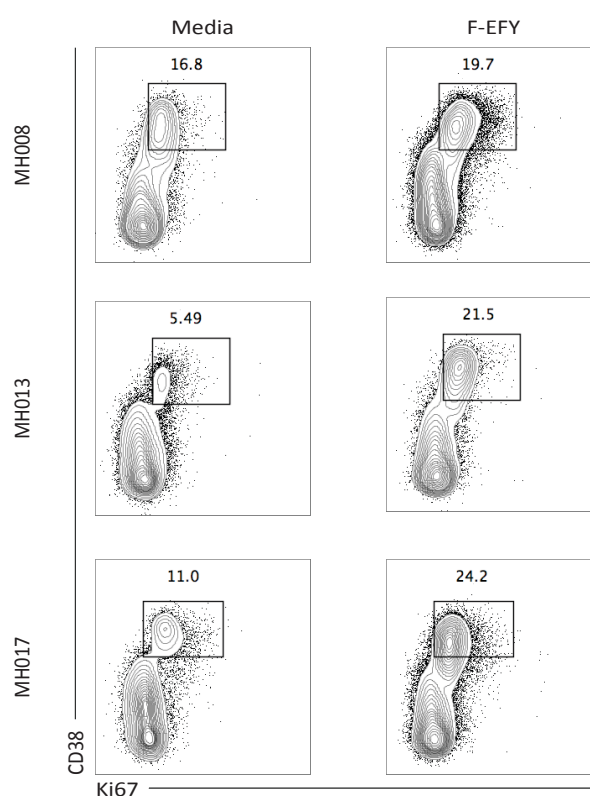


Figure 3.5.10 MIMIC culture induces non-specific CD4⁺ T cell proliferation. HLA-matched baseline PBMCs from study subjects (n=3) were used to set up MIMIC as described previously with F-EFY. Cells were stained with anti- CD3, CD4, CD8, CD38, Ki67 and LiveDead marker prior to analysis. FACS plots gated on live CD4⁺ lymphocytes show the frequency of Ki67⁺CD38⁺ cells as a percentage of CD4⁺ cells in response to media (negative control) and F-EFY peptide.

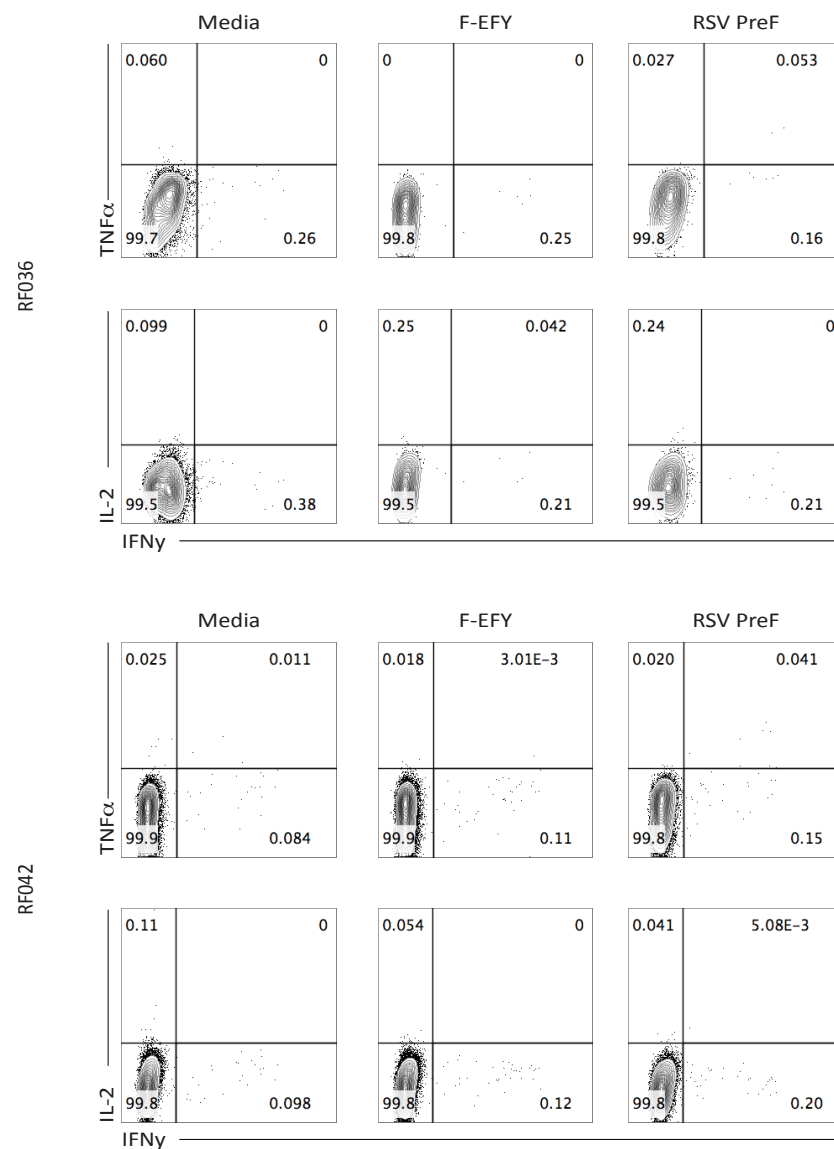


Figure 3.5.11 MIMIC poorly induces cytokine-producing RSV-specific CD4+ T cells. HLA-matched PBMCs from study subjects at baseline (n=2) were used to set up MIMIC as described previously with F-EFY and RSV preF protein. At day 14, all conditions were re-stimulated with F-EFY peptide for 6 hours with Brefeldin A added in the last 4 hours of the incubation. Cells were then surface stained with anti- CD3, CD4, CD8, and LiveDead marker prior to intracellular cytokine staining with IFN- γ , IL-2 and TNF- α . Gated on live, CD3+CD4+ lymphocytes, FACS plots show the frequencies of the 3 cytokines in subject RF036 (top panels) and subject RF042 (lower panels).

3.5.6 Discussion

Tfh cells are a distinct subset of CD4⁺ T cells that are specialised in providing help to B cells in GCs for affinity maturation, class switching and memory B cell differentiation (Crotty, 2011). These cells are commonly identified by the expression of CXCR5, ICOS, PD-1 and Bcl-6 in human tonsils and murine lymphoid tissues (Baumjohann et al., 2013; Bentebibel et al., 2011; Harker et al., 2011; Hatzi et al., 2015). Limitations on accessing lymphoid tissues from humans have resulted in a search for Tfh cells in blood but the exact peripheral analogue of GC Tfh cells has yet to be clarified.

Studies in mouse models have shown that prophylactic treatment with an anti-G protein monoclonal antibody can enhance the frequency of CD4⁺ T cells expressing CXCR5 and Bcl-6 in the spleen and is associated with the increased number of B cells and higher titre of antibodies late after infection (Boyoglu-Barnum et al., 2014). To date, there have been no reports studying Tfh cells in human RSV infection. However, Tfh-like cells in blood characterised by the expression of CXCR5, CXCR3, ICOS and PD-1 have been shown to correlate with antibody titre (Bentebibel et al., 2013) and more recently with the generation of high-avidity antibodies following influenza vaccination (Bentebibel et al., 2016). These studies provided the rationale to investigate Tfh-like cells following RSV infection.

Tfh cells have been previously shown to have distinct profiles between lymphoid tissues and peripheral blood (Morita et al., 2011; Bentebibel et al., 2013; Hatzi et al., 2015; He et al., 2013). These studies suggested that CD4⁺ T cells expressing the surface proteins CXCR5, PD-1 and ICOS were present at significantly higher frequencies in tonsils, and that Bcl-6 expression could not be detected in peripheral blood. Furthermore, a comprehensive analysis by Hatzi and colleagues of CD4⁺ T cells in tonsils divided these into non-Tfh (CXCR5-PD1⁺), Tfh (CXCR5+PD1⁺) and GC Tfh (CXCR5+PD-1^{Hi}) subsets and found Bcl-6 expression was highest in the GC Tfh subset (Hatzi et al., 2015). Since this study focuses primarily on peripheral blood from RSV infected subjects, tonsillar Tfh cells were not extensively investigated. However, our results comparing the frequencies of CXCR5, PD-1, ICOS and Bcl-6 expressing CD4⁺ T cells agreed with what has been published by others.

With the absence of Bcl-6 expression, identification of a phenotype defining functional Tfh-like cells in blood has been difficult. As a canonical marker of true Tfh cells, CXCR5 expression alone was initially proposed as a marker of Tfh-like cells in blood. Rivino and colleagues showed detectable CXCR5+CD4+ T cells in blood up to 4 years after a TT booster vaccination providing evidence that memory cells expressing CXCR5+CD4+ circulate in peripheral blood (Rivino et al., 2004). However, this was complicated by the results of *in vitro* experiments that showed short term upregulation of CXCR5 following cross-linking of naïve CD4+ T cell receptors, suggesting that CXCR5 expression might also be a feature of transiently activated CD4+ T cells (Schaerli et al., 2001). In addition, the majority of CXCR5+CD4+ cells in healthy subjects were found not to express CD69, ICOS and PD-1, other markers of T cell activation (Ma et al., 2009; Simpson et al., 2010). Earlier studies had reported that CXCR5+ and CXCR5-CD4+ T cells had a similar capacity to help B cells *in vitro* (Kim et al., 2001). This study had technical limitations, as T cells were activated using plate-bound anti-CD3 and soluble anti-CD28 in the absence of antigen, which overrides cognate interaction with autologous B cells. In contrast, more recent studies used cognate antigen for the interaction of T and B cells (along with super-antigen to increase the strength of T-B interaction) and showed greater helper capacity by the blood CXCR5+CD4+ population (Bentebibel et al., 2013; Morita et al., 2011).

The phenotype of functional CXCR5+CD4+ Tfh-like cells in blood was further refined by taking into account the expression of ICOS, PD-1 and CXCR3 (Bentebibel et al., 2013, 2016; Matsui et al., 2015). Using two combinations of these phenotypic markers previously suggested as defining functional Tfh-like cells, we investigated the changes in surface marker expression (ICOS and PD-1 or ICOS and CXCR3) of CXCR5+CD4+ T cells through the course of RSV infection. The frequencies of both subsets increased only in the infected subjects at day 10 post-infection. While neither showed a statistically significant correlation with antibody secreting cell numbers, a positive trend was observed between the frequency of CXCR5+ICOS+CXCR3+ Tfh-like cells and antibody secreting numbers in RSV infection that may have reached statistical significance with increased sample size. Sorted CXCR5+ICOS+CXCR3+CD4+ T cells from blood of influenza vaccinated subjects have been shown to help memory B cells *in vitro* and correlate with the increase in antibody titres (Bentebibel et al., 2013). However, with the relatively low frequencies of CXCR5+CD4+ICOS+CXCR3+ cells and

limitations the number of PBMCs available following the RSV challenge study, we were not able to sort these Tfh-like cells to assess their functionality and this remains planned for future work.

Tfh cells in GCs have previously been shown to produce IL-21 and IL-4 to help B cells in the follicles (Bryant et al., 2007; Bentebibel et al., 2013; Yusuf et al., 2010). However, following the stimulation of antigen-specific CD4⁺ T cells in the tonsils, we found little production of IL-21 or IFN- γ and no IL-4. One possible explanation for this outcome could be a low frequency of RSV-specific cells. Tonsillar samples were obtained from children undergoing elective tonsillectomy. Although infection with RSV occurs in almost every child by the age of 2, multiple repeated infections are necessary before antibody levels reach adult levels. Although CD4⁺ T cells have not been studied in detail in this population, it is possible that memory CD4⁺ T cells against RSV also remain at very low frequencies until boosted by several RSV infections. Another possibility could be due to intrinsic limitations within GC Tfh cells, as the main role of these cells is to deliver localised help to B cells rather than secreting large amounts of cytokine (Havenar-Daughton et al., 2016). Recently, a cytokine-independent method was reported for detection of antigen-specific Tfh cells in GCs and in human blood using CD25 and OX40L expression (Havenar-Daughton et al., 2016). Using this approach, the authors identified HIV-specific GC Tfh cells at 10-fold higher frequencies in HIV Env protein immunized macaques when compared with short-term stimulation and intracellular cytokine staining. This method could be utilised in future experiments to investigate the RSV-specific Tfh-like cells in tonsils and blood following stimulation with immunodominant F-EFY and G-DDF epitopes described here.

As an alternative approach to *in vitro* co-culture assays that involve sorting of T and B cells, we aimed to optimise the MIMIC system in collaboration with GSK Vaccines (Rixensart, Belgium). Originally developed by scientists at Vaxdesign (Florida, USA), MIMIC was shown to recapitulate vaccine-induced antigen-specific IgG serum titres (Byers et al., 2009; Higbee et al., 2009). This technique might have provided us with a system that more closely resembled the physiological microenvironment, and which might allow study of the proliferative capacity and functionality of Tfh-like cells in response to RSV. However, our efforts in trying to optimise this technique were hampered by high levels of non-specific

proliferation. To date, there have been no reports that show the use of the MIMIC system for the investigation of T cell proliferation and the mechanisms by which non-specific expansion of T cells occurs remains unclear. In the literature, reports that have used this technique to assess antibody production as a functional readout have suggested a significant advantage of MIMIC over using classical co-culture assays (Byers et al., 2009; Higbee et al., 2009). In contrast, we found low levels of IFN- γ producing CD4⁺ T cells stimulated with F-EFY and RSV preF protein and in some cases these were comparable to frequencies in the negative controls. Although we did not use antibody production as a readout, we concluded that despite extensive troubleshooting, non-specific levels of proliferation and cytokine production made this technique inadequate in comparison to classical culture of PBMCs and antigen *in vitro*. The MIMIC method used was developed in-house by GSK Vaccines, and may have had differences compared to the original proprietary protocol developed by Vaxdesign. For this reason, we cannot directly compare or comment on the reproducibility of our results with what has been published by others (Byers et al., 2009; Higbee et al., 2009).

In conclusion, we showed for the first time the induction of Tfh-like cells in blood following RSV infection, defined as ICOS⁺CXCR3⁺CXCR5⁺ CD4⁺ T cells. The positive trend between the frequencies of Tfh-like cells and antibody-secreting cells suggest that Tfh-like cells in the blood may be useful in predicting antibody responses but more studies need to be done to overcome the limitations of small sample size and low precursor frequencies. In addition, more sensitive techniques for the detection and tracking of antigen-specific CD4⁺ T cells, such as class II tetramers, may enhance the analysis of Tfh-like cells in blood.

3.6 Phenotypic characterisation of RSV-specific CD4⁺ T cells using class II tetramers

3.6.1 Introduction

An important breakthrough in the study of antigen-specific T cells occurred with the work of Altman and colleagues and the development of MHC class I/peptide tetramers (Altman et al., 1996). Using immunodominant epitopes in HIV gag and pol, they showed that antigen-specific CD8⁺ T cells labelled with tetramers expressed phenotypic markers effector and memory cells in HIV infected subjects (Altman et al., 1996). Since then, class I tetramers have been used for characterisation of CD8⁺ T cells in a number of infectious diseases including influenza (Danke and Kwok, 2003; He et al., 1999, 2008; Novak et al., 1999), HIV (Angin et al., 2012; Scott-Algara et al., 2001), hepatitis C (Day et al., 2003; López-Labrador et al., 2004) and RSV (Jozwik et al., 2015). Although methods for producing class II tetramers appeared not long after (Novak et al., 1999), broad application of these tools has been slow due to difficulties in manufacturing class II-based reagents, low frequencies of antigen-specific CD4⁺ T cells in many biological contexts and difficulties caused by low TCR avidity (Vollers and Stern, 2008). Nevertheless, class II tetramers have provided insights that have included the proliferative capacity and phenotype of HA-specific CD4⁺ T cells in influenza infection (Danke and Kwok, 2003; Lucas et al., 2004; Novak et al., 1999).

MHC class I/peptide complexes are typically produced by overexpression of the heavy chain and $\beta 2m$ at high levels in *E. coli*. This leads to formation of protein aggregates which can be purified in large quantities and folded *in vitro* in the presence of defined peptides (Garboczi et al., 1992). However, production of class II/peptide complexes in this way requires folding conditions for individual allotypes to be optimised and there are only 3 different HLAs that have been produced using this method; HLA-DR1, DR2a and DR4 (Cunliffe et al., 2002; Li et al., 2000; Stöckel et al., 1994). Instead, the most common method for producing the MHC class II/peptide complex is co-expression of α and β -subunits in stably transformed insect cells using Baculovirus expression systems (Cecconi et al., 2008; Sloan et al., 1995). The lack of antigen processing and loading machinery as well as assembly of stable MHC protein in

the absence of peptide allows isolation of peptide-free complexes, which can then be loaded with defined peptides *in vitro* (Vollers and Stern, 2008).

Using class I tetramers, our group previously showed that almost all tetramer-labelled RSV-specific CD8⁺ T cells in BAL displayed a resident memory phenotype, which was not seen in matched blood samples, and accumulated to strikingly high frequencies post-infection (Jozwik et al., 2015). Conversely, there are no reports on the characteristics of human RSV-specific CD4⁺ T cells, mainly due to the lack of appropriate reagents. Therefore, having shown that the epitopes F-EFY and G-DDF are immunodominant and restricted to MHC class II molecules, we produced novel class II tetramers; HLA-DRB1*04:01/F-EFY, HLA-DRB1*09:01/F-EFY, HLA-DRB1:11*01/F-EFY and HLA-DPA1*03:01/HLA-DPB1*04:01/G-DDF in collaboration with the NIH tetramer core facility at Emory University (Georgia, USA) and optimised staining with them.

3.6.2 HA-PRY stimulation and cytokine supplementation of PBMCs drive expansion of influenza-specific CD4⁺ T cells

The frequency of antigen-specific CD4⁺ T cells in blood are low, estimated to be in the 1:1,000 – 1:20,000 range after vaccination, and 1:20,000 – 1:100,000 without recent boosting for influenza-specific CD4⁺ T cells (Nepom, 2012). As detection of these rare populations can be challenging, we aimed to optimise the staining of the novel tetramers with cells expanded using peptide and cytokine supplementation, which we have shown to be effective in enhancing the proliferation of antigen-specific cells (Section 3.4.5). Having a robust method for generating high frequencies of antigen-specific CD4⁺ T cells was necessary in order to optimise the staining conditions for tetramers against RSV-specific epitopes as these can be highly variable between reagents. Influenza HA-specific class II tetramers with the peptide PRYVKQNTLKLAT (HA-PRY) and HLA-DRB1*01:01, 04:01 and 07:01 were optimised previously (Danke and Kwok, 2003; Novak et al., 1999) and so these were used to assess the optimum culturing conditions using peptide and cytokine supplementation to expand cells of interest *in vitro*.

Baseline samples from HLA matched subjects (MH010 – DRB1*04:01, MH015 – DRB1*04:01 and MH035 – DRB1*07:01) were cultured with DMSO or HA-PRY peptide and supplemented with IL-2 and IL-7 for 14 days (Figure 3.6.1a). A similar method had previously been reported for the expansion of CD4⁺ T cells followed by staining with DRB1*11:01 tetramers (Cecconi et al., 2008). Cells were then labelled with HA-PRY tetramers using conditions previously reported (Danke and Kwok, 2003; Novak et al., 1999). To serve as a negative control, a fraction of cultured cells were labelled with a tetramer made with the irrelevant human CLIP peptide (PVSKMRMATPLLMQA). Following culture with DMSO, no antigen-specific cells were detected in the context of any of these HLAs HA-PRY tetramers (Figure 3.6.1b). In contrast, HA-PRY specific CD4⁺ T cells were detected with tetramer following culture with HA-PRY peptide (0.33% with DRB1*01:01, 0.79% with DRB1*04:01 and 0.41 % with DRB1*07:01). Staining of HA-PRY cultured cells with CLIP tetramer resulted in no detectable populations, ruling out non-specific staining (Figure 3.6.1b).

Thus, PBMCs cultured with peptide and cytokine supplementation for 14 days resulted in expansion of antigen-specific CD4⁺ T cells that were detectable with class II tetramers and supported a similar strategy for the optimisation of novel RSV tetramers.

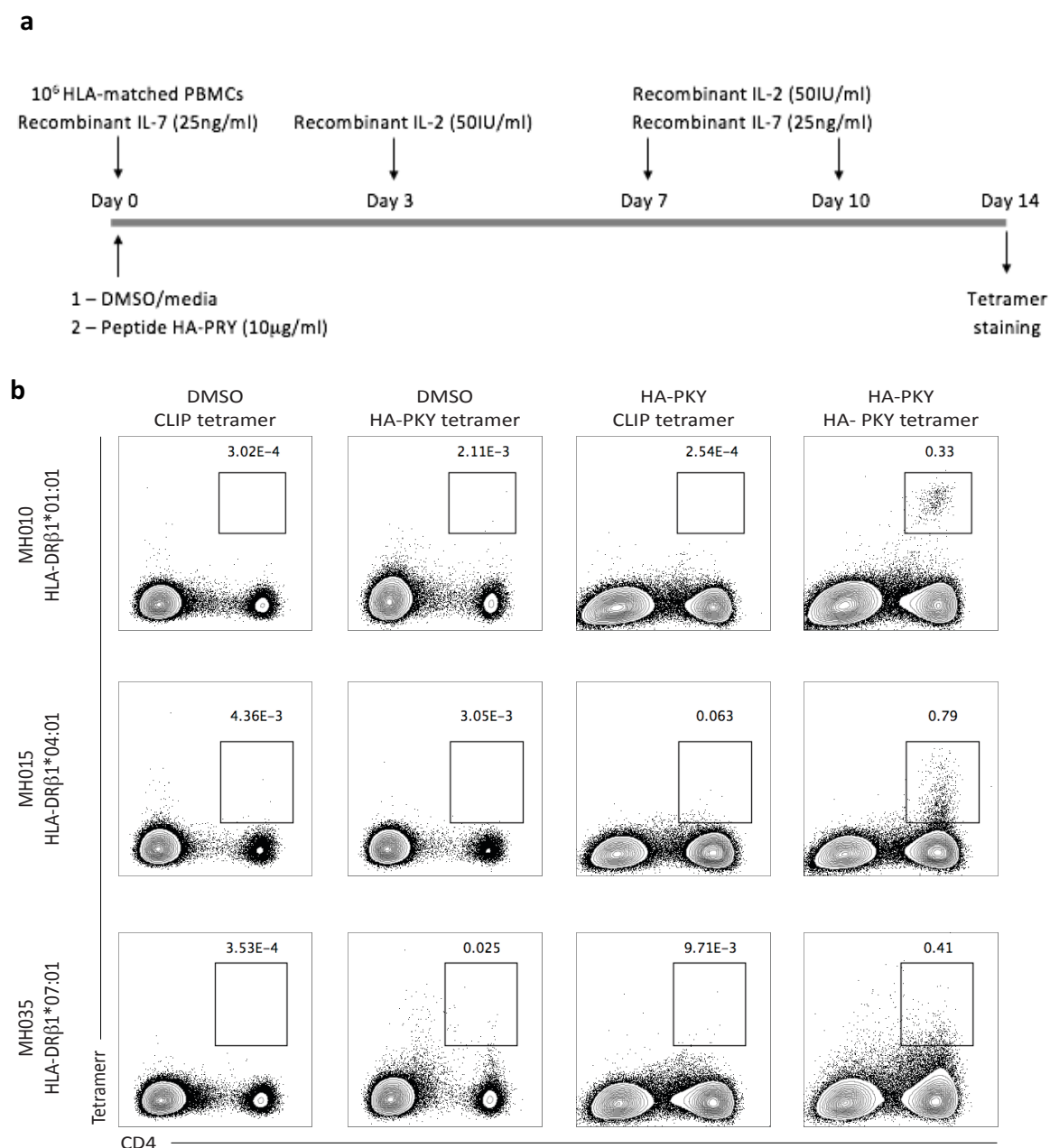


Figure 3.6.1 Culture of HLA-matched PBMCs with peptide and cytokine supplementation induces expansion of antigen-specific CD4+ T cells. (a) 1x10⁶ CFSE labelled PBMCs from HLA-matched study subjects at baseline were cultured with DMSO or 10µg/ml of HA-PRY at 37°C for 14 days. The cultures were supplemented with recombinant IL-7 (25ng/ml) at days 0, 7, 10 and recombinant IL-2 (50IU/ml) at days 3,7 and 10. At day 14, cells were stained with tetramer for 20 minutes at 4°C prior to surface staining with anti- CD3, CD4, CD8 and Live/Dead marker to assess antigen-specific CD4+ T cells. (b) FACS plots are gated on live CD3+ lymphocytes in three representative subjects with different HLA types.

3.6.3 *In vitro* expanded RSV-specific CD4⁺ T cells can be detected with F-EFY and G-DDF tetramers

Using HA-PRY tetramer, we found that *in vitro* culture of PBMCs with peptide and cytokine supplementation was effective in expanding CD4⁺ T cells. Using the same approach, we sought to optimise the staining conditions for class II tetramers against F-EFY and G-DDF. This required testing of each reagent with varying incubation temperature (4°C, 20°C and 37°C), time (30', 90' and 180') and tetramer concentration (2µg/ml, 6µg/ml and 18µg/ml) as shown in Table 3.6.1 In all experiments, we included dasatinib, a reversible protein kinase inhibitor that has been shown to improve the staining intensity of T cells with class I and class II tetramers by at least 50% without any increases in background staining (Lissina et al., 2009).

	4°C		20°C		37°C	
	Control (CLIP)	Experimental	Control (CLIP)	Experimental	Control (CLIP)	Experimental
30 minutes	2µg/ml	2µg/ml	2µg/ml	2µg/ml	2µg/ml	2µg/ml
	6µg/ml	6µg/ml	6µg/ml	6µg/ml	6µg/ml	6µg/ml
	18µg/ml	18µg/ml	18µg/ml	18µg/ml	18µg/ml	18µg/ml
90 minutes	2µg/ml	2µg/ml	2µg/ml	2µg/ml	2µg/ml	2µg/ml
	6µg/ml	6µg/ml	6µg/ml	6µg/ml	6µg/ml	6µg/ml
	18µg/ml	18µg/ml	18µg/ml	18µg/ml	18µg/ml	18µg/ml
180 minutes	2µg/ml	2µg/ml	2µg/ml	2µg/ml	2µg/ml	2µg/ml
	6µg/ml	6µg/ml	6µg/ml	6µg/ml	6µg/ml	6µg/ml
	18µg/ml	18µg/ml	18µg/ml	18µg/ml	18µg/ml	18µg/ml

Table 3.6.1 Conditions for optimisation of class II tetramer staining with varying incubation temperature, time and concentration.

For initial optimisation experiments, PBMCs from one individual expressing each appropriate HLA were expanded using F-EFY or G-DDF for 14 days with cytokine supplementation as described in previous section (Figure 3.6.2a). Cells were then stained with varying concentrations of tetramer for analysis by flow cytometry. While 2µg/ml of tetramer against the different HLA-types produced low levels of non-specific staining with CLIP tetramer (<0.1%), antigen-specific CD4⁺ T cells were detectable with frequencies of CD4⁺ T cells of 0.26% for DRB1*04:01/F-EFY, 1.16% for DRB1*11:01/F-EFY and 0.35% for DPB1*04:01/G-DDF (Figure 3.6.2b). There was no advantage in increasing the tetramer concentration as 6µg/ml and 18µg/ml resulted in increased background staining by both the actual tetramers

and negative control CLIP tetramer. Despite having produced the DRB1*09:01/F-EFY tetramer, we were limited by low numbers of cells from donors of this HLA-type and so this was not tested. On the other hand, there were technical difficulties in expressing the DPB1*02:01/G-DDF tetramer and this reagent was not available in time for testing.

After determining the optimum tetramer concentration, we expanded PBMCs to test varying time and temperature conditions. For the DRB1*04:01/F-EFY tetramer, we found staining at 4°C for 30 minutes to be optimum with 0.047% of CD4+ T cells labelled non-specifically by negative control compared with 0.22% by tetramer (Figure 3.6.2c). Although increasing the incubation time and temperature resulted in higher frequencies of antigen-specific cells detected by the F-EFY tetramer (up to 0.31% at 4°C for 180 minutes), we also observed an increase in non-specific labelling of CD4- cells. Next, we found staining at 4°C for 30 minutes was optimum for the DRB1*11:01/F-EFY tetramer with a frequency of 1.09% and background staining by the negative control below 0.1% (Figure 3.6.2d). Similarly, increasing the time and temperature enhanced the frequency of detectable antigen-specific cells (up to 1.68% at 37°C for 180 minutes) but these conditions also resulted in higher background. Finally, we found the optimum staining conditions for DPB1*04:01/G-DDF tetramer following incubation at 20°C for 30 minutes with background frequency of 0.045% and G-DDF tetramer+ CD4+ T cells at 1.39% (Figure 3.6.2e). As was the case with other tetramers tested, varying time and temperature could enhance frequency of antigen-specific cells to a certain extent but the increased background levels made these conditions less suitable.

We next validated these optimum staining conditions for each tetramer using expanded baseline samples from 3 subjects for each HLA (Figure 3.6.2f). Reassuringly, non-specific staining with CLIP tetramer remained below 0.05% in all donors. In contrast, the frequency of DRB*04:01/F-EFY tetramer-binding CD4+ T cells was found to be between 0.03% and 0.09%; DRB1*11:01/F-EFY tetramer binding between 0.40% and 0.82%; and DPB1*04:01/G-DDF tetramer binding between 0.15% and 0.22% (Figure 3.6.2f).

Non-specific staining using a negative control class II tetramer or within the non-CD4+ T cell population have been observed using different reagents in different settings such that a level of background staining between 0.1-0.3% is generally accepted as a limitation of the reagent

(Braendstrup et al., 2014; Holland et al., 2015; Schmidt et al., 2013). In a few cases, we found non-specific staining of the CD3⁺CD4⁻ population with the G-DDF tetramer and investigated the source of this using by back-gating (Figure 3.6.2g). As illustrated in the representative example (Figure 3.6.2h), we found that half of the non-specific staining from binding to CD8⁺ cells (mean 0.085% $\pm$ s.e.m. 0.003%) and the remaining background staining was from double negative CD3⁺ cells (mean 0.07% $\pm$ s.e.m. 0.002%; Figure 3.6.2i).

In conclusion, we optimised and validated staining conditions for three F-EFY and G-DDF tetramers. For all 3 reagents, a concentration of 2 μ g/ml and incubation period of 30 minutes achieved the optimum balance between sensitivity and non-specific background staining with temperatures of 4°C for F-EFY/DRB1*04:01 and F-EFY/DRB1*11:01, and 37°C for G-DDF/DPB1*04:01.

a

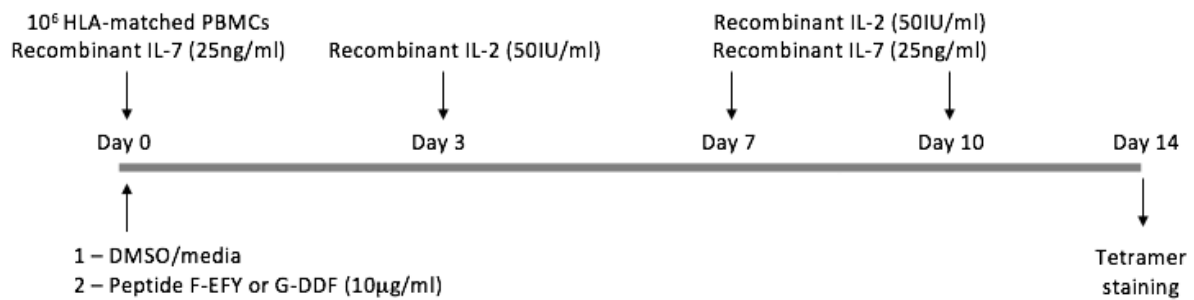
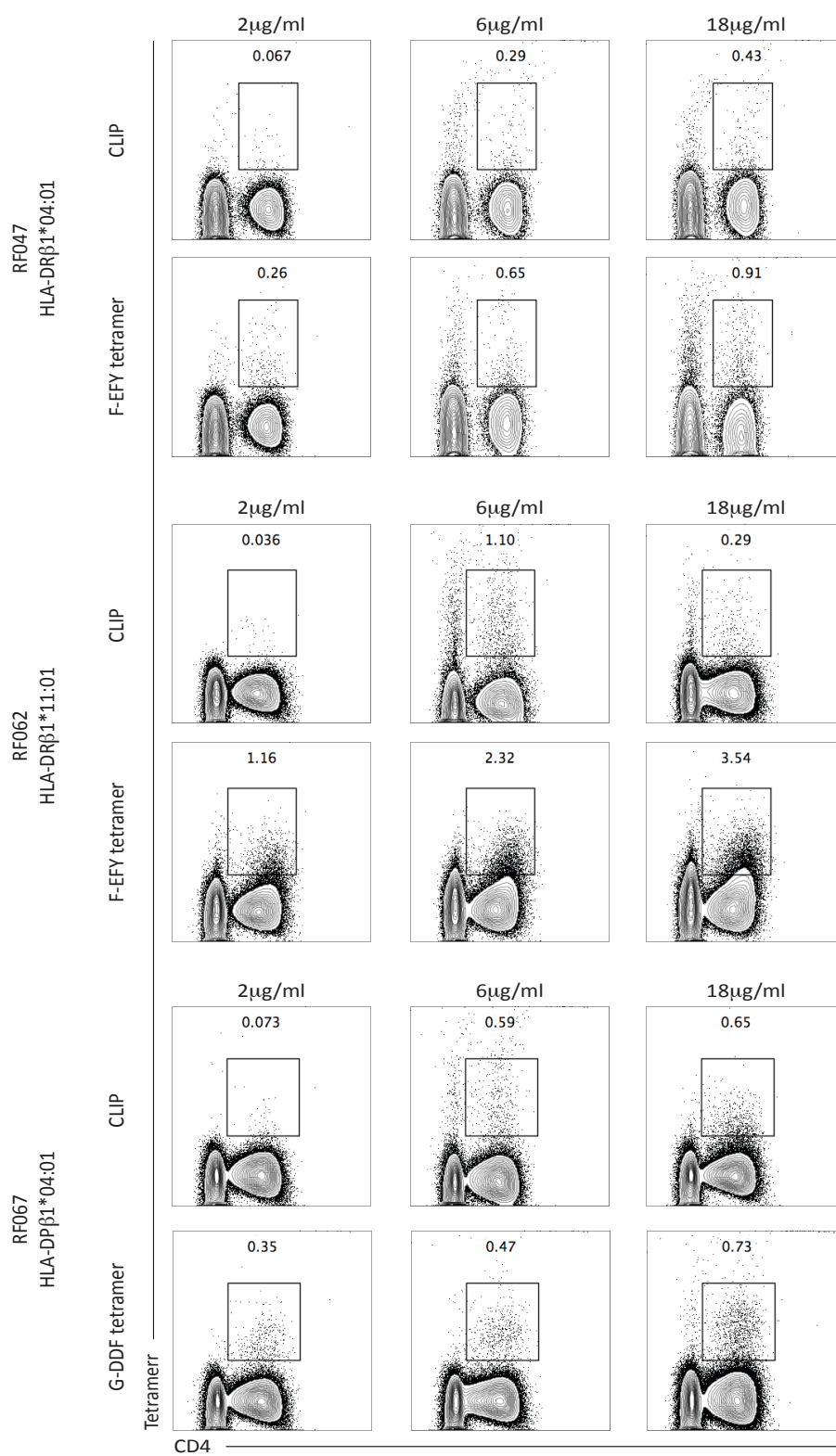
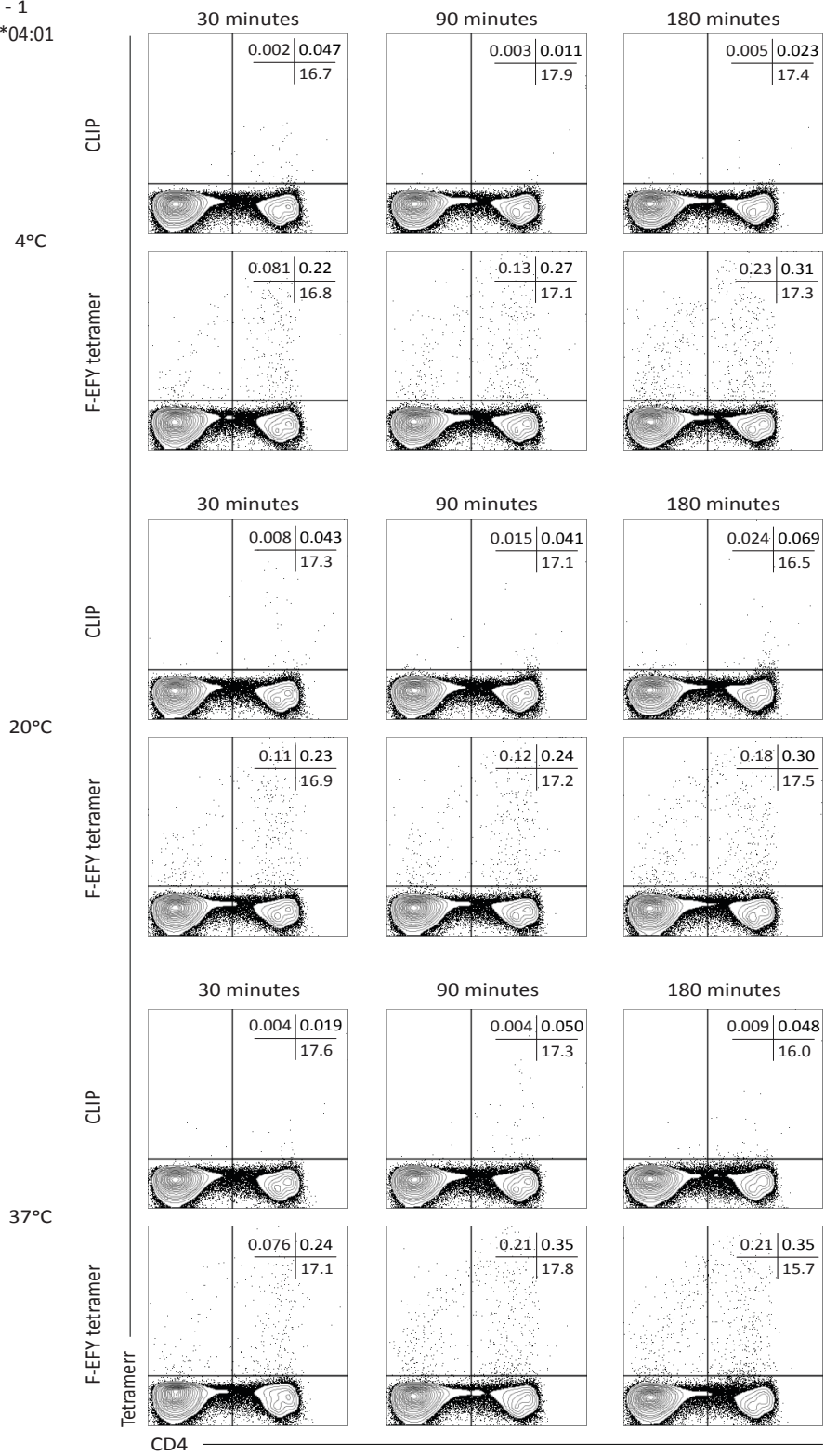


Figure 3.6.2 RSV-specific CD4⁺ T cells can be detected with class II tetramers following *in vitro* expansion. (a) 1×10^6 CFSE labelled baseline PBMCs from HLA-matched study subjects were cultured with DMSO and 10µg/ml of F-EFY or G-DDF at 37°C for 14 days. The cultures were supplemented with recombinant IL-7 at days 0, 7, 10 and recombinant IL-2 at days 3, 7 and 10. At day 14, cells were treated with dasatinib and stained with tetramer with varying conditions described in Table 3.6.1 prior to surface staining with anti- CD3, CD4, CD8 and Live/Dead marker to assess antigen-specific CD4⁺ T cells. (b) FACS plots show tetramer staining with varying concentrations (2µg/ml, 6µg/ml and 18µg/ml) on cells gated on live CD3⁺ lymphocytes in three subjects with different HLA types (DRB1*04:01 and -11:01 for F-EFY and DPB1*04:01 for G-DDF). FACS plots show tetramer staining (2µg/ml) with varying incubation temperature (4°C, 20°C and 37°C) and time (30', 90' and 180') for F-EFY tetramer against (c) DRB1*04:01, (d) DRB1*11:01 and G-DDF tetramer against (e) DPB1*04:01. (f) To validate the optimum conditions, cultured PBMCs from three subjects for each tetramer were stained with F-EFY tetramer against DRB1*04:01 at 4°C for 30 minutes (top panel), DRB1*11:01 at 4°C for 30 minutes (middle panel) and G-DDF tetramer against DPB1*04:01 at 20°C for 30 minutes (lower panel). FACS plots show the (g) back-gating and (h) full gating strategy on RF036 (DPB1*04:01/G-DDF) to investigate the source of background staining within the CD3⁺CD4⁻ population. (i) Mean $\pm$ s.e.m. of frequencies of tetramer-stained CD3⁺ T cells are shown.

b

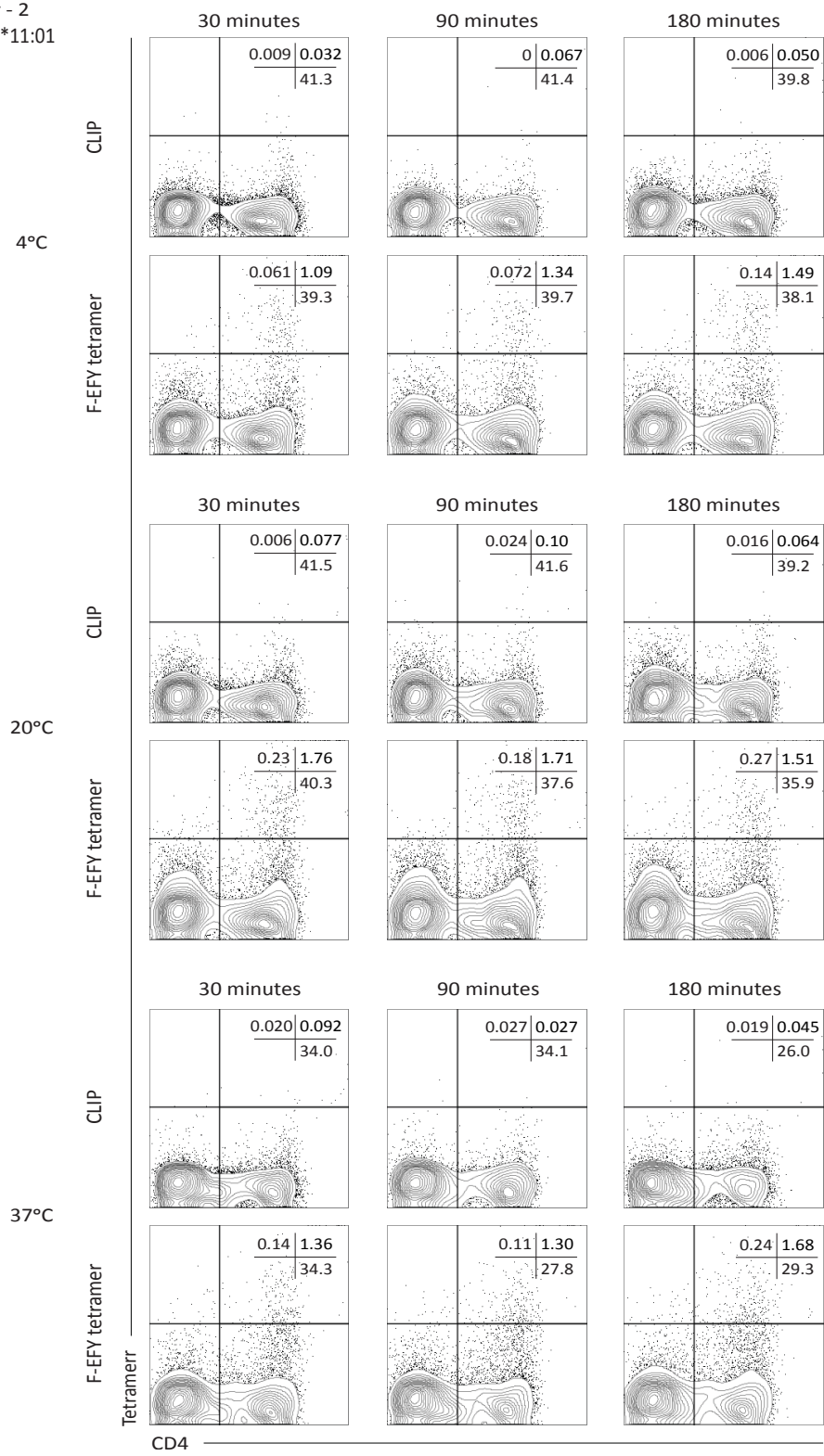
c

Donor - 1
HLA-DR β 1*04:01



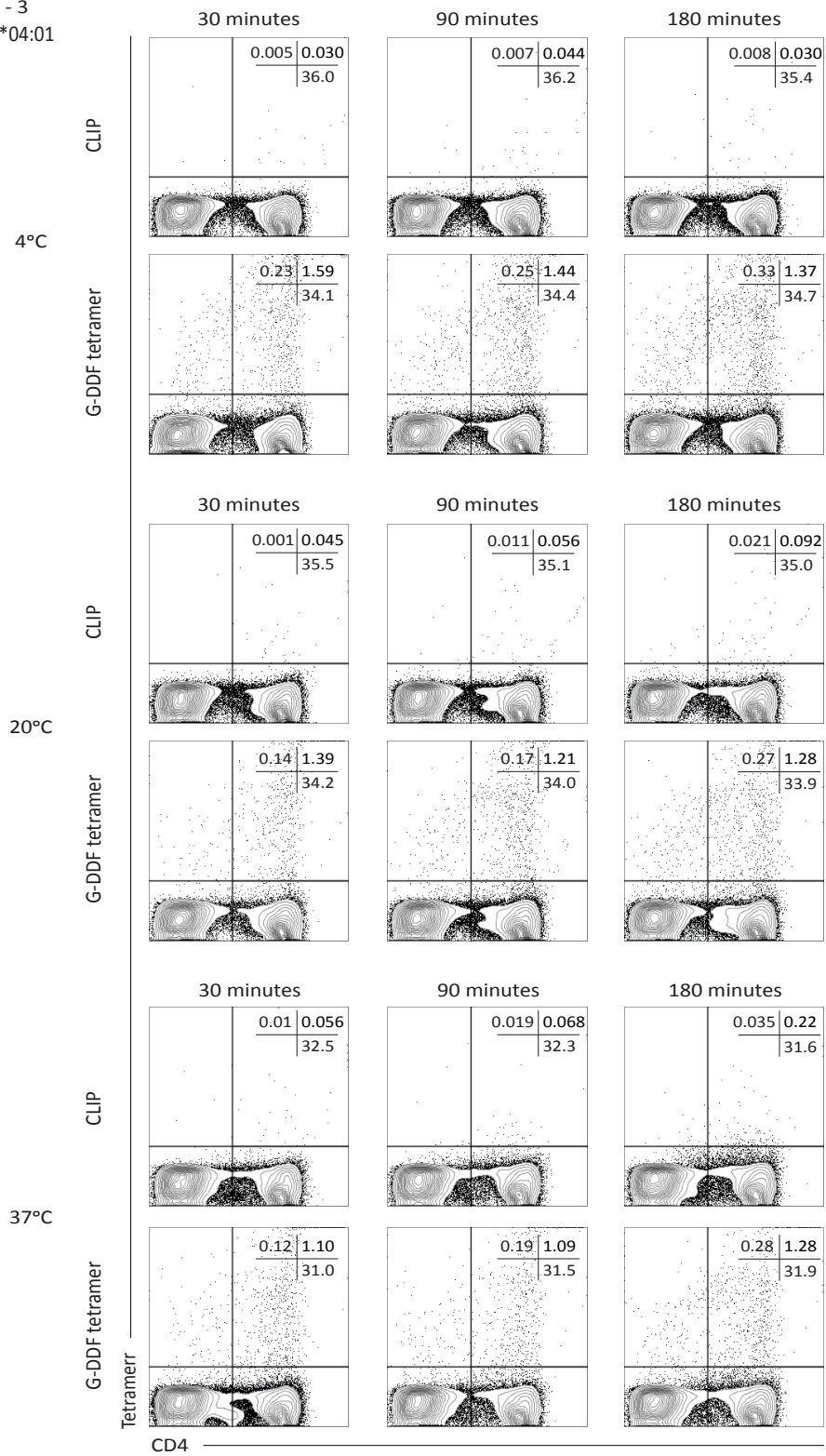
d

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HLA-DR β 1*11:01

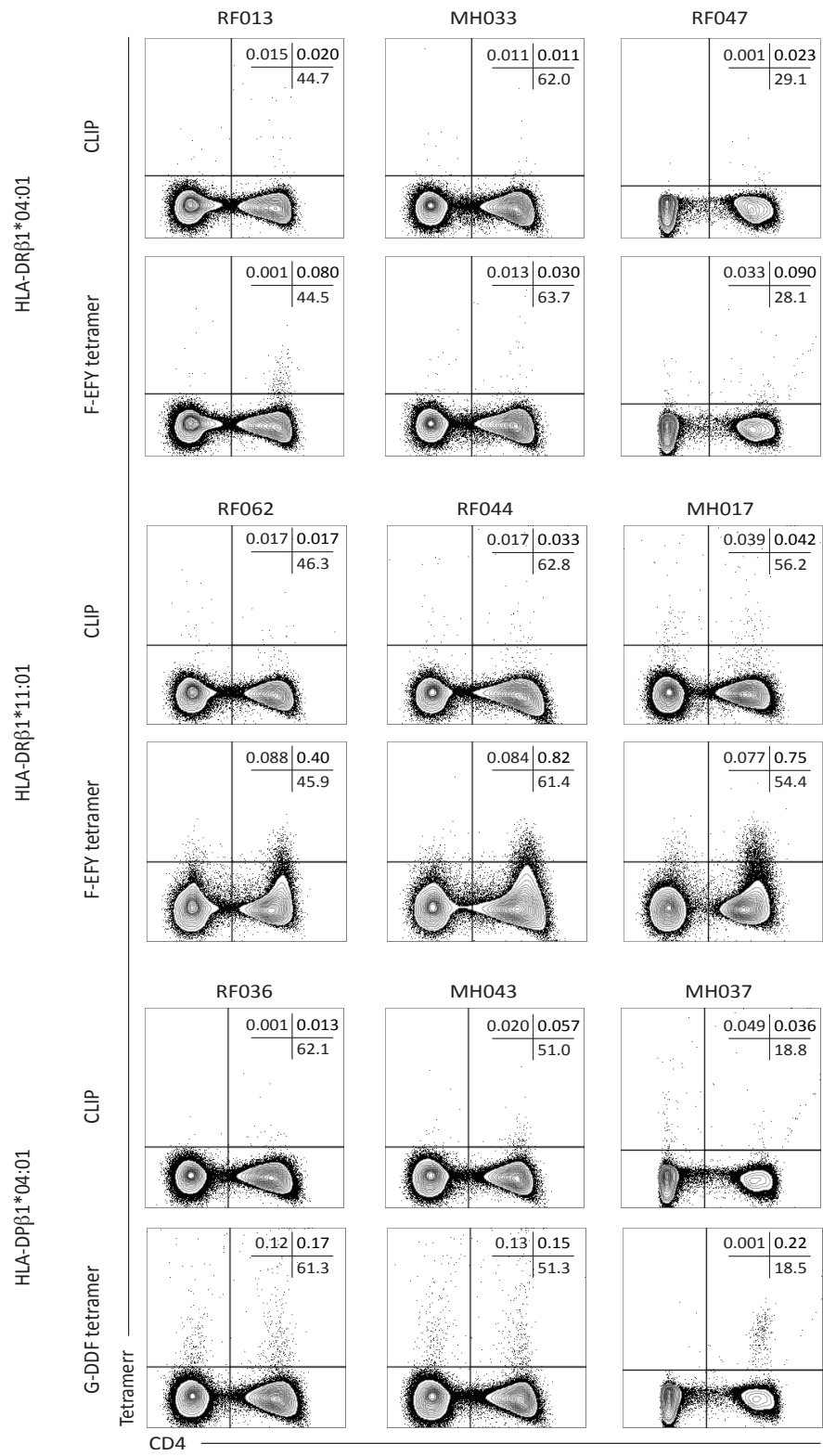


e

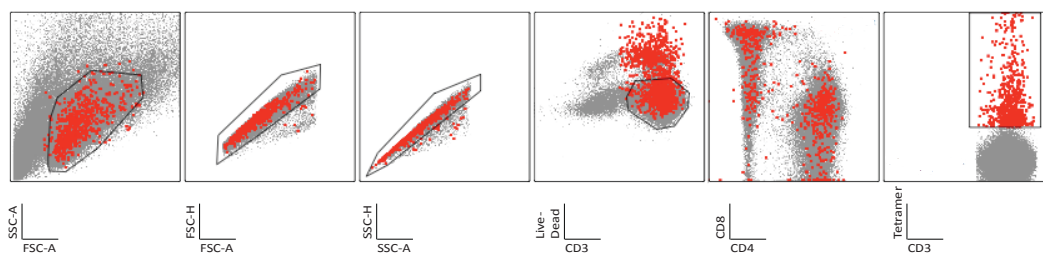
Donor - 3
HLA-DPβ1*04:01



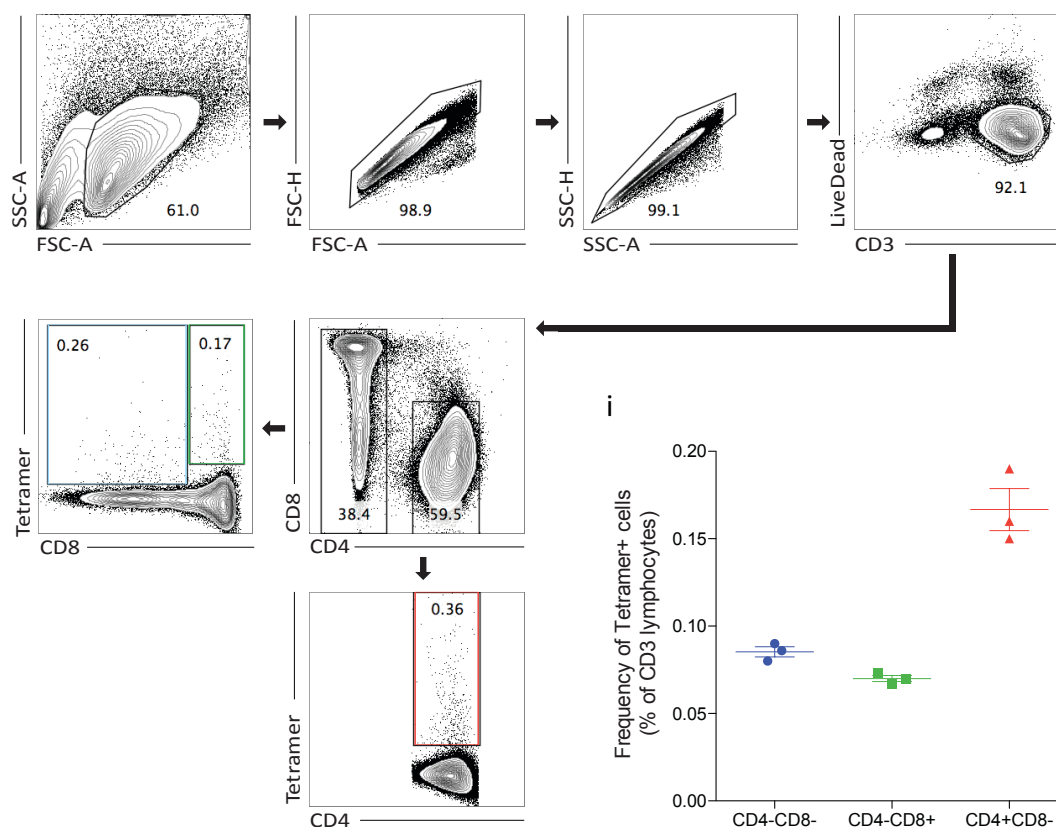
f



g



h



3.6.4 RSV-specific CD4+ T cells can be detected *ex vivo* using the G-DDF/DPB1*04:01 tetramer

T cell recognition of antigen triggers a programme of differentiation, which is required both for immediate effector function and long-lived memory. A number of essential signals are required for this programme to occur optimally and subsequent differentiation can be tracked using phenotypic markers. RSV is relatively poorly immunogenic, inducing low levels of antibody that rapidly wane. One contributor might be impaired CD4+ T cell activation and therefore helper function, but the phenotype of antigen-specific CD4+ T cells responding acutely to RSV infection is poorly understood. We have shown previously that the activation and proliferation of blood CD4+ T cells peaks at day 10 in infected subjects (Section 3.2.1). Therefore, the highest frequency of antigen-specific CD4+ T cells is likely to be at this time-point.

Due to limitations in remaining samples with appropriate HLAs, we could only use the G-DDF/DPB1*04:01 tetramer for phenotypic analysis. Thawed PBMCs from 6 subjects at baseline and 4 subjects at day 10 post infection were therefore stained with the CLIP (negative control) and G-DDF/DPB1*04:01 tetramer (Figure 3.6.3a). The frequencies of CD4+ T cells binding the CLIP tetramer were negligible for all conditions. At baseline, there were very few RSV-specific CD4+ T cells (mean 0.019% $\pm$ s.e.m. 0.003; Figure 3.6.3b). In contrast, there was a significant increase ($p=0.0436$) in detectable G-DDF specific blood CD4+ T cells *ex vivo* at day 10 of infected subjects (mean 0.108% $\pm$ s.e.m. 0.026; Figure 3.6.3b) Thus, epitope-specific CD4+ T cells could be detected in blood *ex vivo* following RSV infection using this tetramer without further enrichment.

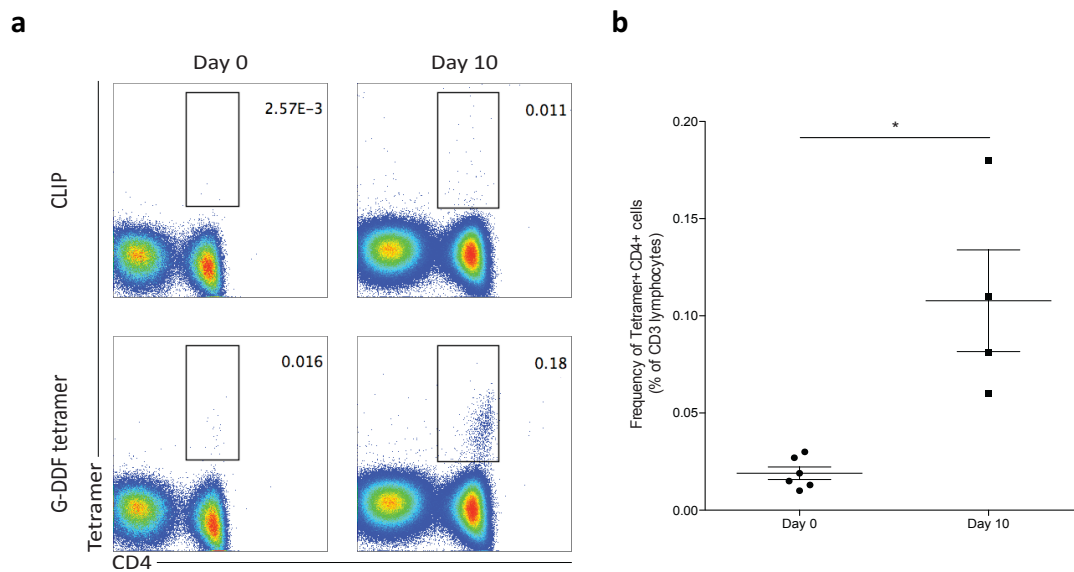


Figure 3.6.3 RSV-specific CD4+ T cells in blood can be detected with G-DDF/DPB1*04:01 tetramer *ex vivo*. Day 0 (n=6) and day 10 (n=4) HLA-matched PBMCs from study subjects were treated with dasatinib and stained with G-DDF/DPB1*04:01 tetramer *ex vivo* prior to surface staining with anti- CD3, CD4, CD8 and Live/Dead marker. Tetramer containing the CLIP peptide was used as a negative control. (a) Representative flow cytometry plots gated on live CD3+ lymphocytes are shown and numbers represent the frequency of CD4+Tetramer+ cells a day 0 (left panel) and day 10 (right panel). (b) Mean $\pm$ s.e.m. of epitope-specific CD4+ T cell responses are shown. P values for unpaired T-test with Welch's correction are shown (* p<0.05).

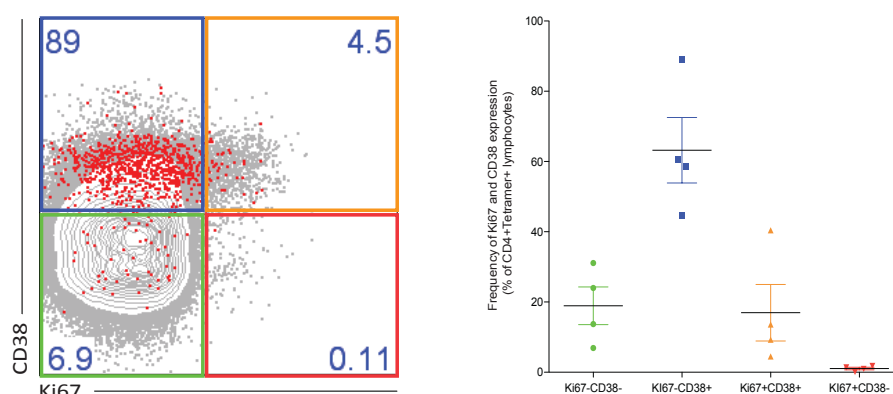
3.6.5 RSV-specific CD4⁺ T cells in blood display an activated, effector memory phenotype with high expression of co-stimulatory molecules

By co-staining tetramer-labelled cells, we next investigated their activation and proliferation status using CD38 and Ki67, respectively. The majority of RSV-specific CD4⁺ T cells expressed CD38 alone (mean 63.2% $\pm$ s.e.m. 4.659), indicative of activation but no proliferation, with a smaller proportion co-expressing CD38 and Ki67 (mean 16.9% $\pm$ s.e.m. 4.018; Figure 3.6.4a). Next, CD45RA and CCR7 expression were analysed to explore their memory subsets. The combination of CD45RA and CCR7 allows categorisation of T cells into four sub-populations; naïve T cells are CD45RA⁺CCR7⁺, central memory T (T_{cm}) cells are CD45RA⁻CCR7⁺, effector memory T (T_{em}) cells are CD45RA⁺CCR7⁻ and terminally differentiated effector memory (T_{emra}) cells are CD45RA⁺CCR7⁻ (Sallusto et al., 1999). Our findings showed that the majority of RSV-specific blood CD4⁺ T cells at this time-point displayed an effector/effector memory phenotype (mean 62.1 % $\pm$ s.e.m. 4.048; Figure 3.6.4b). In addition, a proportion expressed markers that classified them into central memory (mean 19.3% $\pm$ s.e.m. 2.003) and naïve phenotypes (mean 16.7% $\pm$ s.e.m. 2.922).

Using the markers CD27 and CD28, we then investigated the expression of co-stimulatory molecules by antigen-specific CD4⁺ T cells (Figure 3.6.5a). The vast majority of these cells were CD27⁺CD28⁺ (mean 88.5% $\pm$ s.e.m. 0.893) with a smaller population that downregulated the expression of CD27 (mean 11.3 % $\pm$ s.e.m. 0.881). Finally, we assessed the expression of homing markers, CD62L and CCR5 (Figure 3.6.5b). Almost all RSV-specific CD4⁺ T cells at this time-point had upregulated CCR5, with most downregulating CD62L expression (mean 62.9% $\pm$ s.e.m. 1.366), although a proportion cells were shown to co-express CD62L and CCR5 (mean 25.6% $\pm$ s.e.m. 1.404).

In conclusion, by using *ex vivo* tetramer staining to track RSV-specific CD4⁺ T cells, we have shown for the first time the phenotype of these acutely-responding epitope-specific cells, the majority of which displayed an activated, effector/effector memory phenotype with high expression of co-stimulatory molecules and CCR5 expression that is associated with chemotaxis to the site of infection.

a



b

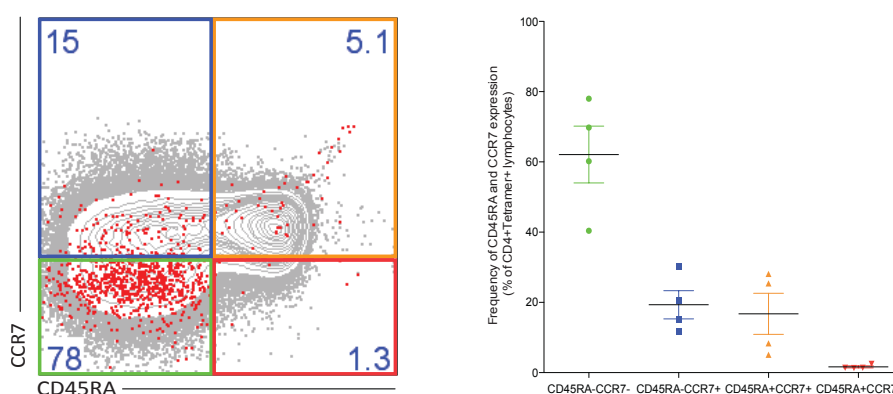


Figure 3.6.4 RSV-specific CD4+ T cells in blood are mainly effector memory and express the activation marker CD38. Day 10 PBMCs from 4 infected and HLA-matched study subjects were treated with dasatinib and stained with G-DDF/DPB1*04:01 tetramer *ex vivo* prior to surface staining with anti- CD3, CD4, CD8, CD38, CD45RA, CCR7 and Live/Dead marker and intracellular staining with Ki67 to assess the activation and memory profile antigen-specific CD4+ T cells. (a) A representative plot shows Ki67 and CD38 expression and summary graph shows frequency of expression for Ki67 and CD38 for all 4 subjects. (b) Representative plot shows CD45RA and CCR7 expression and summary graph shows frequency of expression for CD45RA and CCR7 for all 4 subjects. CD4+Tetramer+ cells (red dot plot) was overlaid onto CD4+ T cells (grey contour plots). The numbers show the frequency of expression as a percentage of CD4+Tetramer+ cells.

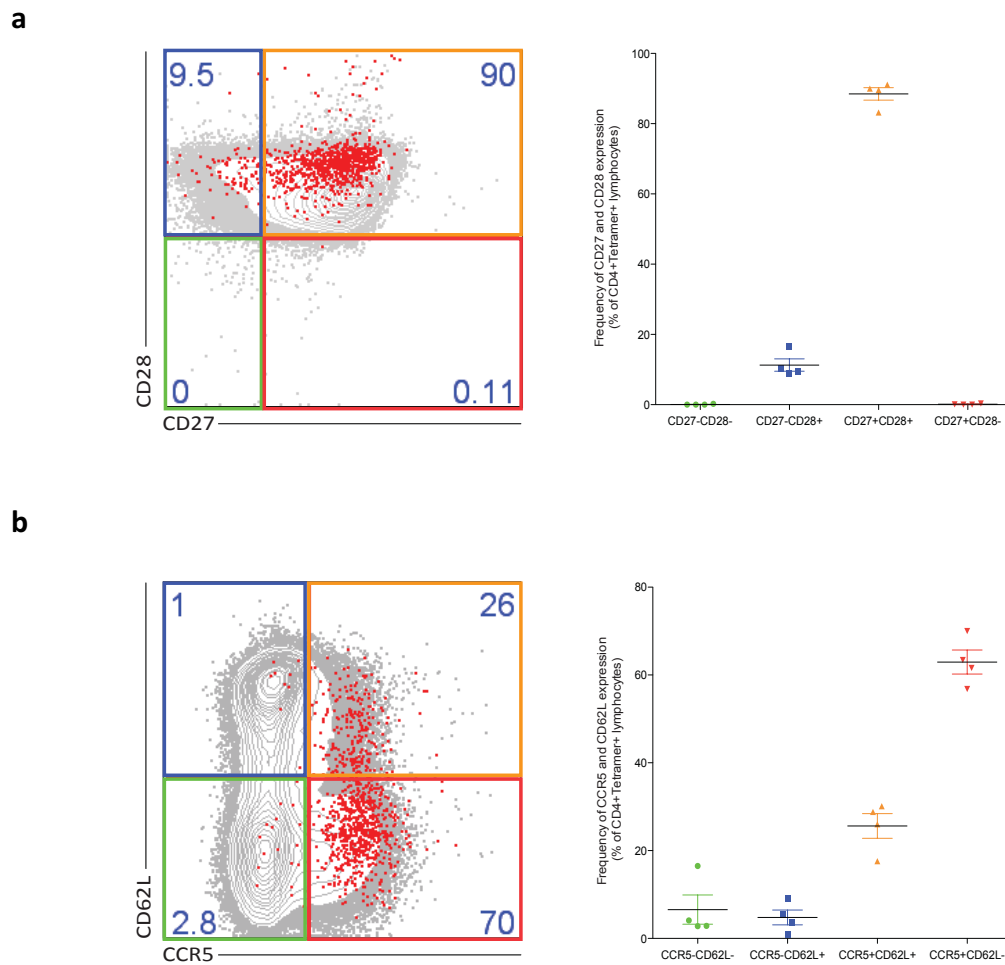


Figure 3.6.5 RSV-specific CD4⁺ T cells in blood at day 10 post-infection express costimulatory molecules and CCR5. Day 10 PBMCs from 4 infected and HLA-matched study subjects were treated with dasatinib and stained with G-DDF/DPB1*04:01 tetramer *ex vivo* prior to surface staining with anti- CD3, CD4, CD8, CD27, CD28, CD62L, CCR5 and Live/Dead marker assess the costimulatory and homing profile of antigen-specific CD4⁺ T cells. (a) Representative plot shows CD27 and CD28 expression and summary graph shows frequency of expression for CD27 and CD28 for all 4 subjects. (b) Representative plot shows CCR5 and CD62L expression and summary graph shows frequency of expression for CCR5 and CD62L for all 4 subjects. CD4⁺Tetramer⁺ cells (red dot plot) was overlaid onto CD4⁺ T cells (grey contour plots). The numbers show the frequency of expression as a percentage of CD4⁺Tetramer⁺ cells.

3.6.6 Discussion

MHC tetramer technology was first developed by Altman and colleagues 2 decades ago and has been central to identification and characterisation of antigen-specific T cells during the course of an immune response (Altman et al., 1996). In the context of respiratory infections, class I tetramers have been used for comprehensive analysis of CD8⁺ T cells in influenza (Hemann et al., 2013; Hoji and Rinaldo, 2005; Kosor Krnic et al., 2008) and more recently RSV (Jozwik et al., 2015). MHC class II tetramers have been less frequently used but tetramers made with an epitope in HA protein of influenza virus have been used to phenotype influenza-specific CD4⁺ T cells (Danke and Kwok, 2003; Lucas et al., 2004; Novak et al., 1999). To date, there have not been any publications using class II tetramers to study antigen-specific CD4⁺ T cells in human RSV infection.

Class II tetramers have several practical limitations which makes their use more challenging compared with class I tetramers. Firstly, identification of class II-restricted immunodominant epitopes is difficult as the specificities of CD4⁺ T cell responses are very broad. McDermott and colleagues showed in Balb/c mice that CD4⁺ T cells targeted 6 of the 11 RSV-derived proteins whereas CD8⁺ T cell responses were much narrower targeting only 3 of the RSV proteins (McDermott et al., 2014). CD4⁺ T cells of each specificity are also less abundant. While CD8⁺ T cells recognising RSV epitopes accounted for 45% of all CD8⁺ T cells in the lung at the peak of RSV infection, CD4⁺ T cells recognising RSV only represented 10% of the total (McDermott et al., 2014). Similar disparity has been reported by others for LCMV and vaccinia virus (Dow et al., 2008; Mothé et al., 2007; Moutaftsi et al., 2007). In human influenza infection, CD4⁺ T cells were shown to recognise 5 of the 8 influenza derived proteins (Wilkinson et al., 2012) and in our study, we also observed a large range of CD4⁺ T cell specificities in infected subjects (section 3.3.3). These findings indicate that multiple epitopes of different specificities stimulate individually modest expansions in CD4⁺ T cells that collectively contribute to an overall response whereas CD8⁺ T cells are focused on relatively few immunodominant epitopes that each account for a large proportion of the antigen-specific CD8⁺ T cell population.

The next limitation is due to differences in the peptide binding groove; class II molecules can accommodate larger peptides than class I, which generally results in class II peptides having multiple binding registers (Bordner, 2010). In addition, peptide binding to class II molecules is known to be promiscuous due to similarities between the MHC alleles (Nielsen et al., 2010). These factors make it difficult to predict high affinity binding peptides for any given class II molecule with accuracy. Technically, although class I and class II molecules overlap in a number of characteristics, the structural differences in the complex that forms the binding groove makes the construction of class II tetramers more challenging (Nepom et al., 2002). Whilst the class I α chain molecule can be efficiently folded in the presence of β 2-microglobulin and antigenic peptide to yield a stable class I monomer (Denkberg et al., 2000), the non-covalent dimerization of α and β chains of class II have a variable range of stability and solubility (Nepom et al., 2002). Having said that, more stable class II complexes can be generated by expressing engineered cDNAs in insect cells, using Baculovirus expression systems (Cecconi et al., 2008). With recent advances, the peptide/MHC reagents are now commonly manufactured in research laboratories or available for purchase from a number of commercial sources.

However, there are still limitations to detection of individual CD4⁺ T cells based on their antigen specificity; (i) the low frequency of such T cells in human peripheral blood and (ii) the low avidity of binding between the TCR and the MHC-peptide complex (Cecconi et al., 2008; Nepom, 2012). Even shortly after boosting by vaccination, the frequency of influenza-specific CD4⁺ T cells is only around 0.01% (Kwok et al., 2012; Nepom, 2012). Furthermore, high avidity of binding between TCR and class II tetramer is essential for efficient staining of antigen-specific CD4⁺ T cells. This has been demonstrated in a number of studies where low avidity interaction between the TCR and tetramer resulted in inability to stain CD4⁺ cells of interest, including T cell clones (Gebe et al., 2003; Mallet-Designé et al., 2003; Mallone et al., 2005).

Following the identification of immunodominant CD4⁺ T cell epitopes in RSV and their class II restriction, we collaborated with the NIH tetramer core facility at Emory University (Georgia, USA) core to manufacture several novel tetramers. Since concentration, incubation time and temperature can all have dramatic effects on tetramer binding, it was necessary to

optimise the staining conditions for each tetramer individually and use expanded epitope-specific CD4⁺ T cells in order to do so (Wooldridge et al., 2009). Several methods have been used to enrich or expand these cells *in vitro*. T cell clones generated against peptides of interest have been used for tetramer optimisation and as positive controls but their production can be complex and labour-intensive (Godkin et al., 2002; Scriba et al., 2005a; de Waal et al., 2004; Holland et al., 2015). Alternatively, enrichment methods have been used where the fluorescent label (typically PE or APC) of tetramer-positive cells is used as a capture tag for magnetic beads (Lemaître et al., 2004; Scriba et al., 2005a, 2005b). Due to the complexity of these methods, we chose to expand antigen-specific cells *in vitro* using cell culture supplemented with recombinant IL-2 and IL-7 that had previously been shown to effectively in expanding antigen-specific CD4⁺ T cells (Cecconi et al., 2008; Fogg et al., 2013). Although addition of these cytokines also induced the non-specific expansion of T cells, we have shown that addition of peptide to the culture selectively increases the frequency of antigen-specific cells (Section 3.4.5).

We tested the success of this method using a previously reported class II tetramer against an epitope in influenza HA protein (Danke and Kwok, 2003; Lucas et al., 2004; Novak et al., 1999) and detected influenza-specific CD4⁺ T cells using these HA-PRY tetramers with negligible background staining using the CLIP tetramer. Different frequencies of tetramer⁺ cells were found with different reagents between individuals, despite having specificity against the same epitope. This was likely to have been due to differences in (i) recent antigen exposure, (ii) vaccination history and (iii) the avidity of the tetramer to its respective HLA molecule. These frequency of these influenza-specific cells were in line with findings of Novak and colleagues, who found *in vitro* expanded CD4⁺ T cells frequencies between 0.4% - 1.5% (Novak et al., 1999). Lucas and colleagues, who used *ex vivo* staining in 6 donors, showed tetramer⁺ cells to make up an approximate average of 0.002% of CD4⁺ T cells, highlighting the low abundance of these cells in peripheral blood at rest. These results indicated that virus-specific CD4⁺ T cells could be detected after expansion in healthy adult PBMCs.

Whether tetramer staining is successful and which cells are stained is dependent on adjustable staining conditions, including tetramer concentration, length of incubation and

temperature. The relevance of these variables has been reviewed extensively (Wooldridge et al., 2009) and it is important to appreciate that optimal staining with different reagents has been shown to require substantially different conditions; Danke and Kwok stained with HA-PRY tetramer at 37°C for 15 minutes while Rastogi et al and Radassi et al used incubation time of 30 minutes and 3 hours, respectively (Raddassi et al., 2011; Rastogi et al., 2007). The amount of tetramer used is important particularly if the avidity of TCR/pMHC interactions fall near or below the dissociation constant for tetramer binding (Wooldridge et al., 2009). Although higher concentrations of tetramer may be preferable, it can also result in increased background staining. This was relevant to the novel reagents we tested, as increasing the tetramer concentration caused a significant rise in staining with the negative control CLIP tetramer. Ultimately, the optimum concentration of 2µg/ml demonstrated for F-EFY and G-DDF tetramers was similar to what had been used for HA-PRY tetramers (1µg/ml by Novak et al. and 1-3µg/ml by Danke et al.). Each tetramer molecule is thought to need to engage three TCRs for optimal binding (Wooldridge et al., 2005), therefore having a higher density of TCRs on the cell surface may enhance staining. This can be achieved by using dasatinib, which is a non-toxic, reversible protein kinase inhibitor (Lissina et al., 2009). Dasatinib has been shown to enhance class I tetramer staining in particular, with a 4-fold increase in tetramer MFI (Dolton et al., 2014). Addition of protein kinase inhibitors for class II tetramer staining has not been systematically evaluate, but is also believed to enhance the surface availability of TCRs in this context. In a study by Holland and colleagues showing that manipulation of the peptide sequence of the flanking residues on the tetramer enhances tetramer labelling, dasatinib was successfully used to enhance staining (Holland et al., 2015). We therefore followed this approach to staining of our samples with F-EFY and G-DDF tetramers.

The temperature at which tetramers are used can have dramatic effects on staining, potentially affecting internalisation by cognate T cells (Whelan et al., 1999). It has been suggested that any tetramer can associate with and become internalised by the T cell at 37°C with lower temperatures preferentially encouraging surface-binding without internalisation of tetramers (Wooldridge et al., 2009). We therefore tested a range of different staining temperatures but found higher background staining at 37°C. Similarly, we found longer incubation times to result in more non-specific binding and concluded that staining for 30

minutes was optimum for all three reagents tested. Thus, for F-EFY/DRB1*04:01 and F-EFY/DRB1*11:01 tetramers, staining at 4°C for 30 minutes and for G-DDF/DPB1*04:01 staining at 20°C for 30 minutes was found to be optimum in providing a balance between the lowest background staining and highest frequency labelled CD4+ T cells. Although some non-specific binding of the experimental tetramers to non-CD4+ T cells remained, these were within the frequencies that observed using other class II tetramers (Braendstrup et al., 2014; Holland et al., 2015; Schmidt et al., 2013).

The main goal in construction and optimisation of class II tetramers with the immunodominant epitopes F-EFY and G-DDF was to characterise antigen-specific CD4+ T cells during human RSV infection. The lack of such reagents to track these cells has been a major hurdle in trying to establish the immunobiology of RSV-specific CD4+ T cells during infection. Using the G-DDF/DPB1*04:01 tetramer, we successfully detected RSV-specific CD4+ T cells by *ex vivo* staining of PBMCs from infected subjects 10 days post-inoculation. The majority of antigen-specific cells at this time-point were CD38+ indicating that they had been activated, but only a fraction of them showed proliferation as assessed by Ki67 expression, unlike RSV-specific CD8+ T cells which almost all express both markers at the acute time-point (Jozwik et al., 2015; Zaunders et al., 2005). Majority of these cells displayed an effector memory phenotype, but we have also found sub-populations that express markers of central memory and naïve T cells (Chattopadhyay and Roederer, 2010). Similarly, majority of RSV-specific CD8+ T cells in blood at this time-point expressed markers of an effector memory phenotype (Jozwik et al., 2015). In contrast, a fraction of RSV-specific CD8+ T cells showed a Temra phenotype, with low frequencies of central memory and naïve cells (Jozwik et al., 2015). Effector memory cells have been recognised for their ability to display immediate function at inflamed tissues, whereas central memory cells home to secondary lymphoid organs with reduced effector function but bearing the capacity to proliferate and differentiate into effector cells following antigenic stimulation (Sallusto et al., 2004). It has been reported that effector memory T cells dominate the memory pool at early times after infection but are less effective at mediating recall responses (Roberts et al., 2005; Wherry et al., 2003) and replenish a long-lived memory CD4+ T cell response (Harrington et al., 2008; Löhning et al., 2008). Our findings on the expression of the homing markers may support this phenomenon; majority of the antigen-specific cells have downregulated CD62L, allowing

homing to sites of inflammation and away from lymphoid tissues which would be ascribed to effector memory T cells. However, ~25% of the cells still had CD62L expression and this may coincide with the central memory T cells having the ability to travel to lymphoid tissues. We observed a similar trend with RSV-specific CD8+ T cells, where CD62L was downregulated following activation (Jozwik et al., 2015). An important functional effect of this has been shown in influenza infection, where CD4+CD62L^{lo} cells correlated with highly-activated IFN- γ producing CD4+ T cells and those cells with little CD62L downregulation showed low IFN- γ production (Jelley-Gibbs et al., 2008). Finally, a large proportion of the antigen-specific CD4+ T cells expressed costimulatory molecules, which may indicate that they have the potential to help other arms of the adaptive immune system (de Bree et al., 2007).

One important strength of this *ex vivo* analysis of RSV-specific CD4+ T cells is minimisation of the manipulation that occurs with *in vitro* stimulation so that the phenotype of the cells of interest is less likely to change. Several studies in the context of influenza have used *in vitro* expansion to overcome the limitations of low CD4+ T cell frequency. Danke and colleagues stimulated PBMCs with the HA₃₀₆₋₃₁₈ peptide for 6 days in order to expand low-frequency influenza-specific CD4+ T cells in blood and using class II tetramers, they characterised these cells as CD45RA-, CD27+, CD28+ and CD62L- (Danke and Kwok, 2003). Conversely, Lucas and colleagues used the same tetramer to study influenza-specific CD4+ T cells *ex vivo* and found a predominantly central memory phenotype with CD45RA-, CD27+, CD28+ and CD62L+ (Lucas et al., 2004). The differential expression of CD62L between these independent studies was probably the result of *in vitro* expansion. In another example, de Bree and colleagues studied the RSV-specific CD4+ T cells in a group of healthy adults following *in vitro* stimulation with RSV and reported a central memory phenotype with high CD27 expression suggesting helper potential (de Bree et al., 2007). This was in contrast to what we have found but the design of detecting the antigen-specific cells could be responsible for the different outcomes.

In conclusion, we have for the first time constructed and optimised RSV class II tetramers using the immunodominant epitopes F-EFY and G-DDF that have allowed us to phenotype RSV-specific CD4+ T cells *ex vivo* at the peak of the acute immune response. We found the majority of these cells to be effector and effector memory T cells that were highly activated but showed less evidence of proliferation. These reagents will allow us to track the T helper

response in multiple tissue compartments including the human airway and enable further investigation of the role of RSV-specific CD4⁺ T cell subsets during infection.

4. Summary

RSV is a major cause of upper respiratory tract infection and is a significant cause of morbidity and mortality in infants, elderly and immunocompromised patients. Despite relatively little antigenic variance, recurrent RSV infections are common. To date, there are no approved vaccines or antiviral drugs available against RSV. This is partially due to the limited understanding of correlates of protection in human RSV infection. To address this, experimental human challenge studies with RSV M37 have been developed to investigate the roles of the cellular and humoral immunity in protection by longitudinal sampling of the airway, nasal fluids and peripheral blood. Using this model, our group previously reported two significant findings; (i) nasal IgA levels significantly correlated with protection against RSV infection and (ii) RSV-specific CD8⁺ T cells in the airways prior to infection correlated with reduced disease severity (Habibi et al., 2015; Jozwik et al., 2015). CD4⁺ T cells are essential as helpers for cytotoxic CD8⁺ T cell and B cell responses but little is known about their role in human RSV infection. Clinical studies with severely ill infants are limited to detection of cytokines in upper and lower airways, and results from these studies have been conflicting. While Th2 and Th17 cytokines have mainly associated with immunopathology, there is also evidence for their role in facilitating resolution during convalescence (Faber et al., 2012; Garofalo et al., 2001; Mukherjee et al., 2011; Pinto et al., 2006). This study has therefore undertaken for the first time a comprehensive analysis of CD4⁺ T cells in human RSV infection, addressing their kinetics, phenotype, specificity and functionality in an experimental human challenge model with RSV.

For this project, 49 healthy adult volunteers were enrolled without pre-selection according to virus-specific antibody levels and challenged with the RSV M37 strain. Of the 49, 26 (53%) volunteers developed RSV infection based on detectable viral load on two consecutive days between day 2 and day 10 post inoculation. Within the infected subjects, 17 (65%) developed cold-like symptoms that resemble an upper respiratory tract infection. The peak of self-reported symptoms and viral load was found to be day 8, and there associated with a positive correlation.

The design of the study gave us the unique opportunity to investigate the characteristics of the polyclonal CD4⁺ T cell response throughout the different stages of RSV infection in peripheral blood and the airways. Short-lived expansion of CD4⁺ T cells was seen at day 10 and the frequencies of the activated and proliferating cells were significantly higher in airways compared to peripheral blood. Moreover, there were distinct expression patterns of costimulatory, memory and homing markers by CD4⁺ T cells in the different compartments. However, the low frequencies of RSV-specific cells were swamped by cells of irrelevant specificities, preventing further conclusions. This highlighted the requirement for more sensitive techniques for tracking of RSV-specific CD4⁺ T cells. Stimulation of PBMCs from infected subjects with whole virus resulted in production IFN- γ and TNF- α by CD4⁺ T cells with no Th2 or Th17 cell cytokines, but the frequency of IFN- γ producing memory CD4⁺ T cells in blood was not associated with lower viral load on subsequent infection.

Earlier work from our group had implicated nasal IgA as a correlate of protection from RSV infection. To provide a better understanding of the role of Tfh cells in RSV infection and their potential association with antibody responses, we investigated these cells in PBMCs of challenged individuals. Following RSV infection, there was an induction of Tfh cells at the peak of the CD4⁺ T cell response. To investigate them in more detail, we also analysed tonsils from children undergoing routine tonsillectomy. However, the low frequency of functional RSV-specific CD4⁺ T cells was a major limitation in pursuing this further. We therefore attempted to optimise an *in vitro* method (MIMIC) that could, in theory, better recapitulate *in vivo* antigen presentation. However, we found little benefit in using this over conventional co-culture methods and more detailed investigation of Tfh cells in RSV infection remains to be undertaken.

Efforts for development of reagents, such as MHC class II/peptide tetramers to track antigen-specific CD4⁺ T cells in humans have been hampered difficulties with epitope discovery in RSV. To address this, we mapped epitopes derived from the entire RSV proteome using overlapping peptides and IFN- γ ELISpot. While stimulation of PBMCs with peptide pools shown large degrees of inter-individual variability in both size and breadth of RSV-specific T cell responses, the most common targets were epitopes within NS1/2, M, F and G proteins. Deconvolution of these confirmed previously reported CD8⁺ T cell epitopes. More

importantly, novel immunodominant CD4⁺ T cell epitopes in F and G proteins were identified and their HLA-restriction confirmed by *in silico* prediction, measured binding affinity *in vitro*, proliferation and cytokine production. These immunodominant epitopes and their class II restriction data were used to construct a set of novel class II tetramers capable of labeling RSV-specific CD4⁺ T cells. Using these reagents, we tracked and characterised for the first time virus-specific CD4⁺ T cells responding to RSV infection, demonstrating their activated, effector memory phenotype and high expression of co-stimulatory molecules.

In influenza infection, antigen-specific IFN- γ producing memory CD4⁺ T cells correlated with protection (Wilkinson et al., 2012). Although such a relationship was not observed with RSV infection, effective resolution of the infection was associated with a strongly biased antiviral Th1 response. This was probably mediated via the production of IFN- γ and TNF- α by a substantial CD8⁺ T cell response, especially in the lung. Moreover, absence of Th2 and Th17 cell cytokines, which are associated with RSV-induced immunopathology in infants, confirmed that these subsets are not an essential feature of RSV disease and are not necessary for disease resolution. This study is the first to systematically investigate the breadth of the T cell response to RSV infection using a relatively unbiased approach to antigen discovery. It is also the first to identify immunodominant CD4⁺ T cell epitopes from both major RSV surface antigens with detailed characterisation of their HLA restriction. Together, these data contribute to a better overall understanding of CD4⁺ T cell responses in human RSV infection and provides novel tools to track antigen-specific CD4⁺ T cells with high sensitivity that may be used in natural infection field studies as well as with future experimental challenge model.

5. Future directions

Polyclonal CD4⁺ T cell phenotypes were notably different between blood and airways. While the construction of class II tetramers allowed us to investigate RSV-specific CD4⁺ T cells in detail in blood, this may not represent what occurs in the lung. This was the case with antigen-specific CD8⁺ T cells and while blood CD8⁺ T cells were not associated with disease, pre-existing CD8⁺ memory T cells in the airways did correlate with reduced symptoms on RSV infection (Jozwik et al., 2015). This highlights the importance of studying RSV-specific CD4⁺ T cells in the airways in future studies, which will now be possible with the reagents available. Moreover, tetramers can be used to stain limited volumes of blood from infants and other naturally infected patients, where the profile of CD4⁺ T cells will provide insights to their role in immunopathology.

Comparison of the antigen-specific CD4⁺ T cells in RSV and influenza infections may also help understanding the contribution that antiviral cellular responses make to optimal broad long-term immunity and this may help future vaccine studies. Ongoing work using the experimental human challenge model with influenza infection (where class II tetramers are already well characterised) will make this possible, with samples from this study being directly compared to those obtained from RSV-infected volunteers using the previously described class II tetramers.

Future studies should also aim to elucidate the function of other T helper subsets in RSV infection. The role of Tregs in preventing severe pathology is well demonstrated in animal models. Although they were not investigated in this project, future RSV challenge studies should address the regulation of RSV-specific responses by Tregs, particularly at the site of infection in view of the demonstrated role of immunopathological responses in animal models of disease. Furthermore, as was shown by others in the context of influenza vaccination, Tfh cells in PBMCs can be sorted by flow cytometry and evaluated for their potential in delivering cognate help to antibody producing B cells in RSV infection. All these investigations will be facilitated by the discovery of RSV antigens achieved in this study and the detailed analysis of individual cells labelled using tetramers that will now be possible.

6. References

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Appendix

Symptom	-14	0	1	2	3	4	5	6	7	8	9	10	14
Sneezing													
Nasal discharge													
Nasal obstruction													
Sore throat													
Hoarseness													
Watery eyes													
Cough													
Difficulty breathing													
Chest discomfort													
Wheezy chest													
Headache													
Malaise													
Fever													
Diarrhoea													
Muscle aches													
Feeling/being sick													
Total score													

Supplementary Figure S1. Subject symptom diary. Subjects were asked to fill in a diary throughout the study to assess systemic, lower and upper respiratory tract symptoms. The grading of the symptoms was scored as follows; 0: absent, 1: mild – present, but does not affect normal daily activities, 2: moderate – some interference with or limitation of normal daily activities, 3: severe – prevents some normal daily activities.

a

NS1 protein

RSV_M37	1	MGSNSLSMIKVRLQNLFDNDEVALLKITCYTDKLIHLTNALAKAVIHTIKLNGIVFVHVI
RSV_A2	1	MGSNSLSMIKVRLQNLFDNDEVALLKITCYTDKLIHLTNALAKAVIHTIKLNGIVFVHVI
RSV_B	1	MGCSNSLSMIKVRLQNLFDNDEVALLKITCYTDKLIHLTNALAKAVIHTIKLNGIVFVHVI
RSV_M37	61	TSSDICPNNNIVVKSNTTTPVQLONGGYIWEMMELTHCSQLNGLMDDNCEIKFSKRLSDS
RSV_A2	61	TSSDICPNNNIVVKSNTTTPVQLONGGYIWEMMELTHCSQLNGLMDDNCEIKFSKRLSDS
RSV_B	61	TSSDVCPDNNIVVKSNTTTPVQLONGGYIWEELTHCSQLNGLMDDNCEIKFSKRLSDS
RSV_M37	121	VMTNYMNQISDLLGLDLNS
RSV_A2	121	TMTNYMNQISDLLGLDLNP
RSV_B	121	VMTNYMNQISDLLGLDLNS

b

NS2 protein

RSV_M37	1	MDTTENGTPQRLMITDMRPLSLETIITSLTRDIITHKFIYILNHECIVRKLDERQATFT
RSV_A2	1	MDTTENDNTPQRLMITDMRPLSLETIITSLTRDIITHKFIYILNHECIVRKLDERQATFT
RSV_B	1	MSTTNDNTTMQRLMITDMRPLSMDSIITSLTRKEIITHKFIYILNHECIVRKLDERQATFT
RSV_M37	61	FLVNYEMKLLHKVGSTKYKKYTEYNTRYGTFFMPPIFINHDFLECIIGIKPTKHTPIIYKY
RSV_A2	61	FLVNYEMKLLHKVGSTKYKKYTEYNTRYGTFFMPPIFINHDFLECIIGIKPTKHTPIIYKY
RSV_B	61	FLVNYEMKLLHKVGSTKYKKYTEYNTRYGTFFMPPIFINEGDFLECIIGIKPTKHTPIIYKY
RSV_M37	121	DLNP
RSV_A2	121	DLNP
RSV_B	121	DLNP

c

N protein

RSV_M37	1	MALSKVKLNDTLNKDQLSSSKYTIQRSTGDSIDTPNYDVQKHINKLCGMILLITEDANHK
RSV_A2	1	MALSKVKLNDTLNKDQLSSSKYTIQRSTGDSIDTPNYDVQKHINKLCGMILLITEDANHK
RSV_B	1	MALSKVKLNDTLNKDQLSSSKYTIQRSTGDNIDTPNYDVQKHINKLCGMILLITEDANHK
RSV_M37	61	FTGLIGMLYAMSRLGREDTIKILRDAGYHVKANGVDVTTHRODINGKEMKFEVLTSLASLT
RSV_A2	61	FTGLIGMLYAMSRLGREDTIKILRDAGYHVKANGVDVTTHRODINGKEMKFEVLTSLASLT
RSV_B	61	FTGLIGMLYAMSRLGREDTIKILRDAGYHVKANGVDVTTHRODINGKEMKFEVLTSLASLT
RSV_M37	121	TEIQINIEIESRKSYSKMLKEMGEVAPEYRHDSPCGMIILCIAALVITKLAAGDRSGLT
RSV_A2	121	TEIQINIEIESRKSYSKMLKEMGEVAPEYRHDSPCGMIILCIAALVITKLAAGDRSGLT
RSV_B	121	SEIQVNIIEIESRKSYSKMLKEMGEVAPEYRHDSPCGMIILCIAALVITKLAAGDRSGLT
RSV_M37	181	AVIRRANNVLKNEMKRYKGLLPKDANSFYEVFEKHPHFIDVFVHFGIAQSSTRGGSRVE
RSV_A2	181	AVIRRANNVLKNEMKRYKGLLPKDANSFYEVFEKHPHFIDVFVHFGIAQSSTRGGSRVE
RSV_B	181	AVIRRANNVLKNEKRYKGLLPKDANSFYEVFEKHPHFIDVFVHFGIAQSSTRGGSRVE
RSV_M37	241	GIFAGLFMNAYGAGQVMLRWGLAKSVKNIMLGHASVQAEMEQQVEVYEAQKLGGEAGF
RSV_A2	241	GIFAGLFMNAYGAGQVMLRWGLAKSVKNIMLGHASVQAEMEQQVEVYEAQKLGGEAGF
RSV_B	241	GIFAGLFMNAYGSGQVMLRWGLAKSVKNIMLGHASVQAEMEQQVEVYEAQKLGGEAGF
RSV_M37	301	YHILNNPKASLLSLTQFPHFSSVVLGNAAGLGIMGEYRGTPRNQDLYDAAKAYAEQLKEN
RSV_A2	301	YHILNNPKASLLSLTQFPHFSSVVLGNAAGLGIMGEYRGTPRNQDLYDAAKAYAEQLKEN
RSV_B	301	YHILNNPKASLLSLTQFPNHFSSVVLGNAAGLGIMGEYRGTPRNQDLYDAAKAYAEQLKEN
RSV_M37	361	GVINYSVLDLTAELEAIAKHQLNPKDNDVEL
RSV_A2	361	GVINYSVLDLTAELEAIAKHQLNPKDNDVEL
RSV_B	361	GVINYSVLDLTAELEAIAKHQLNPKDNDVEL

d

P protein

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RSV_M37    1  MEKFAPEFHGEDANNRATKFLSIRKGFSTSPKDPKKKDSIISVNSIDIEVTKESPITSNS
RSV_A2     1  MEKFAPEFHGEDANNRATKFLSIRKGFSTSPKDPKKKDSIISVNSIDIEVTKESPITSNS
RSV_B      1  MEKFAPEFHGEDANNRATKFLSIRKGFSTSPKDPKKKDSIISVNSIDIEVTKESPITSNS

RSV_M37    61  TIINPTNETDDTAGNKPNYQKPLVSFKEDPTPSDNPFPSKLYKETIETFDNNEEESSYSY
RSV_A2     61  TIINPTNETDDTAGNKPNYQKPLVSFKEDPTPSDNPFPSKLYKETIETFDNNEEESSYSY
RSV_B      61  NIINPTSEADSTPETKANYPRKPLVSFKEDPTPSDNPFPSKLYKETIETFDNNEEESSYSY

RSV_M37    121  EEINDQTNDNITARLDRIDEKLSEILGMLHTLVVASAGPTSARDGIRDAMVGLREEMIEK
RSV_A2     121  EEINDQTNDNITARLDRIDEKLSEILGMLHTLVVASAGPTSARDGIRDAMVGLREEMIEK
RSV_B      121  EEINDQTNDNITARLDRIDEKLSEILGMLHTLVVASAGPTSARDGIRDAMVGLREEMIEK

RSV_M37    181  IRTEALMTNDRLEAMARLRNEESEKMAKDTSEVSLNPTSEKLNLLLEGNDSDNDLSLED
RSV_A2     181  IRTEALMTNDRLEAMARLRNEESEKMAKDTSEVSLNPTSEKLNLLLEGNDSDNDLSLED
RSV_B      181  IRTEALMTNDRLEAMARLRNEESEKMAKDTSEVSLNPTSEKLNLLLEGNDSDNDLSLED

RSV_M37    241  F
RSV_A2     241  F
RSV_B      241  F

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e

M protein

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RSV_M37    1  METYVNKLHEGSTYTAQVYNVLEKDDDPASLTIVVPMFQSSMPADLLIKELANVNILVK
RSV_A2     1  METYVNKLHEGSTYTAQVYNVLEKDDDPASLTIVVPMFQSSMPADLLIKELANVNILVK
RSV_B      1  METYVNKLHEGSTYTAQVYNVLEKDDDPASLTIVVPMFQSSMPADLLIKELANVNILVK

RSV_M37    61  QISTPKGPSLRVMINSRSVLAQMPKFTICANVSLDERSKLAYDVTTTPCEIKACSLTCL
RSV_A2     61  QISTPKGPSLRVMINSRSVLAQMPKFTICANVSLDERSKLAYDVTTTPCEIKACSLTCL
RSV_B      61  QISTPKGPSLRVTINSRSVLAQMPKFTICANVSLDERSKLAYDVTTTPCEIKACSLTCL

RSV_M37    121  KSKNMLTTVKDLTMKTLNPTHTDIALCEFENIVTSKKVVIPTYLRSISVRNKDLNTLENI
RSV_A2     121  KSKNMLTTVKDLTMKTLNPTHTDIALCEFENIVTSKKVVIPTYLRSISVRNKDLNTLENI
RSV_B      121  KVKSMMLTTVKDLTMKTLNPTHTDIALCEFENIVTSKKVVIPTYLRSISVRNKDLNTLENI

RSV_M37    181  TTTEFKNAITNAKIIPYSGLLLVITVTDNKGAFKYIKPQSQFIVDLGAYLEKESIYYVTT
RSV_A2     181  TTTEFKNAITNAKIIPYSGLLLVITVTDNKGAFKYIKPQSQFIVDLGAYLEKESIYYVTT
RSV_B      181  ATTEFKNAITNAKIIPYAGLVITVTDNKGAFKYIKPQSQFIVDLGAYLEKESIYYVTT

RSV_M37    241  NWKHTATRFAIKPMED
RSV_A2     241  NWKHTATRFAIKPMED
RSV_B      241  NWKHTATRFSIKPMED

```

f

SH protein

RSV_M37	1	MENTSITIEFSSKFWPYFTLIHMITTIISLLIIISIMIAILNKLCEYNVFNKTFELPRA
RSV_A2	1	MENTSITIEFSSKFWPYFTLIHMITTIISLLIIISIMIAILNKLCEYNVFNKTFELPRA
RSV_B	1	MGN ~~T~~ SITIEF ~~T~~ SKFWPYFTLIHMI ~~L~~ ~~T~~ ISLLII ~~I~~ IMIAILNKLSEHKTF ~~C~~ NN ~~T~~ LELGQM
RSV_M37	61	R-VNT
RSV_A2	61	R ~~V~~ N ~~T~~ -
RSV_B	61	HQ ~~I~~ N ~~T~~

g

M2 protein

RSV_M37	1	MSRRNPCKFEIRGHCLNGKRCHFSHNYFEWPPHALLVRQNFMLNRILKSMDKSIDTLSEI
RSV_A2	1	MSRRNPCKFEIRGHCLNGKRCHFSHNYFEWPPHALLVRQNFMLNRILKSMDKSIDTLSEI
RSV_B	1	MSRRNPCKFEIRGHCLNG ~~R~~ CH ~~V~~ SHNYFEWPPHALLVRQNFMLN ~~K~~ ILKSMDKSIDTLSEI
RSV_M37	61	SGAAELDRTEEYALGVVGVLESYIGSINNITKQSACVAMSKLLTELNSDDIKKLRDNEEP
RSV_A2	61	SGAAELDRTEEYALGVVGVLESYIGSINNITKQSACVAMSKLLTELNSDDIKKLRDNEE ~~L~~
RSV_B	61	SGAAELDRTEEYALG ~~V~~ GVLESYIGSINNITKQSACVAMSKLL ~~E~~ ~~I~~ NSDDIKKLRDNEEP
RSV_M37	121	NSPKIRVYNTVISYIESNRKNNKQTIHLLKRLPADVLKKTIKNTLDIHKSITINNPKEST
RSV_A2	121	NSPKIRVYNTVISYIESNRKNNKQTIHLLKRLPADVLKKTIKNTLDIHKSITINNPKEST
RSV_B	121	NSPKIRVYNTVISYIESNRKNNKQTIHLLKRLPADVLKKTIKNTLDIHKS ~~I~~ ~~I~~ SNPKEST
RSV_M37	181	VNDTNDHAKNNDTT-
RSV_A2	181	VSDTNDHAKNNDTT-
RSV_B	181	VNDQNDQTKNNDITG

h

L protein

RSV_M37	1	MDPIINGNSANVYLTDSYLKGVISFSECNALGSYIFNGPYLKNDYTNLISRQNPLIEHNM
RSV_A2	1	MDPIINGNSANVYLTDSYLKGVISFSECNALGSYIFNGPYLKNDYTNLISRQNPLIEHNM
RSV_B	1	MDPIINGNSANVYLTDSYLKGVISFSECNALGSYIFNGPYLKNDYTNLISRQSPLEHNM
RSV_M37	61	LKKLMITQSLISKYHKGKIKLEPTYPQSLLMTYKSMTSSEQIATTNLLKKIIRRAIEIS
RSV_A2	61	LKKLMITQSLISKYHKGKIKLEPTYPQSLLMTYKSMTSSEQIATTNLLKKIIRRAIEIS
RSV_B	61	LKKLMITQSLISKYHKGKIKLEPTYPQSLLMTYKSMTSSEQIATTNLLKKIIRRAIEIS
RSV_M37	121	DVKVYAILNKLGLKEKDKIKSNNGQDENNSVITTIKDDILSAVKDNQSHLKAQKNESTK
RSV_A2	121	DVKVYAILNKLGLKEKDKIKSNNGQDENNSVITTIKDDILSAVKDNQSHLKAQKNESTK
RSV_B	121	DVKVYAILNKLGLKEKDKIKSNNGQDENNSVITTIKDDILSAVESMQSYTNSDKNEHVN
RSV_M37	181	QKDTIKTTLKKLMCSMQHPPSWLIWFNLYTKLNNILTQYRSNEVKNEGFILIDNQTLN
RSV_A2	181	QKDTIKTTLKKLMCSMQHPPSWLIWFNLYTKLNNILTQYRSNEVKNEGFILIDNQTLN
RSV_B	181	QKDTIKTTLKKLMCSMQHPPSWLIWFNLYTKLNNILTQYRSNEVKNEGFILIDNQTLN
RSV_M37	241	GFQFILNQYGCIVYHKLKRIITVTYTNQFLTWKDISLSRLNVCLITWISNCLNTLNKSLG
RSV_A2	241	GFQFILNQYGCIVYHKLKRIITVTYTNQFLTWKDISLSRLNVCLITWISNCLNTLNKSLG
RSV_B	241	GFQFILNQYGCIVYHKLKRIITVTYTNQFLTWKDISLSRLNVCLITWISNCLNTLNKSLG
RSV_M37	301	LRCGFNNVILTQLFLYGDICILKLFNEGFYIIKEVEGFIMSLILNITEEDQFRKRFYNSM
RSV_A2	301	LRCGFNNVILTQLFLYGDICILKLFNEGFYIIKEVEGFIMSLILNITEEDQFRKRFYNSM
RSV_B	301	LRCGFNNVILTQLFLYGDICILKLFNEGFYIIKEVEGFIMSLILNITEEDQFRKRFYNSM
RSV_M37	361	LNNITDAANKAQKNLLSRVCHTLLDKTVSDNIINGRWIILLKSKFLKLIKLAGDNNLNNLS
RSV_A2	361	LNNITDAANKAQKNLLSRVCHTLLDKTVSDNIINGRWIILLKSKFLKLIKLAGDNNLNNLS
RSV_B	361	LNNITDAANKAQKNLLSRVCHTLLDKTVSDNIINGRWIILLKSKFLKLIKLAGDNNLNNLS
RSV_M37	421	ELYFLFRIFGHPMVDERQAMDAVKINCNETKPYLLSSLSMLRGAFIYRIIKGFVNNYNRW
RSV_A2	421	ELYFLFRIFGHPMVDERQAMDAVKINCNETKPYLLSSLSMLRGAFIYRIIKGFVNNYNRW
RSV_B	421	ELYFLFRIFGHPMVDERQAMDAVKINCNETKPYLLSSLSMLRGAFIYRIIKGFVNNYNRW
RSV_M37	481	PTLRNAIVLPLRWLTYYKLNTYPSLLETERDLIVLSGLRFYREFPLPKKVDLEMIINDK
RSV_A2	481	PTLRNAIVLPLRWLTYYKLNTYPSLLETERDLIVLSGLRFYREFPLPKKVDLEMIINDK
RSV_B	481	PTLRNAIVLPLRWLTYYKLNTYPSLLETERDLIVLSGLRFYREFPLPKKVDLEMIINDK
RSV_M37	541	AISPPKNLIWTSFPPRNYPMSHIQNYIEHEKLPSESSEKSRVLEYLLRDNKNFNECDLYNC
RSV_A2	541	AISPPKNLIWTSFPPRNYPMSHIQNYIEHEKLPSESSEKSRVLEYLLRDNKNFNECDLYNC
RSV_B	541	AISPPKNLIWTSFPPRNYPMSHIQNYIEHEKLPSESSEKSRVLEYLLRDNKNFNECDLYNC
RSV_M37	601	VVNQSYLNNPNEVVSITGKERELSVGRMPAMQPGMFRQVQILAEXMIAENILOFPFESLT
RSV_A2	601	VVNQSYLNNPNEVVSITGKERELSVGRMPAMQPGMFRQVQILAEXMIAENILOFPFESLT
RSV_B	601	VVNQSYLNNPNEVVSITGKERELSVGRMPAMQPGMFRQVQILAEXMIAENILOFPFESLT
RSV_M37	661	RYGDLELQKILELKAGISNKSNNRYNDNYYNYSKCSIIITDLKFPNQAFRYETSCICSDVL
RSV_A2	661	RYGDLELQKILELKAGISNKSNNRYNDNYYNYSKCSIIITDLKFPNQAFRYETSCICSDVL
RSV_B	661	RYGDLELQKILELKAGISNKSNNRYNDNYYNYSKCSIIITDLKFPNQAFRYETSCICSDVL
RSV_M37	721	DELHGVQSLPSWLHLTIPHTIICTYRHAPPYIRDEIVDLNNVDEQSGLYRYEMGGIEGW
RSV_A2	721	DELHGVQSLPSWLHLTIPHTIICTYRHAPPYIRDEIVDLNNVDEQSGLYRYEMGGIEGW
RSV_B	721	DELHGVQSLPSWLHLTIPHTIICTYRHAPPYIRDEIVDLNNVDEQSGLYRYEMGGIEGW
RSV_M37	781	CQKLWTIEAISLLDLISLKGKPSITALINGDNQSIDISKPVRLMEGQTHAQADYLLALNS
RSV_A2	781	CQKLWTIEAISLLDLISLKGKPSITALINGDNQSIDISKPVRLMEGQTHAQADYLLALNS
RSV_B	781	CQKLWTIEAISLLDLISLKGKPSITALINGDNQSIDISKPVRLMEGQTHAQADYLLALNS
RSV_M37	841	LKLLYKEYAGIGHKLKGTETYSRDMQPMSTIQHNGVYYPASIKKVLRVGPWINTILDD
RSV_A2	841	LKLLYKEYAGIGHKLKGTETYSRDMQPMSTIQHNGVYYPASIKKVLRVGPWINTILDD
RSV_B	841	LKLLYKEYAGIGHKLKGTETYSRDMQPMSTIQHNGVYYPASIKKVLRVGPWINTILDD

RSV_M37	901	FKVSLESIGSLTQELEIRGESLLCSLIFRNWVLYNQIALQKNEHALCENKLYLDILKVLK
RSV_A2	901	FKVSLESIGSLTQELEIRGESLLCSLIFRNWVLYNQIALQKNEHALCENKLYLDILKVLK
RSV_B	901	FKVSLESIGSLTQELEIRGESLLCSLIFRNWVLYNQIALQKNEHALCENKLYLDILKVLK
RSV_M37	961	HLKTPFNLDNIDTALTLYMNLPMFLGGGDPNLLYRSFYRRTDPDLTEAIVHSVPILSYIT
RSV_A2	961	HLKTPFNLDNIDTALTLYMNLPMFLGGGDPNLLYRSFYRRTDPDLTEAIVHSVPILSYIT
RSV_B	961	HLKTPFNLDNIDTALTLYMNLPMFLGGGDPNLLYRSFYRRTDPDLTEAIVHSVPILSYIT
RSV_M37	1021	NHDLKDKLQDLSDDRLNKFLTCIITFDKNPNAEFVTLMRDPQALGSEKQAKITSEINRLA
RSV_A2	1021	NHDLKDKLQDLSDDRLNKFLTCIITFDKNPNAEFVTLMRDPQALGSEKQAKITSEINRLA
RSV_B	1021	NHDLKDKLQDLSDDRLNKFLTCIITFDKNPNAEFVTLMRDPQALGSEKQAKITSEINRLA
RSV_M37	1081	VTEVLSTAPNKPFSKSAQHYYTTTEIDLNDIMQNIETPEGLRVVYESLPFYKAEKIVNL
RSV_A2	1081	VTEVLSTAPNKPFSKSAQHYYTTTEIDLNDIMQNIETPEGLRVVYESLPFYKAEKIVNL
RSV_B	1081	VTEVLSTAPNKPFSKSAQHYYTTTEIDLNDIMQNIETPEGLRVVYESLPFYKAEKIVNL
RSV_M37	1141	ISGTSITNILEKTSIDLTDIRATEMRKNITLLIRILPLDCNRDKREILSMENLSIT
RSV_A2	1141	ISGTSITNILEKTSIDLTDIRATEMRKNITLLIRILPLDCNRDKREILSMENLSIT
RSV_B	1141	ISGTSITNILEKTSIDLTDIRATEMRKNITLLIRILPLDCNRDKREILSMENLSIT
RSV_M37	1201	ELSKYVRERSWSLSNIVGVTSPTSIMYTMIDIKYTTSTIASGIIIEKYNVNSLTRGERGPTK
RSV_A2	1201	ELSKYVRERSWSLSNIVGVTSPTSIMYTMIDIKYTTSTIASGIIIEKYNVNSLTRGERGPTK
RSV_B	1201	ELSKYVRERSWSLSNIVGVTSPTSIMYTMIDIKYTTSTIASGIIIEKYNVNSLTRGERGPTK
RSV_M37	1261	PMVGSSTQEKKTMPVYNRQVLTKKQRDQIDLLAKLDWVYASIDNKDEFMEELSIGTLGLT
RSV_A2	1261	PMVGSSTQEKKTMPVYNRQVLTKKQRDQIDLLAKLDWVYASIDNKDEFMEELSIGTLGLT
RSV_B	1261	PMVGSSTQEKKTMPVYNRQVLTKKQRDQIDLLAKLDWVYASIDNKDEFMEELSIGTLGLT
RSV_M37	1321	YEKAKKLPQYLSVNYLHRLTVSSRPCEFPASIPAYRTTNYHFDTSPIRILTEKYGDED
RSV_A2	1321	YEKAKKLPQYLSVNYLHRLTVSSRPCEFPASIPAYRTTNYHFDTSPIRILTEKYGDED
RSV_B	1321	YEKAKKLPQYLSVNYLHRLTVSSRPCEFPASIPAYRTTNYHFDTSPIRILTEKYGDED
RSV_M37	1381	IDIVFQNCISFGLSLMSVVEQFTNVCNRIILIPKLNEIHLMKPPIFTGDVDIEKLKQVI
RSV_A2	1381	IDIVFQNCISFGLSLMSVVEQFTNVCNRIILIPKLNEIHLMKPPIFTGDVDIEKLKQVI
RSV_B	1381	IDIVFQNCISFGLSLMSVVEQFTNVCNRIILIPKLNEIHLMKPPIFTGDVDIEKLKQVI
RSV_M37	1441	QKQHMFLPDKISLTQYVELFLSNKTLKSGSHVNSNLILAEKISDYFENTYILSTNLAGEW
RSV_A2	1441	QKQHMFLPDKISLTQYVELFLSNKTLKSGSHVNSNLILAEKISDYFENTYILSTNLAGEW
RSV_B	1441	QKQHMFLPDKISLTQYVELFLSNKTLKSGSHVNSNLILAEKISDYFENTYILSTNLAGEW
RSV_M37	1501	ILIIQLMKDSKGIFEKDWGEGYITDHFINLVFFNAYKTYLLCFHKGYGAKLECDMNT
RSV_A2	1501	ILIIQLMKDSKGIFEKDWGEGYITDHFINLVFFNAYKTYLLCFHKGYGAKLECDMNT
RSV_B	1501	ILIIQLMKDSKGIFEKDWGEGYITDHFINLVFFNAYKTYLLCFHKGYGAKLECDMNT
RSV_M37	1561	SDLLCVLELIDSSYNKSMKVPLEQKVIKYLSDASLHRVKGCHSPKLMFLKRLNVAEP
RSV_A2	1561	SDLLCVLELIDSSYNKSMKVPLEQKVIKYLSDASLHRVKGCHSPKLMFLKRLNVAEP
RSV_B	1561	SDLLCVLELIDSSYNKSMKVPLEQKVIKYLSDASLHRVKGCHSPKLMFLKRLNVAEP
RSV_M37	1621	TVCNPNVNIIDYHPTMKAILTYIDLVRMGLINIDRIYIKNKKKPNDEFYTSNLPYIYNF
RSV_A2	1621	TVCNPNVNIIDYHPTMKAILTYIDLVRMGLINIDRIYIKNKKKPNDEFYTSNLPYIYNF
RSV_B	1621	TVCNPNVNIIDYHPTMKAILTYIDLVRMGLINIDRIYIKNKKKPNDEFYTSNLPYIYNF
RSV_M37	1681	SDNTHLLTKHIRIANSELENNYNKLYHPTPETLENILNPVKCDPKKTLNDYICIGKNVDS
RSV_A2	1681	SDNTHLLTKHIRIANSELENNYNKLYHPTPETLENILNPVKCDPKKTLNDYICIGKNVDS
RSV_B	1681	SDNTHLLTKHIRIANSELENNYNKLYHPTPETLENILNPVKCDPKKTLNDYICIGKNVDS

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RSV_M37 1741 IMLPLLSNKKLIKSSSTMIR-TNYSKQDLYNLFPPTVVIDKIIDHSGNTAKSNQLYTTTSHQ
RSV_A2 1741 IMLPLLSNKKLIKSSAMIRTNYSKQDLYNLFPMPVIDRIIDHSGNTAKSNQLYTTTSHQI
RSV_B 1740 IMNSTFSNKKMHKSSSTVTTTRFNYSKQDLYNLFPPTVVIDKIIDHSGNTAKSNQLYTTTSHQ

RSV_M37 1800 ISLVHNSISLYCMLPWHHINRFPNFVFSSTGCKISIEYILKDLKIDPNCIAFIGEGAGNL
RSV_A2 1801 ISLVHNSTSLYCMLPWHHINRFPNFVFSSTGCKISIEYILKDLKIDPNCIAFIGEGAGNL
RSV_B 1800 ISLVHNSISLYCMLPWHHINRFPNFVFSSTGCKISIEYILKDLKIDPNCIAFIGEGAGNL

RSV_M37 1860 LLRTVVELHPDIKYIYRSLKDCNDHSLPIEFRLRYNGHINIDYGENLTIPATDATNNIHW
RSV_A2 1861 LLRTVVELHPDIRYIYRSLKDCNDHSLPIEFRLRYNGHINIDYGENLTIPATDATNNIHW
RSV_B 1860 LLRTVVELHPDIKYIYRSLKDCNDHSLPIEFRLRYNGHINIDYGENLTIPATDATNNIHW

RSV_M37 1920 SYLHIKFAEPISIFVCDALPVTANWSKIIIEWSKHVRKCKYCSSLVCKLIVKYHAQDD
RSV_A2 1921 YLHIKFAEPISISLFVCDALSVTVNWSKIIIEWSKHVRKCKYCSSLVNCMLIVKYHAQDDH
RSV_B 1920 SYLHIKFAEPISIFVCDALPVTANWSKIIIEWSKHVRKCKYCSSLVCKLIVKYHAQDD

RSV_M37 1980 IDPKLDNITILKTYVCLGSKLKGSEVYLVLITIGPANVFPVFNWQNAKLISRTKNPIMP
RSV_A2 1981 DPKLDNITILKTYVCLGSKLKGSEVYLVLITIGPANIFPVPFNWQNAKLISRTKNPIMP
RSV_B 1980 IDPKLDNITILKTYVCLGSKLKGSEVYLVLITIGPANILPVPFNWQNAKLISRTKNPIMP

RSV_M37 2040 KKADKESIDANIKSLIPFLCYPITKKGINTALSKLKSVMGDIISYSIAGRNEVFSNKL
RSV_A2 2041 KADKESIDANIKSLIPFLCYPITKGIN TALSKLKSVMGDIISYSIAGRNEVFSNKLIN
RSV_B 2040 KKTADKESIDANIKSLIPFLCYPITKKGINTALSKLKSVMGDIISYSIAGRNEVFSNKL

RSV_M37 2100 HKHNMNILKWFNHLNFRSTELNHNHLYMVESTYPYLSELLNSLTNELKLIKITGSL
RSV_A2 2101 HKHNMNILKWFNHLNFRSTELNHNHLYMVESTYPYLSELLNSLTNELKLIKITGSL
RSV_B 2100 HKHNMNILKMLDHLNFRSAELNHNHLYMVESTYPYLSELLNSLTNELKLIKITGSL

RSV_M37 2160 YLPHNE-
RSV_A2 2161 NPHNE--
RSV_B 2160 YLPLNEQ

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Figure S2. Alignment of the translated amino acid sequence for RSV M37 proteins with their counterparts in RSV A2 and B strains. Multiple sequence alignment tool ClustalW (version 1.83) was used to align the (a) NS1, (b) NS2, (c) N, (d) P, (e) M, (f) SH, (g) M2 and (h) L protein sequences of RSV M37 against RSV A2 (accession number M74568.1) and B1 (accession number NC_001781.1). Black box indicates positions of a fully conserved residue; grey box indicates conservation between groups of strongly similar properties – scoring > 0.5 in the Gonnet PAM 250 matrix; white box indicates conservation between groups of weakly similar properties – scoring ≤ 0.5 in the Gonnet PAM 250 matrix.

Peptide	Sequence	Protein	Peptide	Sequence	Protein
1	MELPILKTNAITL	F	43	SCSISNIETVIEFQQ	F
2	LKTNAITTLAAVTL	F	44	NIETVIEFQQKNNRL	F
3	ITTLAAVTLCFASS	F	45	IEFQQKNNRLEITR	F
4	AAVTLCFASSQNITE	F	46	KNNRLEITREFSVN	F
5	CFASSQNITEEFYQS	F	47	LEITREFSVNAGVTT	F
6	QNITEEFYQSTCSAV	F	48	EFSVNAGVTTTPVSTY	F
7	EFYQSTCSAVSKGYL	F	49	AGVTTTPVSTYMLTNS	F
8	TCSAVSKGYLSALRT	F	50	PVSTYMLTNSELLSL	F
9	SKGYLSALRTGWYTS	F	51	MLTNSELLSLINDMP	F
10	SALRTGWYTSVITIE	F	52	ELLSLINDMPITNDQ	F
11	GWYTSVITIELSNIK	F	53	INDMPITNDQKKLMS	F
12	VITIELSNIKENKCN	F	54	ITNDQKKLMSNNVQI	F
13	LSNIKENKCNGTDAK	F	55	KKLMSNNVQIVRQQS	F
14	ENKCNGTDAKVLIK	F	56	NNVQIVRQQSYSIMS	F
15	GTDAAKVLIKQELDK	F	57	VRQQSYSIMSIIKEE	F
16	VKLIKQELDKYKNAV	F	58	YSIMSIIKEEVLAYV	F
17	QELDKYKNAVTELQL	F	59	IIKEEVLAYVVQLPL	F
18	YKNAVTELQLLMQST	F	60	VLAYVVQLPLYGVID	F
19	TELQLLMQSTPAANN	F	61	VQLPLYGVIDTPCWK	F
20	LMQSTPAANNRARRE	F	62	YGVIDTPCWKLHTSP	F
21	PAANNRARRELPRFM	F	63	TPCWKLHTSPLCTTN	F
22	RARRELPRFMNYTLN	F	64	LHTSPLCTTNTKEGS	F
23	LPRFMNYTLNNTKNN	F	65	LCTTNTKEGSNICLT	F
24	NYTLNNTKNNNVTLS	F	66	TKEGSNICLTRTRDRG	F
25	NTKNNNVTLSSKKRKR	F	67	NICLTRTRDRGWYCDN	F
26	NVTLSSKKRKRFLGF	F	68	RTDRGWYCDNAGSVS	F
27	KKRKRKRFLGFLLGVG	F	69	WYCDNAGSVSFFPQA	F
28	RFLGFLLGVGSAIAS	F	70	AGSVSFFPQAETCKV	F
29	LLGVGSAIASGIAVS	F	71	FFPQAETCKVQSNRV	F
30	SAIASGIAVSKVLHL	F	72	ETCKVQSNRVFCDTM	F
31	GIAVSKVLHLEGEVN	F	73	QSNRVFCDTMNSLTL	F
32	KVLHLEGEVNKIKNA	F	74	FCDTMNSLTLPSEVN	F
33	EGEVNLIKKNALLSTN	F	75	NSLTLPSEVNLNID	F
34	KIKKNALLSTNKAVVS	F	76	PSEVNLNIDIFNPK	F
35	LLSTNKAVVSLSNGV	F	77	LCNIDIFNPKYDCKI	F
36	KAVVSLSNGVSVLTS	F	78	IFNPKYDCKIMTSKT	F
37	LSNGVSVLTSKVLDL	F	79	YDCKIMTSKTDVSSS	F
38	SVLTSKVLDLKNYID	F	80	MTSKTDVSSSVITSL	F
39	KVLDLKNYIDKQLLP	F	81	DVSSSVITSLGAIVS	F
40	KNYIDKQLLPVKNKQ	F	82	VITSLGAIVSCYGKT	F
41	KQLLPVKNKQSCSIS	F	83	GAIVSCYGKTKCTAS	F
42	IVNKQSCSISNIETV	F	84	CYGKTKCTASKNKRG	F

Peptide	Sequence	Protein	Peptide	Sequence	Protein
85	KCTASNKNRGIKTF	F	127	NNKVTLTTAIIQDAT	G
86	NKNRGIKTFSNLCD	F	128	LTTAIIQDATSQIKN	G
87	IIKTFSNLCDYVSNK	F	129	IQDATSQIKNTTPTY	G
88	SNLCDYVSNKGVDTV	F	130	SQIKNTTPTYLTQNP	G
89	YVSNKGVDTVSVGNT	F	131	TTPTYLTQNPQLGIS	G
90	GVDTVSVGNTLYYVN	F	132	LTQNPQLGISFFNLS	G
91	SVGNTLYYVNVKQEGK	F	133	QLGISFFNLSGTISQ	G
92	LYYVNVKQEGKSLYVK	F	134	FFNLSGTISQTTAIL	G
93	KQEGKSLYVKGEPII	F	135	GTISQTTAILALTP	G
94	SLYVKGEPIINFYDP	F	136	TTAILALTPSVESI	G
95	GEPIINFYDPLVFPS	F	137	ALTPSVESILQSTT	G
96	NFYDPLVFPSDEFDA	F	138	SVESILQSTTVKTKN	G
97	LVFPSDEFDASISQV	F	139	LQSTTVKTKNTTTTQ	G
98	DEFDASISQVNEKIN	F	140	VKTKNTTTTQIQPSK	G
99	SISQVNEKINQSLAF	F	141	TTTTQIQPSKPTTKQ	G
100	NEKINQSLAFIRKSD	F	142	IQPSKPTTKQRQKNP	G
101	QSLAFIRKSDELLHN	F	143	PTTKQRQKNKPPNKP	G
102	IRKSDELLHNVNVGK	F	144	RQNKPPNKPNDDFHF	G
103	ELLHNVNVGKSTTNI	F	145	PNKPNDDFHFEVFN	G
104	VNVGKSTTNIMITTI	F	146	DDFHFEVFNFVPCSI	G
105	STTNIMITTIIVII	F	147	EVFNFVPCSICSNNP	G
106	MITTIIVIIIVILL	F	148	VPCSICSNNPTCWA	G
107	IIVIIIVILLIIAVG	F	149	CSNNPTCWAICKRIP	G
108	VILLIIAVGLFLYC	F	150	TCWAICKRIPSKKPG	G
109	LIAVGLFLYCKARST	F	151	CKRIPSKKPGKKT	G
110	LFLYCKARSTPVTLS	F	152	SKKPGKKTTPKTKK	G
111	KARSTPVTLSKDQLS	F	153	KKTTPKTKKPTIKT	G
112	PVTLSKDQLSGINNI	F	154	KPTKTKPTIKTKKDL	G
113	KDQLSGINNIAFSN	F	155	PTIKTKKDLKPQTT	G
114	MSKTKDQRTAKTLEK	G	156	TKKDLKPQTTKPKEA	G
115	DQRTAKTLEKTWDTL	G	157	KPQTTKPKEAPTTKP	G
116	KTLEKTWDTLNHLLF	G	158	KPKEAPTTKPTDKPT	G
117	TWDTLNHLLFISSCL	G	159	PTTKPTDKPTINITK	G
118	NHLLFISSCLYKLN	G	160	TDKPTINITKPNIRT	G
119	ISSCLYKLNLSIAQ	G	161	INITKPNIRTTLLTN	G
120	YKLNLSIAQITLSI	G	162	PNIRTTLLTNSTTGN	G
121	KSIAQITLSILAMII	G	163	TLLTNSTTGNPEHTS	G
122	ITLSILAMIIISTSLI	G	164	STTGNPEHTSQEETL	G
123	LAMIIISTSLIIVAI	G	165	PEHTSQEETLHSTSS	G
124	STSLIIVAIIFIASA	G	166	QEETLHSTSSSEGNTS	G
125	IVAIIFIASANNKVT	G	167	HSTSSSEGNTSPSQIY	G
126	FIASANNKVTLTAI	G	168	EGNTSPSQIYTTSEY	G

Peptide	Sequence	Protein	Peptide	Sequence	Protein
169	PSQIYTTSEYLSQPP	G	211	SMQHPPSWLIHWFNL	L
170	TTSEYLSQPPSPSNI	G	212	PSWLIHWFNLYTKLN	L
171	LSQPPSPSNITDQ	G	213	HWFNLYTKLNNILTQ	L
172	MDPIINGNSANVYLT	L	214	YTKLNNILTQYRSNE	L
173	NGNSANVYLTDSYLK	L	215	NILTQYRSNEVKNHG	L
174	NVYLTDSYLKGVISF	L	216	YRSNEVKNHGFILID	L
175	DSYLKGVISFSECNA	L	217	VKNHGFILIDNQTLLN	L
176	GVISFSECNALGSYI	L	218	FILIDNQTLLNGFQFI	L
177	SECNALGSYIFNGPY	L	219	NQTLLNGFQFILNQYG	L
178	LGSYIFNGPYLKNDY	L	220	GFQFILNQYGCIVYH	L
179	FNGPYLKNDYTNLIS	L	221	LNQYGCIVYHKELKR	L
180	LKNDYTNLISRQNPL	L	222	CIVYHKELKRITVTT	L
181	TNLISRQNPPIEHIN	L	223	KELKRITVTTYNQFL	L
182	RQNPLIEHINLKKLN	L	224	ITVTTYNQFLTWKDI	L
183	IEHINLKKLNITQSL	L	225	YNQFLTWKDISLSRL	L
184	LKKLNITQSLISKYH	L	226	TWKDISLSRLNVCLI	L
185	ITQSLISKYHKGEIK	L	227	SLSRLNVCLITWISN	L
186	ISKYHKGEIKIEPT	L	228	NVCLITWISNCLNTL	L
187	KGEIKIEPTYFQSL	L	229	TWISNCLNTLNKSLG	L
188	IEEPTYFQSLLMTYK	L	230	CLNTLNKSLGLRCGF	L
189	YFQSLLMTYKSMTSS	L	231	NKSLGLRCGFNNVIL	L
190	LMTYKSMTSSEQITT	L	232	LRCGFNNVILTQLFL	L
191	SMTSSEQITTTNLLK	L	233	NNVILTQLFLYGDCI	L
192	EQITTTNLLKKIIRR	L	234	TQLFLYGDCILKLFH	L
193	TNLLKKIIRRAIEIS	L	235	YGDCILKLFHNEGFI	L
194	KIIRRAIEISDVKVY	L	236	LKLFHNEGFIYIIEV	L
195	AIEISDVKVYAILNK	L	237	NEGFIYIIEVEGFIM	L
196	DVKVYAILNKLGLKE	L	238	IIEVEGFIMSLILN	L
197	AILNKLGLKEKDKIK	L	239	EGFIMSLILNITEED	L
198	LGLKEKDKIKSNGGQ	L	240	SLILNITEEDQFRKR	L
199	KDKIKSNGGQDENNS	L	241	ITEEDQFRKRFYNSM	L
200	SNGGQDENNSVITTI	L	242	QFRKRFYNSMLNNIT	L
201	DENNSVITTIKDDI	L	243	FYNSMLNNITDAANK	L
202	VITTIKDDILLAVK	L	244	LNNITDAANKAQKNL	L
203	IKDDILLAVKDNQSH	L	245	DAANKAQKNLLSRVC	L
204	LLAVKDNQSHLKAGK	L	246	AQKNLLSRVCHTLTD	L
205	DNQSHLKAGKNHSTK	L	247	LSRVCHTLTDKTVSD	L
206	LKAGKNHSTKQKDTI	L	248	HTLLDKTVSDNIING	L
207	NHSTKQKDTIKTTLL	L	249	KTVSDNIINGRWIIL	L
208	QKDTIKTTLLKKLMC	L	250	NIINGRWIILLKFL	L
209	KTTLLKKLMCSMQHP	L	251	RWIILLKFLKLIK	L
210	KKLMCSMQHPPSWLI	L	252	LSKFLKLIKLAGDNN	L

Peptide	Sequence	Protein	Peptide	Sequence	Protein
253	KLIKLAGDNNLNLS	L	295	LTGKERELSVGRMFA	L
254	AGDNNLNLSLEYFL	L	296	RELSVGRMFAMQPGM	L
255	LNNLSLEYFLFRIFG	L	297	GRMFAMQPGMFRQVQ	L
256	ELYFLFRIFGHMVD	L	298	MQPGMFRQVQILA EK	L
257	FRIFGHMVDERQAM	L	299	FRQVQILA EK MIAEN	L
258	HPMVDERQAMDAVKV	L	300	ILA EK MIAENILQFF	L
259	ERQAMDAVKVNCNET	L	301	MIAENILQFFPESLT	L
260	DAVKVNCNETKFYLL	L	302	ILQFFPESLTRYGDL	L
261	NCNETKFYLLSSLSM	L	303	PESLTRYGDLELQKI	L
262	KFYLLSSLSMLRGAF	L	304	RYGDLELQKILELKA	L
263	SSLSMLRGAFIYRII	L	305	ELQKILELKAGISNK	L
264	LRGAFIYRIIKGFVN	L	306	LELKAGISNKS NRYN	L
265	IYRIIKGFVNNYNRW	L	307	GISNKS NRYNDN YNN	L
266	KGFVNNYNRWPTLRN	L	308	SNRYNDN YNNYISKC	L
267	NYNRWPTLRNAIVLP	L	309	DN YNNYISKCSIITD	L
268	PTLRNAIVLPLRWLT	L	310	YISKCSIITDLSKFN	L
269	AIVLPLRWLTYYKLN	L	311	SIITDLSKFNQAFRY	L
270	LRWLTYYKLNTYPSL	L	312	LSKFNQAFRYETSCI	L
271	YYKLNTYPSLLELTE	L	313	QAFRYETSCICSDVL	L
272	TYPSLLELTERDLIV	L	314	ETSCICSDVLDELHG	L
273	LELTERDLIVLSGLR	L	315	CSDVLDELHGVQSLF	L
274	RDLIVLSGLRFYREF	L	316	DELHGVQSLFSWLHL	L
275	LSGLRFYREFRLPKK	L	317	VQSLFSWLHLTIPHV	L
276	FYREFRLPKKVDLEM	L	318	SWLHLTIPHV TICT	L
277	RLPKKVDLEMIINDK	L	319	TIPHV TICTYRHAP	L
278	VDLEMIINDKAISPP	L	320	TICTYRHAPPYIRD	L
279	IINDKAISPPKNLIW	L	321	YRHAPPYIRDHIVDL	L
280	AISPPKNLIWTSFPR	L	322	PYIRDHIVDLNNVDE	L
281	KNLIWTSFPRNYMPS	L	323	HIVDLNNVDEQSGLY	L
282	TSFPRNYMP SHIQNY	L	324	NNVDEQSGLYRYHMG	L
283	NYMP SHIQNYIEHEK	L	325	QSGLYRYHMG GIEGW	L
284	HIQNYIEHEKLFSE	L	326	RYHMG GIEGW CQKLW	L
285	IEHEKLFSESDKSR	L	327	GIEGW CQKLW TIEAI	L
286	LKFSESDKSRRVLEY	L	328	CQKLW TIEAISLLDL	L
287	SDKSRRVLEY YLRDN	L	329	TIEAISLLDLISLKG	L
288	RVLEY YLRDNKFNEC	L	330	SLLDLISLKGKFSIT	L
289	YLRDNKFNECDLYNC	L	331	ISLKGKFSIT ALING	L
290	KFNECDLYNCVVNQ S	L	332	KFSIT ALINGDNQSI	L
291	DLYNCVVNQSYLNNP	L	333	ALINGDNQSIDISK P	L
292	VVNQSYLNNPNHVVS	L	334	DNQSIDISK P VRLME	L
293	YLNNPNHVVS LTGKE	L	335	DISK P VRLMEGQTHA	L
294	NHVVS LTGKERELSV	L	336	VRLMEGQTHA QADYL	L

Peptide	Sequence	Protein	Peptide	Sequence	Protein
337	GQTHAQADYLLALNS	L	379	LNKFLTCIITFDKNP	L
338	QADYLLALNSLKLLY	L	380	TCIITFDKNPNAEFV	L
339	LALNSLKLLYKEYAG	L	381	FDKNPNAEFVTLMRD	L
340	LKLLYKEYAGIGHKL	L	382	NAEFVTLMRDPQALG	L
341	KEYAGIGHKLKGTET	L	383	TLMRDPQALGSERQA	L
342	IGHKLKGTETYISRD	L	384	PQALGSERQAKITSE	L
343	KGTETYISRDMQFMS	L	385	SERQAKITSEINRLA	L
344	YISRDMQFMSKTIQH	L	386	KITSEINRLAVTEVL	L
345	MQFMSKTIQHNGVYY	L	387	INRLAVTEVLSTAPN	L
346	KTIQHNGVYYPASIK	L	388	VTEVLSTAPNKIFSK	L
347	NGVYYPASIKKVLRV	L	389	STAPNKIFSKSAQHY	L
348	PASIKKVLRVGPWIN	L	390	KIFSKSAQHYTTTEI	L
349	KVLRVGPWINTILDD	L	391	SAQHYTTTEIDLNDI	L
350	GPWINTILDDFKVSL	L	392	TTTEIDLNDIMQNI	L
351	TILDDFKVSLESIGS	L	393	DLNDIMQNIPTYPH	L
352	FKVSLESIGSLTQEL	L	394	MQNIPTYPHGLRVV	L
353	ESIGSLTQELEYRGE	L	395	PTYPHGLRVVYESLP	L
354	LTQELEYRGESLLCS	L	396	GLRVVYESLPFYKAE	L
355	EYRGESLLCSLIFRN	L	397	YESLPFYKAEKIVNL	L
356	SLLCSLIFRNVWLYN	L	398	FYKAEKIVNLISGTK	L
357	LIFRNVWLYNQIALQ	L	399	KIVNLISGTKSITNI	L
358	VWLYNQIALQLKNHA	L	400	ISGTKSITNILEKTS	L
359	QIALQLKNHALCENN	L	401	SITNILEKTSIDL	L
360	LKNHALCENNKLYLDI	L	402	LEKTSIDLTDIDRA	L
361	LCNNKLYLDILKVLK	L	403	AIDLTDIDRATEMMR	L
362	LYLDILKVLKHLKTF	L	404	DIDRATEMMRKNITL	L
363	LKVLKHLKTFNLDN	L	405	TEMMRKNITLLIRIF	L
364	HLKTFNLDNIDTAL	L	406	KNITLLIRIFPLDCN	L
365	FNLDNIDTALTLYMN	L	407	LIRIFPLDCNRDKRE	L
366	IDTALTLYMNLPMFL	L	408	PLDCNRDKREILSME	L
367	TLYMNLPMFLGGGDP	L	409	RDKREILSMENLSIT	L
368	LPMLFGGGDPNLLYR	L	410	ILSMENLSITELSKY	L
369	GGGDPNLLYRSFYRR	L	411	NLSITELSKYVRERS	L
370	NLLYRSFYRRTPDFL	L	412	ELSKYVRERSWSLSN	L
371	SFYRRTPDFLTEAIV	L	413	VRERSWSLSNIVGVT	L
372	TPDFLTEAIVHSVFI	L	414	WSLSNIVGVTSPSIM	L
373	TEAIVHSVFILSYYT	L	415	IVGVTSPSIMYTMDI	L
374	HSVFILSYYTNHDLK	L	416	SPSIMYTMDIKYTTS	L
375	LSYYTNHDLKDKLQD	L	417	YTMDIKYTSTIASG	L
376	NHDLKDKLQDLSDDR	L	418	KYTTSTIASGIIIEK	L
377	DKLQDLSDDRNLKFL	L	419	TIASGIIIEKYNVNS	L
378	LSDDRNLKFLTCIIT	L	420	IIIEKYNVNSLTRGE	L

Peptide	Sequence	Protein	Peptide	Sequence	Protein
421	YNVNSLTRGERGPTK	L	463	YVELFLSNKTLKSGS	L
422	LTRGERGPTKPWVGS	L	464	LSNKTLSGSHVNSN	L
423	RGPTKPWVGSSTQEK	L	465	LKSGSHVNSNLILAH	L
424	PWVGSSTQEKTMPV	L	466	HVNSNLILAHKISDY	L
425	STQEKTMPVYNRQV	L	467	LILAHKISDYFHNTY	L
426	KTMPVYNRQVLTKKQ	L	468	KISDYFHNTYILSTN	L
427	YNRQVLTKKQRDQID	L	469	FHNTYILSTNLAGHW	L
428	LTKKQRDQIDLLAKL	L	470	ILSTNLAGHWILIIQ	L
429	RDQIDLLAKLDWVYA	L	471	LAGHWILIIQLMKDS	L
430	LLAKLDWVYASIDNK	L	472	ILIIQLMKDSKGIFE	L
431	DWVYASIDNKDEFME	L	473	LMKDSKGIFEKDWGE	L
432	SIDNKDEFMEELSIG	L	474	KGIFEKDWGEGYITD	L
433	DEFMEELSIGTLGLT	L	475	KDWGEGYITDHMFIN	L
434	ELSIGTLGLTYEKAK	L	476	GYITDHMFINKVFF	L
435	TLGLTYEKAKKLFPQ	L	477	HMFINKVFFNAYKT	L
436	YEKAKKLFPQYLSVN	L	478	LKVFFNAYKTYLLCF	L
437	KLFPQYLSVNYLHRL	L	479	NAYKTYLLCFHKGYG	L
438	YLSVNYLHRLTVSSR	L	480	YLLCFHKGYGRAKLE	L
439	YLHRLTVSSRPCEFP	L	481	HKGYGRAKLECDMNT	L
440	TVSSRPCEFPASIPA	L	482	RAKLECDMNTSDLLC	L
441	PCEFPASIPAYRTTN	L	483	CDMNTSDLLCVLELI	L
442	ASIPAYRTTNYHFDT	L	484	SDLLCVLELIDSSYW	L
443	YRTTNYHFDTSPINR	L	485	VLELIDSSYWKSMSK	L
444	YHFDTSPINRILTEK	L	486	DSSYWKSMSKVLEQ	L
445	SPINRILTEKYGED	L	487	KSMSKVLEQKVIKY	L
446	ILTEKYGDEDIDIVF	L	488	VFLEQKVIKYILSQD	L
447	YGDEDIDIVFQNCIS	L	489	KVIKYILSQDASLHR	L
448	IDIVFQNCISFGLSL	L	490	ILSQDASLHRVKGCH	L
449	QNCISFGLSLMSVVE	L	491	ASLHRVKGCHSFKLW	L
450	FGLSLMSVVEQFTNV	L	492	VKGCHSFKLWFLKRL	L
451	MSVVEQFTNVCPNRI	L	493	SFKLWFLKRLNVAEF	L
452	QFTNVCPNRIILIPK	L	494	FLKRLNVAEFTVCPW	L
453	CPNRIILIPKLNEIH	L	495	NVAEFTVCPWVVNID	L
454	ILIPKLNEIHLMKPP	L	496	TVCPWVVNIDYHPTH	L
455	LNEIHLMKPPIFTGD	L	497	VVNIDYHPTHMKAIL	L
456	LMKPPIFTGDVDIHK	L	498	YHPTHMKAILTYIDL	L
457	IFTGDVDIHKLKQVI	L	499	MKAILTYIDLVRMGL	L
458	VDIHKLKQVIQKQHM	L	500	TYIDLVRMGLINIDR	L
459	LKQVIQKQHMFLPDK	L	501	VRMGLINIDRIYIKN	L
460	QKQHMFLPDKISLTQ	L	502	INIDRIYIKNKHKN	L
461	FLPDKISLTQYVELF	L	503	IYIKNKHKNFDEFYT	L
462	ISLTQYVELFLSNKT	L	504	KHKFNDEFYTSNLFY	L

Peptide	Sequence	Protein	Peptide	Sequence	Protein
505	DEFYTSNLFYINYNF	L	547	RSLKDCNDHSLPIEF	L
506	SNLFYINYNFSDNTH	L	548	CNDHSLPIEFLRLYN	L
507	INYNFSDNTHLLTKH	L	549	LPIEFLRLYNGHINI	L
508	SDNTHLLTKHIRIAN	L	550	LRLYNGHINIDYGEN	L
509	LLTKHIRIANSELEN	L	551	GHINIDYGENLTIPA	L
510	IRIANSELENNYNKL	L	552	DYGENLTIPATDATN	L
511	SELENNYNKLYHPTP	L	553	LTIPATDATNNIHWS	L
512	NYNKLYHPTPETLEN	L	554	TDATNNIHWSYLHIK	L
513	YHPTPETLENILTNP	L	555	NIHWSYLHIKFAEPI	L
514	ETLENILTNPVKCDD	L	556	YLHIKFAEPISLFVC	L
515	ILTNPVKCDDKKTLN	L	557	FAEPISLFVCDAELP	L
516	VKCDDKKTLDNYCIG	L	558	SLFVCDAELPVTNVW	L
517	KKTLDNYCIGKNVDS	L	559	DAELPVTNVWSKIII	L
518	DYCIGKNVDSIMLPL	L	560	VTVNWSKIIIEWSKH	L
519	KNVDSIMLPLLSNKK	L	561	SKIIIEWSKHVRKCK	L
520	IMLPLLSNKKLIKSS	L	562	EWSKHVRKCKYCSSLV	L
521	LSNKKLIKSSMTIRT	L	563	VRKCKYCSSLVNKCTL	L
522	LKSSMTIRTNYSKQ	L	564	YCSSLVNKCTLIVKYH	L
523	TMIRTNYSKQDLYNL	L	565	NKCTLIVKYHAQDDI	L
524	NYSKQDLYNLFPTVV	L	566	IVKYHAQDDIDFKLD	L
525	DLYNLFPTVVIDKII	L	567	AQDDIDFKLDNITIL	L
526	FPTVVIDKIIDHSGN	L	568	DFKLDNITILKTYVC	L
527	IDKIIDHSGNTAKSN	L	569	NITILKTYVCLGSKL	L
528	DHSGNTAKSNQLYTT	L	570	KTYVCLGSKLKGSEV	L
529	TAKSNQLYTTTSHQI	L	571	LGSKLKGSEVYLVLT	L
530	QLYTTTSHQISLVHN	L	572	KGSEVYLVLTIGPAN	L
531	TSHQISLVHNSTSLY	L	573	YLVLTIGPANVFPVF	L
532	SLVHNSTSLYCMLPW	L	574	IGPANVFPVFNVVQN	L
533	STSLYCMLPWHHINR	L	575	VFPVFNVVQNAKLIL	L
534	CMLPWHHINRFNFVF	L	576	NVVQNAKLILSRTKN	L
535	HHINRFNFVFSSTGC	L	577	AKLILSRTKNFIMPK	L
536	FNFVFSSTGCKISIE	L	578	SRTKNFIMPKKADKE	L
537	SSTGCKISIEYILKD	L	579	FIMPKKADKESIDAN	L
538	KISIEYILKDLIKD	L	580	KADKESIDANIKSLI	L
539	YILKDLIKDPNCIA	L	581	SIDANIKSLIPFLCY	L
540	LKIKDPNCIAFIGEG	L	582	IKSLIPFLCYPITKK	L
541	PNCIAFIGEGAGNLL	L	583	PFLCYPITKKGINTA	L
542	FIGEGAGNLLLRTVV	L	584	PITKKGINTALSCLK	L
543	AGNLLLRTVVELHPD	L	585	GINTALSCLKSVVSG	L
544	LRTVVELHPDIKIYI	L	586	LSCLKSVVSGDILSY	L
545	ELHPDIKIYIRSLKD	L	587	SVVSGDILSYSIAGR	L
546	IKIYIRSLKDCNDHS	L	588	DILSYSIAGRNEVFS	L

Peptide	Sequence	Protein	Peptide	Sequence	Protein
589	SIAGRNEVFSNKLIN	L	631	ESNRKNNKQTIHLLK	M2.1
590	NEVFSNKLINHKHMN	L	632	NNKQTIHLLKRLPAD	M2.1
591	NKLINHKHMNILKWF	L	633	IHLLKRLPADVLKKT	M2.1
592	HKHMNILKWFNHVLN	L	634	RLPADVLKKTIKNTL	M2.1
593	ILKWFNHVLNFRSTE	L	635	VLKKTIKNTLDIHS	M2.1
594	NHVLNFRSTELNYNH	L	636	IKNTLDIHSITINN	M2.1
595	FRSTELNYNHLYMVE	L	637	DIHSITINNPKEST	M2.1
596	LNYNHLYMVESTYPY	L	638	ITINNPKESTVNDTN	M2.1
597	LYMVESTYPYSELL	L	639	PKESTVNDTNDHAKN	M2.1
598	STYPYSELLNSLTT	L	640	VNDTNDHAKNNDTT	M2.1
599	LSELLNSLTTNELKK	L	641	MTMPKIMILPDKYPC	M2.2
600	NSLTTNELKKLIKIT	L	642	IMILPDKYPCSINSI	M2.2
601	NELKKLIKITGSLLY	L	643	DKYPCSINSILITSS	M2.2
602	LKITGSLLYNFHNE	L	644	SINSILITSSCRVTM	M2.2
603	GSLLYNFHNE	L	645	LITSSCRVTMYNQKN	M2.2
604	MSRRNPCKFEIRGHC	M2.1	646	CRVTMYNQKNTLYFN	M2.2
605	PCKFEIRGHCLNGKR	M2.1	647	YNQKNTLYFNQNNQN	M2.2
606	IRGHCLNGKRCHFSH	M2.1	648	TLYFNQNNQNNHTYS	M2.2
607	LNGKRCHFSHNYFEW	M2.1	649	QNNQNNHTYSPNQPF	M2.2
608	CHFSHNYFEWPPHAL	M2.1	650	NHTYSPNQPFNEIHW	M2.2
609	NYFEWPPHALLVRQN	M2.1	651	PNQPFNEIHWTSQDL	M2.2
610	PPHALLVRQNFMLNR	M2.1	652	NEIHWTSQDLIDAIQ	M2.2
611	LVRQNFMLNRILKSM	M2.1	653	TSQDLIDAIQNFLQH	M2.2
612	FMLNRILKSMDKSID	M2.1	654	IDAIQNFLQHLGITD	M2.2
613	ILKSMDKSIDTLSEI	M2.1	655	NFLQHLGITDDIYTI	M2.2
614	DKSIDTLSEISGAEE	M2.1	656	LGITDDIYTIYILVS	M2.2
615	TLSEISGAEEELDRTE	M2.1	657	DIYTIYILVS	M2.2
616	SGAAELDRTEEYALG	M2.1	658	METYVNKLHEGSTYT	M
617	LDRTEEYALGVVGVL	M2.1	659	NKLHEGSTYTAHVQY	M
618	EYALGVVGVLSEYIG	M2.1	660	GSTYTAHVQYNVLEK	M
619	VVGVLSEYIGSINNI	M2.1	661	AAVQYNVLEKDDDDPA	M
620	ESYIGSINNITKQSA	M2.1	662	NVLEKDDDDPASLTIW	M
621	SINNITKQSACVAMS	M2.1	663	DDDDPASLTIWVPMFQ	M
622	TKQSACVAMSKLLTE	M2.1	664	SLTIWVPMFQSSMPA	M
623	CVAMSKLLTELNSDD	M2.1	665	VPMFQSSMPADLLIK	M
624	KLLTELNSDDIKKLR	M2.1	666	SSMPADLLIKELANV	M
625	LNSDDIKKLRDNEEP	M2.1	667	DLLIKELANVNILVK	M
626	IKKLRDNEEPNSPKI	M2.1	668	ELANVNILVKQISTP	M
627	DNEEPNSPKIRVYNT	M2.1	669	NILVKQISTPKGPSL	M
628	NSPKIRVYNTVISYI	M2.1	670	QISTPKGPSLRVMIN	M
629	RVYNTVISYIESNRK	M2.1	671	KGPSLRVMINRSASV	M
630	VISYIESNRKNNKQT	M2.1	672	RVMINRSASVLAQMP	M

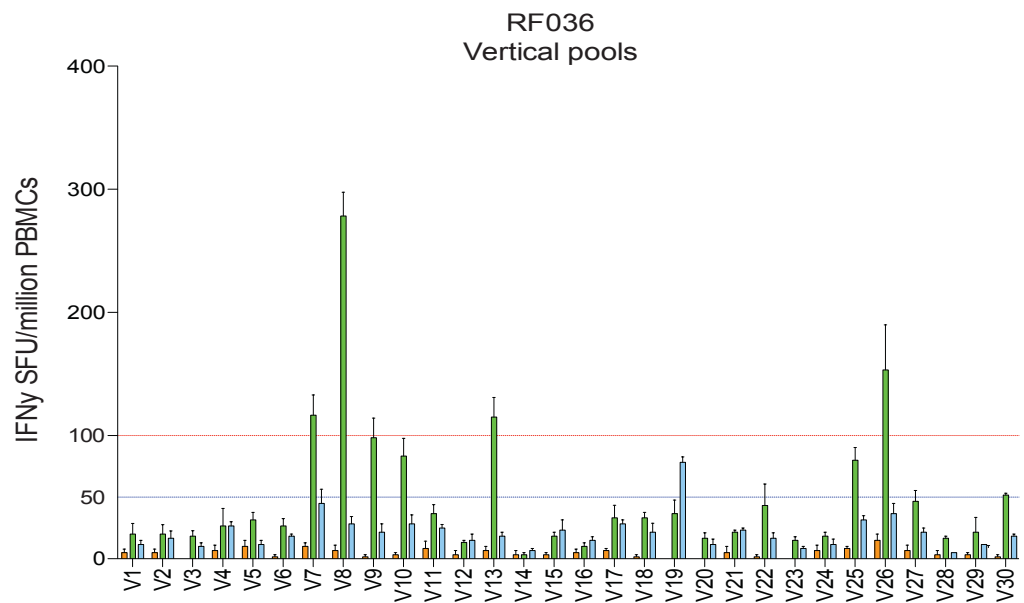
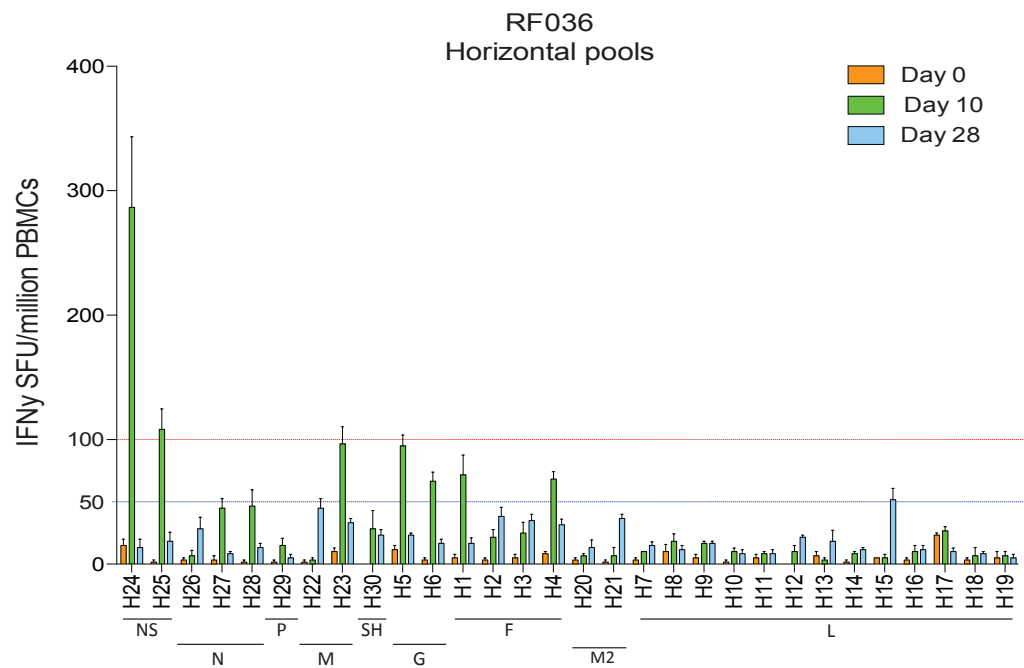
Peptide	Sequence	Protein	Peptide	Sequence	Protein
673	SRSAVLAQMPSKFTI	M	715	HLTNALAKAVIHTIK	NS1
674	LAQMPSKFTICANVS	M	716	LAKAVIHTIKLNGIV	NS1
675	SKFTICANVSLDERS	M	717	IHTIKLNGIVFVHVI	NS1
676	CANVSLDERSKLAYD	M	718	LNGIVFVHVITSSDI	NS1
677	LDERSKLAYDVTTTPC	M	719	FVHVITSSDICPNNN	NS1
678	KLAYDVTTTPCEIKAC	M	720	TSSDICPNNNIVVKS	NS1
679	VTTTPCEIKACSLTCL	M	721	CPNNNIVVKSNTFTM	NS1
680	EIKACSLTCLKSKNM	M	722	IVVKSNTFTMPVLQN	NS1
681	SLTCLKSKNMLTTVK	M	723	NFTTMPVLQNGGYIW	NS1
682	KSKNMLTTVKDLTMK	M	724	PVLQNGGYIWEMMEL	NS1
683	LTTVKDLTMKTLNPT	M	725	GGYIWEMMELTHCSQ	NS1
684	DLTMKTLNPTHDIIA	M	726	EMMELTHCSQPNGLI	NS1
685	TLNPTHDIIALCEFE	M	727	THCSQPNGLIDDNCE	NS1
686	HDIIALCEFENIVTS	M	728	PNGLIDDNCEIKFSK	NS1
687	LCEFENIVTSKKVII	M	729	DDNCEIKFSKKLSDS	NS1
688	NIVTSKKVIIPTYLR	M	730	IKFSKKLSDSTMTNY	NS1
689	KKVIIPTYLRSISVR	M	731	KLSDSTMTNYMNQLS	NS1
690	PTYLRSISVRNKDLN	M	732	TMTNYMNQLSELLGF	NS1
691	SISVRNKDLNTLENI	M	733	MNQLSELLGFDLNP	NS1
692	NKDLNTLENITTEF	M	734	MDTTHNGTTPQRLMI	NS2
693	TLENITTEFKNAIT	M	735	NGTTPQRLMITDMRP	NS2
694	TTTEFKNAITNAKII	M	736	QRLMITDMRPLSLET	NS2
695	KNAITNAKIIPYSG	M	737	TDMRPLSLETIITSL	NS2
696	NAKIIPYSGLLLVIT	M	738	LSLETIITSLTRDII	NS2
697	PYSGLLLVITVTDNK	M	739	IITSLTRDIITHRFI	NS2
698	LLVITVTDNKGAFKY	M	740	TRDIITHRFIYLINH	NS2
699	VTDNKGAFKYIKPQS	M	741	THRFIYLINHECIVR	NS2
700	GAFKYIKPQSQFIVD	M	742	YLINHECIVRKLDER	NS2
701	IKPQSQFIVDLGAYL	M	743	ECIVRKLDERQATFT	NS2
702	QFIVDLGAYLEKESI	M	744	KLDERQATFTFLVNY	NS2
703	LGAYLEKESIYYVTT	M	745	QATFTFLVNYEMKLL	NS2
704	EKESIYYVTTNWKHT	M	746	FLVNYEMKLLHKVGS	NS2
705	YYVTTNWKHTATRFA	M	747	EMKLLHKVGSTKYKK	NS2
706	NWKHTATRFAIKPME	M	748	HKVGSTKYKKYTEYN	NS2
707	ATRFAIKPMED	M	749	TKYKKYTEYNTKYGT	NS2
708	MGSNSLSMIKVRQLN	NS1	750	YTEYNTKYGTFPMPPI	NS2
709	LSMIKVRQLNLFND	NS1	751	TKYGTFPMPPIFINHD	NS2
710	VRLQNLFNDNEVALL	NS1	752	FPMPPIFINHDGFLEC	NS2
711	LFDNDEVALLKITCY	NS1	753	FINHDGFLECIGIKP	NS2
712	EVALLKITCYTDKLI	NS1	754	GFLECIGIKPTKHTP	NS2
713	KITCYTDKLIHLTNA	NS1	755	IGIKPTKHTPIIYKY	NS2
714	TDKLIHLTNALAKAV	NS1	756	TKHTPIIYKYDLNP	NS2

Peptide	Sequence	Protein	Peptide	Sequence	Protein
757	MALSKVKLNDTLNKD	N	799	EVFEKYPHFIDVFVH	N
758	VKLNDTLNKDQLLS	N	800	YPHFIDVFVHFGIAQ	N
759	TLNKDQLLSSSKYTI	N	801	DVFVHFGIAQSSTRG	N
760	QLLSSSKYTIQRSTG	N	802	FGIAQSSTRGGSRVE	N
761	SKYTIQRSTGDSIDT	N	803	SSTRGGSRVEGIFAG	N
762	QRSTGDSIDTPNYDV	N	804	GSRVEGIFAGLFMNA	N
763	DSIDTPNYDVQKHIN	N	805	GIFAGLFMNAYGAGQ	N
764	PNYDVQKHINKLCGM	N	806	LFMNAYGAGQVMLRW	N
765	QKHINKLCGMMLITE	N	807	YGAGQVMLRWGVLAKE	N
766	KLCGMMLITEDANHK	N	808	VMLRWGVLAKEVKN	N
767	LLITEDANHKFTGVI	N	809	GVLAKEVKNIMLGHA	N
768	DANHKFTGVIGMLYA	N	810	SVKNIMLGHASVQAE	N
769	FTGVIGMLYAMSRLG	N	811	MLGHASVQAEMEQQV	N
770	GMLYAMSRLGREDTI	N	812	SVQAEMEQQVEVVEY	N
771	MSRLGREDTIKILRD	N	813	MEQVVEVVEYQAQKL	N
772	REDTIKILRDAGYHV	N	814	EVVEYQAQKLGGGAG	N
773	KILRDAGYHVKANGV	N	815	AQKLGGGAGFYHILN	N
774	AGYHVKANGVDVTTH	N	816	GEAGFYHILNNPKAS	N
775	KANGVDVTTHRQDIN	N	817	YHILNNPKASLLSLT	N
776	DVTTHRQDINGKEMK	N	818	NPKASLLSLTQFPHF	N
777	RQDINGKEMKFEVLT	N	819	LLSLTQFPHFSSVVL	N
778	GKEMKFEVLTLASLT	N	820	QFPHFSSVVLGNAAG	N
779	FEVLTLASLTTEIQI	N	821	SSVVLGNAAGLGIMG	N
780	LASLTTEIQINIEIE	N	822	GNAAGLGIMGEYRGT	N
781	TEIQINIEIESRKS	N	823	LGIMGEYRGTPRNQD	N
782	NIEIESRKSYYKMLK	N	824	EYRGTPRNQDLYDAA	N
783	SRKSYYKMLKEMGEV	N	825	PRNQDLYDAAKAYAE	N
784	KKMLKEMGEVAPEYR	N	826	LYDAAKAYAEQLKEN	N
785	EMGEVAPEYRHDSPE	N	827	KAYAEQLKENGVIN	N
786	APEYRHDSPEDCGMII	N	828	QLKENGVINYSVLDL	N
787	HDSPEDCGMIIICIAA	N	829	GVINYSVLDLTAEEL	N
788	CGMIICIAALVITK	N	830	SVLDLTAEELIAIKH	N
789	LCIAALVITKLAAGD	N	831	TAEELIAIKHQLNPK	N
790	LVITKLAAGDRSGLT	N	832	EAIKHQLNPKDNDVE	N
791	LAAGDRSGLTAVIRR	N	833	QLNPKDNDVEL	N
792	RSGLTAVIRRANNVL	N	834	MEKFAPEFHGEDANN	P
793	AVIRRANNVLKNEMK	N	835	PEFHGEDANNRATKF	P
794	ANNVLKNEMKRYKGL	N	836	EDANNRATKFLESIK	P
795	KNEMKRYKGLLPKDI	N	837	RATKFLESIKGKFTS	P
796	RYKGLLPKDIANSFY	N	838	LESIKGKFTSPKDPK	P
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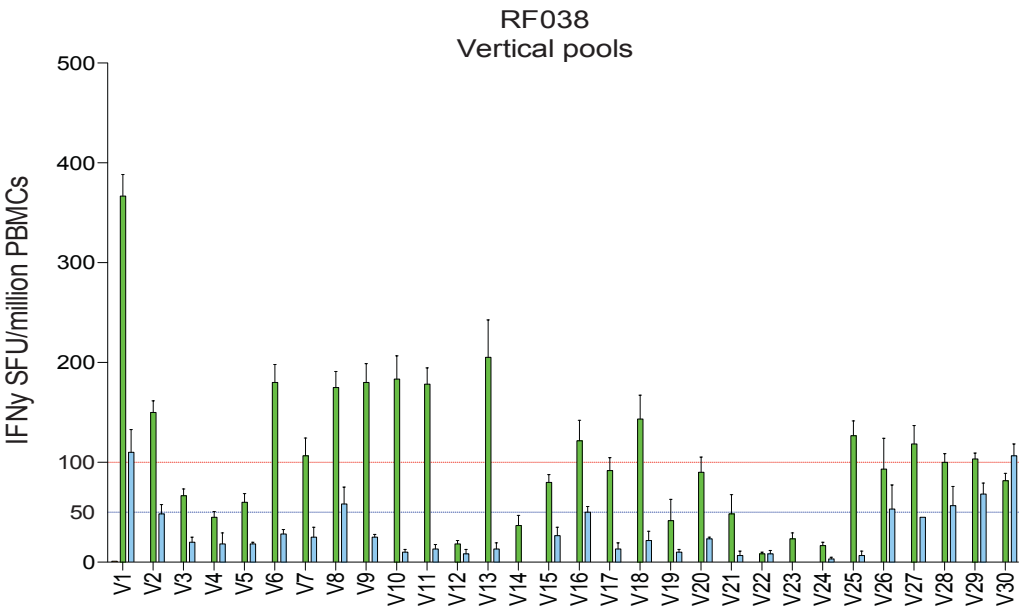
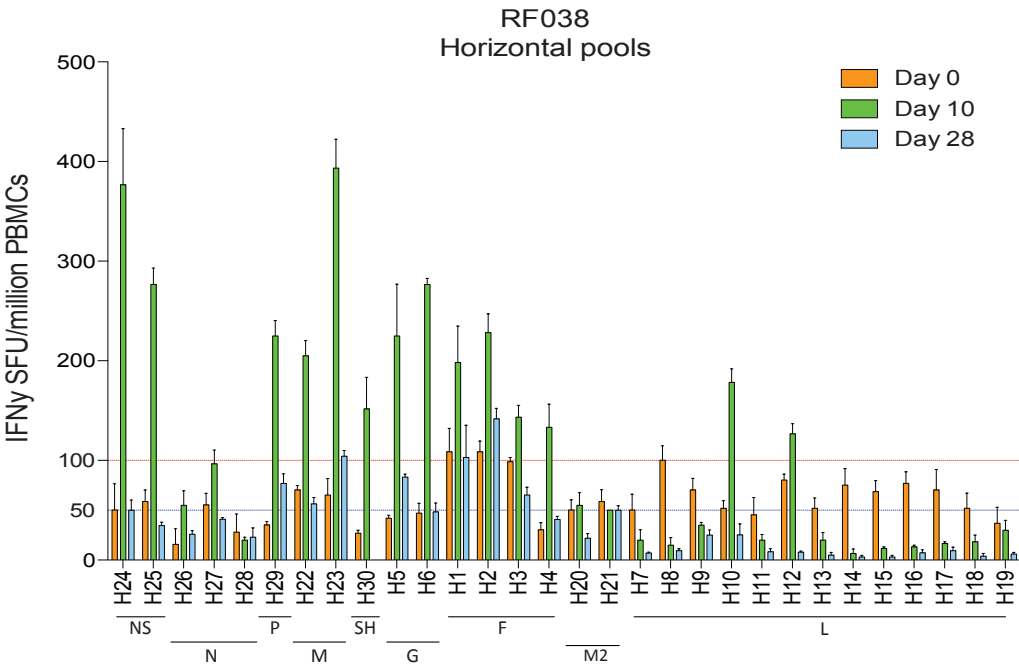
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843	IDIEVTKESPITSNS	P	869	EMIEKIRTEALMTND	P
844	TKESPITSNSTIINP	P	870	IRTEALMTNDRLEAM	P
845	ITSNSTIINPTNETD	P	871	LMTNDRLEAMARLRN	P
846	TIINPTNETDDTAGN	P	872	RLEAMARLRNEESEK	P
847	TNETDDTAGNKPNYQ	P	873	ARLRNEESEKMAKDT	P
848	DTAGNKPNYQRKPLV	P	874	EESEKMAKDTSDDEV	P
849	KPNYQRKPLVSFKED	P	875	MAKDTSDDEVSLNPTS	P
850	RKPLVSFKEDPTPSD	P	876	SDEVSLNPTSEKLNN	P
851	SFKEDPTPSDNPFSK	P	877	LNPTSEKLNNLLEGN	P
852	PTPSDNPFSKLYKET	P	878	EKLNNLLEGNDSDND	P
853	NPFSKLYKETIETFD	P	879	LLEGNDSDNDLSLED	P
854	LYKETIETFDNNEEE	P	880	DSDNDLSLED	P
855	IETFDNNEEESSYSY	P	881	MENTSITIEFSSKFW	SH
856	NNEEESSYSYEEIND	P	882	ITIEFSSKFWPYFTL	SH
857	SSYSYEEINDQTNND	P	883	SSKFWPYFTLIHMIT	SH
858	EEINDQTNNDNITARL	P	884	PYFTLIHMITTIISL	SH
859	QTNNDNITARLDRIE	P	885	IHMITTIISLLIIS	SH
860	ITARLDRIEKLSEI	P	886	TIISLLIISIMIAI	SH
861	DRIDEKLSEILGMLH	P	887	LIISIMIAILNKLC	SH
862	KLSEILGMLHTLVVA	P	888	IMIAILNKLCYENVF	SH
863	LGMLHTLVVASAGPT	P	889	LNKLCEYNVFNKTF	SH
864	TLVVASAGPTSARDG	P	890	EYNVFNKTFELPRA	SH
865	SAGPTSARDGIRDAM	P	891	HNKTFELPRARVNT	SH
866	SARDGIRDAMVGLRE	P			

Figure S3. The amino acid sequences of the 891 peptides that was tested as part of T cell epitope mapping. Peptide numbers, amino acid sequence and their originating protein are presented. Peptides 1 – 113 from F, 114 – 171 from G, 172 – 603 from L, 604 – 657 from M2, 658 – 707 from M, 708 – 756 from NS1/2, 757 – 833 from N, 834 – 880 from P and 881 – 891 from SH proteins.

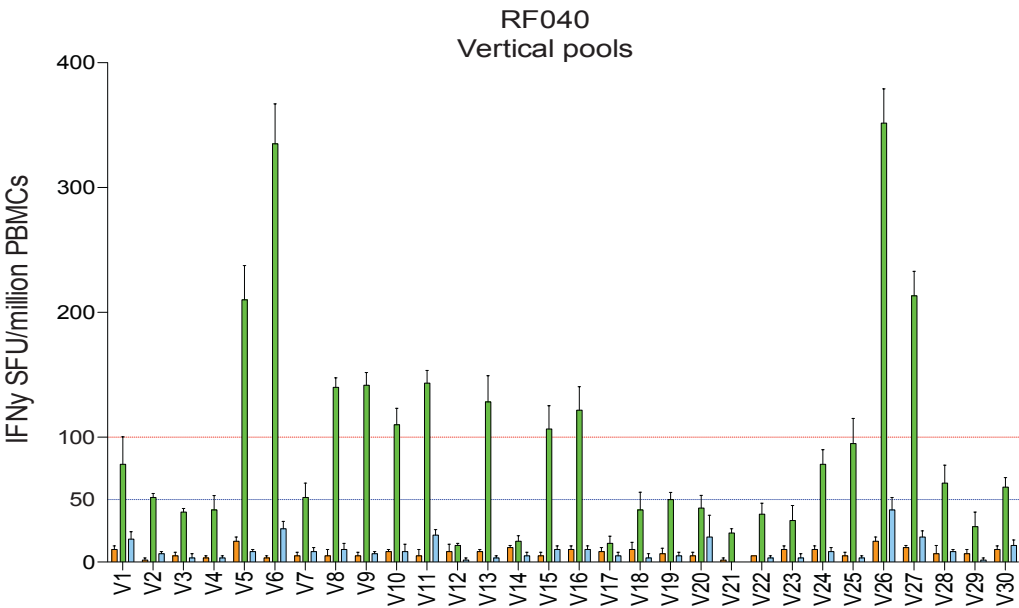
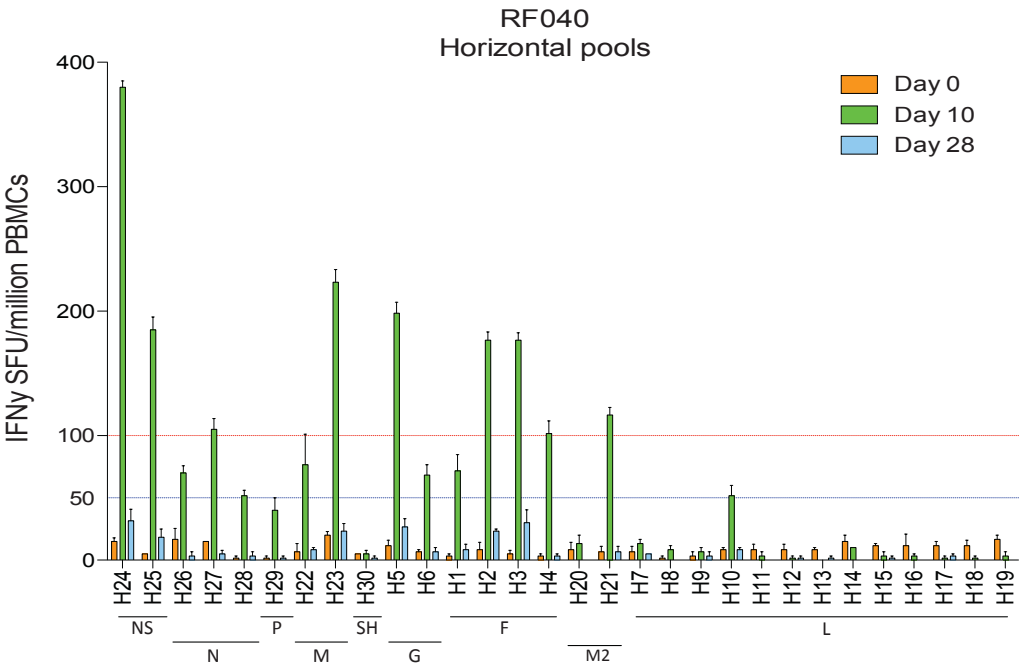
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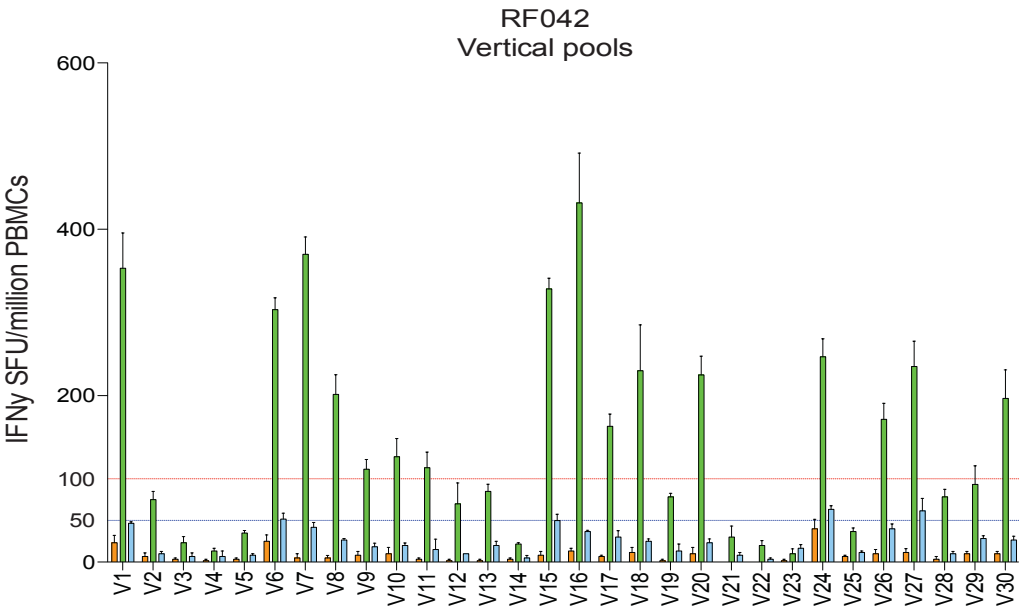
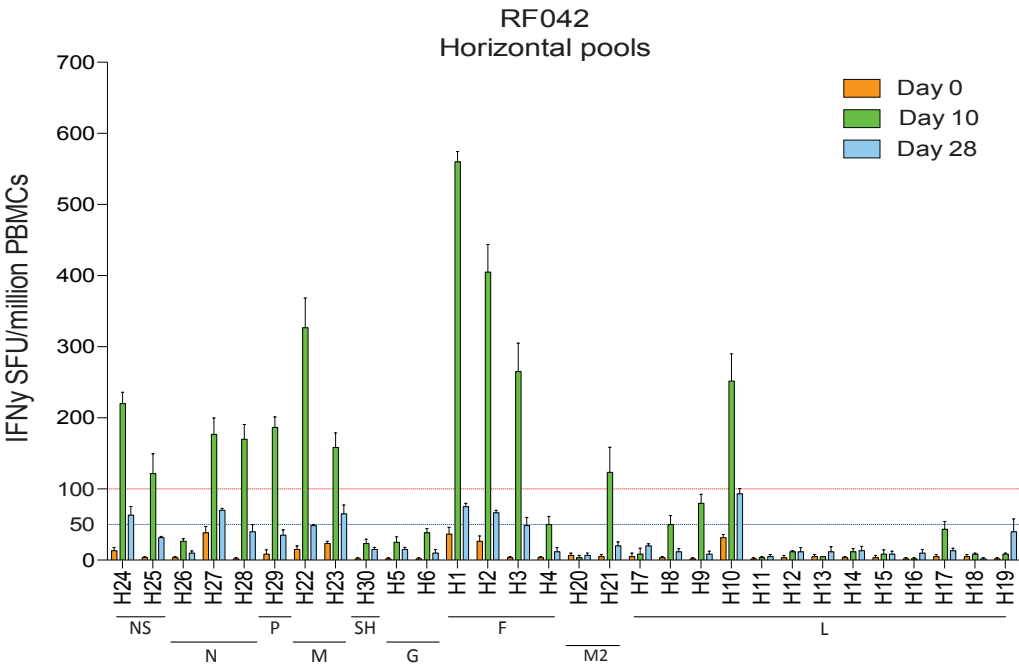
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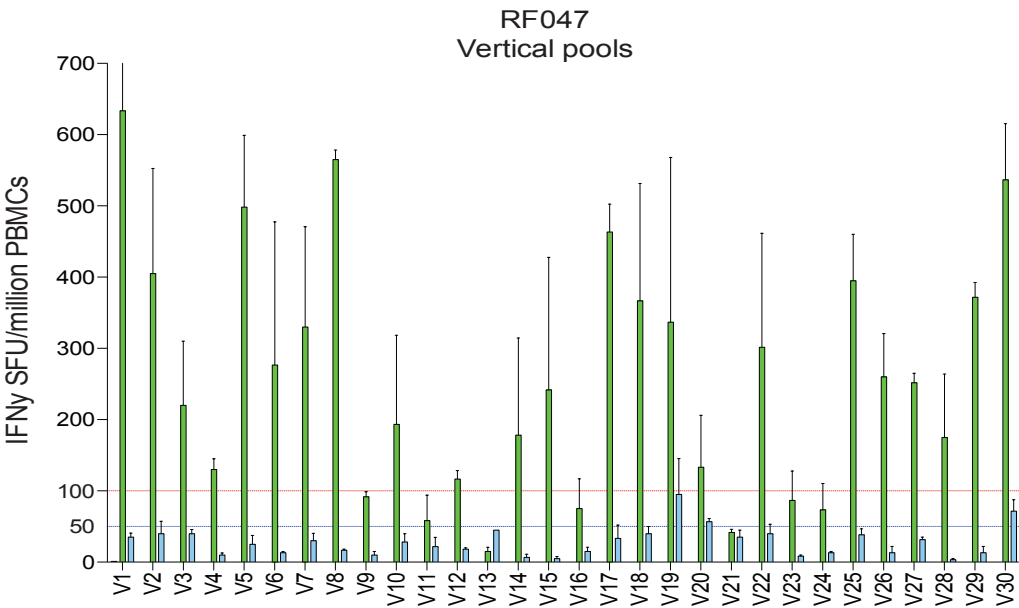
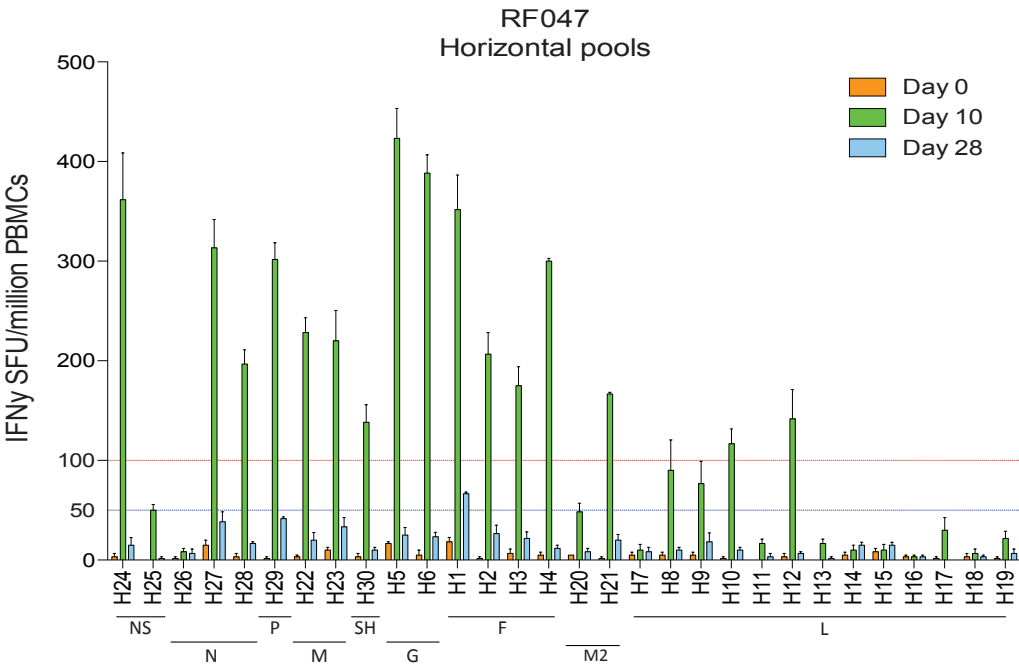
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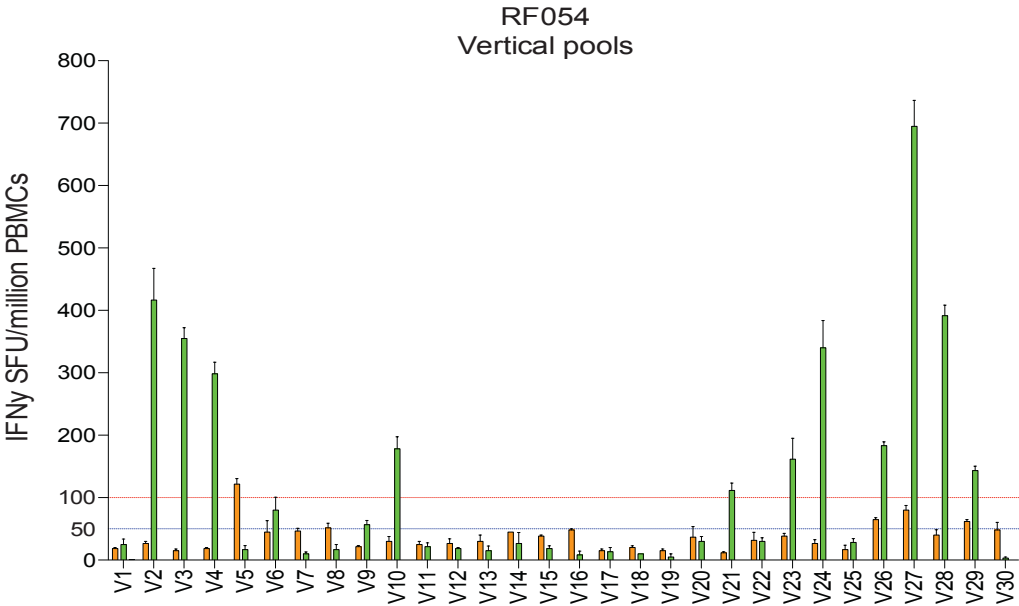
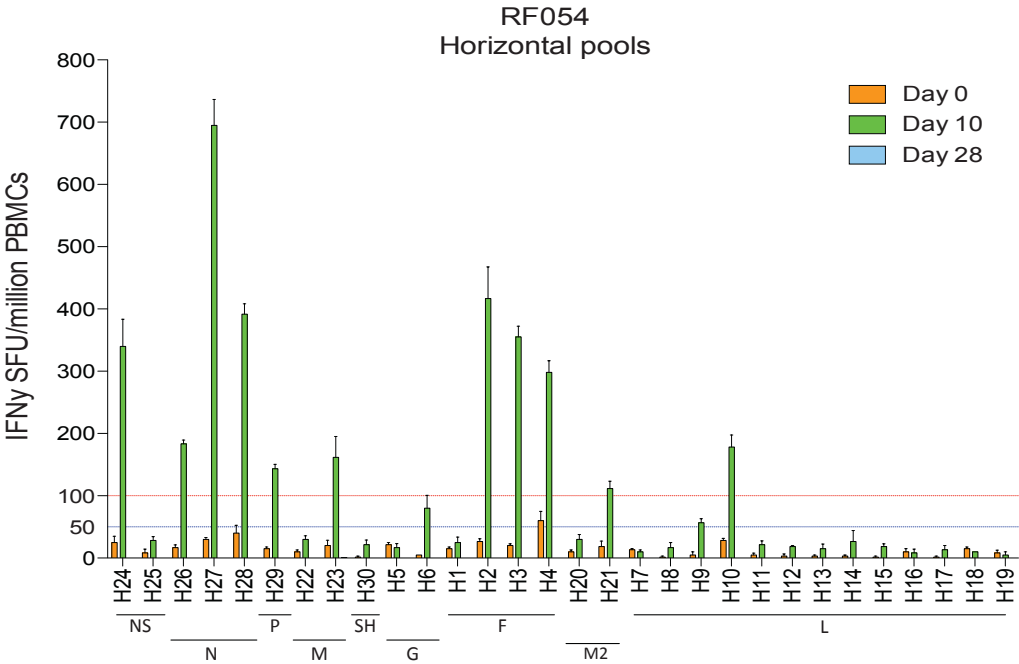
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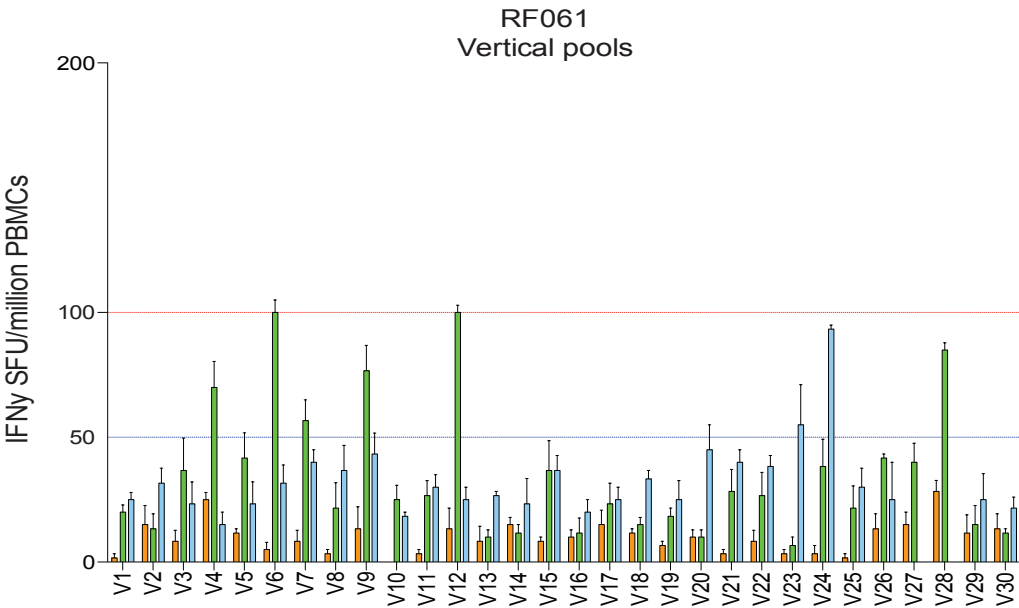
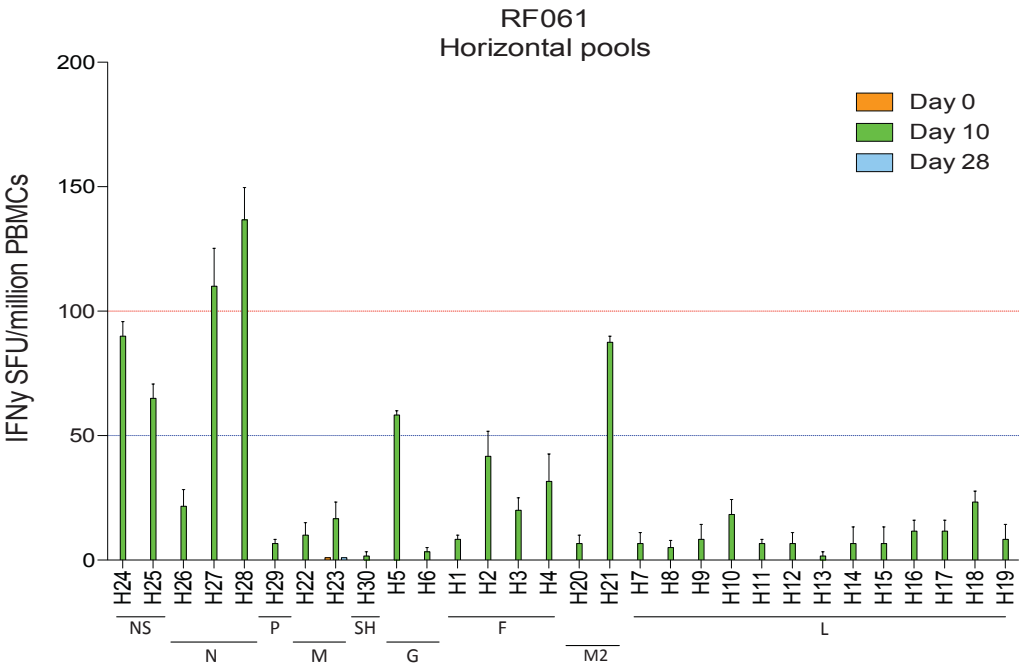
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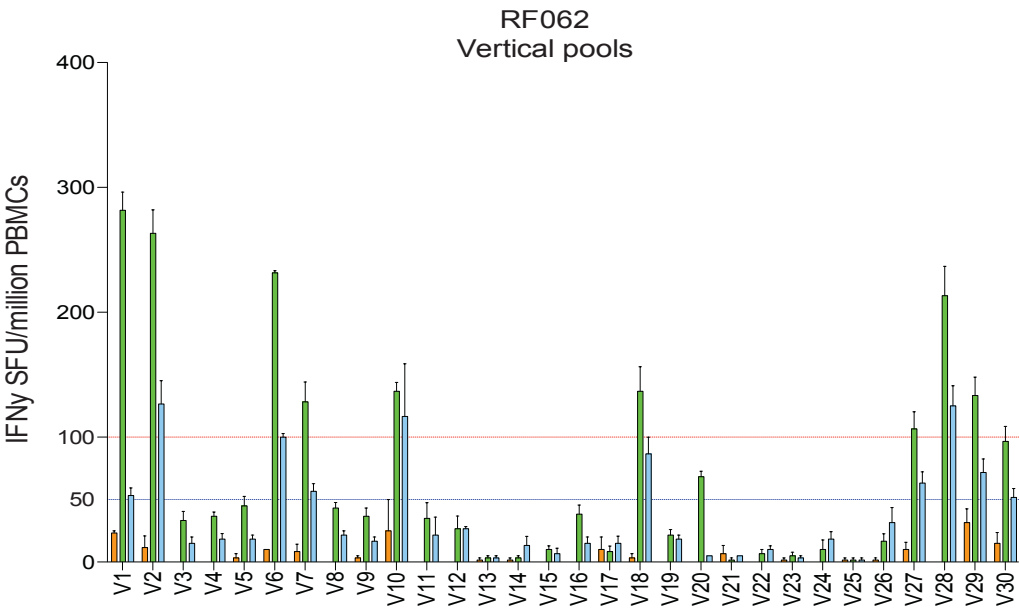
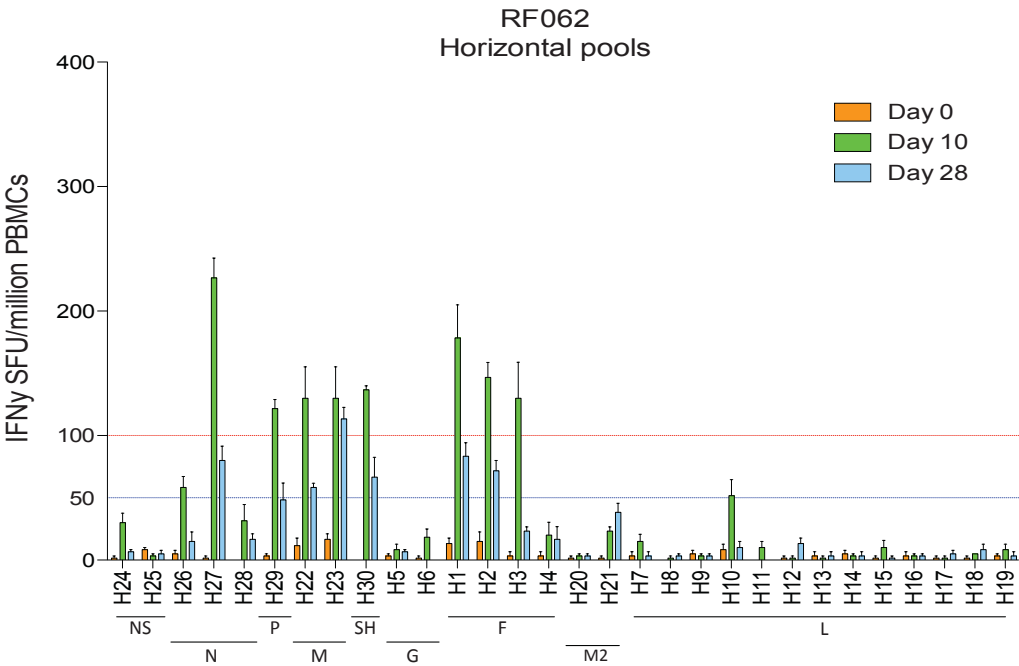
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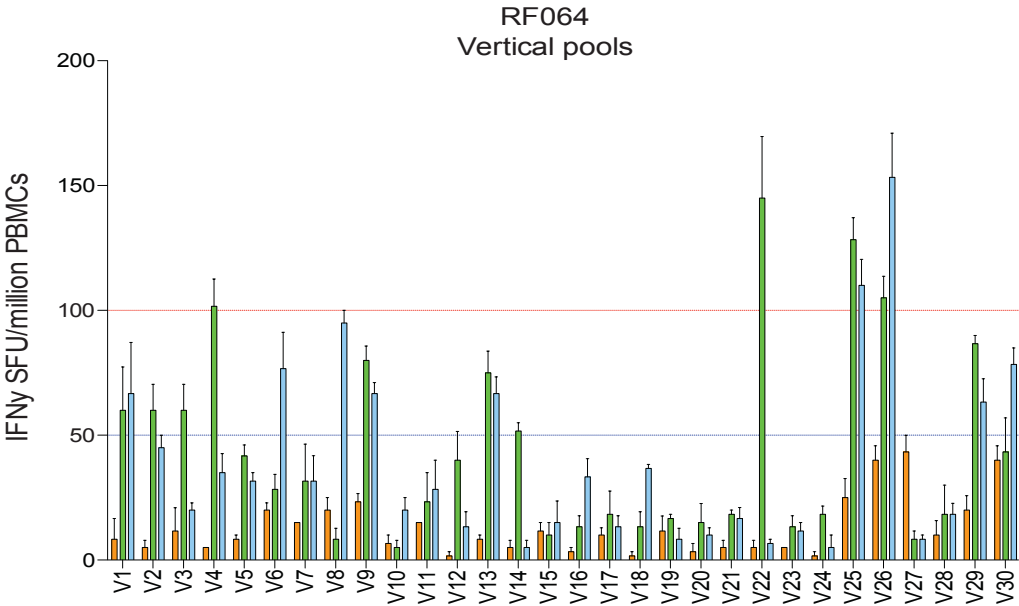
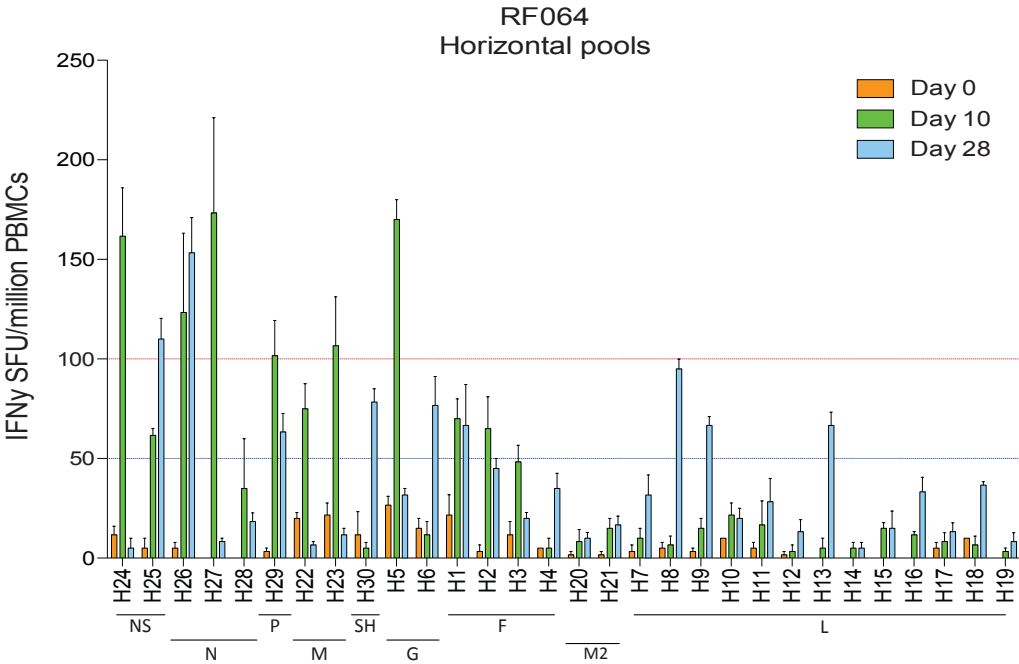
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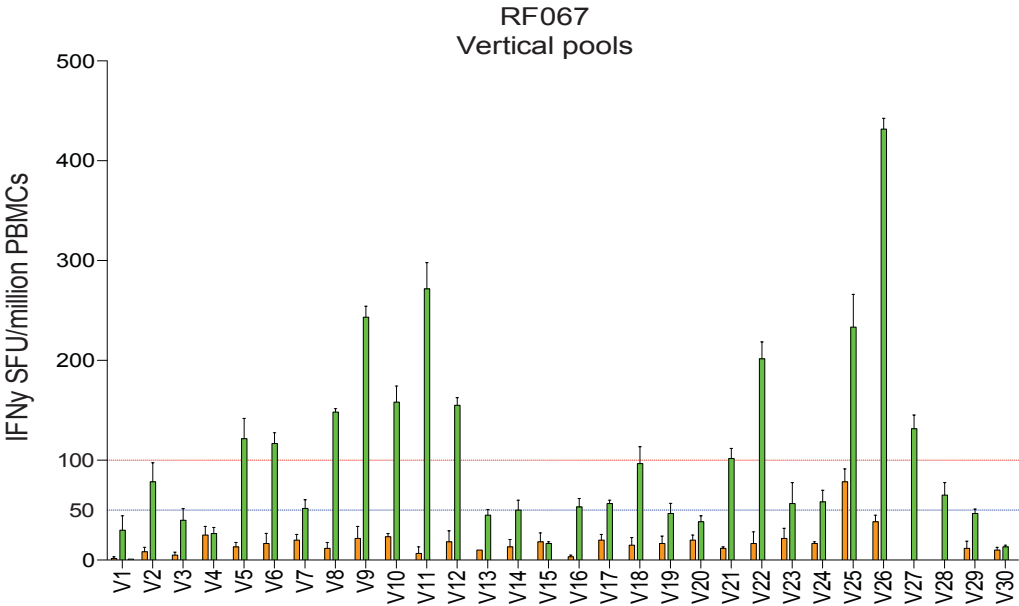
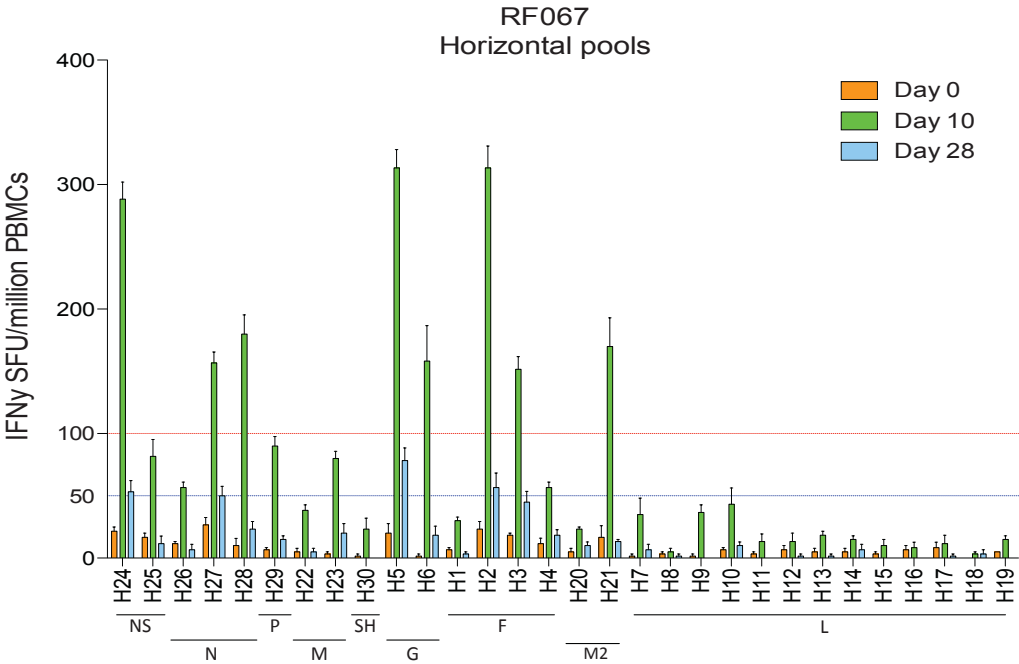
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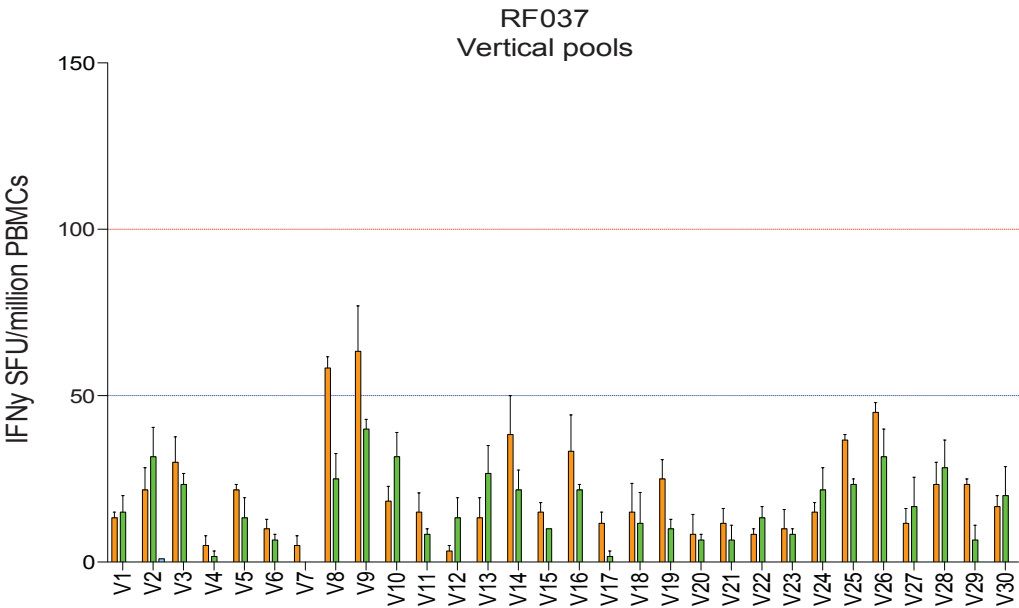
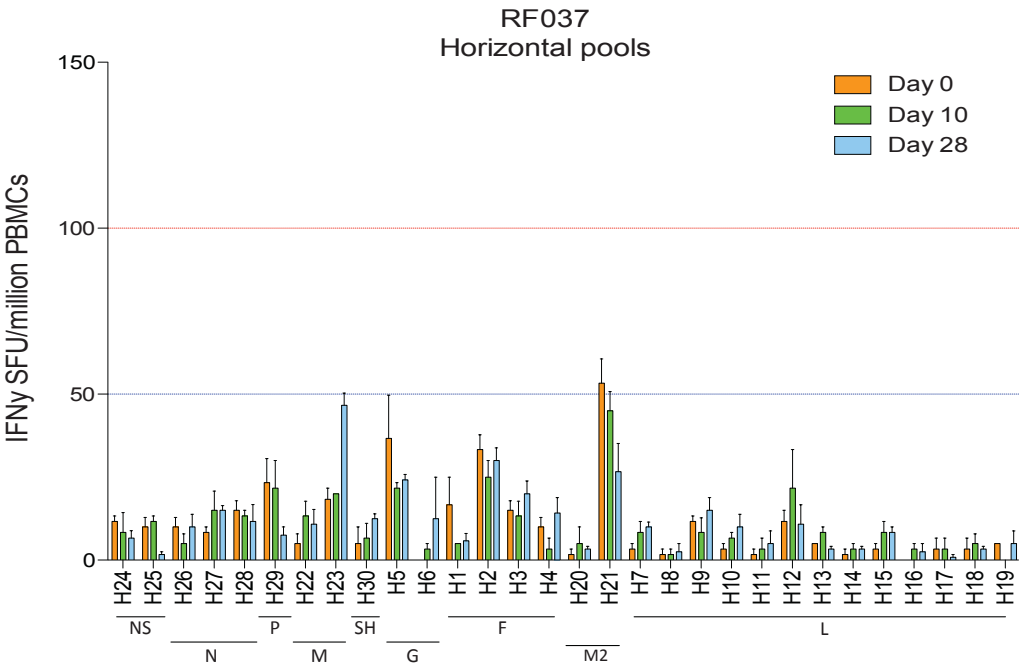
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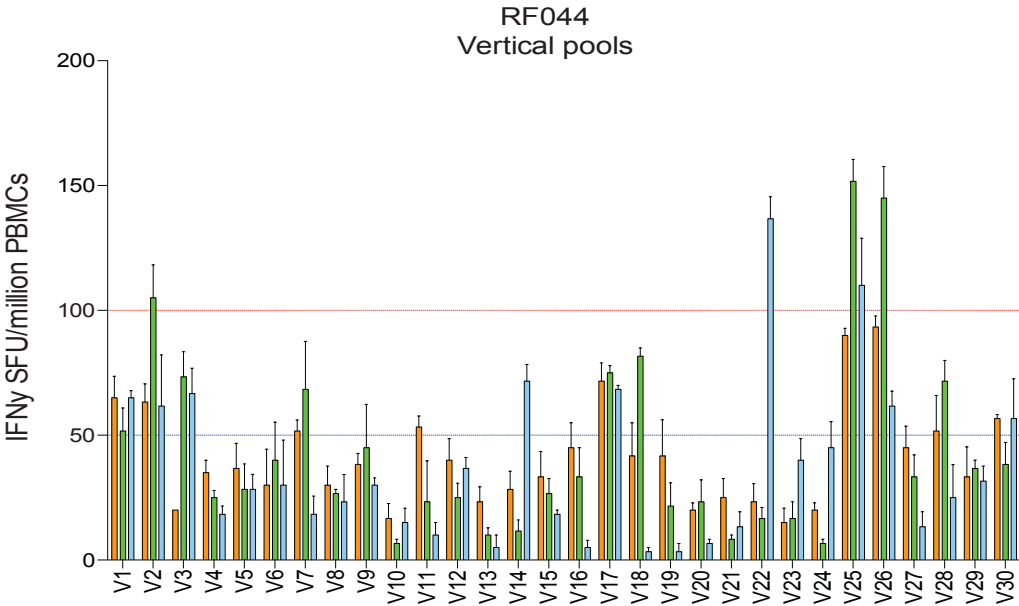
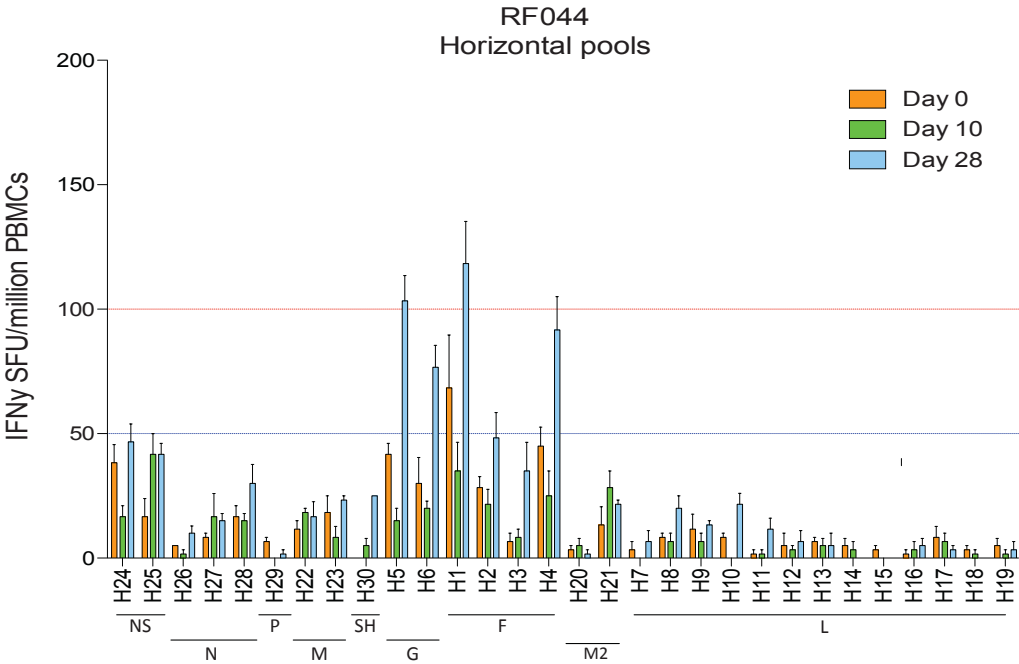
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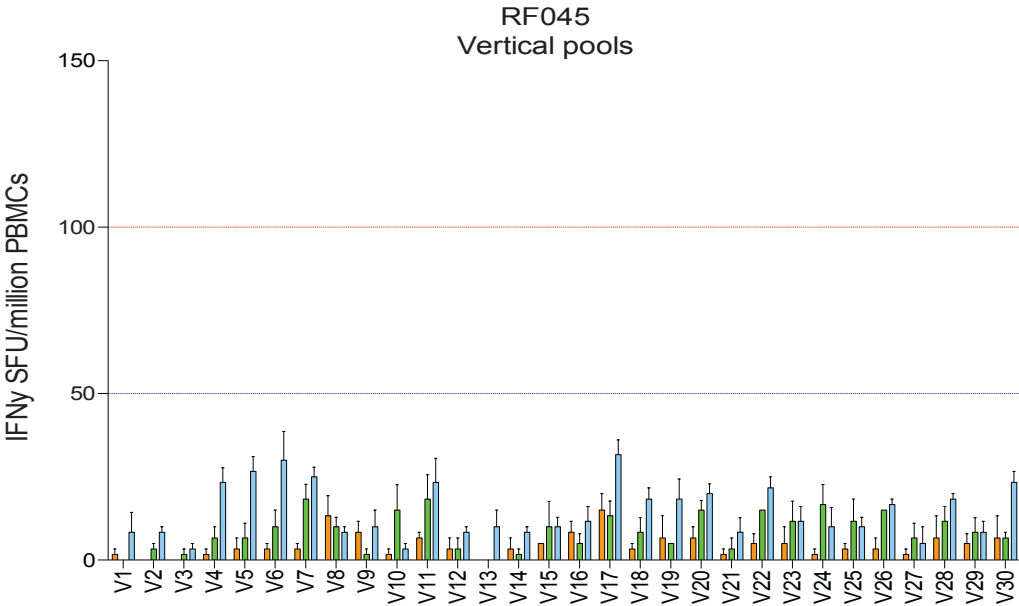
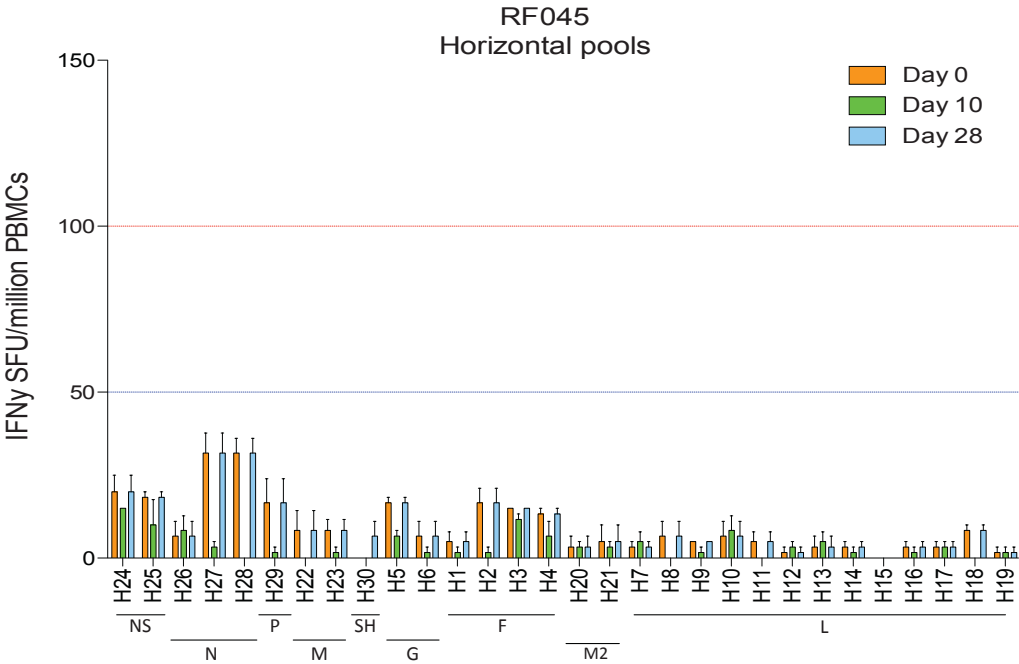
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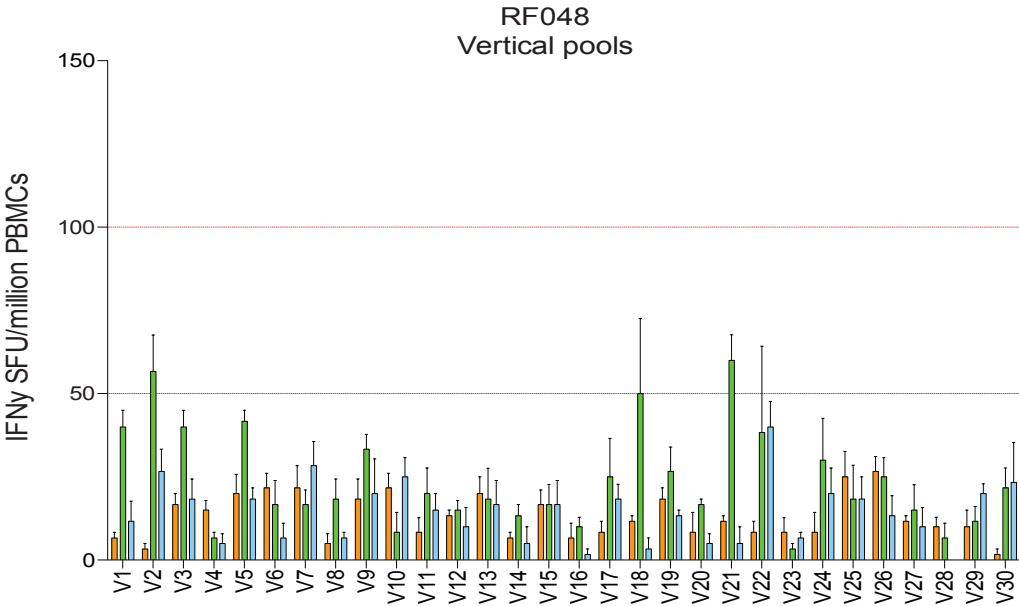
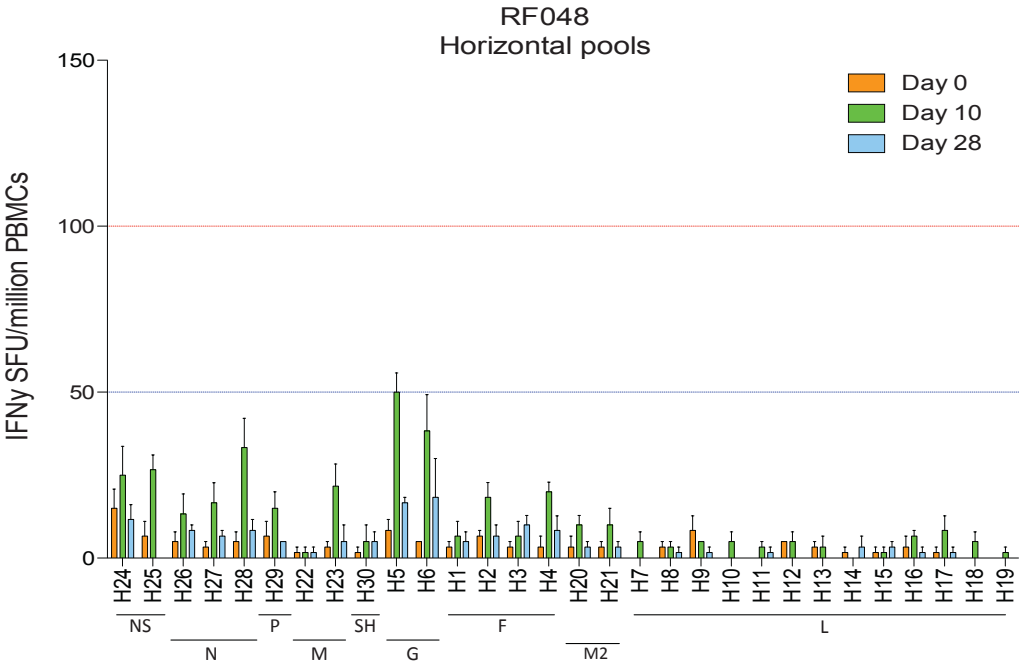
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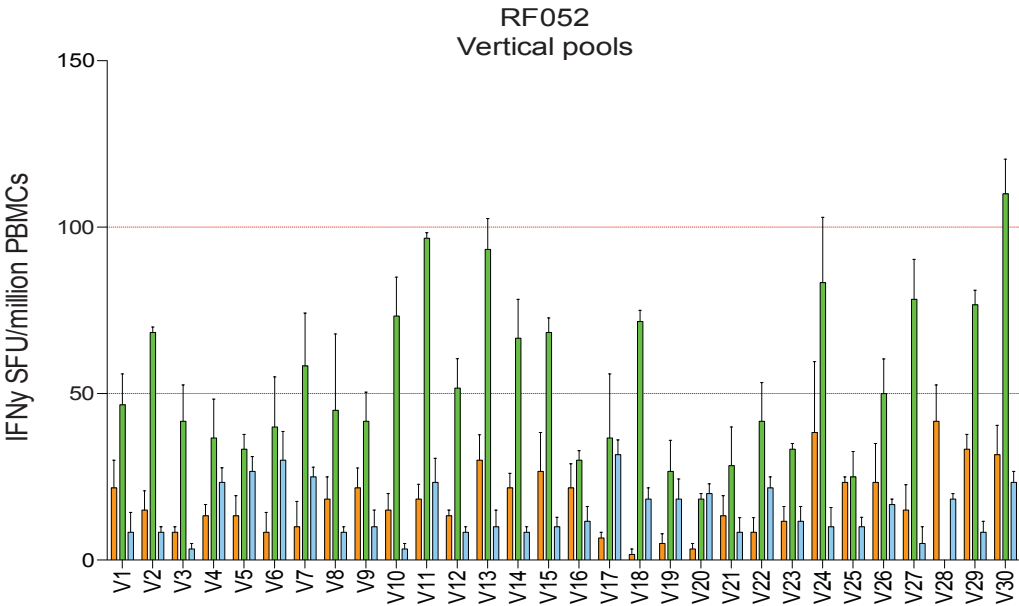
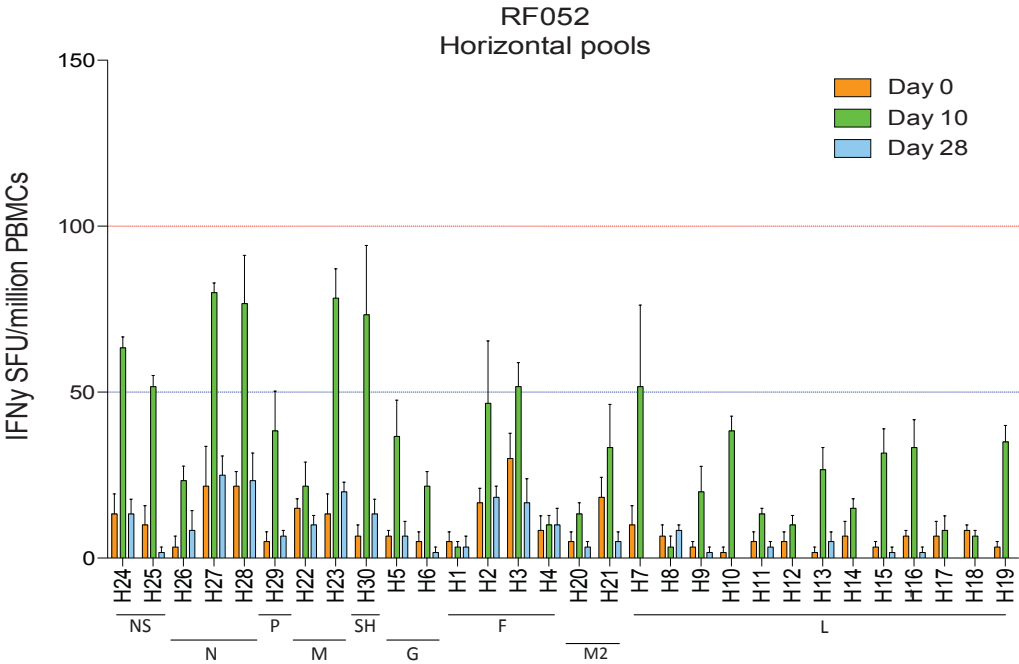
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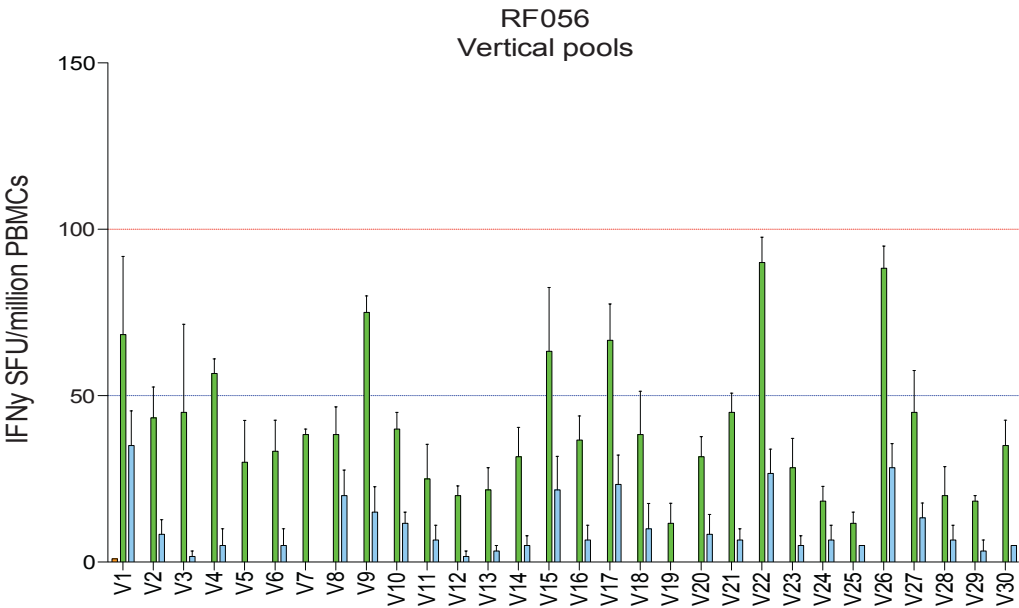
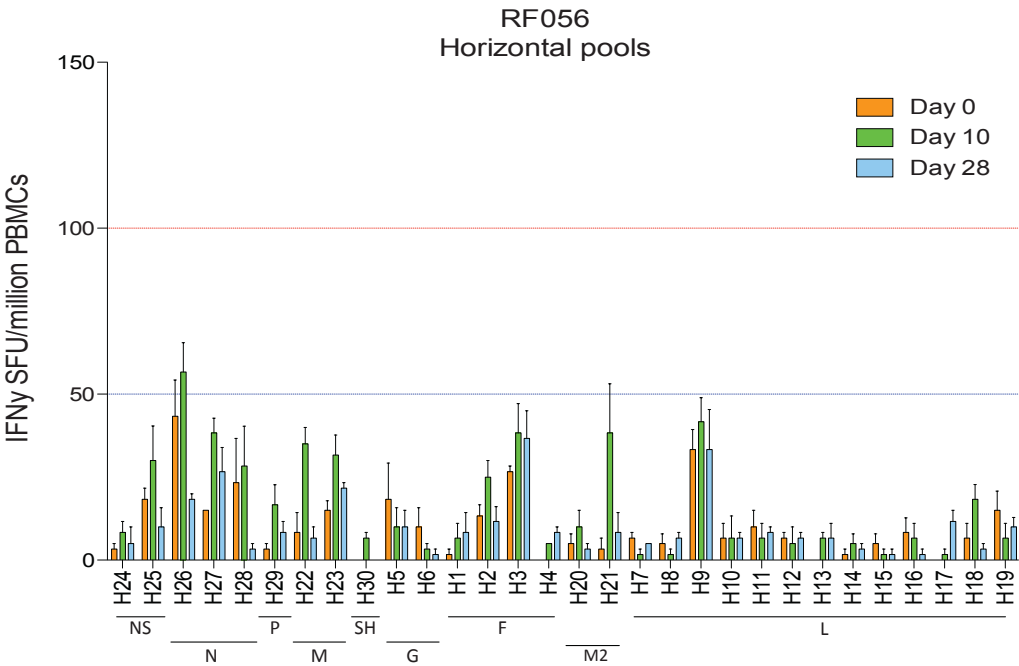
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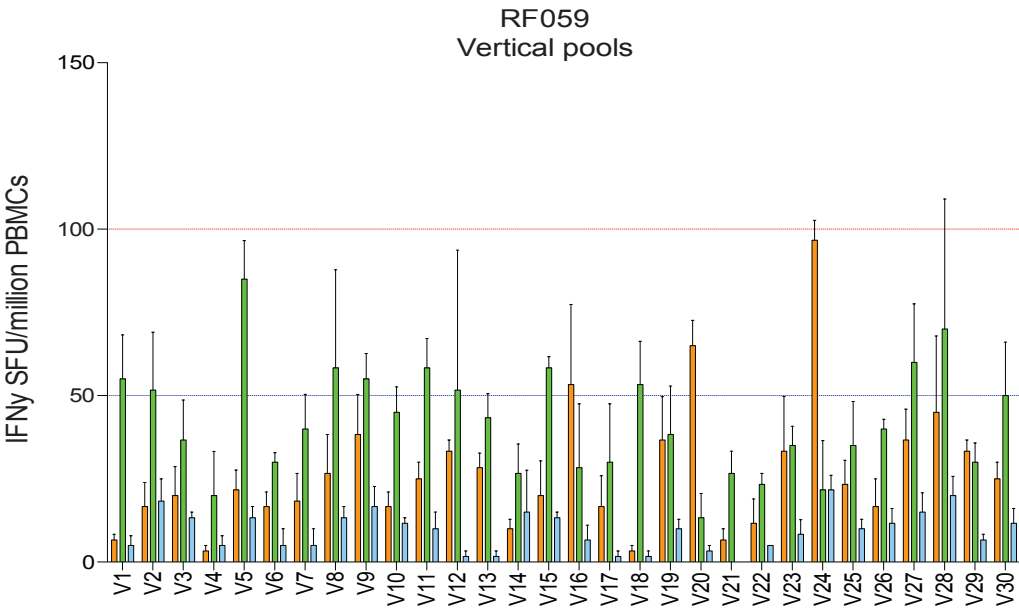
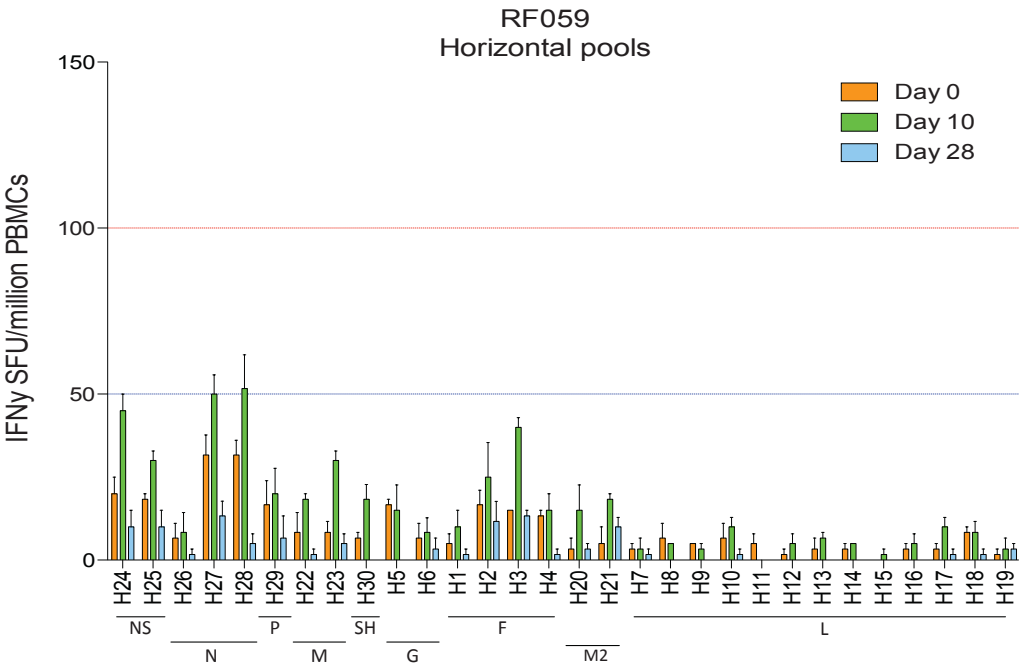
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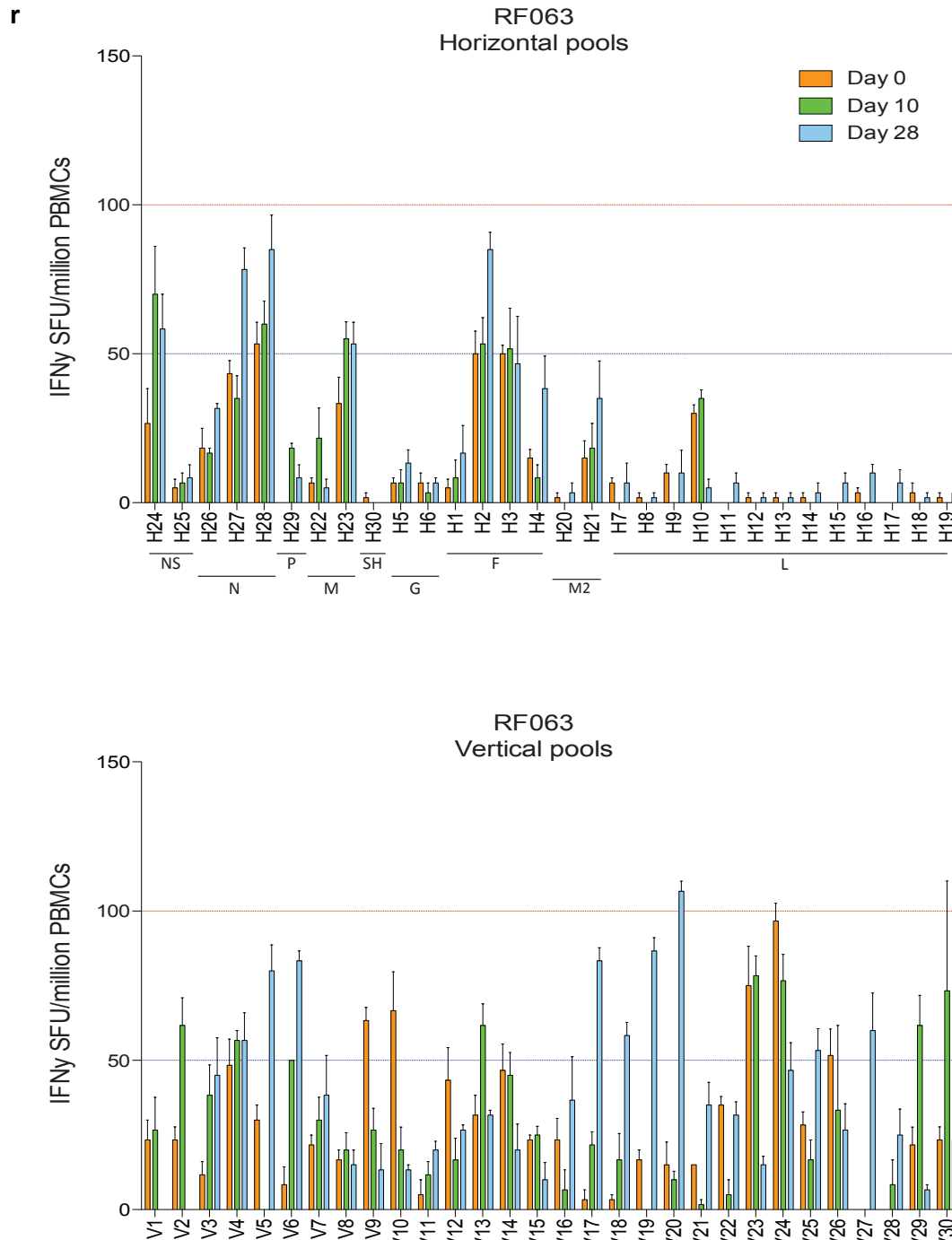
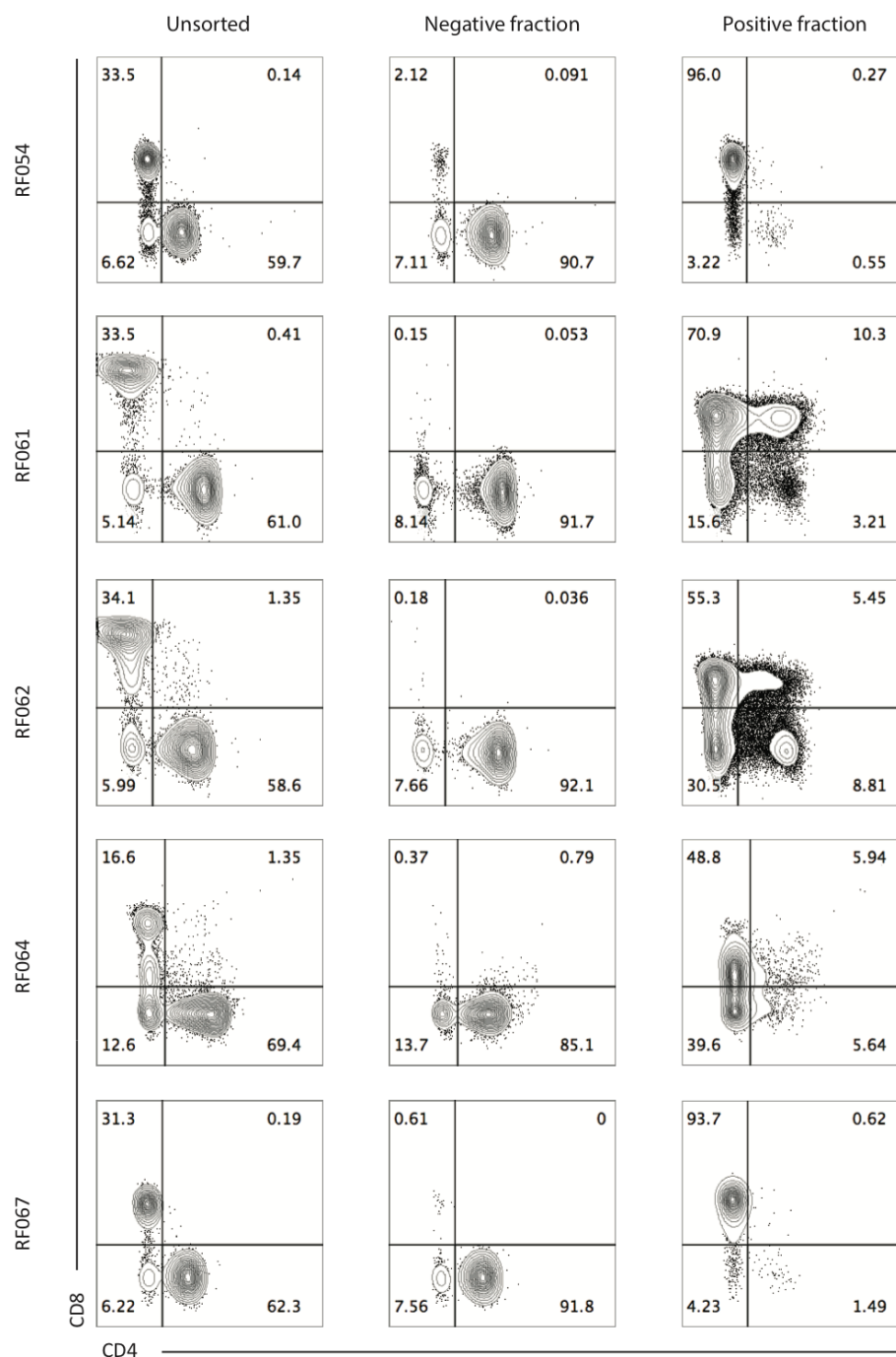


Figure S4. T cell responses to overlapping peptide pools in PBMCs of infected and uninfected subjects. 2×10^5 PBMCs from infected subjects (A – J; $n=10$) and uninfected (K – R; $n=8$) were cultured for 20 hours with overlapping peptide pools at days 0 (orange), 10 (green) and 28 (blue). Graphs show the mean $\pm$ s.e.m. IFN- γ spot forming cells responsive to peptides in horizontal and vertical pools. Horizontal pools were reorganised in the order of the genomic sequence. Proteins were annotated on the basis of contributing the most peptides to the pools. All pools were tested in triplicate and DMSO stimulation was used as a negative control.



Supplementary Figure S5. CD8+ T cell depletion from PBMCs was achieved with at least 98% purity. CD8+ T cells from freshly isolated PBMCs were labelled with micro-beads for depletion using MACS columns. A small proportion of cells were then stained with anti- CD3, CD4 and CD8 to check the purity of the negative fraction. Plots show expression of CD4 and CD8 gated on CD3+ lymphocytes in unsorted (left) negative fraction which was depleted of the CD8+ cells (middle) and positive fraction which was labelled cells bound to the column (right).