A crucial role for IL-21 in controlling CD4 T cell responses to respiratory viral infection

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Declaration of originality: I confirm that the work included in this thesis is my own, and the work of all others has been referenced appropriately.

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B. Abstract.

Respiratory syncytial virus (RSV) is a pneumovirus that infects almost all children by the age of three, and causes an intense pulmonary infiltrate termed bronchiolitis. The tissue damage caused by this immune response significantly reduces lung function such that hospitalisation and mechanical ventilation may be required. There is no licensed vaccine against RSV, partly because the exact immunological mechanism responsible for bronchiolitis remains unclear, though CD4 and CD8 T cells are known to be essential.

Interleukin-21 (IL-21) is a recently identified member of the γ_c chain cytokine family, important in autoimmunity, cancer, and chronic viral infections. Produced mainly by CD4 T cells, IL-21 affects the responses of several cell types but is particularly important for enhancing activation and survival of CD8 T cells. As such, it was hypothesised that IL-21 could be targeted therapeutically to reduce anti-RSV CD8 T cell responses and reduce the incidence of bronchiolitis.

This hypothesis was tested in three models of RSV disease. Here, it is shown that IL-21 is critical for control of CD4 T cell responses rather than CD8. IL-21 depletion increases T cell responses, including cell recruitment and cytokine production, thereby increasing disease. Conversely, it reduced regulatory T cell influx and antibody production. In contrast, IL-21 over-expression ablates the anti-viral T cell response and RSV disease without affecting regulatory T cells. Also, early chemokine production by infected epithelial cells is inhibited and that DC migration is affected, possibly reducing T cell activation and influx. Antibody

production is also reduced, and consequently lymphocyte memory development is blocked resulting in no protection against viral rechallenge.

Therefore, IL-21 plays a crucial role in the development of anti-viral pulmonary immunity and should be considered as part of a therapy to alleviate primary RSV disease in conjunction with other factors to boost anti-viral memory. [296 words]

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E. List of Abbreviations

Abbreviation	Full Name
Bgal	β-galactosidase
µl	Microlitre
Ab	Antibody
ADCC	Antibody-dependent cell cytoxicity
AHR	Aryl hydrocarbon receptor
AICD	Activation-induced cell death
AID	Activation-induced deaminase
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
APC	Antigen-presenting cell
APC	Allophycocyanin
ARDS	Acute Respiratory Distress Syndrome
ATP	Adenosine tri-phosphate
Bad	Bcl-2-associated death promoter
BALF	Bronchoalveolar lavage fluid
Bax	Bcl-2-associated X
Bcl	B-cell lymphoma
BMDC	Bone-marrow-derived dendritic cell
BP	Binding protein
BSA	Bovine serum albumin
CCL	(C-C)-motif-containing ligand
CCR	(C-C)-motif-binding receptor

Abbreviation	Full Name
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester
CIA	Collagen-induced arthritis
c-maf	Musculoaponeurotic fibrosarcoma
COPD	Chronic obstructive pulmonary disease
CPE	Cytopathic effect
CSR	Class-switch recombination
Ct	Transcription cycle
CTLA	Cytotoxic-T-lymphocyte-associated
CXCL	(C-X-C)-motif-containing ligand
CX3CL	(C-X-X-X-C)-motif-containing ligand
CXCR	(C-X-C)-motif-binding receptor
CX3CR	(C-X-X-X-C)-motif-binding receptor
d	Days
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DI	Defective interfering
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eomes	Eomesodermin
Eos	Eosinophil
F	Fusion
FACS	Fluorescence-activated cell sorting

Abbreviation	Full Name
FBS	Foetal bovine serum
Fig	Figure
FI-RSV	Formalin-inactivated respiratory syncytial virus
FITC	Fluorescein isothiocyanate
Fox	Forkhead box
G	Attachment
GAG	Glycosaminoglycan
gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GC	Germinal centre
GM-CSF	Granulocyte/macrophage-colony-stimulating factor
H	Haemagglutinin
HAART	Highly active retroviral therapy
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HMPV	Human metapneumovirus
HN	Haemagglutinin-neuraminidase
HPIV	Human parainfluenza virus
HRP	Horseradish peroxidase
hrs	hours
iBALT	inducible broncho-associated lymphoid tissue
IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
ICOS	Immune costimulatory
IFN	Interferon
Ig	Immunoglobulin

Abbreviation	Full Name
IL	Interleukin
i.n	Intranasal
i.p	Intraperitoneal
i.t	Intratracheal
i.v	Intravascular
IRF	Interferon regulatory factor
ISGF	Interferon-stimulated gamma factor
iTreg	inducible regulatory T cell
JAK	Janus kinase
Kb	kilobase
KO	Knockout
L	Polymerase
LCMV	Lymphocytic choriomeningitis virus
LFA	Leukocyte functional-associated
LPS	Lipopolysaccharide
LRTI	Lower respiratory tract infection
LT	Lymphotoxin
LTB	Leukotriene-B
M	Matrix
M	Molar
M2	Matrix protein 2
Macro	Macrophage
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
Mcl	Myeloid cell leukemia sequence

Abbreviation	Full Name
MCP	Monocyte chemotactic protein
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mins	Minutes
MIP	Macrophage inflammatory protein
ml	Millilitre
mm	Millimetre
mmol	Millimole
MMP	Matrix metalloproteinase
MOG	Myelin oligodendrocyte glycoprotein
MOI	Multiplicity of infection
MRL	Murphy roths large
mRNA	Messenger ribonucleic acid
MUC	Mucin
N	Nucleoprotein
NALT	Nasal-associated lymphoid tissue
NaN3	Sodium azide
NFAT	Nuclear factor of activated T cells
NK	Natural killer
NKT	Natural killer T
nm	Nanometre
nM	Nanomolar
NOD	Non-obese diabetic
NS	Non-structural
nTreg	natural regulatory T cell

Abbreviation	Full Name
OAS	Oligoadenylate synthetase
OD	Optical density
OPD	o-phenylenediamine
ORF	Open reading frame
OVA	Ovalbumin
P	Phosphoprotein
PAMP	Pathogen-associated molecular pattern
PB	Pacific blue
PBS	Phosphate-buffered saline
p.c	Post challenge
PD	Programmed death
pDC	plasmacytoid dendritic cell
PE	Phycoerythrin
PE-Cy	Phycoerythrin-cychrome
pfu	Plaque-forming units
PKR	Protein kinase R
PLP	Polylipopeptide
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear
prdm	PR domain zinc finger protein
PRR	Pattern recognition receptor
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RAG	Recombination activating gene
RANTES	Regulated upon activation, normal T-cell expressed, and secreted

Abbreviation	Full Name
RBC	Red blood cell
RDA	Representation difference analysis
rm	Recombinant murine
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediate
ROR	Retinoic-acid-related orphan receptor
RPMI	Roswell park memorial institute
RQ	Relative quantity
RSV	Respiratory syncytial virus
runx	Runt-related transcription factor
rVV	Recombinant vaccinia virus
s	Seconds
SARS	Severe acute respiratory syndrome
SH	Small hydrophobic
SHM	Somatic hypermutation
STAT	Signal transducer and activator of transcription
T-bet	T-box
Tc	Cytotoxic T
TCGF	T cell growth factor
TCR	T cell receptor
Tfh	Follicular T helper cell
TGF	Transforming growth factor
Th	Helper T
tk	Thymidine kinase
TLR	Toll-like receptor

Abbreviation	Full Name
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
URTI	Upper respiratory tract infection
V	Variable
v/v	Volume-by-volume
w/v	Weight-by-volume
WT	Wild-type
X-SCID	X-linked severe combined immunodeficiency
XBP	X-box binding protein

F. Introduction.

1. The respiratory system

1.1. Lung structure

In mammals and more complex animals, the lungs are located near the backbone on either side of the heart. Mammalian lungs have a soft, spongy texture and the exposed internal surface covered with epithelium. The internal surface is folded upon itself, like the microvilli of the intestine, maximising the total surface area compared to the outer surface area of the lung itself. Human lungs are a typical example. Though similar, the two lungs are not identical. For example, there are three lobes on the right and two on the left. Further, the left lung contains an indentation (the cardiac notch) where the heart resides. The lobes are individually-contained within pleural cavities, and bathed in pleural fluid. This aids lubrication, as well as aiding and maintaining contact with the rib cage (1).

Air enters the lungs through the oral and nasal cavities. It flows through the pharynx, the larynx, the trachea (the 'upper' respiratory tract), and then a progressively subdividing system of bronchi. The bronchial tree continues branching until it reaches the level of terminal bronchioles, which lead to alveolar sacs. Alveolar sacs are made up of clusters of alveoli, the individual alveoli are tightly wrapped in blood vessels to aid gas exchange [Fig.1; (2)].

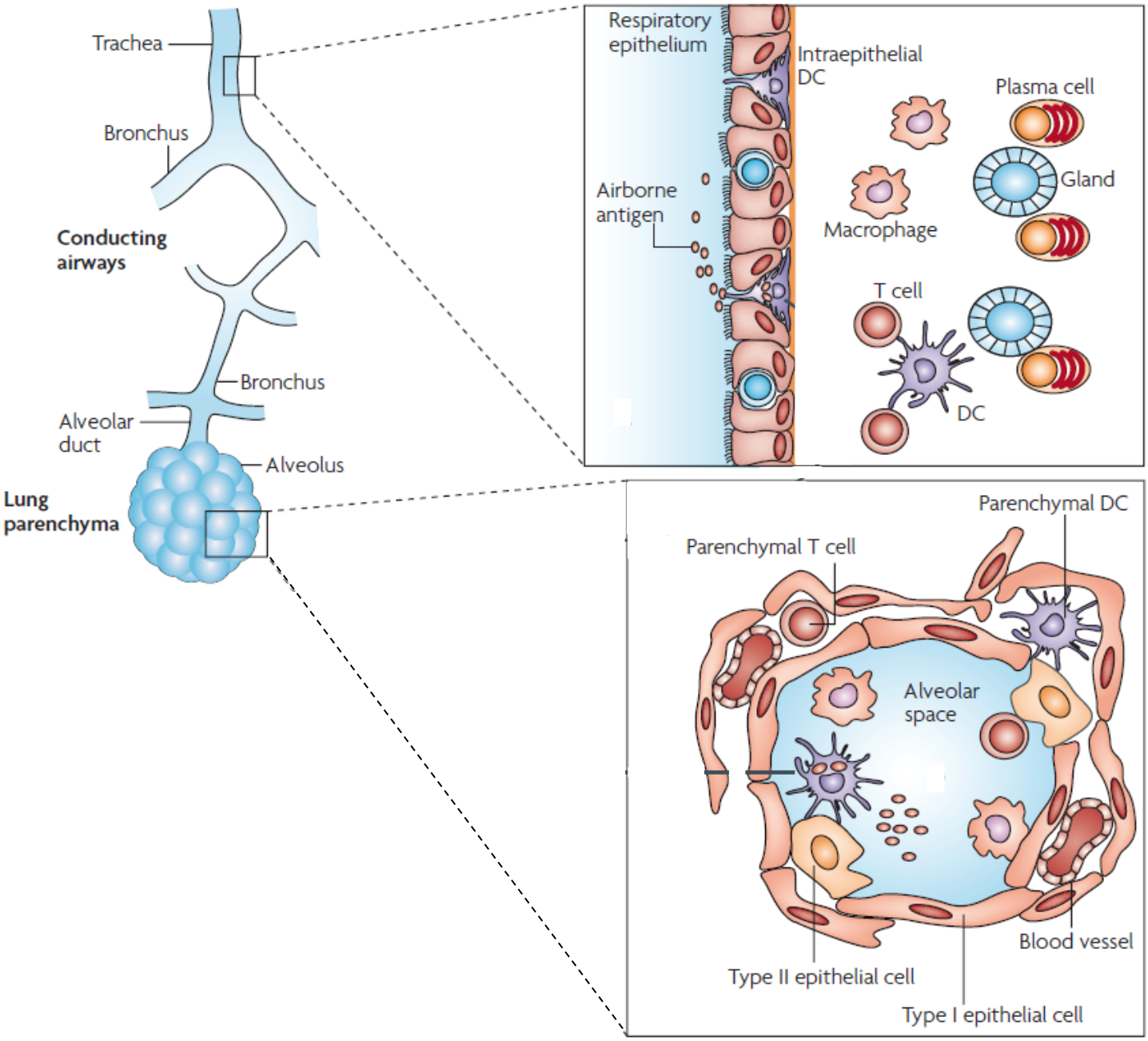

Figure 1. Lung structure and immune induction in the lungs. Air enters the lungs via the trachea, and then a progressively subdividing system of bronchi. The bronchial tree continues branching until it reaches the level of terminal bronchioles, which lead to alveolar sacs. Alveolar sacs are made up of clusters of alveoli, like individual grapes within a bunch. Antigens in the airway are sampled by dendritic cells (DCs) and they migrate via the lymphatics to the draining lymph nodes where they present to naïve T cells (adapted from Holt PG et al. Nature Rev Immunol 2008 8 142).

1.2. Lung function

The lungs are essential for respiration in all land-based animals, as well as a few fish species. Their primary function is to exchange oxygen in the atmosphere for carbon dioxide in the bloodstream in approximately 700 million tiny, thin-walled alveolar sacs. Deoxygenated blood is pumped via the heart through the pulmonary artery to the lungs, where oxygen is captured by haemoglobin within red blood cells. The oxygen-rich blood then returns to the heart via the pulmonary veins for distribution via the circulation.

In addition to their main function, the lungs also perform other secondary tasks such as maintenance of blood pH, filtration of venous blood, catabolism of peptides, and acting as a physical barrier to protect the heart (1).

1.3. The immunological challenges for the lung

In order to optimally perform its primary function, the largest possible surface area of the lungs must be exposed to the external environment in order to maximise the amount of oxygen in the air that can be consumed, and carbon dioxide from the blood exhaled. However, as a result this maximises the exposure of the lungs to pathogenic material and allergic irritants in the atmosphere. Despite this, the lower respiratory tract (the bronchi, bronchioles, and alveoli) must be maintained to allow for efficient lung function. Moreover, the environment of the lung is very moist and fluid, excellent growing conditions for many species of microbes. Consequently, most respiratory illnesses are the result of bacterial or viral infection of the lungs. Therefore, the lungs have to constantly defend themselves against microbial infection and prevent pathogens from entering the body (2). The

mechanisms (both immunological and non-immunological) that have developed to provide this defence are discussed in the following sections.

2. The immune system

The immune system is a network of biological structures comprising cells and soluble factors whose function is to protect the host from damage caused by foreign bodies (pathogens: disease-causing agents). These foreign bodies range from viruses to parasitic worms, and the immune system must recognise these distinctly from the host's own tissue. Almost all living things possess an 'immune system'; even bacteria contain enzymes that protect them against bacteriophage infection (3).

The development of the immune system placed an evolutionary selection pressure on pathogens that needed to invade a host in order to replicate, grow, and survive. Consequently, pathogens have devised many methods of subverting the immune system (e.g. antigenic variation, immune factor mimicry, inhibiting immune factor expression) which in turn placed a selection pressure on the immune system, leading to evolution and expansion of the immune system. This dynamic process has shaped development of our immune system into its current form (4,5).

The mammalian immune system is organised into two distinct parts, with distinct properties (3). These are the innate immune system and the adaptive immune system. A table of their characteristics and differences are shown (Table 1).

Table 1. A comparison of the innate and adaptive immune systems

Innate	Adaptive
Found in nearly all organisms	Found only in jawed vertebrates
Response is non-specific	Response is pathogen and antigen-specific
Response time hours	Response time - days
Comprises both cell and soluble factors	
No immunological memory formed	Immunological memory formed

Each branch of the immune system will be described in turn.

2.1. The innate immune system

The innate system is the more ancient in evolutionary terms. It is found in all plant and animal species, but also forms the immune systems of insects, fungi, and smaller multi-cellular organisms. It responds to foreign agents in a non-specific manner. The response is rapid, but does not confer any immunological memory against the agent, meaning it will respond in an identical manner if the agent were encountered again (3). The major components of the innate immune system are described below with an emphasis on the respiratory tract (Fig.2.).

2.2. Cells of the innate immune system

2.2.1. Epithelial cells

Epithelial cells are a crucial component of the innate immune system because they form a continuous barrier against pathogen entry into the circulation and deeper tissues. Within the lung for example, the type I and type II alveolar epithelial cells and conducting airway

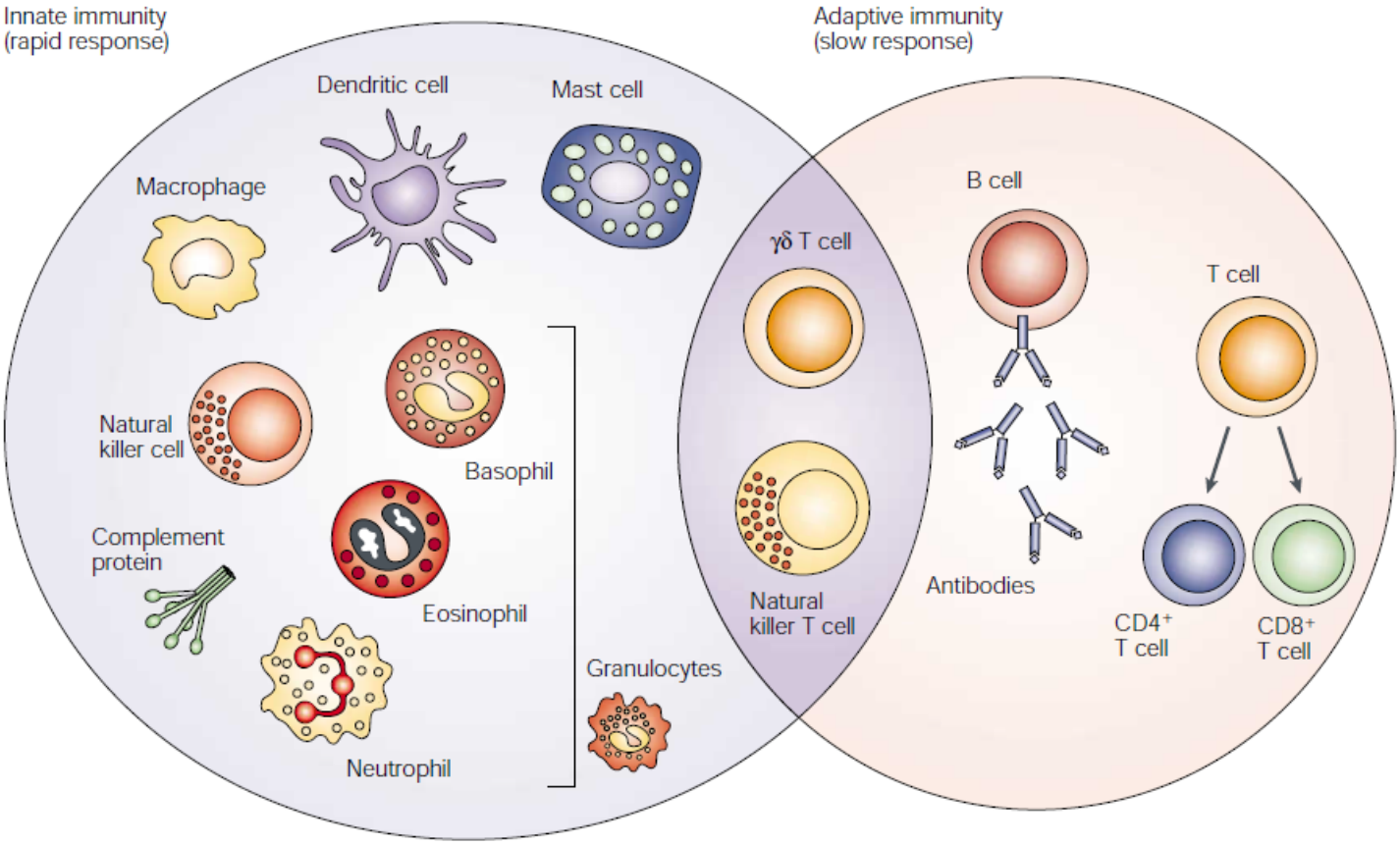

Figure 2. Components of innate and adaptive immunity. Innate immunity is rapid but non-specific, responding in an identical manner with each exposure. Cellular components include macrophages, dendritic cells, mast cells, NK cells, and granulocytes. Soluble factors include complement proteins. Adaptive immunity is slower to respond but antigen-specific, forming memory after primary exposure. Therefore, further exposures are responded to with greater expediency by these specific cells. Cellular components include B cells and T cells, soluble factors include antibodies. γδ T cells and NKT cells share characteristics and features of both innate and adaptive immune systems. (adapted from Dranoff G et al. Nat Rev Cancer 2004 4 11).

epithelial cells are of particular importance because they are the primary targets of many respiratory pathogens, particularly respiratory viruses. These include Coronaviruses [e.g. SARS; (6)], influenza A [e.g. pandemic H1N1 and avian H5N1 strains; (7,8)], influenza B, rhinoviruses (9), and respiratory syncytial virus (RSV) (10). As these cells are essential to efficient gas exchange, infection can compromise respiratory function and lead to acute respiratory distress syndrome [ARDS; (11)]. Terminally-differentiated type I alveolar epithelium accounts for only 10% of the alveolar cell population yet covers 95% of the surface alveolar cells cover (12). Their primary role is gas exchange. In contrast, type II alveolar epithelium accounts for only 5% of the surface covered. They constitutively produce surfactant protein that aids efficient gas and fluid exchange, anti-microbial peptides that maintain mucosal immunity, and act as stem cells for both themselves and type I alveolar epithelial cells (13).

Upon infection, epithelial cells secrete a broad spectrum of factors to mobilise further anti-microbial immune components. One of the first families of proteins to be produced are the interferons (IFNs) whose main function is to induce neighbouring epithelial cells into an anti-viral state. Epithelial cells produce type I IFNs (e.g. IFN-α, IFN-β, IFN-κ, IFN-ε, and limitin) that signal through the ubiquitously expressed IFN-α/β receptor (14), and type III IFNs (e.g. IFN-λ1, IFN-λ2, and IFN-λ3 in humans; IL-28A and B in mice) that signal though IL-28R (15). The importance of the type I interferons as an anti-viral immune mechanism is evidenced by the susceptibility of IFN-α/βR knock-out (KO) mice to many respiratory viral infections (16). Binding of type I interferons to their cognate receptor induces the transcription factor ISGF3 (IFN-stimulated gamma factor 3) to initiate transcription of several genes whose proteins possess potent anti-viral activity. These include 2’-5’ oligoadenylate synthetases (OAS),

protein kinase R (PKR), and orthomyxovirus resistant GTPases (the Mx family) (17). OAS degrades all cellular, including viral, RNA; PKR inhibits RNA translation; Mx1 inhibits transcription (18); and MxA inhibits posttranslational processing (19-21). The type III IFNs appear to play a lesser role than type I IFNs in anti-viral immunity , because mice deficient in the IL-28R are less susceptible to respiratory virus infection than IFN-α/βRKO mice (22). The limited expression of IL-28R compared to IFN-α/βR may in part account for this difference (23). However, type III IFNs have been shown to play a non-redundant role in protection against respiratory viral challenge where mice lacking receptors for both type I and III IFNs are significantly more susceptible to challenge than mice lacking just one (15).

Upon infection, epithelial cells are the first cellular source of pro-inflammatory cytokines and chemokines including: TNF (24), IL-6 (25), IL-8 in humans (26), MCP-1 (26), MIP-1 (26), IP-10 (25), and RANTES (25), which trigger downstream inflammatory responses. . This cocktail of cytokines and chemokines produced during the first 48hrs after infection is associated with symptoms of fever, sleeplessness, and loss of appetite. One major function of these soluble factors is to increase expression of adhesion molecules on the endothelium which enables other cells of the innate immune system including macrophages, monocytes, dendritic cells (DCs), and neutrophils to infiltrate lung tissue (27,28). In the context of pathogen challenge, these infiltrating cells become activated upon exposure to the cocktail of inflammatory mediators and binding of pathogen-associated- molecular-patterns (PAMPS) to pattern recognition receptors (PRRs) expressed by the cell. However, in addition to aiding host defence, these factors can also damage host tissue, either directly inducing cell apoptosis, or indirectly by recruiting inflammatory cells, that further amplify the inflammatory process. Therefore there is a trade-off between an effective anti-microbial

response and damage limitation. This is evident in disease conditions such as ARDS where the immune response and not the pathogen is responsible for much of the pathology (11).Finally, epithelial cells can assist in reducing viral spread by inducing 'self-apoptosis'. This may occur via cell-intrinsic mechanisms [e.g. caspase-8 induction; (29)], or extrinsic mechanisms [e.g. expression of TNF-related apoptosis-inducing ligand (TRAIL) (30)].

2.2.2. Macrophages

Macrophages are phagocytes and a crucial component of the host innate immune system. They phagocytose and process pathogens, produce inflammatory mediators, thereby making a significant contribution to both innate and the adaptive immunity. Macrophages are a heterogeneous population based on their anatomical location and function. In addition to the heterogeneity based on their location, macrophage heterogeneity is also observed within a single organ. The different tissue populations are replenished by new monocytes that proliferate to maintain a steady state (31). This process is dependent on chemokine release and migration of different monocyte subsets expressing distinct repertoires of chemokine receptors. It is unknown if tissue-resident macrophages are terminally differentiated, or if they remain functionally-flexible to respond to different stimuli or alter their effector function according to changes in the microenvironment (32).

Within the lung, there are alveolar, interstitial, and intravascular macrophage subsets (33), and each subset performs specific functions within the lung. For example, alveolar macrophages reside in the alveoli ingesting irritants and microbes from the alveolar space. Intravascular macrophages perform the same function for the circulation. Interstitial macrophages reside within the interstitial spaces and help limit inflammation and fibrosis

(34). As mentioned above, it is unclear if these subsets are derived from a common monocyte pool following a specific inflammatory signal, or whether each subset has its own unique precursor so that distinct populations develop in response to different stimuli at specific locations.

In order to effectively perform their function, alveolar macrophages have the greatest expression of pattern recognition receptors (PRRs) and scavenger receptors. They are long-lived cells (35), and have been observed to possess both pro-inflammatory and anti-inflammatory functions. Their function *in vivo* is regulated by their continuous exposure to lipids and surfactant proteins which form a significant part of their microenvironment (36). Surfactant proteins are known to modulate the expression of inflammatory mediators by alveolar macrophages by reducing TLR-agonist-mediated activation via Toll-like receptors (TLRs) (37). However, alveolar macrophages express a broad TLR repertoire and are rendered insensitive to IL-10 exposure upon TLR stimulation, inducing pro-inflammatory cytokine production [e.g. IL-1β, IL-6, IL-8, IL-12, IFN-γ, and TNF-α (38)]. This is particularly important for immune induction as interstitial macrophages increase IL-10 production at this time (see below) (39). Secretion of pro-inflammatory mediators drives monocyte and neutrophil recruitment into the airways. Macrophage activation also drives the production of reactive oxygen species and nitric oxide synthase (40,41), allowing for the effective killing of ingested microorganisms.

Interstitial macrophages reside in the narrow space between the alveolar epithelium and the vascular endothelium. In contrast to alveolar macrophages, these cells are located in a relatively sterile environment surrounded by extracellular matrix. Despite the technical challenges in isolating this subset, recent studies have shown that interstitial macrophages

may modulate the functions of dendritic cells to prevent allergic inflammation. Further, this subset appears more capable than alveolar macrophages at antigen presentation (42,43). This population also exhibits potent anti-inflammatory properties by producing cytokines such as IL-10 (44). Despite this progress, there is a great need to further characterise this subset, in particular to determine the functional interrelationship of interstitial macrophages with dendritic cells and alveolar macrophages in the lung under both steady-state and pro-inflammatory conditions.

Intravascular macrophages are a newly-identified member of the pulmonary macrophage family (45). They are located in the capillaries in the alveolar septa in some species (e.g. cattle) but have not been identified in rodent or primate species. Studies to date have demonstrated that intravascular macrophages initiate lung inflammation, and have potential as a therapeutic target for modulating lung inflammation (46). However, more studies are required to fully understand the immunological role this subset plays in lung homeostasis and protection against disease.

2.2.3. Dendritic cells (DCs)

Lung DCs can be divided into at least five subsets depending on origin, anatomic location, and function. At baseline, the airways are lined with an intraepithelial, highly dendritic network of $MHCII^{int}CD11c^{hi}$ cells that are $CD11b^{-}$ and, at least in mice, express langerin and the mucosal integrin CD103 (αEβ7) (47). This subset extends dendrites into the airway lumen forming tight junctions with bronchial epithelial cells (23, 26–28). It is very likely that airway epithelial cells are crucial in controlling the surveillance function and activation of this DC subset in the lungs (38). Immediately beneath the airway, the lamina propria

contains MHCII^{hi}CD11c^{hi} cells that highly express CD11b and produce inflammatory chemokines such as CCL17 and CCL22 (23, 29, 30). This CD11b^{+}CD103^{-} subset also expresses the SIRPα molecule, a ligand of CD47 involved in DC migration (31). A similar division into CD11b^{+} and CD11b^{-} subsets can also be applied to interstitial DCs obtained by enzymatic digestion of peripheral lung tissue (25, 26). The alveolar space also contains CD11c^{hi}MHCII^{hi} DCs. At least in rodents and man, alveolar DCs highly express CD103. Both CD11b^{+} and CD11b^{-} subsets express high levels of CD11c and are termed conventional DCs (cDCs). These contrast with CD11c^{int} plasmacytoid DCs (pDCs) that express Siglec-H, and bone-marrow stromal antigen-1 (48). The exact anatomic location of lung pDCs is unknown although they have been identified lining the alveolar septa and recovered from enzymatic digests of the large airways (24, 32). Finally, under inflammatory conditions [e.g. viral infection, allergen challenge, or lipopolysaccharide (LPS) administration] there is recruitment of CD11b^{+} monocyte-derived DCs that rapidly upregulate CD11c (49).

DCs perform a unique sentinel function in the lung in that they recognize inhaled antigens through expression of PRRs such as Toll-like receptors, NOD-like receptors, and C-type lectin receptors. These receptors recognise conserved motifs on virtually all pathogens and allergens (14, 15). Because DCs are able to sense the presence of danger as well as process antigen and migrate to the draining lymph nodes, these cells form the bridge between innate and adaptive immunity in the lung. Lung DCs also express a broad range of receptors for inflammatory mediators that are released upon tissue damage [damage-associated molecular patterns (DAMPs; e.g. ATP, uric acid, and high mobility group box 1)] by pathogens, trauma, or necrosis (50,51). DCs also express neuropeptide receptors that can respond to neurotransmitters released in response to efferent neural responses (52).

Interestingly, lung DCs have been observed to bind unmyelinated nerve endings around the airway mucosa, suggesting neural responses play a role in lung DC effector responses (17, 18).

The type of immune response elicited in the lung depends on the nature of the local tissue environment. It is therefore very likely that immune recognition by barrier cells such as epithelial cells determines the functional properties of resident DCs, thereby shaping the phenotype of antigen-specific immunity (38). This concept is of critical importance for the regulation of mucosal homeostasis and for the initiation of innate and adaptive immune responses in the lung.

2.2.4. Neutrophils

Neutrophils are one of the most predominant haematopoietic cells in the human body with ~5×10^9/l in peripheral blood. Those isolated from peripheral blood are short-lived cells with a lifespan of only 5-6 days and tissue-derived neutrophils 1-2 days (53). The population has to be replenished daily via continuous CXCR4-dependent release of new cells form the bone marrow (54). Neutrophil influx into the pulmonary compartment occurs in many diseases of the lung, and neutrophils are believed to be fundamental in dysregulated inflammatory responses [e.g. COPD (55) and asthma (56)]. Neutrophil influx is principally controlled by binding of leukotriene-B_4 and/or IL-8 (KC in mice) binding to their cognate receptors (LTB_4R and CXCR1 or CXCR2 respectively) (57,58). However, other chemokines such as CXCL5 (ENA-78) may also contribute.

Upon recruitment and activation, neutrophils can carry out multiple effector functions to enhance pathogen clearance. Neutrophils phagocytose invading pathogens and cellular

debris prior to destroying phagocytosed material by enzymatic digestion [e.g. elastase (59)]. They also undergo a degranulation and respiratory burst process which destroys pathogenic (and host) material in the immediate environment. Neutrophils possess four granule types that contain an array of cytotoxic and immunoregulatory components: secretory, tertiary, specific, and azurophilic (60). More recently, neutrophils have been shown to release 'NETs' (neutrophil extracellular traps): a dense web of chromatin complexed with primary and secondary granule components that trap and kill bacteria and fungi (61). Neutrophils are also a significant early source of IP-10 that recruits NK cells and later Th1 cells (62). They have also been shown to process and present antigens to dendritic cells for downstream presentation to T cells.

Neutrophils possess potent anti-microbial effector mechanisms that can also damage host tissue. Therefore, regulation of neutrophil activity is essential to limit unwanted damage. This occurs primarily via neutrophil apoptosis and uptake of neutrophil debris by inflammatory macrophages. This phagocytic process induces release of the anti-inflammatory molecules IL-10 and TGF-β that aid response resolution (63). This not only allows the safe removal of a cell type capable of extensive damage to host tissue if left unregulated, but also ensures that the activity of other immune cells in the environment is kept in check.

2.2.5. Eosinophils

Eosinophils develop and mature in the bone marrow from myeloid precursors. They comprise 1-6% of white blood cells in man. In a healthy individual they are found in the

thymus medulla, lower intestinal tract, lymph nodes, and spleen (64). They circulate in the blood and migrate into infected/inflamed tissues in response to eotaxin-1, -2, or -3, RANTES, or leukotrienes (e.g. leukotriene B_4) (65). Within inflamed sites they are activated by type 2 cytokines, particularly IL-5 (66). Their cytoplasm contains granules comprising a variety of enzymes and chemicals including histamine, eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPO), major basic protein (MBP), eosinophil cationic protein (ECP), plasminogen, lipase, and nucleases (67). Upon degranulation, these factors are released into the environment where ECP creates channels that disrupt plasma membranes of large, extracellular pathogens such as helminths. ECP also stimulates mast cell degranulation and mucus production by fibroblasts. EPO forms reactive oxygen species (e.g. superoxide and peroxide) and reactive nitrogen species that enter target cells, increase oxidative stress, and cause death by apoptosis or necrosis. However, they also toxic to host tissues and may cause unwanted damage. MBP induces mast cell and basophil degranulation. ECP and EDN are ribonucleases and have demonstrated anti-viral activity (68). Activated eosinophils also produce eicosanoids that recruit other immune cells, enzymes such as elastase, and a wide array of cytokines (69). They may also present antigen to T cells via MHCII (70). Eosinophils, with basophils and mast cells, are considered important in wound healing, allergy, and asthma pathogenesis (71), however the limited efficacy of anti-IL-5 therapy (mepoluzimab or reslizumab) in asthma and allergy has raised questions about their exact role in these conditions (72,73).

2.2.6. Basophils

Basophils are the least common circulating white blood cell in man, comprising 0.01-0.3% (74). They share many characteristics with mast cells and eosinophils. For example, they possess large cytoplasmic granules that contain chemicals and enzymes designed to combat large extracellular parasites such as worms, and are considered important in allergy and asthma. They express $F_c\varepsilon RI$ that enables them to bind environmental allergens (e.g. pollens) via IgE (75). Their granules contain histamine, heparin and chondroitin, as well as enzymes such as elastase and lysophospholipase. Like eosinophils, they also secrete eicosanoids as well as numerous cytokines (76).

2.2.7. Mast cells

Mast cells share many structural features of basophils and are derived from the same progenitor cell. Like eosinophils, they are important in allergy, asthma (77,78), and wound healing (79) but are also crucial in anaphylaxis (80), and implicated in autoimmune conditions such as diabetes (81) and rheumatoid arthritis (82). They also possess large cytoplasmic granules but these are particularly rich in histamine and heparin (83,84). They too bind antigens fixed by IgE via $F_c\varepsilon RI$, but unlike basophils they are resident in many tissues of the body rather than a circulating population of cells. They reside in close proximity to blood vessels and nerves as well as surfaces exposed to the environment such as the nasal and lung mucosa (85,86). There are considered to be two types of mast cell depending on the site they reside in, connective and mucosal.

Mast cells can be activated by binding IgE, direct physical or chemical insult (e.g. opoids or alcohols), or by activated complement proteins (87). They respond, as eosinophils and basophils do with degranulation. As well as histamine and heparin, mast cells secrete tryptases and serotonin (88-90). Like eosinophils and basophils they also produce eicosanoids and several cytokines (91,92).

2.2.8. Natural Killer (NK) cells

NK cells are innate lymphocytes comprising 10% of lymphocyte numbers in the lung (93), and are a critical first line of defence against respiratory pathogens (94). Their importance in respiratory viral infection is clear because their depletion increases morbidity and mortality (95). In the healthy lung they are maintained by IL-15 secretion by bronchial epithelium (96), but suppressed by anti-inflammatory factors secreted by alveolar macrophages [e.g. IL-10 and TGF-β (97,98)]. Large numbers of NK cells are recruited from the circulation within 24hrs of pathogen challenge. They can be activated by pro-inflammatory cytokines [e.g. IFN-α, IL-2, IL-12, and IL-18 (99-101)] released by infected epithelial cells and macrophages (102), as well as by binding of an array of activatory and inhibitory ligands on infected cells. In order to bind these ligands, NK cells express a diverse spectrum of complementary receptors. The activatory family include the Ly49 homodimers (an ancient family of C-type lectin molecules), the NCR (natural cytotoxicity receptors), the CD94-NKG2D heterodimers, and $Fc_{\gamma}RIII$ (CD16) that binds IgG and mediates antibody-dependent cytotoxicity (ADCC). Inhibitory receptors include KIRs (killer-cell immunoglobulin-like receptors) that recognise both classical and non-classical MHCI molecules, the Ly49 homodimers, and the more recently discovered LIRs (leukocyte inhibitory receptors). The balance of these signals

determines whether NK cells remain dormant and inactive, or become activated and carry out their effector functions which include cytokine production [primarily IFN-γ (100)], and cytotoxicity (103).

NK cell function is also important for induction of adaptive immunity. In particular, NK cells are important for the activation of cytotoxic T cell responses during responses to respiratory viral infection (104).

2.2.9. Natural Killer T (NKT) cells

NKT cells are a numerically minor (~0.1% of peripheral blood T cells in man), heterogeneous cell population, so-called because they share features of both T cells (a αβ T cell receptor) and NK cells [e.g. CD56, CD16 (both in man) and CD161] (105). However, unlike conventional T cells their TCR is of limited diversity (mouse NKT cells express $V_{\alpha}14/V_{\beta}2,7$, or 8, human NKT cells express $V_{\alpha}24/V_{\beta}11$), and recognise non-peptide ligands in the context of an oligomorphic CD1d molecule (106,107). There are three main subsets of NKT cells: Type 1 that have a semi-invariant TCR, and recognise glycolipid antigens in the context of CD1d; Type 2 that have a diverse TCR, and recognise ceramide-like molecules (e.g. sulfatide) in the context of CD1d; and Type 3 that have a diverse TCR, and do not recognise CD1d (108,109). Unlike conventional T cells, they also express a memory phenotype (i.e. $CD44^{hi}$ in mice, CD45RA-/CD45RO+ in man) and rely on the transcriptional regulator promyelocytic leukaemia zinc finger (PLZF) for their development (110).

Upon activation NKT cells can release significant amounts of a wide array of cytokines (e.g. IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-17 and IL-21) and chemokines (e.g. TNF) (111,112). They also

exhibit cytotoxic potential through expression of Fas/FasL and granzyme B (113). Given their considerable effector functions, NKT cells have been shown to play important roles in responses to a wide array of infectious diseases (e.g. *M.tuberculosis*) (114), chronic diseases (e.g. asthma) (115), cancers (116), and autoimmune conditions (e.g. RA, diabetes, and EAE)(117). The best described role of NKT cells in immunity is their recognition of glycolipid antigens from many species of bacteria (118-120).

2.2.10. γδ T cells

γδ T cells constitute a minor T cell population (<5%) in the secondary lymphoid organs, but are a major component (up to 60% of T cells) of organs which possess epithelium such as skin, lung, and gut (121). Interestingly, resident γδ T cells within each organ express a biased TCR repertoire, suggesting that they are adapted to operate within their unique environment. For example, those in the skin preferentially express $V_\gamma5$, those in the gut express $V_\gamma7$, and those in the lung express $V_\gamma6$ (122,123). How this preferential homing of γδ T cell subsets occurs is unknown. It is known that specific V_γ chains are not necessary to populate a specific tissue, as mice with specific chain depletions have tissues populated by alternative V_γ-chain-expressing T cells (124). However, specific γδ T cell subsets can affect disease as observed in cocksackie-B3-mediated myocarditis. In this model it has been found that $V_\gamma1^+$ T cells suppress and $V_\gamma4^+$ T cells exacerbate the development of this condition (125). Furthermore, in *L.monocytogenes* infection, $V_\gamma1^+$ T cells reduce disease resistance despite γδ T cells as a whole being protective (126).

γδ T cells play a significant role in both the development and inhibition of airway disease. In models of OVA-induced tolerance, a regulatory $CD8^+$ γδ T cell population was induced that produced IFN-γ and inhibited IgE production (127). Moreover, these cells only tolerised against OVA, and not against an unrelated protein such as Derp1, suggesting that the γδ T cells were acting in an antigen-specific manner. Another study in non-obese diabetic (NOD) mice found a similar effect by γδ T cells but here they produced IL-4 and IL-10 (128). They observed that, after the onset of disease in NOD mice, repeated exposure to aerosolised human insulin reduced the incidence of insulin-dependent diabetes mellitus and inhibited pancreatic islet destruction. This alleviation from disease was mediated by $CD8^+$ γδ T cells, and they reduced IFN-γ production by autoreactive αβ TCR^+ T cells. As for the previous study, the antigen-specificity of these γδ T cells is unknown. There was some evidence that they were responding to insulin, as denatured insulin failed to induce these regulatory cells (129). Moreover, a single amino acid change that prevented insulin binding to its receptor still elicited regulatory CD8 γδ T cells.

Other studies using OVA immunisation and challenge as a model of allergic inflammation in γδ-T-cell-deficient mice found that γδ T cells enhanced pulmonary CD4 and CD8 T cells, as well as eosinophilia (130). Addition of IL-4 to these mice reversed the observed losses, suggesting that γδ T cells boosted allergic inflammation by producing IL-4 directly or signalling its production by other cells (e.g. basophils, NKT cells, or CD4 T cells). γδ T cells also regulate airway hyperreactivity as responses to methacholine have demonstrated in OVA immunised and challenged mice. Mice depleted of γδ T cells exhibit increased responses to methacholine, suggesting that they play a negative regulatory role in this process (131). This role is independent of αβ TCR^+ T cells.

Pulmonary γδ T cells have been shown to be critical for protection against bacterial challenge to the lung. Intranasal challenge of WT mice with the gram-positive bacteria *N.asteroides* results in damage to the non-ciliated tracheal-bronchial epithelium that elicits a strong neutrophilia (132). In the absence of γδ T cells, the damage to the lung is significantly increased with increased bacterial replication, and mice die within 14 days of infection. Similar observations have been made in mouse models of lung damage caused by exposure to ozone. In these models, γδ T cells clearly play a crucial regulatory role in limiting potentially damaging immune responses while simultaneously curtailing bacterial growth. IL-22 production by responding γδ T cells has been shown to protect against potentially damaging immune responses. In a murine model of hypersensitivity pneumonitis induced by repeated exposure to *B.subtilis*, responding Vγ6+Vδ1+ γδ T cells expressed aryl hydrocarbon receptor (AhR), IL-17 and IL-22. If mice were depleted of γδ T cells, AhR function was blocked, or IL-22 was neutralised, they failed to effectively clear the pathogen, resulting in recruitment of CXCR3+ CD4 T cells, excessive collagen deposition, and pulmonary fibrosis. However, IL-22 treatment of TCRδKO mice was sufficient to protect against αβ-T-cell-mediated fibrosis by reducing CXCL9 secretion that recruited the pathological CD4 T cells (133).

2.2.11. Innate Lymphoid cells (ILC)

Innate lymphoid cells (ILCs) are a recently discovered group of innate lymphocytes, including NK cells, that lack the rearranged antigen-receptors expressed by conventional T and B cells of the adaptive immune system (134,135). This cell subset requires the transcription factor Id2 and the γ_c chain (CD132) for their development (136). However, they differ in their

cytokine requirements: as NK cells require IL-15, and all other ILCs require IL-7 for their development (137,138). As described earlier NK cells are important for destruction of tumours and virally-infected cells (139), however other ILCs regulate an array of homeostatic processes and immunological mechanisms in several organs by secreting different cytokines (140).

At the most basic level ILCs are identified as being $Lin^{-}CD127^{+}$ (141). They are primarily located at mucosal sites and have been divided into subsets based on their cytokine profile. For example, one subset (termed 'ILC1') that expresses T-bet and produces IFN-γ (but is not an NK cell as it is absent in $IL\text{-}7R^{-/-}$ mice) has been found in the human gut (142). Another population ('ILC2') that expresses GATA3 and RORα and produces IL-5 and IL-13 has been identified in the human lung and intestine (143,144). A third population ('ILC3') that express RORγt and produces IL-17 and IL-22 is found in lymph nodes and mucosal sites (145). There are clear parallels between ILCs and helper T cell subsets with ILC1, ILC2, and ILC3 being the innate equivalents of Th1, Th2, and Th17/Th22 cells respectively. Moreover, they utilise similar transcriptional programs that control their development and function (140). The functional significance of this is as yet unclear but it seems logical that ILCs support development of the optimal immune environment for differentiation of helper T cells.

ILC1s are derived from a $ROR\gamma t^{+}$ precursor but shift to express T-bet, and rapidly produce IFN-γ upon IL-12/-18 exposure (142,146,147). This suggests that there is plasticity between ILC subsets as has been observed for conventional helper T cell subsets. ILC1s are prominent at mucosal sites and their development is thought to be partly-controlled by colonisation of these sites by microbial flora (148). They are thought to be causative in colitis as their depletion ameliorated disease and adoptive transfer into recipients was enough to induce

disease. They have also been found infiltrating the inflamed ileum of human patients suggesting they play a role in clinical disease.

ILC2s are thought to develop from a distinct precursor compared to ILC1s and ILC3s. This precursor has been identified as $Lin^{-}CD127^{+}Flt3^{+}$ and develops upon exposure to IL-7 and IL-33 (149). As a result they express GATA-3 and RORα (143,144), but are not reliant on RORγt as ILC1s and ILC3s are (150). ILC2s are also found at mucosal sites but have been shown to produce type 2 cytokines upon exposure to IL-25, particularly IL-33. They can be identified as being $Lin^{-}CD127^{+}Sca\text{-}1^{+}ST2^{+}$ (151). Functionally, they have been shown to be crucial for expulsion of helminths in the intestine (149), as well as for induction of airway hyperreactivity and tissue repair after respiratory viral infection (152).

ILC3s are very similar to lymphoid tissue inducer (LTi) cells (153). Like ILC3s, LTi cells require Id2, RORγt, and CD132 for their development. They also produce IL-17 and IL-22 upon stimulation (154). It is unclear whether ILC3s develop from LTi cells as contrasting data have been produced. In two studies, an NKp46-reporter system was used to track $NKp46^{+}$ $ROR\gamma t^{-}$ and $ROR\gamma t^{+}$ ILCs. When the progeny of $ROR\gamma t^{+}$ cells were tracked, one group concluded that IL-22 producing $ROR\gamma t^{+}$ cells were not derived from LTi cells but from a $ROR\gamma t^{+}$ liver precursor that could independently generate all $ROR\gamma t^{+}$ ILCs present in mice (LTi cells and ILC3s) (155). However, another report showed that LTi cells adoptively transferred into mice generated $NKp46^{+}ROR\gamma t^{+}$ ILCs capable of producing IL-22. Interestingly, RORγt expression was not stable in these cells, as a proportion lost RORγt expression and IL-22 production, and began to produce IFN-γ (147). This suggested that ILC1s may be derived from an LTi or $ROR\gamma t^{+}$ population. However, ILC3s are not derived from NK cells. In one study, a population of $CD3^{-}NKp46^{+}$ cells was shown to consist of $NK1.1^{+}Ly49^{+}ROR\gamma t^{-}$ NK cells and $NK1.1^{-}$ $CD127^{+}ROR\gamma t^{+}$ ILC3s, and only ILC3s produced IL-22 (138). Further fate-mapping

experiments tracked the RORγt$^+$ cells and found that ILC3s were not precursors of NK cells. One of the major functions of ILC3s seems to be the maintenance of epithelial cell integrity and barrier function, especially in the intestine. For example, ILC3s have been shown to be a critical IL-22 source in *C.rodentium* infection (156,157), and IL-22 production is at least partly regulated by expression of the aryl hydrocarbon receptor (AHR) transcription factor in ILC3s (158-160).

2.3. Soluble factors of the innate immune system

2.3.1. Mucus

Mucus is an extracellular gel matrix comprising water, heavily glycosylated proteins, and several antimicrobial compounds (e.g. lactoferrin, lysozyme, and lactoperoxidase) (161). It forms a fluid, physical barrier that protects the epithelial layer from exposure to inhaled pathogens, particles, and toxins, immobilising them, and carrying them out of the lungs by means of ciliary beating (described in the next section) and coughing. Although a deficient mucus barrier leaves the lungs vulnerable, excessive mucus production or impaired mucus clearance can also lead to many common airway diseases (e.g. asthma) (162). Therefore, mucus production is tightly controlled at the transcriptional level.

Pulmonary epithelium is composed of two principal cell types: ciliated and secretory. Secretory cells can be further divided into different subtypes based on their microscopic appearance (e.g. Clara, goblet, and serous cells) (163). Secretory cells release a broad range

of antimicrobial molecules (e.g. defensins, lysozyme, and IgA) with mucins, and these become incorporated into its substructure, increasing its anti-microbial potential (164).

There are 17 genes encoding mucins in the human genome, seven of which are secreted proteins and ten membrane-bound. The most important immunologically are MUC5AC and MUC5B. They are strongly expressed in the airways and are detected in similar quantities in human mucus (165).

Mucins trap pathogens by providing a promiscuous glycoprotein array capable of interacting and binding with a broad spectrum of microbial proteins. This array of glycoproteins changes during inflammation to aid pathogen capture and clearance (166), as well as acting as a solid physical barrier. However, mucus is not a uniform, solid structure. It is a matrix, containing many pores that are sufficiently large (approximately 500 nm) to be traversed by small viruses with hydrophilic structural proteins and envelopes (165).

The proportion of MUC5AC and MUC5B that comprise mucus varies with health status. Many pathogens increase mucin expression such as viruses (167), as well as multiple stimuli such as cytokines (e.g. interleukin (IL)-4, IL-9, IL-13, IL-17, and IL-25) (168). At baseline, MUC5AC is produced predominantly by surface goblet cells in the airways, whereas MUC5B is produced by secretory cells (165). In mice, only MUC5B is produced by airway secretory cells, and mice with MUC5B deletion die from dysregulated lung inflammation (161). This suggests that MUC5B is essential as a barrier in mice, mediating pathogen clearance, and probably plays a similar role in human airways (169).

2.3.2. Defensins

Defensins are small (18-45 amino acids) cationic peptides found in numerous cell types in both plants and animals. They all contain a conserved 6-8 cysteine-rich motif that is crucial to their function as anti-microbial agents (170). They are found in cells typically involved in phagocytosing and killing bacteria such as neutrophils and other granulocytes, macrophages, epithelial cells, NK cells, and T cell subsets (171). There are three families of defensins: α-defensins, β-defensins, and θ-defensins. α-defensins are produced mainly by neutrophils, NK cells, and T cells; β-defensins are very common being produced by most leukocytes and epithelial cells; θ-defensins are very rare and have only been identified in rhesus macaques to date (171,172). They function by binding to microbial cell walls and forming pores that allow internal components to leak out, and studies have demonstrated them effective against bacteria, fungi, and viruses (173).

2.3.3. Alarmins

Alarmins are endogenous molecules that are constitutively available and released upon tissue damage to activate the immune system (174). Alarmin members are structurally diverse and evolutionarily unrelated. They include High Mobility Group Box Protein (HMG) B1 (175), Heat Shock Protein (HSP) 60/70 (176) , β-defensins (177), cathelicidin (178), S100B (179), and S100A8 (humans only) (180). They are released by necrotic cells upon infection or tissue injury, but epithelial cells and stimulated leukocytes also secrete them. Once released, they recruit and activate innate cells, including dendritic cells (DCs), and their ability to activate DCs makes them important in the induction of adaptive immune

responses (51). Excessive alarmin release can lead to exacerbations of inflammatory processes leading to tissue damage and induction of autoimmunity (181). In contrast, alarmins also play important roles in tissue homeostasis and repair when transiently-expressed (182). For example, cutaneous application of recombinant alarmins to the skin has been shown to be beneficial in wound repair by recruiting, activating, and inducing differentiation of mesenchymal stem cells (183).

The potential of alarmins for initiating an inflammatory cascade is exemplified by S100A8/9. These two proteins are the most abundant cytosolic proteins in neutrophils and macrophages (184). Upon exposure of these cells to very low doses of lipopolysaccharide (LPS) they immediately release S100A8/9 which bind to the TLR4 receptor, and increase secretion of TNF and other pro-inflammatory factors (185). S100A8/9 levels are inversely correlated with survival from sepsis and toxic shock, and blockade of S100A8/9 is associated with reduced mortality (186). These proteins are also highly expressed in phagocytes within the joints of rheumatoid arthritis (RA) sufferers (187). In contrast, HSP60 and 70 have been shown to induce regulatory T cell influx and activation that may be beneficial to RA patients (188).

Alarmins also have contradictory effects on the development and spread of various cancers. S100A8/9 has been shown to promote the proliferation and survival of tumour cells *in vitro* (189). They have also been shown to suppress T cells responses an increase immune evasion of malignant cells (190). These effects may be due to the ability of alarmins to promote migration and angiogenesis (191,192). HMGB1 is upregulated in melanoma, prostate cancer, breast cancer, and pancreatic cancer (193). Alarmin depletion has been shown to reduce cancer growth (194). Despite this some defensins have been shown to exhibit tumour-

suppressive properties, and HMGB1 released from dying tumour cells activate DCs to present cancer antigens to CTL by cross-presentation (195). Therefore the effects of alarmins on different cancers may be context-dependent.

2.3.4. Pentraxins

Pentraxins are a class of pattern recognition receptor with a conserved 8-amino acid sequence (the pentraxin signature) that bind their ligands in a calcium-dependent manner (196). They are divided into two families: the short pentraxins [C-Reactive Protein (CRP), and Serum Amyloid P protein (SAP)] and long pentraxins (Pentraxin-3 plus several neuronal pentraxins) (197,198). CRP resembles antibody in terms of function: it promotes agglutination of foreign matter, activates complement via the classical pathway, and promotes bacterial phagocytosis (199). SAP binds lipoprotein and is thought to be important in the development of atherosclerosis and amyloidosis (200).

2.3.5. Complement

Complement is a family of ~25 serum proteins that are produced as inactive precursors mainly in the liver, but are also produced by macrophages, monocytes and epithelial cells. Upon infection, a subset of these proteins is activated by cleavage which leads to a massive amplification of activated complement proteins (201). The end product of this amplification is assembly of the membrane attack complex that binds microbes and ruptures their membranes (201). Activation of the complement system can occur in several ways that are

separated into pathways: the classical pathway, the alternate pathway, and the lectin pathway.

The classical pathway is activated by the C1 complex. The C1 complex forms when C1q binds foreign bodies directly, or to IgG or IgM bound to the surface of foreign bodies. This induces a conformational change in C1q that activates C1r, which in turn activates C1s by cleavage. One molecule of C1q, and two molecules of C1r and C1s each form the C1q complex (202). The C1 complex then cleaves C4 (into C4a and b) and C2 (into C2a and b), and the cleavage products C4b and C2a form a C3-convertase (203). The lectin pathway works in an analogous manner but uses mannose binding lectin (MBL) and ficolins instead of the C1 complex (204). Upon pathogen binding, MBL and ficoloins activate two MBL-associated serine proteases (MASP-1 and -2) that cleave C4 and C2 into C4a and C4b plus C2a and b as described above (205).

The alternative pathway does not involve antibodies. In the serum the C3 molecule continuously breaks apart into C3a and b, but is rapidly inactivated by factors H and I (negative regulators of the complement system) (206). However, in the presence of a foreign body cleaved C3b can bind to its surface, protecting it from inactivation. Factor D cleaves C3b into Ba and Bb, and Bb binds more C3b to create C3bBb. C3bBb is stabilised by factor P and together this forms a C3-convertase that amplifies the cleavage of C3 into C3a and b (207).

All three pathways form C3-convertases that cleave the C3 protein into C3a and b (208). As well as leading to activation of other complement proteins C3a induces degranulation of mast cells and increases vascular permeability to allow greater cell influx, whereas C3b binds to foreign material opsonising it for phagocytosis (209). The membrane attack

complex forms when C3b binds to C4bC2a to cleave C5 into C5a and b. C5a acts as a chemoattractant for macrophages and neutrophils. C5b results in cleaveage and activation of C6, C7, C8, and C9. The membrane attack complex is formed of activated C5b, C6, C7, C8, and C9 molecules (210).

2.3.6. Cytokines

Cytokines are small molecules that are essential for effective intercellular communication. They are produced by virtually all nucleated cells (which distinguish them from hormones) and act on virtually all nucleated cells. Many cytokines are expressed during both innate and adaptive immune responses, making categorisation difficult. However, a selection have been chosen that are more associated with innate than adaptive immunity and are described below (Fig.3).

G-CSF (Granulocyte-colony stimulating factor): This is a cytokine produced by endothelium and macrophages as well as several other tissues to drive granulocyte and stem cell development in the bone marrow (211). The receptor is expressed by progenitor cells in the bone marrow and by neurons in the spinal chord and brain. It stimulates the survival of neutrophils and neurons, and neurogenesis (212).

GM-CSF (Granulocyte macrophage-colony stimulating factor): This is produced by several cell types including macrophages, T cells, NK cells, mast cells, endothelium, and fibroblasts (213). It stimulates granulocyte and macrophage development in the bone marrow where the receptor is mainly expressed (214).

	mass (kDa)	assembly	pdb	source(s)	target(s)
IL1	17	monomer	3O4O	macrophages, endothelia, epithelia	endothelia (↑ coagulation, ↑ inflammation) hepatocytes (↑ acute phase proteins), hypothalamus (↑ fever)
IL18	17	monomer		macrophages	NK lymphocytes (↑ IFN-II γ), T lymphocytes (↑ IFN-II γ)
TNF	17	homotrimer	1TNF, 3ALQ	macrophages, T lymphocytes	endothelia (↑ coagulation, ↑ inflammation) hepatocytes (↑ acute phase proteins), neutrophils (↑ activation), hypothalamus (↑ fever)
IL6	26	homodimer	1P9M (complex)	macrophages, endothelia, T lymphocytes	hepatocytes (↑ acute phase proteins), B lymphocytes (↑ proliferation)
IL15	13	monomer		macrophages	NK lymphocytes (↑ proliferation), T lymphocytes (↑ proliferation
IL12	35/40	heterodimer	1F45 3DUH	macrophages, dendritic cells	Th1 lymphocytes (↑ differentiation), Tc lymphocytes (↑ IFN-II γ), NK lymphocytes (↑ IFN-II γ)
IL23	19/40	heterodimer		macrophages, dendritic cells	T lymphocytes (↑ IL17)
IL27	28/13	heterodimer		macrophages, dendritic cells	Th1 lymphocytes (inhibition and/or differentiation), NK lymphocytes (↑ IFN-II γ),
IL10	18	homodimer	2H24 1Y6K	macrophages, T lymphocytes	macrophages, dendritic cells (↓ IL12)
INF-I (α)	21	homodimer		macrophages	all cells (↑ viral immunity, ↑ MHC class I), NK lymphocytes (↑ activation)
INF-I (β)	25	homodimer	1AU1	fibroblasts	all cells (↑ viral immunity, ↑ MHC class I), NK lymphocytes (↑ activation)
INF-III			3OG4	under study	all cells (↑ viral immunity, ↑ MHC class I), NK lymphocytes (↑ activation)
chemo-kines	8-12	monomer		macrophages, endothelia, fibroblasts, epithelia	phagocyte (↑ migration), B lymphocytes (↑ migration), T lymphocytes (↑ migration), ↑ wound repair

Figure 3. Soluble factors of the innate immune system. This table lists cytokines that are important in induction, maintenance, and inhibition of innate immunity. Their structure, molecular weights, sources, and known effects are listed. (adapted from http://nfs.unipv.it/nfs/minf/dispense/immunology/lectures/files/immune_network.html).

IL-1α and β: IL-1α is produce by macrophages, neutrophils, epithelial cells (constitutively in an inactive form), and endothelial cells (215). Its major function is in the maintenance of barrier function at sites directly exposed to the environment. In its precursor form it also acts as an alarmin, acting as a danger signal. It binds to IL-1RI. IL-1α and TNF also combine to promote fever in the earliest stages of microbial infection to initiate immune responses (216). IL-1α is central to many immune processes that are beyond the scope of this section. IL-1β is produced by macrophages in an inactive form that is cleaved into an active form by caspase-1 activated as part of the inflammasome (217). It is a central mediator of immune responses, regulating cell activation, proliferation, differentiation and apoptosis (218). In man, it is known to induce the differentiation and activation of Th17 cells in conjunction with IL-6, IL-21, and IL-23 (219).

IL-6: IL-6 is a member of the gp130 cytokine family that all utilise gp130 as part of its cytokine receptor. The IL-6-specific receptor counterpart is IL-6Rα (CD126) (220). IL-6 is secreted by T cells and macrophages [in response to microbial Pathogen-associated molecular patterns (PAMPs)] to stimulate immune responses (221). It is a critical factor in fever induction. IL-6 can cross the blood-brain barrier and signal the hypothalamus to change the set point temperature of the host via prostaglandin E_2 production (222). IL-6 also induces acute phase responses by stimulating production of C-reactive protein (CRP) (223). It boosts neutrophil development in the bone marrow and inhibits regulatory T cell suppression. It also plays an important role in CD4 T cell differentiation into Th17 cells that are important in anti-bacterial responses but also the development of autoimmunity (224,225).

IL-6 has been shown to be crucial in many immune responses and diseases including diabetes (226), rheumatoid arthritis (224), systemic lupus erythrematosus (SLE) (227), as well as cancer (228).

IL-12: IL-12 is produced by activated macrophages, dendritic cells, and B cells (229). It is a heterodimer, composed of p35 and p40 subunits (230). Its receptor is composed of IL-12Rβ1 and IL-12Rβ2 chains and signals via STAT4, which are particularly important for T cell differentiation into Th1 cells (231). It stimulates IFN-γ and TNF (and reduces IL-4) production from NK cells as well as T cells (232). IL-12, in conjunction with IL-2, also enhanced cytotoxicity functions of stimulated NK cells and CD8 T cells. IL-12 is particularly important in anti-viral responses and immunity against intracellular pathogens. However, dysregulation of IL-12 production is associated with the development of autoimmunity (231).

IL-15: Please see section on γ_c chain cytokines.

IL-25: Also known as IL-17E, IL-25 shares significant sequence homology to IL-17 (233). It is produced by epithelial cells, mast cells, and Th2 cells and increases production of IL-8 and type 2 cytokines such as IL-4, -5, and -13 to drive eosinophilic responses (234,235). It is particularly important in responses in the gut and the development of chronic gut inflammation (236). It may also play a role in the development of asthma.

IL-33: Like IL-1β, IL-33 is produced as a precursor that can act as an alarmin, binding its receptor ST2 (237,238). It is cleaved into its mature form by cathepsin G and elastase, however caspase-1 inactivates it upon cleavage (239). IL-33 is constitutively produced in an inactive form by endothelial cells and upon release induces cytokine production by ILC2s, Th2 cells, mast cells, basophils, eosinophils, NK cells, and NKT cells (240). As a type 2 factor it

is strongly associated with allergic and asthmatic responses. IL-33, in conjunction with IL-2, -7, or TSLP upregulates expression of ST2 on the surface of Th2 cells, increasing its activity. Further binding induces IL-13 production, proliferation and activation of Th2 cells, even in the absence of antigen (240). This is analogous to the effects of IL-1 on Th17 cells and IL-18 on Th1 cells.

TNF: This is the signature cytokine of the tumour necrosis factor family of cytokines, whose main function is the induction of apoptosis (241). Monocytes are a primary source of TNF, but it is also produced by T cells, B cells, mast cells, epithelial cells, and endothelial cells (242). It is produced as a trimer that is bound to the monocyte plasma membrane by an anchor peptide (243). The anchor is cleaved and the trimer released where it is proteolytically-cleaved into active monomers (244). TNF induces cachexia (loss of body mass) and fever (either directly or by increasing IL-1 secretion) (245). It can also induce cell proliferation and reduce Th17 differentiation at sites of inflammation (246,247).

TSLP: Thymic stromal lymphopoietin (TSLP) is mainly produced by non-haematopoietic cells such as epithelial cells, fibroblasts, and stromal cells (248). It serves a major function in activating antigen-presenting cells such as DCs, monocytes, and macrophages (249,250). In response, these cells release T-cell-attracting chemokines such as monocyte-derived chemokine (MDC) and thymus and activation related chemokine (TARC) (251). Thymic TSLP induces regulatory T cell differentiation via monocytes and plasmacytoid dendritic cells (PDCs) (252,253). TSLP is heavily implicated in asthma pathogenesis where it induces airway hyperreactivity and infiltration of the lung by Th2 cells (251). It is also a therapeutic target in arthritis because it induces TNF production by T cells which is central to pathogenesis (254).

2.3.7. Chemokines

Chemokines are a family of small proteins that regulate chemotaxis (i.e. movement along a chemical concentration gradient) of immune cells. Some are involved in homeostatic processes (e.g. immune surveillance and angiogenesis) and are constitutively secreted, whereas others are produced during immune responses to pathogen invasion to direct immune cells in the peripheral circulation to the site of inflammation. They share four cysteine residues that are critical for their 3-dimensional structure and are divided into families depending upon their amino acid sequence around these cysteine residues. They exert their effects by binding to G-protein-linked transmembrane receptors. Chemokines are generally expressed earlier than most cytokines as they recruit many immune cells to the site of inflammation, where cytokines are produced and exert their effects (3). However, as for cytokines, classification into 'innate' and 'adaptive' chemokines is difficult. Therefore, the selection presented is a 'best fit' for each type of response.

Eotaxins: There are three eotaxins: eotaxin-1 (CCL11), -2 (CCL24), and -3 (CCL26) that are all chemotactic for eosinophils and are therefore associated with asthmatic responses (255). CCL11 binds CCR2, CCR3, and CCR5 but has greatest affinity for CCR3, and consequently is not chemotactic for neutrophils and macrophages which do not express CCR3 (256). CCL24 and CCL26 also utilise CCR3 and have been shown to be chemotactic for resting T cells and basophils as well as eosinophils (257,258).

Fractalkine: Also known as CX3CL1 and neurotactin (in mice), it is the only known member of the CX3C family (259). It is also unusual in that it is a very large protein (373 amino acids) with a mucin stalk that allows it to bind to the surface of endothelial cells (260). Fractalkine

binds the receptor CX3CR1 and strongly chemoattracts T cells and monocytes, whereas the surface-bound form is chemoattractive for leukocytes (261).

IL-8: IL-8 (CXCL8) is a member of the CXC cytokine family and is produced by macrophages, epithelial cells, and endothelial cells (262). IL-8 binds to CXCR1 and CXCR2 to mediate its effects (263). IL-8 is a potent chemotactic factor, particularly for neutrophils and other granulocytes (264). Moreover, it induces respiratory burst, degranulation, and phagocytosis in recruited granulocytes (265). IL-8 is considered to play a key role in various inflammatory disease including bronchiolitis and psoriasis (266).

MIP-1α: Macrophage inflammatory protein-1α (CCL3) is chemotactic for granulocytes (neutrophils, basophils, eosinophils) (267). It is produced by macrophages, particularly after exposure to microbial products such as LPS. It also increases production of TNF, IL-1, and IL-6 from these cells and therefore amplifies inflammation (268).

MIP-2α: Macrophage inflammatory protein-2α (CXCL2) is produced by monocytes and macrophages and chemoattracts granulocytes and haematopoietic stem cells (269). It binds the CXCR2 receptor (270).

RANTES: Regulated upon activation T cell expressed and secreted (RANTES; CCL5) is produced by T cells as well as other immune cells. It binds to three chemokine receptors: CCR1, CCR3, and CCR5, making it chemotactic for multiple cell types including T cells, eosinophils, and basophils, and other leukocyte populations (271-273). In combination with IL-2 and/or IFN-γ it increases NK cell proliferation and cytotoxicity, and acts as a natural suppressor of HIV (274,275).

2.4. The adaptive immune system

The adaptive immune system developed with vertebrates. It responds to foreign agents in an antigen-specific manner, each antigen being recognised by only a small number of clonal cells. As such the response is much slower than innate immunity because the activated cells must first proliferate to sufficient number before carrying out their effector functions. However, these cells also form the basis of immunological memory such that upon re-exposure they will respond much more rapidly (3). The major components of the adaptive immune system are described in the following sections (Fig.2.).

2.5. Cells of the adaptive immune system

2.5.1. B cells

B cells (so-called because they mature in the Bursa of fabricus in birds and bone marrow in man) are lymphocytes and white blood cells, forming part of the humoral immune system (276). They can be distinguished from other lymphocyte subsets by expression of a B cell receptor (BCR) that is analogous to the TCR expressed on T cells (277). As for the TCR, the BCR is generated by recombination of germline gene segments (V, D and J) to form a clonal receptor with unique specificity (276). However, in contrast to the TCR which recognises a linear peptide epitope complexed with an MHC molecule, the BCR recognises a three dimensional structure comprising several discontinuous epitopes (278). B cells act as APCs, presenting peptides to CD4 T cells in the context of MHCII molecules. This B-cell:CD4-T-cell interaction between TCR and MHCII:peptide (signal 1) results in activation of both B cell and

CD4 T cell in conjunction with costimulatory molecule interaction (signal 2) and cytokine secretion (signal 3) (276). The primary function upon B cell activation is the production of antibody, soluble molecules that are identical to the BCR and bind the same epitope on microbial pathogens (279). This may neutralise the pathogen, preventing invasion of host cells and tissues, or 'tag' the pathogen for destruction by phagocytosis or complement fixation (3,280). Antigens are T-cell-independent (e.g. proteoglycans that crosslink BCRs so do not require CD4 T cells to activate the responding B cell) or -dependent (e.g. most protein antigens that require CD4 T cell activation of B cells to induce and antibody response) (281).

B cells develop continuously in the bone marrow in most mammals. During development, B cells progress through several stages of development (282). At each stage they rearrange their antibody gene loci to generate a unique, clonotypic BCR (283). The stages are early pro (Heavy (H) chain undergoes DJ rearrangement), late pro (H chain undergoes VD rearrangement, expresses CD19), large pre (expresses surface IgM), small pre (Light (L) chain undergoes VJ rearrangement), and immature (loses CD127 expression) (3). If any step fails to produce a productive rearrangement then the B cell dies by clonal deletion (276). As for T cells, B cells are tested for autoreactivity during development. B cells that bind too strongly to self are either clonally deleted, become anergic, or the BCR undergoes receptor editing. Receptor editing is the introduction of point mutations by the recombination activation gene (RAG) 1 &2 proteins in the L chain that changes its affinity for antigen (284). The B cell is then retested, and if the BCR is less autoreactive it can continue development. However, if the BCR remains autoreactive then the B cell is deleted (285). Immature B cells migrate to

the secondary lymphoid organs (they are now called ‘transitional’ B cells) and a proportion express surface IgD and mature into functional B cells (282).

Clonal B cells are continuously produced in the bone marrow and exist in multiple forms. B-1 cells (‘natural’ B cells) are found mainly in the peritoneal and pleural cavities, and express polyspecific, low affinity IgM rather than IgG. These polyspecific IgM molecules often target bacterial polysaccharide antigens, other immunoglobulins, or self-antigens (286). B-2 cells are considered to be the generic ‘B cell’. Plasma cells and memory B cells are generated after primary B cell activation in germinal centres (284,287). Inflammatory plasma cells produce large amounts of antibody at inflamed sites until antigen levels are ablated. These plasma cells then die by apoptosis because of a lack of survival signals derived from soluble factors such colony-stimulating factors (CSFs) (288). CXCR4-expressing long-lived plasma cells migrate in a CXCL12-dependent manner to the bone marrow where they survive for many years in survival niches, producing antibody (289). They are maintained by a diverse array of survival factors such as IL-6, XBP-1, BAFF, CXCL9, and CXCL16 (290). Memory B cells are also long-lived cells but patrol the periphery (rather than migrate into the bone marrow) patrolling for their specific antigen. These cells also undergo affinity maturation via somatic hypermutation (mutation of the complementary-determining regions that increase the antibody affinity for antigen) and clonal selection (survival of only the clones with the greatest affinity) (285,287).

2.5.2. T cells

T cells (so-called because they are derived from the thymus) are white blood cells comprising part of the lymphocyte population. They can be distinguished by the presence of a clonotypic T-cell receptor (TCR) on their cells surface, which is generated by recombination of germline gene segments (V, D, and J) to recognise a unique peptide epitope in the context of major histocompatibility complex (MHC) molecules (291). The TCR signals via a CD3 signalling complex that is attached to it and required for TCR expression. This complex comprises six proteins in two heterodimeric (εγ and εδ) and one homodimeric (ζζ) pair. The ζζ homodimer is particularly crucial as it contains six ITAM (intracellular tyrosine activation motifs) motifs that can be phosphorylated to allow recruitment of intracellular signalling proteins (e.g. Lck and ZAP-70) that initiate signalling cascades resulting in T cell activation (291,292). The importance of T cells for protection against pathogens is evident in AIDS patients, where a lack of functional CD4 T cells leads to failure of CD8 T cell and B cell function and overwhelming susceptibility to pathogen invasion and spread (293,294).

There are several distinct types of T cell, some form part of the innate immune system (NKT cells, γδ T cells) and have already been described, and others form part of the adaptive immune system (CD4 T cells, CD8 T cells, regulatory T cells) and will be described in further detail in the following sections.

2.5.2.1. Thymic development

T cell development begins in the bone marrow but mostly occurs in the thymus (295). The process is initiated by Sca-1^+ KIT^+ haematopoietic stem cells (HSC) which is a heterogeneous

mixture of Flt3- and CD150-expressing cells (296). True HSCs are positive for CD150 and have long term self-renewal capacity, whereas multi-potent progenitors (MPPs) are $CD150^-$ and do not self-renew (297). Some MPPs are $Flt3^{hi}$ and respond to Flt3L to differentiate into lymphoid-primed progenitors (LPPs) (298). These are the first cells to express the recombinase activating genes (RAG-1 and -2) that generate the T cell receptor. These cells subsequently form common lymphoid progenitors (CLPs) and only LPPs and CLPs can migrate to the thymus to develop into T cells. Migration is dependent on expression of CCR7, CCR9, and PSGL1 [P-selectin glycoprotein ligand 1] (299).

Upon thymic entry close to the cortico-medullary junction, CLPs begin differentiation and final commitment to the T cell lineage (300,301). They undergo four stages of differentiation [termed double negative (DN) 1-4] that are characterised by extensive proliferation and expression of CD25 and/or CD44 (302). DN2-3 differentiation occurs in the thymic cortex (303). These four stages precede expression of CD4 and CD8, and involve rearrangement of the VDJ segments to form the TCR β, γ, or δ chains at the DN3 stage (303). Productive rearrangement occurs in the subcapsular zone, the γδ TCR commits a cell to the γδ T cell lineage, whereas a β chain binds the pre-TCRα chain to commit to the αβ T cell lineage. This stage is called 'β selection' (304). Regardless of lineage commitment, both then differentiate to the DN4 stage (αβTCR-committed cells require Notch signalling for this to occur) and express CD4 and CD8 (the 'double-positive' stage) (305). Differentiation to DN4 requires TCR signalling, acting as a checkpoint that productive TCR rearrangement has occurred (303). Double-positive (DP) cells move to the cortex where they rearrange their TCRα genes to produce a TCRα chain, that replaces the pre-TCRα chain, and formation of a clonal TCR heterodimer (306). These cells then interact with thymic epithelial cells and those that

weakly recognise self-antigen:MHC molecules are selected for further development, known as 'positive selection'(307,308). Cells that do not respond to self-antigen:MHC complexes die by neglect, and those that respond too strongly die by negative selection in the medulla (309). Cells that successfully transit this stage become single-positive (SP; either CD4 or CD8), enter the medulla and exit the thymus. Exit from the thymus is regulated by a sphingosine-1-phosphate chemotactic gradient (310). During this time, surviving SP cells mature from $CD69^{hi}$, $CD62L^{lo}$, $CD24^{hi}$ to $CD69^{lo}$, $CD62L^{hi}$, $CD24^{lo}$ and will form part of the naïve T cell compartment (311).

2.5.2.1.1. CD4 T cells

CD4 T cells express a TCR heterodimer, CD3 signalling complex, and CD4 surface molecules as well as a broad repertoire of costimulatory molecules. Upon activation they produce and secrete a wide array of soluble factors. The principal function of CD4 T cells is to orchestrate the action of other immune cell types via the release of cytokines. In particular, CD4 T cells are essential for optimal B cell activation, antibody production, and isotype class switching (312). They are also crucial for optimal activation of CD8 T cells and induction of cytotoxic activity (313). CD4 T cells also increase microbial activity of the innate immune system, particularly macrophages (314). The primary mechanism of this ‘help’ is expression of cognate antigen on MHCII molecules on APCs (B cells, macrophages, and DCs) (315). MHCII molecules primarily express exogenous antigens that are engulfed by cells into vesicles that then fuse with a vesicle array containing enzymes that degrade the proteins into peptides (316). Other vesicles containing newly-synthesised MHCII molecules complexed with CLIP peptide fuse with these peptide-containing vesicles allowing loading of these peptides in

exchange for CLIP. These new complexes are then transported to the surface for recognition by peptide-specific CD4 T cells (316). The peptide-binding cleft of MHC II is open-ended and so can bind longer peptides (12-25 amino acids) than MHCI. This peptide:MHCII complex is recognised using the TCR and CD4 molecules (signal 1), but CD4 T cells also require costimulatory molecule interaction (e.g. CD28, OX40, CD40L; signal 2) and cytokine signalling (signal 3) for full activation (317). As a result, the APC is activated by the CD4 T cell (318). These APCs can activate other cell types (e.g. DCs activate CD8 T cells) or carry out effector functions (e.g. B cells produce antibody). The importance of these functions is evident in AIDS patients who lack a functional CD4 T cell compartment. These patients rapidly succumb to infections they would normally repulse (293,294).

CD4 T cells develop in thymus, and after leaving reside in the secondary lymphoid organs, including lymph nodes throughout the body (319). ~95% form part of the naïve T cell population, but a small fraction form natural 'regulatory T cells' (nTregs) that express the IL-2Rα protein (CD25), the transcription factor FoxP3, and IL-10 (320). Their function is to inhibit activation and effector functions of other immune cells in order to prevent excessive tissue damage. This they achieve using soluble factors (e.g. IL-10) or contact-mediated (e.g. CTLA-4) mechanisms (321). What determines whether a CD4 T cell becomes an nTreg or naïve T cell is unclear. Upon activation, naïve CD4 T cells express CD25 and secrete IL-2 that acts in an autocrine (i.e. upon the cell that produced it) and paracrine (i.e. on neighbouring cells) fashion to induce proliferation (322). During proliferation, CD4 T cells become effector cells and undergo a period of 'transcriptional reprogramming', during which time their specific effector functions are determined. These have been broadly defined on the basis of their cytokine repertoire as Th1, Th2, Th9, Th17, Th22, Tfh and iTreg subsets (323). The

mechanisms that determine the effector CD4 T cell phenotype are immensely complex and an intensely active area of study. As such they are discussed in greater detail in a later section (section 2.7).

Once a pathogen has been cleared and antigen has disappeared then ~95% of effector T cells die by apoptosis (324). The remainder, expressing CD127 (IL-7Rα), survive and form the memory T cell population that protects against re-infection with any pathogen expressing a cross-reactive epitope (325). This memory is dependent on IL-7 and IL-15 for its long-term survival (326). Memory T cells exist as 'effector' or 'central forms. Effector memory patrols the periphery (they are CCR7 and CD62L negative), have limited proliferative capacity but retain their effector functions. In contrast, central memory is retained in the secondary lymphoid organs (they are CCR7 and CD62L positive), proliferate continuously to replenish the memory pool, but retain few effector functions (325). Memory T cells do not have the strict costimulatory requirements as naïve T cells, and as such are able to respond more rapidly to rechallenge. What determines which CD4 T cells become memory (and what type) and which die is not fully understood but may be related to their degree of differentiation (i.e. those that are fully, terminally-differentiated effector cells die and those that are not fully committed become memory) (326).

Given the diverse anti-microbial immune responses that CD4 T cells generate via cytokine secretion, these cells also have the potential to generate aberrant responses such those observed in hypersensitivity reactions (327), and the autoimmune diseases rheumatoid arthritis (328), and diabetes (329).

2.5.2.1.2. CD8 T cells

CD8 T cells also express a clonal TCR that recognises a specific epitope, just as for CD4 T cells, but CD8 T cells recognise their specific peptides (7-9 amino acids) in the context of MHCI molecules (330). This is due to CD8 binding to the α_3 subunit of MHCI. The major effector function of CD8 T cells is the ability to kill infected cells (typically virally-infected, but any intracellular pathogen), preventing pathogen replication and spread (331,332). Microbial proteins that are degraded by the proteasome into peptides are bound to MHCI molecules within vesicles and transported to the surface where they are recognised by peptide-specific CD8 T cells (333). It is also possible for 'cross-presentation' of exogenously-derived antigens to be degraded into peptides and loaded into MHCI molecules for presentation to CD8 T cells (334). As for CD4 T cells, CD8 T cells require three signals for full activation: TCR signalling by recognition of specific peptide in the context of MHCI, costimulatory molecule interaction, and cytokines (335). Upon activation, CD8 T cells proliferate upon exposure to IL-2 which acts as a T cell growth factor and maximises the number of effector CD8 T cells to locate and destroy other virally-infected cells (322). Upon recognition and CD8 T cell activation; there are five major mechanisms that can be used to lyse an infected cell: perforin, granzyme, and granulysin release, Fas/FasL interaction, and cytokine release. Perforin is a pore-forming protein released from granules by activated CD8 T cells. It disrupts the osmotic balance of the infected cell, allowing entry of not only extracellular fluid but also granzyme proteins that activate serine proteases called caspases that induce programmed cell death (apoptosis) (336,337). Granulysin performs a similar function to perforin (338). Fas/FasL interaction also leads to apoptosis of the infected cell (339). Intracellular signals result in the formation and recruitment of the death-inducing

signalling complex (DISC) that recruits the Fas-associated death domain (FADD) (340). This activates the initiator caspases 8 and 10 that results in activation of effector caspases 3, 6, and 7, resulting in apoptosis (341). Finally, CD8 T cells are a potent source of cytokines that can cause cell death, particularly TNF (342). Given that CD8 T cells have potent cytotoxic activity that destroys host cells, they have the potential to cause extensive damage to host tissue which has to be strictly controlled (343). Therefore, many disease states (pathogen-derived and autoimmune) can be attributed to dysregulated CD8 T cell responses (344-346).

Similar to their CD4 counterparts, central and effector memory CD8 T cells form once a pathogen has been cleared and antigen levels decrease (347). However, the survival of this memory is dependent on CD4 T cells and cytokines for its survival, in particular IL-7 and IL-15, but also IL-21 produced by CD4 T cells (348).

2.6. Soluble factors of the adaptive immune system

2.6.1. Cytokines

In this section the effects of several cytokines associated with different helper T cell subsets (i.e. Th1, Th2, Th17, Th22, and regulatory T cells) will be described (Fig.4).

IFN-γ: The only member of the type II IFN family, IFN-γ is a dimer considered essential for protective immunity against intracellular microbes and tumours (349,350). It is produced by NK cells and NKT cells, particularly after IL-12 secretion by macrophages which are classically-activated by IFN-γ (351). It is also produced by CD4 and CD8 T cells during

	mass (kDa)	assembly	pdb	source(s)	target(s)
lympho-toxin	21-24	homotrimer		T lymphocytes	B lymphocytes (↑ development), T lymphocytes (↑ development), neutrophils (↑ migration, ↑ activation),
IL-2	17	monomer	2ERJ	T lymphocytes	T lymphocytes (↑ survival, ↑ proliferation, ↑ cytokines), B lymphocytes (↑ proliferation, ↑ antibody production), NK lymphocytes (↑ proliferation, ↑ activation),
IL-4	17	monomer	1RCB 3BPN	Th2 lymphocytes	B lymphocytes (↑ isotype switch IgE), Th2 lymphocytes (↑ proliferation, ↑ differentiation), macrophages (↓ IFN-II γ response), mast cells (↑ proliferation)
IL-5	26	homodimer	1HUL	Th2 lymphocytes	B lymphocytes (↑ proliferation, ↑ isotype switch IgA), eosinophils (↑ proliferation, ↑ activation)
IL-13	13	monomer	3BPO	Th2 lymphocytes, NK-T lymphocytes, mast cells	B lymphocytes (↑ isotype switch IgE), macrophages (↑ collagen), fibroblasts (↑ collagen), epithelia (↑ mucus),
IL-17	35/40	dimer	3JVF	T lymphocytes	endothelia (↑ chemokines), macrophages (↑ cyto-kines/chemokines), epithelia (↑ G-CSF and GM-CSF),
INF-γ	19/40	homodimer	1HIG, 1FG9	Th1 lymphocytes, Tc lymphocytes, NK lymphocytes	B lymphocytes (↑ isotype switch), Th1 lymphocytes (↑ differentiation), macrophages (↑ activation), various cells (↑ antigen processing and ↑ MHC class I)

Figure 4. Soluble factors of the adaptive immune system. This table lists cytokines that are important in induction, maintenance, and phenotype of adaptive immunity. Their structure, molecular weights, sources, and known effects are listed. (adapted from http://nfs.unipv.it/nfs/minf/dispense/immunology/lectures/files/immune_network.html).

antigen-specific adaptive immune responses, and is the signature cytokine of Th1 cells (350). Macrophages and DCs have also been shown to produce IFN-γ upon infection, possibly as a method of self-activation (350). The biological effects of IFN-γ are mediated by binding a heterodimeric receptor comprising IFNGR1 and IFNGR2 chains, and activation of the JAK-STAT pathway (352). These effects are pleiotropic, to describe a few in brief: it increases antigen presentation, lysosomal activity, and iNOS production by macrophages, increases T-bet expression to enhance Th1 differentiation of CD4 T cells and CTL, increases IgG2a and IgG3 production by activated plasma cells, boosts NK cell cytotoxicity, and promotes adhesion molecule expression to enhance leukocyte migration (350,351). Due to its importance in immunoregulation dysregulated IFN-γ production is considered causative of multiple autoimmune conditions (e.g. diabetes, MS, RA) (353-357).

IL-2: Please see section on γ_c chain cytokines.

IL-4: Please see section on γ_c chain cytokines.

IL-5: IL-5 is a homodimer produced mainly by Th2 CD4 T cells, mast cells, and eosinophils (358-361). Its production is regulated by several transcription factors including GATA-3 (361). Upon binding its specific IL-5Rα, its main effects are increasing B cell activation and antibody production as well as eosinophil maturation, activation, and effector function (362,363). IL-5 has a strong association with diseases characterised by eosinophil activation and recruitment: helminth infection, asthma and allergy being particular examples (363,364). However, IL-5 blockade was ineffective at significantly reducing asthma pathology so it is currently unclear how crucial IL-5 is in this chronic disease (365).

IL-9: Please see section on γ_c chain cytokines.

IL-10: IL-10 is a homodimer and class II cytokine (with IL-19, -20, -22, -24, -26, -28, and -29) (366). It is produced by several cell types including monocytes, T cells (particularly regulatory T cells), B cells, and mastocytes (367-369). IL-10 production is normally triggered by microbial colonisation of mucosal tissues, as part of tissue homeostasis, or by PD-1 binding, as a negative immune signal (367,370). It binds a heterodimeric receptor consisting of IL-10R1 and R2 chains, the latter chain being shared by other IL-10 family members (371). IL-10 is considered particularly critical for inhibition of NK, CD4, and CD8 T cell responses as these cause significant tissue damage if uncontrolled (367,372-374). It also inhibits macrophage activity by reducing IL-12 secretion, as well as reducing MHC II and costimulatory molecule expression (369,375). Many of its anti-inflammatory effects are mediated by reducing NF-κB activation (369). However, it can also be stimulatory: it increases B cell proliferation as well as survival, and increases antibody production by such cells (376). IL-10 appears to be particularly important in intestinal immunoregulation as IL-10KO mice develop chronic conditions (e.g. Crohns disease), and IL-10 administration has been shown to be beneficial in alleviating disease symptoms (375).

IL-13: IL-13 is a type 2 cytokine that is produced by several cell types, but especially by Th2 cells (377). IL-13 shares many effects with IL-4, and this is partly due to it sharing a receptor chain (IL-4Rα) but also utilises at least two IL-13-specific receptor chains (377). Like IL-4, IL-13 signalling acts mainly through STAT6 activation and translocation. However, even more than IL-4, IL-13 is considered to be central to asthma pathogenesis (378-380). The reasons for this are not fully understood but may stem from the fact that while IL-4 acts mainly on the pro-allergic haematopoietic cells, IL-13 acts on the non-immune structural cells (e.g. smooth muscle and goblet cells) that are responsible for the observed clinical symptoms of

asthma (i.e. airway hyperreactivity and mucus plugging) (380). This is supported by the findings that several polymorphisms are associated with asthma development, yet deletion of IL-13 does not significantly affect Th2 cell differentiation or IgE production (379). Despite this, IL-13 is capable of inducing IgE production by activated B cells, and is important for chemokine release that recruits allergen-specific Th2 cells to the lung (378). It is likely therefore that IL-13's effects on non-haematopoietic cells are more important for asthma pathogenesis. As for other type 2 cytokines, IL-13 is important in protective anti-helminth responses. IL-13 induces glycoprotein hypersecretion to enhance parasite detachment from the gut wall, and also increases gut muscle contractions to aid parasite expulsion (377,380).

IL-17: IL-17, otherwise known as IL-17A, is the first member of the IL-17 family of cytokines named A-F (381). It is produced mainly by T cells and is the signature cytokine of Th17 cells (382). Its production is induced by several soluble factors including TGF-β, IL-6 (in mice), IL-1β (in humans) IL-21, and IL-23 (383-387). IL-17A signals via a heterodimeric receptor consisting of IL-17RA and RC chains activating STAT3 (388). The IL-17 family's main function is to degrade invading pathogen matrix, particularly extracellular pathogens (389). Similar to IFN-γ, IL-17A is important as a mediator of delayed-type reactions by inducing chemokine release that recruits monocytes and neutrophils to inflamed sites (390). IL-17 is a critical factor in several diseases including lupus, RA, psoriasis, multiple sclerosis, tumour rejection, allograft rejection, and asthma (391-393). Its wide-ranging effects are partly due to its ability to induce production of many downstream mediators including IL-6, TNF, PGD_2, IL-1β, IL-8, and MCP-1 from several different cell types including epithelial cells, macrophages, fibroblasts, and endothelium. The chemokines induced by IL-17 preferentially recruit neutrophils and not eosinophils (390,393-395).

IL-22: IL-22 is a member of the IL-10 cytokine family and is produced by DCs and T cells, and is particularly important in immunity against bacterial pathogens (366,396). It binds a heterodimeric receptor comprising IL22R1 and IL-10R2, and is negatively regulated by a soluble IL-22 receptor called IL-22BP. It signals via Jak1 and Tyk2, then STAT3 (397). IL-22 is particularly important in maintaining epithelial cell barrier integrity, like IL-10 (398). It stimulates innate immunity against invading microbes by stimulating production of defensins and alarmins (399,400).

2.6.2. Chemokines

In this section I will briefly describe chemokines that are associated with recruitment of Th1, Th2, and Th17 cells.

IP-10: Interferon-gamma induced protein-10 (CXCL10) is produced by monocytes, endothelial cells, and fibroblasts in response to IFN-γ. It binds to the CXCR3 receptor and is chemoattractive for T cells, NK cells, DCs, monocytes, and macrophages (27,401,402).

MDC: Known as Macrophage-derived chemokine or CCL22, this chemokine is secreted primarily by macrophages, DCs, and epithelial cells and binds the CCR4 receptor (403-405). It is important for recruitment of Th2 cells to the site of inflammation (406,407).

TARC: Also known as 'Thymus and Activation Related Chemokine' or CCL17, this chemokine is constitutively expressed in the thymus where it may drive thymocyte migration. However, it is only transiently expressed by stimulated PBMC (408). TARC is specifically chemotactic for T cells by binding the CCR4 receptor (409,410).

MIP-3α: Macrophage inflammatory protein-3 (CCL20) is produced by myeloid cells and lymphocytes and is strongly chemoattractive for lymphocytes expressing the CCR6 chemokine receptor (411,412). It is induced by lipopolysaccharide (LPS), TNF, and IFN-γ and is important in the induction of mucosal lymphoid tissue by attracting T cells and DCs to epithelial cells (413-415). It is also thought to be crucial in disease pathogenesis involving Th17 cells or type 17 responses, particularly gut inflammation and autoimmunity (e.g. IBD & Crohn's disease), as well as disease of the CNS (e.g. multiple sclerosis) (416,417).

2.6.3. **Antibody**

Antibodies (also known as immunoglobulins; Ig) are Y-shaped, globular plasma glycoproteins belonging to the immunoglobulin superfamily (418). They are produced by activated, antigen-specific B cells (primarily long-lived plasma cells that reside in the bone marrow) and their primary function is to bind and neutralise foreign antigens that may be potentially hazardous for host tissue (279). Antigen binding also targets it for destruction either via phagocytosis by macrophages or neutrophils (3), by opsonisation (280), or complement fixation (280). As each antibody molecule can bind two separate epitopes, it can agglutinate antigens enhancing their immunogenicity thereby activating effector cells (3,419). For example, macrophages can bind these complexes using Fc receptors to enhance phagocytosis, neutrophils and mast cells degranulate upon complex binding, and NK cells increase cytokine release and cytotoxic functions upon Fc receptor binding (3). As Fc receptors bind specific antibody isotypes, cellular functions can be tailored to the specific response required (283).

All antibody classes have the same basic structure, composed of two identical heavy (H) chains linked to two identical light (L) chain connected by disulphide bonds (420). Each chain is made of 70-110 amino acid immunoglobulin domains: L chains comprise one IgV and one IgC dmain and H chains have one IgV domain and 3-4 IgC domains (421). There are two types of L chain called lambda (λ) and kappa (κ) and only one is used per antibody (3). There are several different types of H chain from which antibodies derive some of their characteristics. These are known as antibody 'isotypes' of which there are five classes: IgA (found at mucosal surfaces), IgE (protects against helminths and elicits histamine release from mast cells), IgG (dominating isotype; can cross placenta providing protection to foetus), IgD (expressed on B cells prior to antigen exposure; activates basophils and mast cells), and IgM (neutralises foreign material prior to IgG production) (3). At the opposite end lies the variable (V) or Fab (antigen-binding fragment) domain, containing the 'paratope' (283). Each antibody molecule possesses two such domains and each one is formed from the variable regions within β strands [also known as complementarity determining regions (CDR) or idiotypes] of the L and H chain (422,423). Three CDRs from each chain make up the antibody V domain. These regions bind a unique three dimensional epitope, distinguishing them from T cell epitopes that are linear peptide sequences. As such, the V domain is hypervariable, recognising approximately 10^{10} distinct epitopes (278). This antibody repertoire is generated in a similar manner to the TCR repertoire is during T cell development in the thymus: three germline gene segments (V, D, and J) are selected at random from a large array (e.g. there are ~65 different V_H genes) and joined to form the basic paratope structure (276,424). For L chains only V and J genes combine. This process is called somatic recombination and once determined a B cell cannot express a different paratope (known as 'allelic exclusion') (425). This variability is increased during somatic

hypermutation when B cells randomly mutate the V gene after activation (285,287). Antibody genes encoding the Fc (crystallisable fragment) portion of the heavy chain (isotype) can also be changed in a process termed 'class switching' that occurs via class switch recombination at switch regions upstream of each C region gene (426,427). These changes alter the effector functions of the molecule.

2.7. T cell differentiation

The cytokine profiles of CD4 T cells distinguish their functions and have been extensively used as lineage-specific markers [Fig.5; (428)]. Each profile is discussed below.

2.7.1. Th1 cells

Th1 cells mediate delayed type hypersensitivity responses, and are crucial for protective immunity against infection with pathogens that have intracellular life stages such as bacteria, viruses, and certain parasites. The signature cytokine of Th1 cells is IFN-γ but they also produce IL-2 and lymphotoxin (LT). IFN-γ promotes Th1 development by inducing IL-12 production from activated macrophages and IL-12 receptor expression on antigen-activated CD4 T cells, while directly inhibiting the growth of Th2 cells.

The transcription factor that controls Th1 differentiation was first identified using a yeast expression cloning strategy in combination with RDA (representational difference analysis). This resulted in the isolation of a novel protein belonging to the T box family of transcription factors called *T-bet* (*T-b*ox *e*xpressed in *T* cells) since it was thought to be only expressed in thymocytes and Th1 cells. However, it is now known to also be expressed in innate lymphoid

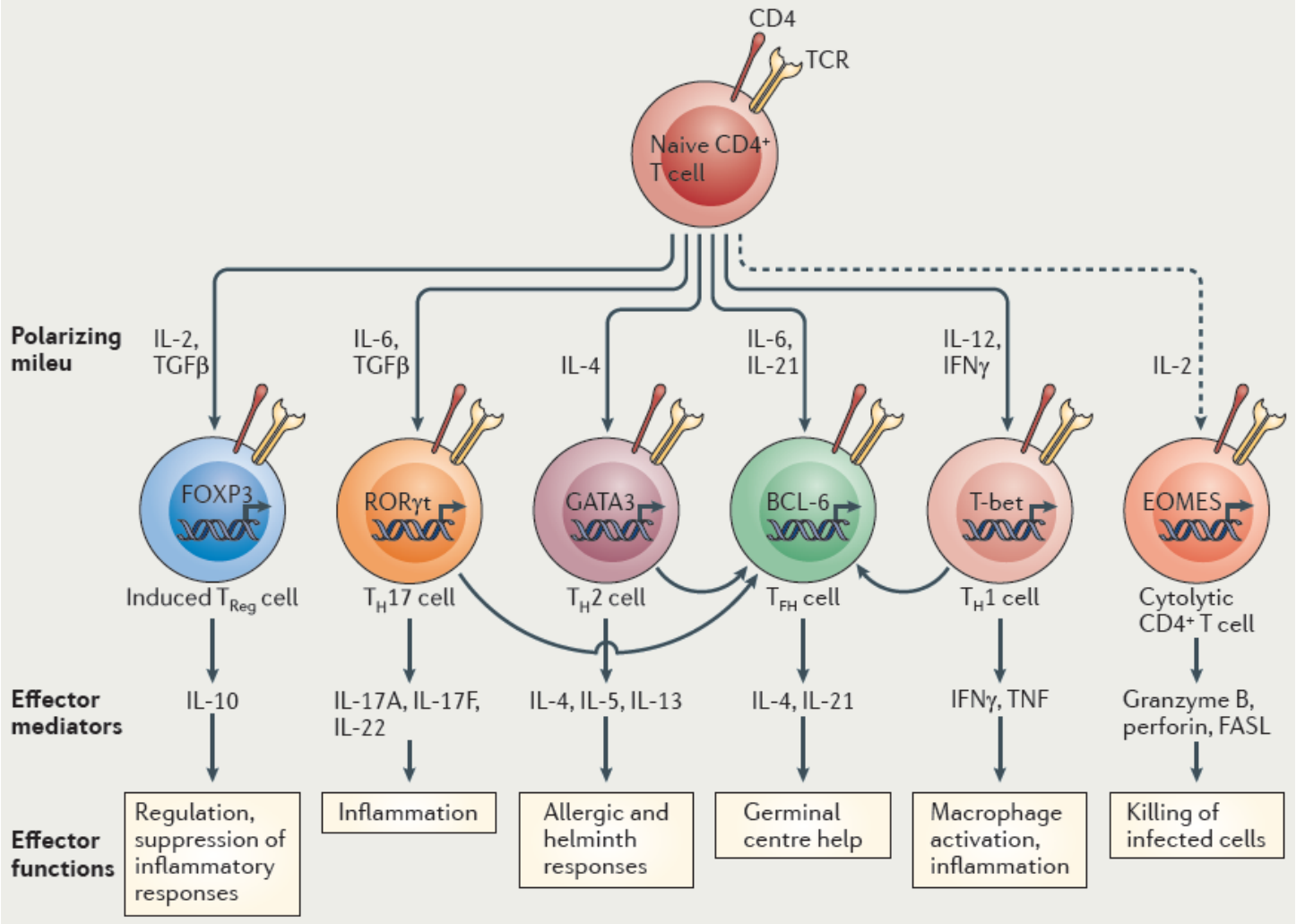

Figure 5. CD4 T cell subsets. Upon recognition of cognate antigen presented by DCs, CD4 T cells begin to proliferate. During this time they begin to differentiate into distinct lineages dependent upon the T-cell-receptor affinity for the antigen:MHCII complex, costimulatory molecule interactions, and cytokines in the microenvironment as shown. Lineage commitment is controlled by expression of unique transcription factors which promotes production of a distinct subset of effector molecules. These molecules, typically cytokines, orchestrate immune responses against infectious pathogens and inert antigens (taken from Swain SL et al. Nature Rev Immunol 2012 12 136).

cells (429), NK cells (430), NKT cells (431), γδ T cells (432), CD8 T cells (433), and DCs (434). T-bet promotes transcription of the *ifng* gene (435) and CXCR3 expression (436) in retrovirally-transduced primary T cells, and redirects Th2 cells into the Th1 pathway. Although beneficial during infection, strong Th1 responses must be regulated to prevent tissue destruction and immunopathology. Failure to do this results in many autoimmune diseases to self-antigens. For example, studies suggest that Th1 cells play a major role in diabetes (437). Blockade of IFN-γ (438) or absence of STAT4 (439) prevent disease, whereas IL-12 accelerates it (440). 'Self-regulation' through interleukin-10 (IL-10) produced by activated Th1 cells is required for limiting immunopathology, and Foxp3+ Treg cells are also essential for effective control of Th1 responses *in vivo*.

2.7.2. Th2 cells

Th2 cells develop following infection with extracellular pathogens such as helminth parasites. Th2 cells produce high levels of IL-4 (their signature cytokine), IL-5, and IL-13. IL-4 acts in a positive-feedback loop by inducing expression of IL-4 receptors on naive CD4 T cells to promote further Th2 differentiation. In addition, IL-4 is a negative Th1 differentiation by decreasing IL-12 receptor expression on naïve T cells, committing them to the Th2 lineage. IL-4 signals via STAT6 and induces the key transcription factors GATA-binding protein 3 (GATA-3), c-maf, and NFATc (441). However, the mechanisms by which Th2 responses are initiated have not been explored extensively. One mouse study suggested that basophils are important in promoting allergen-induced Th2 cell differentiation (442). These cells also have a primary role in IgE-mediated chronic allergic inflammation (443). In humans, the basophil has long been associated with allergic inflammation in chronic

disease, and both human and mouse basophils are able to produce large amounts of Th2 cell-promoting cytokines, like IL-4 and TSLP (442). However, it is unclear how often basophils are the early IL-4 source as others may also be utilised (e.g. innate lymphoid cells, γδ T cells and NKT cells).

Overzealous Th2 responses can also cause inappropriate responses and disease to otherwise innocuous antigens, resulting in allergies and asthma. Such over-active responses are an increasing problem in the western world.

2.7.3. Th17 cells

The signature cytokines produced by Th17 cells are IL-17A and IL-17F. They differentiate from naïve CD4 T cells in response to TGF-β and IL-6 (383-385). STAT3 is fundamental for Th17 development, being involved in IL-6 signalling to induction of IL-21, an autocrine factor implicated in the progression of Th17 development. IL-21, also acting through STAT3, promotes expression of the transcription factors RORγt and RORα, which are essential for Th17 differentiation. IL-23R (444) is induced and maintained by IL-21in developing Th17 cells, and pairs with IL-12Rβ1 chain to bind IL-23 (a heterodimer of IL-23p19 and IL-12p40; (445)). Although IL-23 is dispensable for Th17 differentiation (383,384), its requirement for Th17-mediated immune responses or autoimmunity is well established, by enhancing and maintaining Th17 differentiation (446). Indeed, Th17 cells differentiated by TGF-β and IL-6 in the absence of IL-23 have been shown to protect in a model of EAE (447), consistent with an essential role for IL-23 in pathogenic Th17 responses. Nevertheless, the precise details regarding IL-23 action on Th17 differentiation remain unclear (448).

Th17 cells are considered an important lineage for study because they play a central role in many autoimmune diseases such as EAE (449), rheumatoid arthritis (416,450), and myocarditis (451). Mice with T cells that are unable to respond to TGF-β are Th17-cell-deficient, yet they succumb to autoimmunity with massive infiltration of lung, liver, stomach, pancreatic islets, and thyroid glands (452).

There is considerable plasticity exhibited by Th17 cells, dictated by the local cytokine environment during on-going inflammatory responses. For example, using an adoptive transfer system, Bending *et al* showed that Th17 cells, even when 99% pure increased their expression of the transcription factor T-bet and IFN-γ upon exposure to IL-12 *in vitro*. Further, they upregulated T-bet expression and secreted IFN-γ in NOD mice, causing diabetes (453). Similar conclusions were made in IL-17 reporter mice (454). Thus, these studies demonstrated that the conversion of Th17 cells into Th1 cells determined disease severity, not the presence of Th17 cells themselves.

2.7.4. Th9 cells

The Th9 subset is the one of the most recently identified, being discovered independently by two research groups (455,456). One used gene microarray analysis of highly polarized effector CD4 T cell subsets and found that IL-9 production was associated with Th17 differentiation. Further studies of the conditions in which IL-9 production was promoted demonstrated that IL-9-producing CD4 T cells were a distinct subset from those previously described. They called this new subset Th9 cells (455).

The second demonstrated that IL-4 inhibited TGFβ-induced Foxp3 expression, suppressing the generation of regulatory CD4 T cells. However, the two cytokines together induced IL-

9^+/IL-10^+ CD4 T cells that, unlike regulatory CD4 T cells, had no suppressive capacity despite the fact they produced IL-10. In fact, these Th9 cells induced the development of colitis, as well as peripheral neuritis when adoptively-transferred with CD45RB^{hi} CD4$^+$ effector T cells into recombination activating gene 1-(RAG-1)-deficient mice (456). Another study has identified PU.1 as a putative transcription factor required for Th9 cell differentiation (457). There is little known about the role of this subset in disease, though recent studies suggest they are a clinically relevant marker of chronic asthma and allergic inflammation. Further studies are required to investigate these cells in more detail.

2.7.5. Th22 cells

Th22 cells are the most recently identified lineage in humans (but not rodents) that naïve T cells may become upon activation, having recently become distinct from Th17 cells. The signature cytokine produced by Th22 cells is IL-22 (which can also be produced by Th17 cells), but this lineage also produces TNF, IL-13 and IL-26. Factors that drive differentiation of T cells into the Th22 lineage are IL-6 and TNF, but may also require interaction with pDCs (458). Upon activation in these conditions, naïve T cells upregulate expression of the transcription factor aryl hydrocarbon receptor (AHR) that drives expression of genes associated with Th22 cells such as *il22* (459,460) , but also the chemokine receptors *ccr4*, *ccr6*, and *ccr10* (458). In contrast, IL-22 production by Th17 cells is not regulated by AHR, highlighting a critical distinction between the two lineages (461).

IL-22 (originally called IL-10-related T-cell-derived inducible factor; IL-TIF) is a member of the IL-10 family of cytokines, sharing 22% amino acid sequence homology in mice and 25% in humans. The gene is on chromosome 12q15 in humans and 10 in mice, both are in close proximity to the *ifng* gene. Curiously, there are two *il22* genes in mice, *il22a* and *il22b*. The

major difference between them is a 658 nucleotide deletion in *il22b* that encodes part of the promoter and the first intron. Therefore, it is possible that this gene is non-functional (462). IL-22 mediates its effects via a heterodimeric type II cytokine receptor, comprising IL-22R1 and IL-10R2 chains (463). IL-10R2 is expressed at variable levels on virtually all somatic cells except the brain (464), but IL-22R1 exhibits restricted expression on epithelial cells of the skin, pancreas, liver, kidney, and gut but not on T cells, B cells, monocytes, or DCs (465). Expression in the lung is extremely low. There is also a soluble IL-22R1 protein called IL-22BP, that likely binds soluble IL-22 thereby reducing its bioactivity *in vivo* (463). Binding of IL-22 to its receptor initiates a signalling cascade that begins with phosphorylation of JAK1 and Tyk2 followed by phosphorylation and homodimerisation of STAT1, 3, and 5. This has been demonstrated in several different cell lines *in vitro* (397). The major functions of IL-22 are induction of innate immunity and tissue repair. Its role in repairing tissue has been confirmed in several tissues including skin, liver, kidney, gut, and lung. Its role as a pathogenic factor is less well-defined as this appears to depend on disease location and the other immune factors in the environment (466).

The role of IL-22 has been tested in several different diseases, but the best studied is psoriasis. There is a very strong correlation between serum IL-22 levels and severity of psoriasis (467). Keratinocytes are a central immune cell in this disease and IL-22 is known to boost their immune activity by increasing anti-microbial peptide and MMP-1 production (467). Moreover, IL-22 increases STAT3 expression by keratinocytes, thereby increasing its own signalling potential. TNF blockade is highly efficacious in psoriasis and this appears to be linked to its ability to increases keratinocyte responsiveness to IL-22 by increasing IL-22R1 expression (468). Therefore, as IL-22 blockade does not reduce immunity to bacterial infections as TNF blockade has been known to, and IL-22R1 is not expressed by many

immune cells, so IL-22 appears to be a very promising candidate for therapeutic intervention in this disease. The role of IL-22 in other diseases is less well-studied. In Crohn's disease for example, blood IL-22 levels are significantly increased. However, other studies have demonstrated a protective role for this cytokine as it induced intestinal epithelial cells and subepithelial myofibroblasts to produce a range of anti-inflammatory, and regenerative proteins (469). Bronchoalveolar lavage fluid (BALF) levels are significantly reduced in patients suffering from acute respiratory distress syndrome (ARDS), suggesting a protective role in this disease (470). In contrast, IL-22 seems to play a pathogenic role in rheumatoid arthritis (RA). IL-22 is produced mainly by T cells, NK cells, macrophages, and synovial fibroblasts in RA, and increases proliferation of synovial fibroblasts as well as their chemokine production (467). In RA, the majority of IL-22R1-positive cells are synovial fibroblasts, highlighting the importance of IL-22 in this autoimmune condition. Furthermore, IL-22KO mice are less susceptible to collagen-induced arthritis (CIA) induction and progression with significantly less cytokine and chemokine production in affected tissues and joints (471). Therefore, IL-22 seems to be a crucial target for therapy in arthritic disease (472).

2.7.6. Induced Regulatory T (Treg) cells

Induced Tregs (iTregs) are produced when naïve T cells upregulate FoxP3 upon activation. This distinguishes them from naturally-occurring Tregs (nTregs) that express FoxP3 as thymocytes undergoing T cell development (473,474). Despite their similar immunoregulatory effector functions iTregs and nTregs are functionally non-redundant, though their relative importance in different disease states is not well understood (475,476). For example, nTregs originate from the thymus and are selected for recognition

of self-antigen, whereas iTregs are derived from peripheral conventional T cells that recognise foreign antigen. They should therefore be recruited by different signals to different targets (477,478).

In the periphery iTregs develop upon exposure to TGF-β (which is boosted by retinoic acid) and IL-2 and are inhibited by IL-6, IL-21, and IL-23 (479-482). Aryl hydrocarbon also increases FoxP3 expression (483). iTregs are increased by unfavourable T cell activation conditions, such as antigen presentation by immature DCs, or antigen persistence (484,485). The amalgamation of signals determine the level of FoxP3 transcription: IL-2 induces STAT5 nuclear translocaton that binds to enhancer elements near the FoxP3 promoter (486), TGF-β signalling activates Smad3 (487), whereas STAT3 activation induced by IL-6 and IL-21 competitively inhibit STAT5 binding (482). Despite this iTregs require STAT3 and RORγt to suppress Th17 cells, as they require T-bet, IRF-4, and Bcl-6 to suppress Th1, Th2, and Tfh cells respectively (488). Thus, iTreg formation and activity are balanced by homeostatic and inflammatory signals.

iTregs are particularly important in immunoregulation of the gut because there are few nTregs present that will have been selected on intestinal or microbial antigens in the thymus (489). iTregs have been detected in the gastrointenstinal tract and been shown to protect against the development of colitis in CNS-1-deficient mice (490). These mice lack a conserved nucleotide sequence in the FoxP3 gene that is required for iTreg but not nTreg development.

The role of iTreg in disease of the central nervous system (CNS) are unclear. Both iTreg and nTreg have been shown to be protective against EAE in mice whereas their deletion exacerbates disease (491). However, in CNS-1-deficient mice (which lack iTreg) no difference was observed compared to control animals (492). There are similar unclear effects of iTregs

in diabetes. iTreg are induced in Non Obese Diabetic (NOD) mice when immunised with insulin, and transfer of these blocks disease in recipient mice (493). However, induction of iTreg by administration of DCs expressing an insulin mimitope failed to affect disease progression but did reduce incidence (494). Further, treatment of mice with All trans retinoic acid (ATRA) reduced insulitis, however no significant protection was conferred by treatment in mice deleted of nTregs, suggesting iTregs did not confer protection (495). Further investigation in these disease models is necessary to confirm the role iTregs play in these diseases.

The environments of tumours are commonly immunosuppressive and infiltrated with regulatory T cells (496). However, whether these are iTregs or nTregs appears to be tumour-dependent. In studies of MO-5, TC-1, and murine glioblastoma (GBM) there was no significant overlap in TCR repertoires between effector and regulatory T cells, suggesting the Tregs were not derived from antigen-specific T cells and were derived from nTregs (497-499). However, another study using B16F1 melanoma in mice, and melanoma in humans, suggested considerable overlap and iTreg involvement (500). Further study of the driving factors of iTreg development in different tumour environments is required to understand why such variability exists.

2.7.7. Follicular T helper (Tfh) cells

Tfh cells are a recently discovered CD4 T cell subset that are specialised in supporting germinal centre (GC) formation, B cell activation, and antibody production (501). As for other CD4 T cell subsets, Tfh differentiation requires a distinct set of cytokine and costimulatory signals that drive transcription and expression of specific transcription factors (501). For Tfh cell differentiation, IL-6, IL-21, and IL-27 are all capable of increasing

expression of the transcription factors Bcl-6 and c-maf, the cytokine IL-21 (by a positive feedback loop), and the surface molecules ICOS, OX40, CD40L, BTLA, and PD-1 (502,502-504). It is unclear if there is a hierarchy between these cytokines, or whether they are equally effective at inducing Tfh differentiation as conflicting data has been generated between studies. It is likely that some functional redundancy exists between them. Likewise, failure to engage ICOS, CD40L, and CD28 results in reduced Tfh differentiation (505,506). The SLAM family of receptors is also important as these utilise SAP as part of their signalling pathway, and SAP is essential for Tfh differentiation as it is instrumental for interaction with B cells (507). These factors are all important for optimal B cell activation, proliferation, differentiation, and antibody production, confirmed by studies in knockout or loss-of-function mouse strains. There is a strict requirement for expression of ligands for the surface molecules on the surface of APCs, but the kinetics of the interactions differ for different APCs. Using deletion studies, it has been demonstrated that initial Tfh differentiation is provided by naive T cell interaction with DCs (502). However, it is equally clear that for complete differentiation to occur these same T cells must interact with antigen-specific B cells (508,509). This suggests that the initial differentiation signals are provided by DCs in secondary lymphoid organs resulting in upregulation of surface CXCR5 expression, and loss of CCR7 expression, on T cells. This enables translocation of these cells to the B cell follicles where they undergo full differentiation (510).

As for other T cell subsets, Tfh cell commitment is characterised by expression of specific transcription factors. For Tfh cells this is Bcl-6, a transcriptional repressor (511). Several studies have demonstrated the essential requirement for Bcl-6 in Tfh development, where overexpression increases Tfh numbers and deficiency reduces them (512,513). Bcl-6

expression in activated CD4 T cells inhibits expression of other signature transcription factors (e.g. T-bet, GATA-3, RORγt) that direct differentiation towards other subsets (e.g. Th1, Th2, Th17) (512-514). However, Bcl-6 alone cannot fully differentiate naive CD4 T cells into Tfh cells, other transcription factors are also necessary. c-maf, a transcription factor found in Th2 cells, is also important because it increases IL-21 and CXCR5 expression (515). c-maf expression is also induced by IL-6, IL-21, and IL-27 which supports the idea that co-expression of Bcl-6 and c-maf increases Tfh differentiation. Moreover, expression of c-maf in Bcl-6-expressing CD4 T cells increases CXCR5, ICOS, and PD-1 expression compared to either factor alone (503).

As Bcl-6 and c-maf expression drive Tfh differentiation, so there exist counter mechanisms that inhibit it. This is crucial to prevent aberrant Tfh responses that may predispose to autoimmune conditions, particularly those driven by autoantibodies. The primary counter mechanism is expression of a transcriptional co-repressor, Blimp-1, often driven by IL-2 production and STAT5 expression (516). Inhibition of any of these factors increases Tfh differentiation whereas overexpression has the opposite effect (517,518). The mechanism of inhibition is binding of Bcl-6, blocking its activity and both Blimp-1 and STAT5 have both been shown to bind Bcl-6 (519). Regulatory T cells also inhibit Tfh cells as they do for other CD4 T cell subsets, expressing cellular factors associated with each subset. For example, regulatory T cells that express T-bet specifically inhibit Th1 cells, whereas those that express IRF-4 inhibit Th2 cells (520). For Tfh cells, there is a subset (10-15%) that express FoxP3, suggesting this is the regulatory population for this subset (521). These follicular regulatory T cells share features of both Tfh cells and regulatory T cells, but they do not express CD40L or IL-21 and are derived from naturally occurring regulatory T cells rather than naïve CD4 T

cells (522). Bcl-6 or CXCR5 deletion prevents development and follicular localisation of these cells respectively, which resulted in increases in B cell activation and antibody production (521,523). Therefore, this population is essential for effective Tfh cell regulation. Plasma cells are also thought to inhibit Tfh differentiation as Tfh numbers are increased in plasma-cell-deficient mice and naïve CD4 T cells activated by plasma cells do not upregulate Bcl-6 or IL-21 expression (524). However, the mechanism by which this occurs is unknown.

2.7.8. T cell plasticity

T cell differentiation was initially considered to be a permanent alteration and commitment to a particular transcriptional program and distinct set of effector functions. However, it has become increasingly clear that differentiation is a fluid, dynamic process with T cells switching from one subset to another depending on their environment and cellular interactions (525). Gene expression early (1-3d) after T cell activation is dependent upon the open chromatin structure and accessible, unmethylated genes that will drive T cell differentiation (e.g. *tbx21*, *gata3*, *rorgt*) (526,527). In uncommitted T cells these genes remain active, and transcription continues even as T cells commit to a particular lineage. As a result, 'intermediate' T cell populations exist that express more than one specific transcription factor (e.g. T-bet and Bcl-6, GATA3 and RORγt) (514,528). Therefore, even when a T cell is considered 'differentiated' it remains capable of switching to an alternative lineage if environmental conditions dictate this to be necessary.

T cell differentiation is also affected by the nature of the pathogen and infection process. Acute infectious pathogens induce Th1, Th2, and Th17 differentiation corresponding to increased expression of T-bet, GATA-3, and RORγt (514). Consequently, Bcl-6 expression is

restricted until antigen levels are reduced, and pro-inflammatory transcription factors are no longer required and must be inhibited to prevent unwanted tissue damage. Once transcription factor levels decrease then Bcl-6 expression is no longer repressed. However, in chronic infections the persistent antigen levels inhibit pro-inflammatory transcription factor expression and therefore Bcl-6 expression increases (529). This cross-regulation has been observed most clearly in comparison of acute and chronic LCMV infection (529). Therefore, T cell differentiation is not absolutely controlled by intracellular signalling pathways controlled by master transcription factors but also by the nature of pathogen invasion, and interplay between other immune factors.

2.8. The γ_c chain cytokine family

The γ_c chain cytokine family are a group of type I cytokines that all utilise the γ_c chain (IL-2Rγ chain; CD132) as part of their receptor. There are currently six members: IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, and one pseudo member: TSLP [Fig.6; (530)]. TSLP is a pseudo member as it utilises IL-7Rα chain, but not CD132, as part of its receptor. As it does not use CD132 it will not be discussed here any further. The family is renowned for its importance in T cell development and function as humans who have a non-functional, mutated form of CD132 have no functional T cell compartment (531). This condition, referred to as X-linked severe combined immunodeficiency (X-SCID), has other symptoms beyond those of T cells. Sufferers also possess no functional NK cells and produce no antibodies as B cell development and function is crippled. Therefore, the γ_c chain cytokine family are critical regulators of elements of both innate and adaptive immunity. Despite all signalling through the γ_c chain, each cytokine has different functions and this is attributed to the STAT (signal

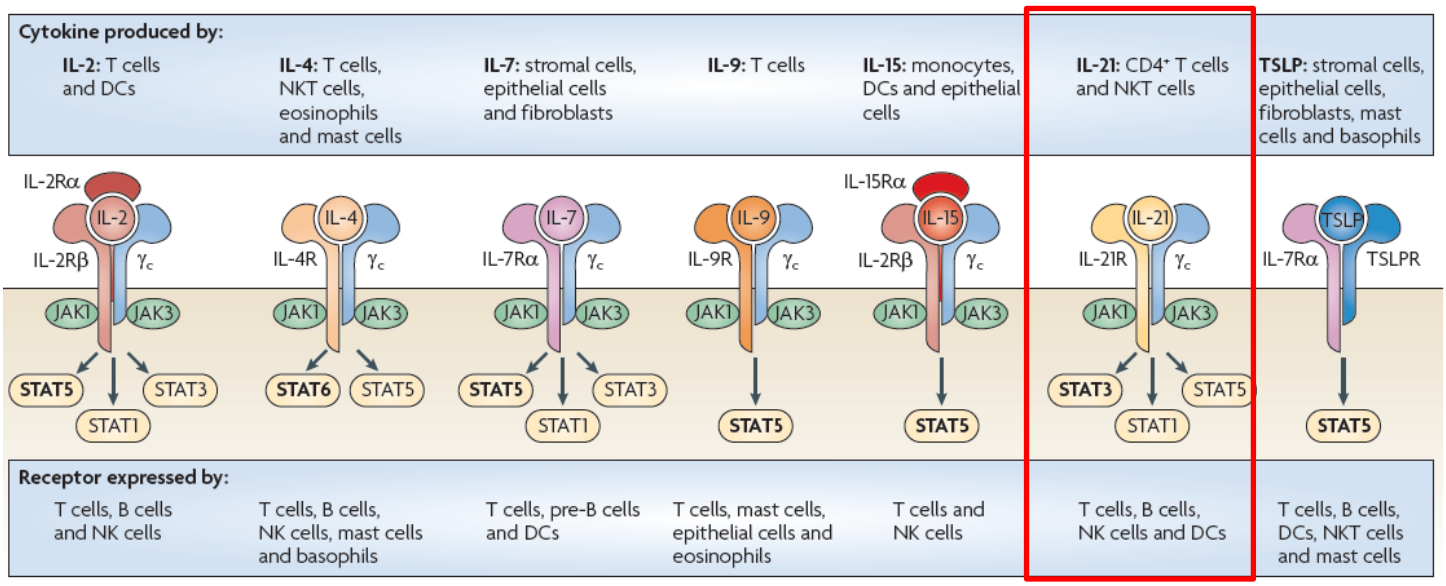

Figure 6. The γ_c chain cytokine family. This family consists of six full members: IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (highlighted) and one pseudomember: TSLP. Apart from TSLP all utilise the γ_c chain as a component of their receptor. All members also use a unique second chain that forms their heterodimeric receptor. IL-2 and IL-15 also use IL-2Rβ chain to form a heterotrimer. Receptor expression and the downstream JAK and STAT signalling molecules that each receptor uses are shown. Cell types that produce each cytokine are also shown (adapted from Rochman Y et al. Nature Rev Immunol 2009 9 480).

transducer and activator of transcription) proteins that they activate. For example, IL-2, IL-7, IL-9, and IL-15 signal via STAT5a and 5b, whereas IL-4 primarily signals via STAT6, and IL-21 signals via STAT3. These differences in signalling may account for the observed differences in function.

2.8.1. Interleukin (IL)-2

IL-2 is the prototypical member of the family as it was the IL-2 receptor where the γ_c chain was first identified (532). The IL-2 receptor is a heterotrimer consisting of IL-2Rα chain (CD25), IL-2Rβ chain (CD122), and CD132. As for all family members, the α chain confers cytokine specificity and the β and γ chains maximise intracellular signalling. IL-2 is produced by T cells (533) was first named T-cell growth factor (TCGF) because of its ability to induce naïve and memory T cell proliferation (534). However, it can also elicit activation-induced cell death [AICD; (535)] of T cells, and aid regulatory T cell development in a STAT5-dependent manner (536,537). Therefore, IL-2 has both pro-inflammatory and anti-inflammatory properties. It also affects other cell types, boosting cytolytic activity of NK cells, and antibody production by B cells (538).

IL-2 is one of the first cytokines produced after T cell activation, and is therefore important in T cell differentiation. It is known to be essential for Th2 differentiation (539,540), yet despite being one of the main cytokine products of Th1 cells it is unclear if it is required for Th1 differentiation (541). As described earlier, IL-2 activates STAT5a and STAT5b signalling proteins upon binding to its receptor and these proteins increase transcription of the *il4ra* gene (540). This increases responsiveness of activated T cells to IL-4, making them more likely to differentiate into Th2 cells. Moreover, activated STAT5 proteins bind to consensus

sequences within DNase-I-hypersensitivity sites throughout the entire Th2 locus, allowing greater access for the transcriptional machinery to bind to the promoters of genes such as *il4*, *il13*, *sept8*, *kif3a*, and *irf1*. Therefore, IL-2 not only enhances responsiveness to IL-4 but also increases production of Th2 cytokines and associated factors (539,540). IL-2 is also known to inhibit Th17 differentiation as IL-2KO mice have an increased frequency of IL-17 production (542,543).

Due to its ability to induce T cell proliferation, IL-2 has already been tested therapeutically to expand T cell populations in HIV (544), and cancer (545), patients.

2.8.2. IL-4

IL-4 is produced by activated T cells (546), NKT cells (547), eosinophils (548), and mast cells (549). It is regarded as the classical Th2 cytokine as it is required for the development and function of Th2 cells. As such it is a crucial factor in the development of asthma and allergy (550), and plays a crucial role in antibody isotype class switching (551). It increases differentiation of DCs from progenitor cells in the presence of Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), and enhances survival of DCs in the periphery (552). However, in the presence of IL-4 DCs maintain an immature state with reduced expression of MHCII and costimulatory molecules, possibly by reducing their responsiveness to type I IFNs (553).

2.8.3. IL-7

IL-7 is a homeostatic cytokine, and is unusual among the γ_c cytokines in that it is constitutively produced by stromal and epithelial cells (554), as well as by fibroblastic reticular cells in the T cell zone of the secondary lymphoid organs (555). However, it is continuously consumed by naïve and memory T cells to maintain their survival; therefore the IL-7 concentration is dictated by the number of T cells consuming it. For example, in lymphopaenic patients the IL-7 concentration is raised (e.g. AIDS patients) and decreases upon increased T cell numbers (e.g. AIDS patients undergoing HAART therapy) (556). Another unusual aspect of IL-7 function is that expression of its receptor (IL-7Rα, CD127) is the reverse of receptors for other γ_c chain cytokines. For the other family members, receptor expression is low on inactive T cells and is upregulated upon T cell activation (e.g. IL-2Rα, CD25). However, IL-7Rα expression is high on inactive T cells (e.g. naïve and memory T cells) and decreases upon T cell activation (557,558). This supports the idea that IL-7 acts mainly on naïve and memory T cells rather than on activated, effector T cells.

IL-7 has a central role in T cell development in both human and rodent species as studies in SCID patients and those with mutations in *il7rα* (559) or *jak3* [JAK3 is a signalling component that is recruited to the γ_c chain; (560)] genes have identified the lack of IL-7 signalling as the reason for the lack of T cells. Further, it also aids memory T cell development and survival after pathogen challenge (554,561). IL-7 enhances T cell survival in two ways; it promotes the expression of anti-apoptotic factors (e.g. Bcl-2 and Mcl1) and reduces expression of pro-apoptotic factors (e.g. Bax and Bad) (562). IL-7 is also important for the development of B cells in rodent species, but not humans, indicating functional differences between species (563).

Based on its ability to enhance both naïve and memory T cell growth and survival, IL-7 (like IL-2) is currently being tested in candidate therapies against HIV (564).

2.8.4. IL-9

IL-9 (originally P40) is produced by activated CD4 T cells (455,456) and is a critical factor in mucus production (565) and mast cell activation (566). It also activates epithelial cells (567,568), T cells (569), B cells (570), and eosinophils (571), and with IL-4 is regarded as a potent inducer of asthma and allergic inflammation (572). More recent studies have highlighted an important role for IL-9 in pathological Th2 responses to Respiratory Syncytial Virus (573).

2.8.5. IL-15

IL-15 is produced by epithelial cells (574), monocytes (575), and DCs (576). It has an essential role in NK cell development: the NK cell deficiency in X-SCID and mutated JAK3 patients is attributed a lack of signalling by this cytokine (563). IL-15 is also an important homeostatic cytokine in memory CD8 T cell maintenance (554), less so in memory CD4 T cell maintenance (577). However, unlike IL-7, IL-15 is not essential for T cell development though it does aid survival of naïve and memory CD8 T cells (578,579). Curiously, IL-15 increases CD8 T cell proliferation and survival when it is 'trans-presented' to them (580). Specifically, IL-15 bound to IL-15Rα on non-T-cells is presented to CD8 T cells expressing the IL-2Rβ and IL-2Rγ chains.

Based on its abilities to boost NK cell and CD8 T cell activity, IL-15 is currently being tested as an adjuvant in vaccine design (545).

2.8.6. IL-21

As IL-21 is the focus of my studies it will be described in greater detail.

2.8.6.1. Sources

IL-21 is a type I cytokine, the newest member of the γ_c chain cytokine family with IL-2, IL-4, IL-7, IL-9, and IL-15. It was cloned and identified as the ligand for IL-21R, which was identified in 2000, and its amino acid sequence most closely resembles IL-2, IL-4, and IL-15 (581). The *il21* gene is adjacent to the *il2* gene and they share very similar gene structure; suggesting one was created by the duplication of the other (581). The source of IL-21 was the CD4 T cell; however subsequent studies more accurately specified that NKT cells (582), Th17 cells (583), and follicular helper T cells [T_{FH} cells; (511)] produce IL-21.

2.8.6.2. Receptor expression

The *il21r* gene was originally identified during genome sequencing as an open reading frame (ORF) encoding a type I cytokine receptor. It is located on human chromosome 16, immediately downstream of the *il4ra* gene (584), and its amino acid sequence most closely resembles the IL-2Rβ chain. Taken together with its restricted expression on haemopoietic cells, these findings suggested that IL-21R belonged to the γ_c chain cytokine receptor family. As for all true members of this family, the γ_c chain (CD132) is essential for signalling to occur once IL-21 has bound IL-21R (585).

IL-21R expression is greatest on B cells, and is expressed constitutively by some B cell lines (586). CD4 and CD8 T cells have low levels of expression, but it is upregulated upon cognate antigen recognition (584). It has also been identified on NK cells, DCs, macrophages, and keratinocytes (584,587).

Upon binding of IL-21 to IL-21R, the cytokine:receptor complex heterodimerises with the γ_c chain. This surface interaction brings the signalling domains on intracellular chains into close proximity, such that phosphorylation at specific tyrosine (Y) residues, allows recruitment of the Janus kinase signalling proteins Jak1 and Jak3 (584,588). Subsequent activation of Jak1 and Jak3 by phosphorylation results in activation of signal transducer and activator of transcription (STAT) proteins. One is STAT3 but STAT1 and STAT5 can also be weakly activated (584,589). This results in phosphorylation and homodimerisation of STAT proteins; followed by their translocation to the nucleus where they bind specific target gene promoter elements. Mutation analysis of the six tyrosine residues on the intracellular domain of IL-21R identified residue Y510 as crucial for STAT1 and 3 activation in CD8 T cells; as well as for optimal B cell proliferation (590). The downstream signalling events are less well characterised, but IL-21/IL-21R interaction has been shown to activate the p52 isoform of Shc (an initiator of the MAPK pathway), and the serine/threonine kinase Akt, a downstream mediator of the PI3K pathway (590).

2.8.6.3. Effects

There has been extensive research into the effects of IL-21 on different immune cell types in several different *in vitro* and *in vivo* models [Fig.7; (591)]. However, a true and complete

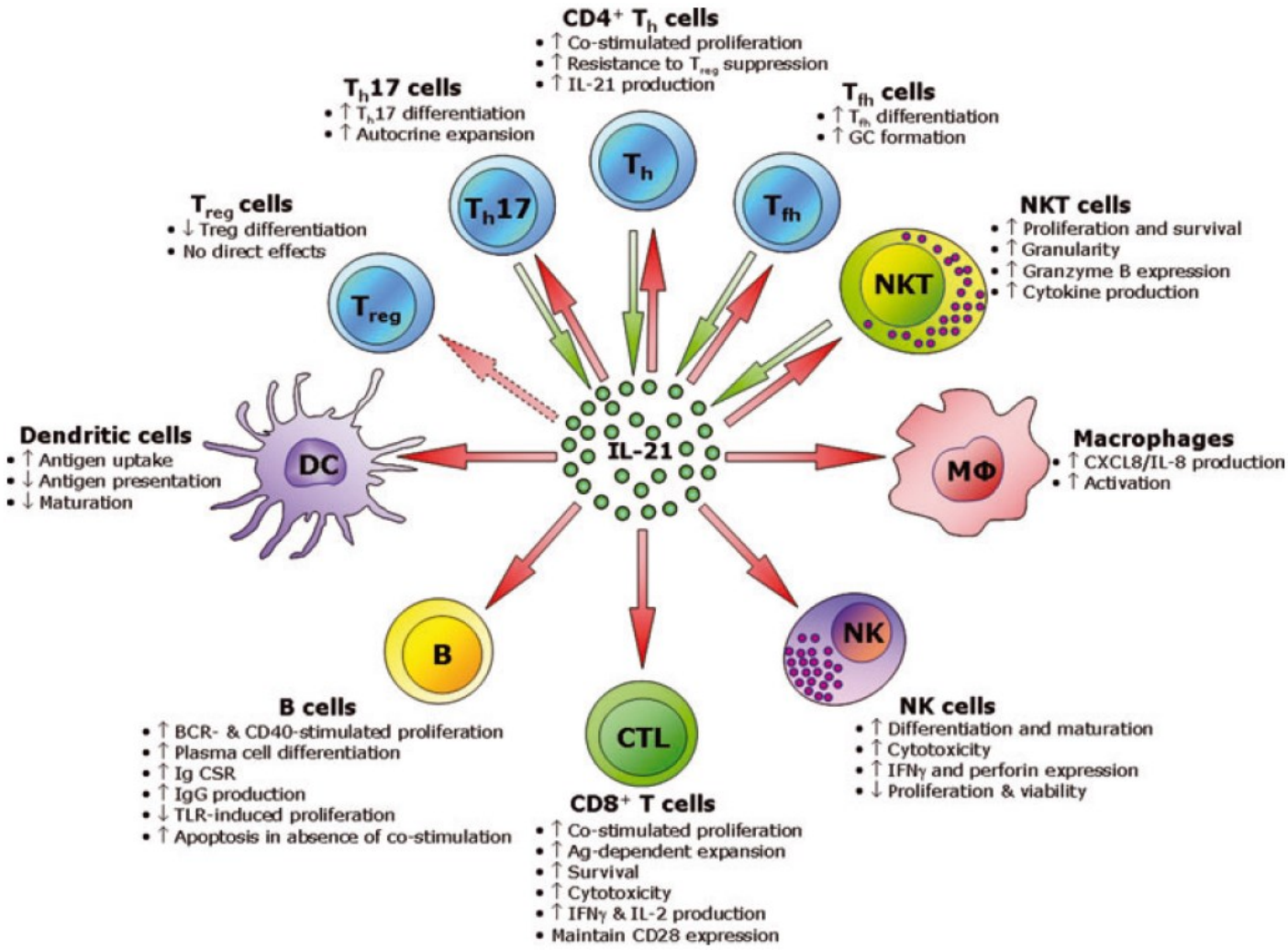

Figure 7. The major effects of IL-21. IL-21 affects a wide range of myeloid and lymphoid immune cells but the effects depend on other signals in the environment. B cells are activated by IL-21 upon BCR ligation, but IL-21 induces B cell apoptosis on its own. IL-21 activates CD8 T cells and NK cells synergistically with IL-15. IL-21 regulates both NKT cell and CD4 T cell activation and differentiation. IL-21 inhibits DC maturation but activates macrophages. IL-21 also reduces the effectiveness of regulatory T cell action (taken from Sondergaard H et al. Tissue Antigens 2009 74 467).

understanding of IL-21 function has been hampered by its context-dependent effects; which will be explained below.

2.8.6.3.1. CD4 T cells

Early studies indicated that IL-21 was mainly produced by Th2 CD4 T cells, and was therefore a Th2 cytokine (592). This was confirmed both *in vitro* by stimulation of naïve CD4 T cells with PMA/Ionomycin in the presence of Th1- or Th2-skewing conditions, and *in vivo* using the *Leishmania major* model (592). However, while IL-21 reduced IFN-γ (but not IL-2 or TNF) production, it did not further promote Th2 cell differentiation, distinguishing it from other Th2 cytokines like IL-4. Moreover, IL-21 did not inhibit expression of the Th1 transcription factor T-bet (592), it inhibited IFN-γ production in an IL-4-dependent manner by reducing STAT4 (592) and Eomes (593) expression. However, one of the most important effects of IL-21 on CD4 T cell function to be elucidated is its effect on the differentiation of naïve CD4 T cells into the Th17 lineage. Three separate studies published in July and September 2007 (two in the same issue of the Nature journal) demonstrated that IL-21 was produced by Th17 CD4 T cells and acted in an autocrine manner to enhance its own production and maintain Th17 development (386,446,583). Th17 differentiation occurs when naïve CD4 T cells are activated in the presence of TGF-β and IL-6, and upregulate expression of the *rorc* (retinoic-acid-related orphan receptor γ) gene (594). IL-6 is crucial for Th17 differentiation as TGF-β alone promotes differentiation of $Foxp3^+$ regulatory CD4 T cells. This was confirmed in IL-6KO mice. However, Th17 cells re-appear when these regulatory CD4 T cells are depleted suggesting that these cells play an active role in inhibiting Th17 differentiation.

Gene expression analysis of *in vitro* generated Th1, Th2, and Th17 cells demonstrated that all three subsets expressed the *il21* gene. However, the greatest expression was observed in Th17 cells (583). IL-21 alone does not induce Th17 development [though it does upregulate expression of *il23r*, *rorc*, *il17*, *il17f*, and *il22* genes (583)]; it works synergistically with TGF-β. IL-6, but not TGF-β, induces production of IL-21 in a STAT3-dependent manner. This was confirmed in IL-6KO mice when activated CD4 T cells do not produce IL-21 or differentiate into Th17 cells. However, CD4 T cells from IL-6KO mice differentiated into Th17 cells normally upon activation in the presence of TGF-β and IL-21, suggesting that IL-21 acts independently of IL-6 once it is produced (583). Further studies in STAT3KO and RORγKO mice demonstrated that STAT3, but not RORγ, was crucial to IL-21 production (583). Th17 differentiation of activated STAT3KO and RORγKO CD4 T cells in the presence of TGF-β and IL-21 was greatly reduced, demonstrating that both these factors are essential to IL-21-driven Th17 differentiation. The same phenotype was also observed when CD4 T cells from IL-21KO mice were tested; Th17 differentiation could be rescued when IL-21 was added exogenously (583). Another downstream effect of IL-21 signalling is the expression of *il23r* mRNA and IL-23R protein (446). Though IL-23 is incapable of inducing Th17 differentiation on its own, it maintains RORγt expression, thus promoting development of the Th17 lineage. The ICOS:ICOSL interaction is crucial for IL-21 action. Studies performed in ICOSKO, ICOSLKO, and c-mafKO mice demonstrated that signalling through ICOS activated c-maf, a transcription factor crucial for the production of IL-21 (515). Therefore, though ICOS:ICOSL interaction is not important for early Th17 differentiation (i.e. the action of TGF-β and IL-6) it is crucial for the later maintenance of Th17 development by IL-21 and IL-23. This was confirmed in deficient mice by demonstrating a lack of c-maf activation, IL-21 production, IL-23R expression, and optimal IL-17 production (515).

In vivo studies have demonstrated the same phenotype, Th17 differentiation is abrogated in the spleens and intestinal lamina propia of IL-21KO mice (446,583). Extensive *in vivo* studies of Th17 differentiation and function have been performed in the mouse model of multiple sclerosis, EAE (Experimental autoimmune encephalomyelitis). Immunisation of mice with MOG_{35-55} (myelin oligodendrocyte glycoprotein) peptide results in a response against the CNS and an autoimmune disease characterised by paralysis of the limbs (583). 5 days after two immunisations WT mice started to exhibit signs of disease that peaked on day 11. In IL-21KO mice disease kinetics was identical but magnitude was reduced suggesting that IL-21 was important for its development. Splenic CD4 T cells isolated from WT mice produced significant levels of IL-17, in IL-21KO mice however these cells produced IFN-γ but no IL-17 (583). A similar defect in Th17 differentiation and disease reduction was observed in IL-6KO mice (386).

Another important function of IL-21 is its role in T follicular helper (Tfh) cell development and function. Tfh cells are a CD4 T cell subset that resides in the secondary lymphoid organs, particularly the spleen. They are critical for optimal germinal centre reactions where B cells are activated, and memory B cell development, as well as plasma cell differentiation. They express the CXCR5 chemokine receptor (595) which binds CXCL13 and localises them close to germinal centres (596). They also express inducible costimulatory receptor (ICOS) which is essential for Tfh cell development (597) and activation of B cells via interaction with ICOSL. ICOS deficiency in humans causes a striking reduction in the number of Tfh cells (598). PD-1 is also highly expressed by Tfh cells (599,600). Like other CD4 T cell subsets that express signature transcription factors, Tfh cells express the transcriptional co-repressor Bcl6, but express high levels of IL-21 rather than IFN-γ, IL-4, IL-10, or IL-17 (599). IL-21 acts in an

autocrine manner on Tfh cells (600), and is important to Tfh cell function as it activates B cells and induces isotype class switching (587). In the absence of IL-21 or IL-21R, few Tfh cells develop after protein immunisation, and as a consequence there is reduced germinal centre B cell activation and antibody production (599). In comparison conventional CD4 T cell functions (e.g. proliferation, IFN-γ production) are normal. The same phenotype is observed in IL-6KO and IL-6RKO mice (599), and as IL-6 is known to induce production of IL-21 (as described above) this suggests that there is an integral link between these two factors, Tfh development, and optimal B cell responses.

2.8.6.3.2. CD8 T cells

In contrast to IL-2 and IL-15 which drive acquisition of effector functions by CD8 T cells, *in vitro* priming of CD8 T cells with specific antigen and IL-21 suppresses the production of IFN-γ, granzyme B and expression of IL-2Rα (601). Expression of the *eomes* gene is also suppressed in CD8 T cells by IL-21. *Eomes* is indicative of induction of cytolytic function, again suggesting that IL-21 inhibits effector functions in CD8 T cells. Moreover, IL-21 suppresses antigen-induced expansion and differentiation of CD8 T cells into memory [as measured by CD44 expression (602)]. Therefore, IL-21 possesses potent negative regulatory effects on *in-vitro*-activated CD8 T cells.

Microarray analysis of gene expression by CD8 T cells primed with antigen and IL-21 demonstrated that IL-21 initiates a distinct genetic program compared with IL-2 and IL-15. Specifically, IL-21 upregulates expression of the genes *Lef1, Sell* (L-selectin)*, Itgae,* and *Tcf7* (associated with immature CD8 T cells; (603)) and downregulates *Gzmb, Il2ra, Ifng,* and

eomes (associated with mature effector CD8 T cells). This is believed to occur because IL-21 primarily signals via STAT1 and 3 whereas IL-2 and IL-15 signal via STAT5a and b (590). Nevertheless, adoptive transfer of IL-21-primed CD8 T cells caused tumour regression in mice, whereas no protection was evident when IL-2- or IL-15-primed CD8 T cells were transferred (601). This is thought to be due to expression of L-selectin by IL-21-primed CD8 T cells which allow migration to the lymph nodes, greater cell expansion, and acquisition of effector function *in vivo*. In contrast, IL-2 or IL-15-primed CD8 T cells lose expression of L-selectin, do not expand or increase in effector function, and are thus unable to kill tumours. Chronic viral infection (e.g. HIV, HBV, HCV, and LCMV) is associated with persistent viral antigen exposure, and continuous T cell activation resulting in clonal deletion or anergy (604). Several models of chronic viral infection have demonstrated that IL-21 is crucial for sustained CD8 T cell responses (605), via CD4 T cell help (606). Studies have shown that in the absence of IL-21, despite enhanced CD4 T cell responses, CD8 T cells are deleted, exacerbating viral persistence. For example, during chronic LCMV infection high levels of IL-21 mRNA are expressed by virus-specific CD4 T cells, whereas IL-2 mRNA is extinguished (605). When WT and IL-21RKO mice were chronically-infected with LCMV no difference in CD8 T cell recruitment or function (IFN-γ and TNF production) was observed during the acute phase (day 8 p.c) of the response. However, during the chronic phase (day 30 p.c) there was a significant decrease in the number of responding virus-specific CD8 T cells in IL-21KO (607) and IL-21RKO mice (605,608) compared to WT controls. Further, the CD8 T cells in IL-21KO and IL-21RKO mice were PD-1^{hi} (indicative of exhaustion), whereas those from WT mice were PD-1^{lo} (607,608). This suggests that IL-21 signalling is crucial to sustain CD8 T cell responses during chronic infection. To confirm this, administration of IL-21 to IL-21KO mice could restore CD8 T cell responses to LCMV, reducing viral titres (607). Reconstitution

of irradiated WT mice with Ly5.1$^+$ WT and Ly5.2$^+$ IL-21RKO bone marrow allowed both WT and IL-21RKO CD8 T cells to respond to chronic LCMV infection in the same host. During the acute phase of the response there was no difference in the number of responding WT or IL-21RKO CD8 T cells. However, by the chronic phase there were significantly more WT than IL-21RKO CD8 T cells responding, demonstrating that IL-21 sustains the CD8 T cell response by acting directly on the cells themselves (605). These differences were not evident when mice were challenged with an acute LCMV strain or other non-persistent viruses (e.g. influenza, vaccinia), suggesting this role of IL-21 in sustaining CD8 T cell responses is restricted to chronic infection.

2.8.6.3.3. B cells

Several studies have highlighted the indirect effect of IL-21 on B cells and antibody production by promoting follicular helper T cell development and function. However, B cells express high levels of IL-21R and can respond to IL-21 directly (586). For example, Ozaki *et al* demonstrated that IL-21 increased B cell proliferation induced by anti-IgM, especially in the presence of anti-CD40. However, this increase required a combination of signals as in the absence of either B cell receptor or anti-CD40 stimulation, IL-21 induced much less proliferation (609). IL-21 also induced expression of Syndecan-1 (CD138, a plasma cell marker) and surface IgG1 on B cells stimulated with anti-IgM antibody. However, only a fraction of the surface IgG1$^+$ cells expressed CD138, indicating that IL-21 increased post-switch B cells as well as plasma cells.

In contrast to its effects on B cell proliferation, IL-21 also induces the apoptosis of primary resting and activated B cells (609,610). Moreover, IL-21 can induce the apoptosis of primary

B cells even in the presence of costimulatory factors such as IL-4, LPS, or anti-CD40 Ab. IL-21-induced apoptosis was shown to correlate with a down-regulation in the expression of Bcl-2 and Bcl-x_L, two antiapoptotic members of the Bcl-2 family (610). However, the latter study found that while mRNA levels for these anti-apoptotic proteins decreased, protein levels remained the same (609). This suggests that other mechanisms may be important. These changes in B cell maturation correlate with changes in transcription factor expression by responding B cells. For example, Blimp-1 is a transcription factor that has been identified as a master regulator of plasma cell differentiation (611), whereas Bcl-6 and Pax5 are required for germinal centre formation (612). Interestingly, Blimp-1 and Bcl-6 can each inhibit expression of each other, and Blimp-1 additionally is an inhibitor of the expression of Pax5 (613). Ozaki *et al* found that IL-21 induced expression of both Blimp-1 and Bcl-6 mRNA in purified splenic B cells, whereas it inhibited expression of Pax5 mRNA, when stimulated with anti-IgM antibody (609). This was confirmed at the protein level by western blotting. Other transcription factors are known to drive plasma cell or memory B cell differentiation. Specifically, IRF-4, and XBP-1 drive plasma cell differentiation (614), and with Blimp-1 these factors inhibit expression of Bach2, MiTF (615,616), and IRF-8 (617). These factors together with Bcl-6 promote memory B cell differentiation. However, it is unknown what effect IL-21 has on expression of these additional factors.

The changes in transcription factor expression attributed to IL-21 have yet to be traced through the signalling pathways elicited by IL-21 binding to its receptor. For example, Zhou *et al* demonstrated that Bcl-6 expression is upregulated by IFN-γ-mediated activation of STAT1 (618). However, this study used Jurkat T cells rather than B cells and it is unclear if the same effect would result in a different cell type. IL-21 is known to induce STAT3 activation and STAT3 is necessary for T-cell-dependent IgG plasma cell differentiation (619).

Additionally, it has been shown that expression of activated STAT3 in fibroblasts leads to upregulation of Bcl-6 expression (620). Therefore, STAT3 activates Bcl-6 in different cell types. Unfortunately, STAT3 activation was not correlated with the expression of Blimp-1 or XBP-1 in these studies but as they are universally required for plasma cell development, it is likely their activation patterns are similar. A role for STAT5 is more controversial as conflicting data have been published. First, Scheeren *et al* demonstrated that in human B cells phosphorylated STAT5 directly induces Bcl-6 expression and that ectopic expression of STAT5 or Bcl-6 in peripheral B cells increased their self-renewal capacity. However, soon afterwards Walker *et al* showed that Stat5 represses Bcl-6 expression in B-lymphoma cells and other haematopoietic cells (519). This difference was attributed to the STAT5-binding site which is not highly conserved among human, rat, and mouse species. Therefore, STAT5 may upregulate Bcl-6 in some cell types of one species and downregulate Bcl-6 expression in the same cell types of a different species. A further study by Kuo *et al* demonstrated that STAT5 mRNA was also increased in human memory B cells and decreased in plasma cells generated in vitro (621). The authors from this study concluded that STAT5 activation negatively correlates with Bcl-6 expression which was low in both cell types. However, none of these studies investigated the effect of IL-21 on STAT5 activation and it remains unclear what molecular mechanisms are used by IL-21 to elicit the observed effects on B cells.

2.8.6.3.4. Dendritic cells (DCs)

Compared to lymphocytes, there have been relatively few studies investigating the effect of IL-21 on development and function of DCs. Like B cells, they express IL-21R and are able to respond to IL-21 in the environment. However, unlike its γ_c cytokine family counterpart IL-

15 [for which DCs express both cytokine (576) and its specific receptor IL-15Rα (622)] IL-21 does not activate DCs, it maintains them in an immature state and suppresses their T-cell stimulatory capacity (622). This has been demonstrated most simply with bone-marrow-derived DCs (BMDCs) which are generated from bone marrow cells grown in the presence of GM-CSF (623). IL-21 does not inhibit the development of BMDCs when added to culture but does reduce their expression of the IL-2Rα and β chains compared to IL-15 (622). It also reduces the expression of MHCII, which is necessary to activate antigen-specific CD4 T cells, and the chemokine receptor CCR7, which is crucial for migration of DCs to the draining lymph nodes where naïve T cells reside (622). Further, DCs cultured in IL-21, but not IL-15, displayed increased antigen uptake (in this case FITC-conjugated dextran particles). Therefore DCs are maintained in an immature state in the presence of IL-21. In contrast, IL-15 matures DCs resulting in their inability to capture antigen and increased capacity to present antigen in the context of MHC molecules (622). These phenotypic and functional differences were maintained even when DCs were exposed to lipopolysaccharide (LPS), a bacterial wall product that binds the toll-like receptor (TLR) 4 and is known to activate DCs and induce their maturation. This lack of DC activation occurred despite similar levels of TLR4 on both cells exposed to IL-15 or IL-21 (622). DCs are known to produce several pro-inflammatory factors including IL-1β, IL-6, IL-12, and TNF upon maturation/activation with LPS (624). Culture of DCs in the presence of IL-21, but not IL-15, reduces production of these factors, further reducing the ability of the DCs to initiate immune responses (622). This has been confirmed *in vivo* by adoptive transfer of DCs cultured in the presence of IL-15 or IL-21 and loaded with the hapten fluorescein isothiocyanate (FITC). Only IL-15-exposed DCs activated antigen-specific T cells. Despite a lack of CCR7 on the DCs exposed to IL-21 the DCs migrated equally into the draining lymph nodes (dLN), demonstrating that the migratory

capacity of IL-21-exposed DCs had not been adversely affected (622). It is possible that other compensatory factors enabled these DCs to migrate, but not activate antigen-specific T cells.

2.8.6.3.5. NK cells

There have been several studies investigating the role of IL-21 in NK cell development and function. It is known that IL-21 is not required for NK cell development as IL-21RKO mice have normal numbers of NK cells (625,626). However, exposure of BM cells in culture to IL-21 boosts NK cell generation, possibly in an IL-15-dependent manner (581,627). The effect appears to be dose-dependent as low doses boosted generation and high doses inhibited it (628). A similar effect, though not identical, has been observed in humans (629). The effect of IL-21 on mature NK cells is also IL-15-dependent. In the presence of IL-15, IL-21 increased the cytolytic activity and IFN-γ production of NK cells, while inhibiting their proliferative capacity (630). This may be due to increased cell apoptosis. It also altered the receptor expression of NK cells, increasing the CD94-NKG2A inhibitory heterodimer as well as CD154 and KLRG1, and reducing expression of NK1.1, Ly49D and Ly49F, as well as NKG2D (630). This was confirmed by demonstrating that IL-21-exposed mature NK cells were less able to lyse NKG2D-sensitive targets (631). These changes in receptor expression are partly due to repression of the DAP10 adaptor protein after IL-21 signalling. Conversely, IL-21 inhibited NKG2D expression on immature NK cells, therefore the effects of IL-21 on NK cells appear to be maturation-dependent. IL-21 also modulates cytokine production by mature NK cells, increasing IFN-γ and IL-10, while reducing GM-CSF, but having no effect on TNF (630). However, these effects only occurred in the presence of IL-2 or IL-15, suggesting that IL-21 promotes effector and regulatory functions of NK cells in the presence of these factors.

2.8.6.3.6. NKT cells

There have been limited studies investigating the effect of IL-21 on NKT cells. As for other lymphocytes, NKT cells constitutively express IL-21R and so are capable of responding to IL-21 in the environment (586). More importantly however, NKT cells are a major source of IL-21 and therefore can regulate their own activity (582). IL-21 production by NKT cells surpassed that of conventional CD4 T cells; therefore under certain conditions NKT cells may be the dominant source. IL-21 alone increases the survival of NKT cells *in vitro*, and can synergise with IL-2, IL-15, or their cognate glycolipid antigen to enhance their proliferation. After antigenic stimulation, IL-21 (in the presence of IL-2) increased the granularity, granzyme B production and expression of NK receptors NKG2A/C/E, Ly49C/I, and CD94 by NKT cells (582). Alone, IL-21 did not alter NK receptor expression. IL-21 also increased IL-4 and IL-13 production by NKT cells after antigenic stimulation, though there was no consistent effect on IFN-γ production. The effect of IL-21 on NKT cells may be subset-dependent as thymic and liver NKT cells responded differently after antigenic stimulation. Therefore further studies may be required to fully elucidate the effect of IL-21 on each subset (582).

2.8.6.3.7 Macrophages and Neutrophils

As for NKT cells, there have been few studies investigating the effect of IL-21 on neutrophils or macrophages. One study used an air pouch model (where air is injected subcutaneously (s.c) into the back of mice) to investigate whether IL-21 played a role in neutrophil recruitment or function. The study found that IL-21 administration to the air pouch

recruited both monocytes/macrophages and neutrophils. However, further studies found that neutrophils do not express IL-21R and cannot respond to its presence. The recruited macrophages did express IL-21R and in response secreted CXCL8 (but not CCL3, CCL5, CXCL5, or IL-6) which recruited the neutrophils. Therefore, neutrophils do not respond directly to IL-21, but can be affected indirectly by the action(s) of IL-21 on other cells (632).

3. Respiratory viruses

3.1. Paramyxoviruses

The paramyxoviruses (Greek: para- beyond, myxo- mucus/slime, virus- poison/slime) are all members of the *paramyxoviridae* family belonging to the *mononegavirales* order. They have been identified in multiple land-based and aquatic species demonstrating a vast host range. They share multiple common features: they are all negative sense, single-stranded, RNA viruses and are responsible for a number of important human and animal diseases. They can be roughly divided into two sub-families: the *paramyxovirinae* and the *pneumovirinae*, though currently there are a number of unassigned viruses (e.g. Beilong virus, J virus, and Tailam virus) (633). The members are organised phylogenetically as shown (Table 2). The features of these viruses will be explained, and each genus will be briefly described in turn.

3.1.1. Common Physical features

The paramyxoviruses are all enveloped viruses but their shape can vary from spherical to

Table 1. The paramyxoviruses, their hosts, and associated clinical diseases

Subfamily	Genus	Species	Natural Host	Disease
Paramyxovirinae	Avulavirus			
	Henipavirus	Hendravirus	Pteropid fruit bats	Causes respiratory infection of horses; occasionally humans
		Nipahvirus	Pteropid fruit bats	Causes human disease with ~50% mortality (with spectrum of symptoms), causes respiratory and neurological disease in pigs
	Morbillivirus	Measles virus	Humans	Causative agent of measles (respiratory infection) in humans
		Rinderpest virus	Unclear, possible cattle	Caused disease of cattle and buffalo with high mortality, now eradicated
		Canine distemper virus	Unclear, possibly several species	Causes respiratory disease in dogs, ferrets, and several wild-life species (mortality: ~15% in dogs)
		Phocine distemper virus	Probably canine species	Causes respiratory disease in pinniped species (primarily seals)
		Ovine rinderpest	Unknown	Causes respiratory infection in sheep with ~80% mortality rate
	Respirovirus	Sendai virus	Probably rodent species	Causes respiratory infection in rodent species, and occassionally pigs
		Human parainfluenza virus 1 & 3	Unknown	Causes upper and lower respiratory tract infection in young children (incl. Croup)
	Rubulavirus	Mumps virus	Humans	Causative agent of mumps in children
		Human parainfluenza virus 2 & 4	Unknown	Causes upper and lower respiratory tract infection in young children (incl. Croup)
		Simian parainflunza virus 5	Unknown	Upper and lower respiratory tract infection
		Menangle virus	Fruit bats	Causes a flu-like illness in humans, causes deformities and still-births in pigs
		Tioman virus	Fruit bats	Currently no evidence it causes disease in animals
		Tuhkovirus 1-3	Unknown	Unknown
	TPMV-like viruses	Tupaia paramyxovirus	Unknown	Unknown
		Mossman virus	Unknown	Unknown
		Nariva virus	Unknown	Unknown
		Salem virus	Unknown	Unknown
Pneumovirinae	Pneumovirus	Human respiratory syncytial virus	Simian species	Causes respiratory disease in humans
		Bovine respiratory syncytial virus	Unknown	Causes respiratory disease in cattle
	Metapneumovirus	Avian pneumovirus	Unknown	Causes respiratory disease in turkeys and chickens
		Human metapneumovirus	Unknown	Second most common cause of respiratory infection in humans, after Respiratory syncytial virus

filamentous. Their surface is dominated by fusion and attachment proteins that allow infection of target cells, and matrix proteins that are located within the envelope and stabilise the virion structure. Internally, the nucleocapsid consists of the genomic RNA bound by nucleocapsid proteins, phosphoproteins, and polymerase proteins (633).

The paramyxoviruses share proteins of similar function. The Nucleocapsid (N) protein binds to hexamers of RNA and protects them from enzymatic digestion. The Phosphoprotein (P) binds together with the nucleocapsid and polymerase (L) proteins to form the polymerase complex necessary for genome replication. The Matrix (M) protein maintains virion structure by holding together the envelope and the nucleocapsid core. The Fusion (F) protein assembles as a trimer on the envelope surface and initiates class I fusion of the viral envelope and target cell membranes prior to cell entry. The H/HN/G (Haemagglutinin/Haemagglutinin Neuraminidase/Glycoprotein) proteins are all necessary for viral attachment to the target cell and mediate cell entry. Morbilliviruses and Henipaviruses express H protein; Respiroviruses, Rubulaviruses, and Avulaviruses express HN protein; and the Pneumovirinae express G protein. Finally, the polymerase (L) protein is the catalytic subunit that forms part of the RNA-dependent, RNA polymerase complex necessary for genome replication (633).

However, not all paramyxvoviruses share the same proteins. For example, the *pneumovirinae* not only express N protein but also two non-structural proteins: NS1 and NS2 that interfere with the host type I interferon response. They also express a second matrix protein: M2 that encodes an elongation factor (M2-1) and a regulator of transcription (M2-2). Such differences indicate that the paramyxoviruses have adopted

different strategies to successfully replicate in their individual hosts (633).

3.1.2. Common Genomic features

The RNA genome of paramyxoviruses is non-segmented and typically 15-19kb in length comprising 6-10 genes. They all share three extracistronic regions: a 3', 50 nucleotide, leader sequence used in transcription initiation; a 5' trailer sequence whose length varies between viral species (50-161 nucleotides), and intergenomic regions that vary in length depending upon the viral species (633).

The typical gene sequence of paramyxoviruses is: nucleocapsid-phosphoprotein-matrix-fusion-attachment-polymerase. As a result of their genome structure, paramyxoviruses exhibit transcriptional polarity: genes closest to the 3' leader sequence are transcribed in greater quantity than those distal to the leader sequence. This is caused by the RNA-dependent, RNA polymerase pausing at the intergenic sequences in the genome. Upon release of the transcribed mRNA from the previous gene there is a chance that the polymerase will dissociate from the genome, when it will have to reattach at the 3' leader sequence. This process continues as the polymerase transcribes towards the 5' trailer sequence. Consequently, the polymerase is more likely to dissociate from the intergenic sequences distal to the 3' leader sequence, reducing the transcriptional frequency of genes distal to the leader sequence. As a result of transcriptional polarity the gene sequence of paramyxoviruses has evolved to maximise chances of successful replication. For example, the *ns1* and *ns2* genes of Respiratory Syncytial Virus (RSV) are first in the genome as they are required to reduce the anti-viral type I interferon response of host epithelial cells. In

contrast, the polymerase gene, *l*, is the most 5' as it is only required for genomic replication (634).

Many paramyxoviruses have genome lengths that are a multiple of 6 (the 'rule of six'). This is because the nucleocapsid (N) protein of the virus binds hexamers of RNA, and these viruses replicate more efficiently when all their RNA is bound by N protein. This rule only applies to paramyxoviruses that encode the N protein. The *pneumovirinae* do not follow this rule (634).

Another process that paramyxoviruses utilise is RNA editing. This applies mainly to the *p* and *m2* genes when multiple proteins are produced by a single gene via alternative stop codons. The alternative P proteins are thought to aid productive viral replication by switching replication from mRNA to anti-genome synthesis. The M2-1 and M2-2 proteins of *pneumovirinae* are involved in regulation of transcription (634).

3.1.3. RSV structure and genome

RSV is an enveloped, negative sense, RNA virus that ranges from 80-200nm in diameter. The variability is due to the pleomorphic virion shape that ranges from spherical to the more common filamentous. As described earlier, the F, G, and SH proteins are located in the virus envelope, the M protein lies on the inner envelope surface, and the genome is encapsulated with the N, P, L, and M2-1 proteins (Fig.8) (633).

RSV can be divided antigenically into two groups: A and B that were created ~350 years ago (635). Members of the two groups co-circulate, and though there is a predominance of one subtype over the other, people can be infected with both simultaneously. The dominance is

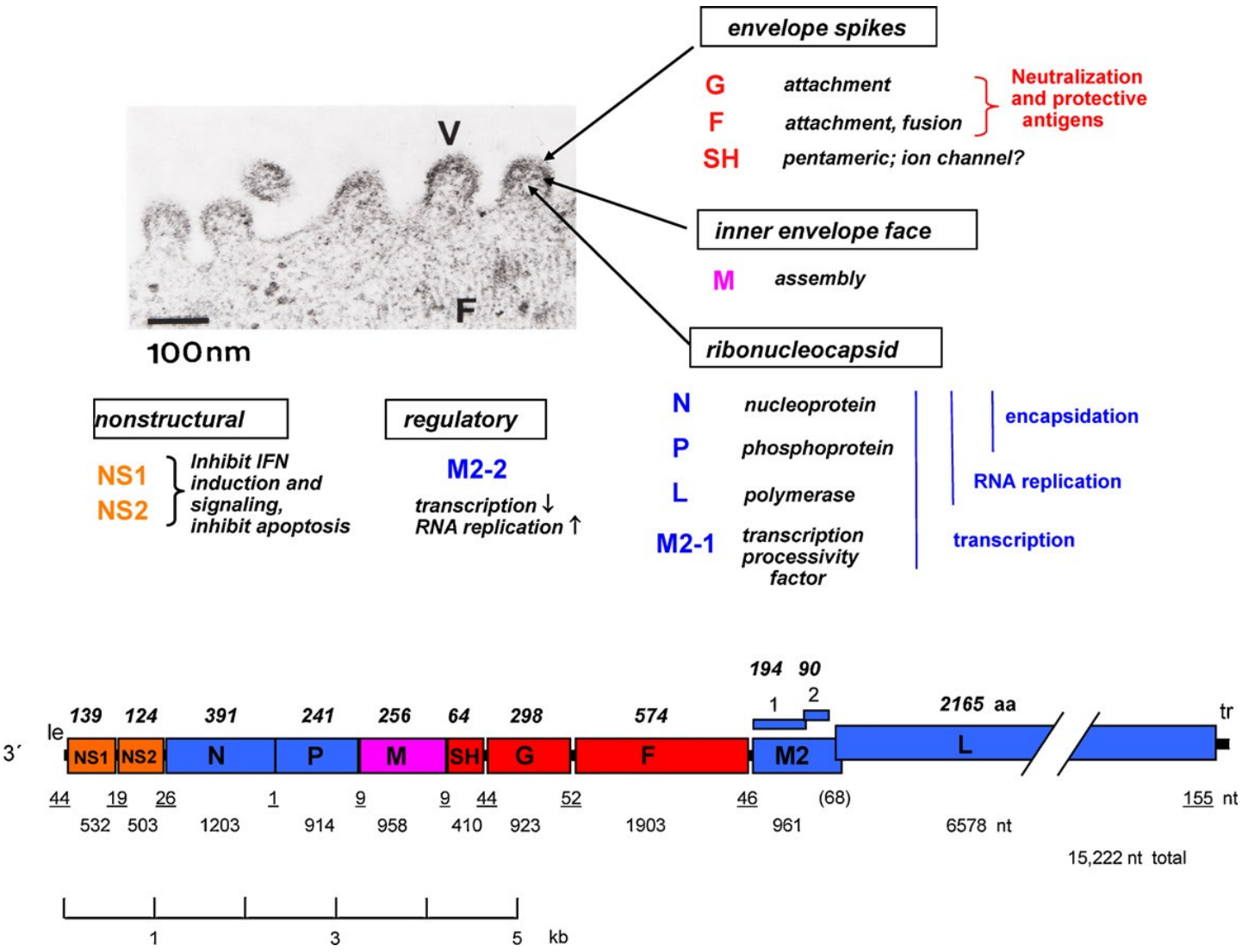

Figure 8. RSV structure, protein composition, and genome. RSV virions (V) are typically captured as spherical structures (see electron micrograph) comprising 11 proteins. F denotes filamentous nucleocapsids within the cytoplasm. The position and function of each protein are indicated. The gene order within the genome is shown with overlapping genes and open-reading frames indicated. Gene nucleotide length is indicated with intergenic sequence lengths underlined (adapted from Collins PL et al. Virus Research 2011 162 80).

caused by sub-optimal immunity against the heterologous strain due to the previous dominance of the subgroup (636). The division is caused by the accumulation of genetic changes in the viral genome, the greatest number of which occur in the two mucin-like domains of the attachment (G) gene (637). Mutation is driven by a selective pressure by host anti-viral antibodies, against the surface attachment (G) and fusion (F) proteins of the virus that are generated after infection. This pressure drives the co-evolvement of several antigenically-distinct strains with several distinct mutations in these proteins (638).

The RSV genome is 15.2kb in length, comprising 10 genes that encode 11 proteins [Fig.8; (639)]. Despite the similar genome length RSV is a complex paramyxovirus, containing additional proteins not present in other paramyxoviruses such as NS1, NS2, SH, M2-1, and M2-2.

3.1.4. Pathogenicity

Several clinically and economically important animal and human diseases are caused by paramyxoviruses. The human diseases will be described in turn.

3.1.4.1. Mumps virus: Mumps

Mumps (or epidemic parotitis) is a contagious disease characterised by painful swelling of the parotid (salivary) glands, typically on both sides of the head. Swelling of the testis (orchitis) and rash can also occur. Despite an effective vaccine in the form of the childhood measles-mumps-rubella immunisation, the disease remains common in third world

countries and sporadic outbreaks still occur in the developed world. The disease is generally self-limiting and life-long immunity is generated from natural infection (640).

3.1.4.2. Measles virus: Measles

Measles (or 'rubeola') is a highly contagious disease of the respiratory organs, and is spread by aerosol transmission. It is characterised by a generalised erythematous (redness of the skin) rash, fever, cough, runny nose, and conjunctivitis that can last up to 12 days from exposure. Complications range from diarrhoea and pneumonia to acute encephalitis (mortality: 15%) and corneal ulceration, though the more severe are only observed in infected adults rather than children. Immunisation against measles is typically administered to children as part of the trivalent measles-mumps-rubella vaccine and is highly effective. As such, measles is now an uncommon disease in developed countries, though it remains endemic in the developing world (641).

3.1.4.3. Parainfluenza virus: Bronchitis and Croup

The parainfluenza viruses are clinically significant because they are the second most common cause of lower respiratory tract infection (LRTI) in young children (after RSV), and they cause ~75% of croup cases. There are four serotypes [HPIV-1: Croup, HPIV-2: Croup, HPIV-3: Bronchiolitis and pneumonia, HPIV-4: General upper respiratory tract infection (URTI) and LRTI]. Like RSV, repeated re-infection with parainfluenza viruses is common, resulting in mild upper respiratory tract infection for between 1-7 days. However, as for most paramyxoviruses infection of immunocompromised individuals can result in

pneumonia and be fatal. Despite extensive research there is still no licensed vaccine against any of the serotypes (642).

3.1.4.4. Metapneumovirus (HMPV): Bronchitis

Metapneumovirus was isolated in 2001 and is closely related to avian metapneumovirus subgroup C. HMPV causes ~10% of respiratory infection cases, and together with RSV and HPIV, is one of the most common causes of LRTI in children. However, in contrast to RSV, HMPV tends to infect slightly older children and cause more severe disease. As for RSV, nearly all children have been infected with HMPV by the age of five years, and co-infections of RSV and HMPV are common. Genetically RSV and HMPV are analogous, though HPV does not encode non-structural proteins and has a slightly different gene order compared to RSV. As for other paramyxoviruses, viral transmission occurs primarily via aerosols (643).

3.1.4.5. Respiratory Syncytial Virus (RSV): Bronchiolitis

RSV was first identified in 1956 (644), its name is derived from the F protein of the virus causing cell membranes to fuse (forming syncytia). It is the most common cause of infant LRTI and hospitalisation in the western world, estimated to cause >30 million LRTI cases worldwide with 10% requiring hospitalisation each year (645). The clinical burden of RSV is made more substantial by the fact that the virus is ubiquitous, and nearly all children are infected with RSV at least once in the first three years of life. Of these, 2-3% will require hospitalisation and mechanical ventilation. Moreover, for unknown reasons, immunity to infection is lost in a short space of time such that individuals can be re-infected with the

same strain in the same year. The long-term consequences of this are significant as there is growing evidence and belief that children who suffer from RSV bronchiolitis (infection of the small airways) are more likely to develop asthma, wheezing, and breathing difficulties later in life (646). It is also being increasingly recognised that RSV infection causes significant disease in the elderly (647).

The symptoms of RSV infection are indistinguishable from those of the common cold: fever, runny nose, sore throat, lethargy, and loss of appetite. However, symptom severity increases in the immunocompromised and there have been cases of fatalities in this population (648). Treatment is mainly supportive as the disease is typically mild and does not warrant anti-virals. For high-risk infants, Palivizumab (Synagis^{TM}) can be administered prophylactically (649). This monoclonal antibody neutralises viral infection by targeting the F protein. It is moderately effective when given prophylactically (i.e. monthly injections over the course of a RSV season) but more limited if infection has already occurred. Moreover, due to the expense of treating patients with the drug for so long it is only used on the immunosuppressed, premature infants, and those with pulmonary and cardiac insufficiency. At present, there is no licensed vaccine against RSV. However, great effort is always being made to test the efficacy of new candidates.

As RSV is the chosen disease model for my studies, I will now describe it's characteristics in greater detail.

3.1.5. RSV infection of the host

The primary target of RSV is the superficial ciliated respiratory epithelium, reducing ciliary function which may be responsible for the airway obstruction characteristic of RSV disease. However, the virus does not invade the underlying layers and does not cause syncytia in these areas, suggesting the virus is not cytopathic compared to more aggressive respiratory viruses such as influenza (650). Virus binding to target cells seems to occur via several distinct mechanisms as multiple receptors for RSV F and G proteins have been identified. F protein can bind TLR4 which is primarily expressed on myelomonocytic leukocytes. The significance of this interaction on viral infection is unclear as there is contradictory data on the effect of TLR deletion on RSV disease (651). More recently, the F protein has been show to bind the nuclear receptor nucleolin (652). This interaction has been shown to be essential for viral replication *in vivo* and may form an attractive target for therapy. Both RSV F and G proteins have been shown to bind glycosaminoglycans (GAGs; e.g. heparin and chondroitin sulphate) that form the glycocalyx on cells (653). Removal of GAGs on cell lines prevents efficient infection by RSV, demonstrating their importance. However, it is unknown how important they are *in vivo*. RSV has also been shown to bind annexin II and L-selectin though, again, how important this is to viral growth *in vivo* is unclear (654).

The RSV genome replicates within the cytoplasm of the cell, creating a positive-sense complimentary genome sequence for the polymerase complex to generate new negative-sense genome sequences for packaging prior to viral budding from the cell. Individual RNA sequences are used to generate new proteins using hijacked cellular machinery. During this process RSV generates reactive oxygen intermediates (ROIs) that drive NF-κB activation and production of pro-apoptotic factors within the cell (655,656) [Fig.9; (657)]. NF-κB is a critical

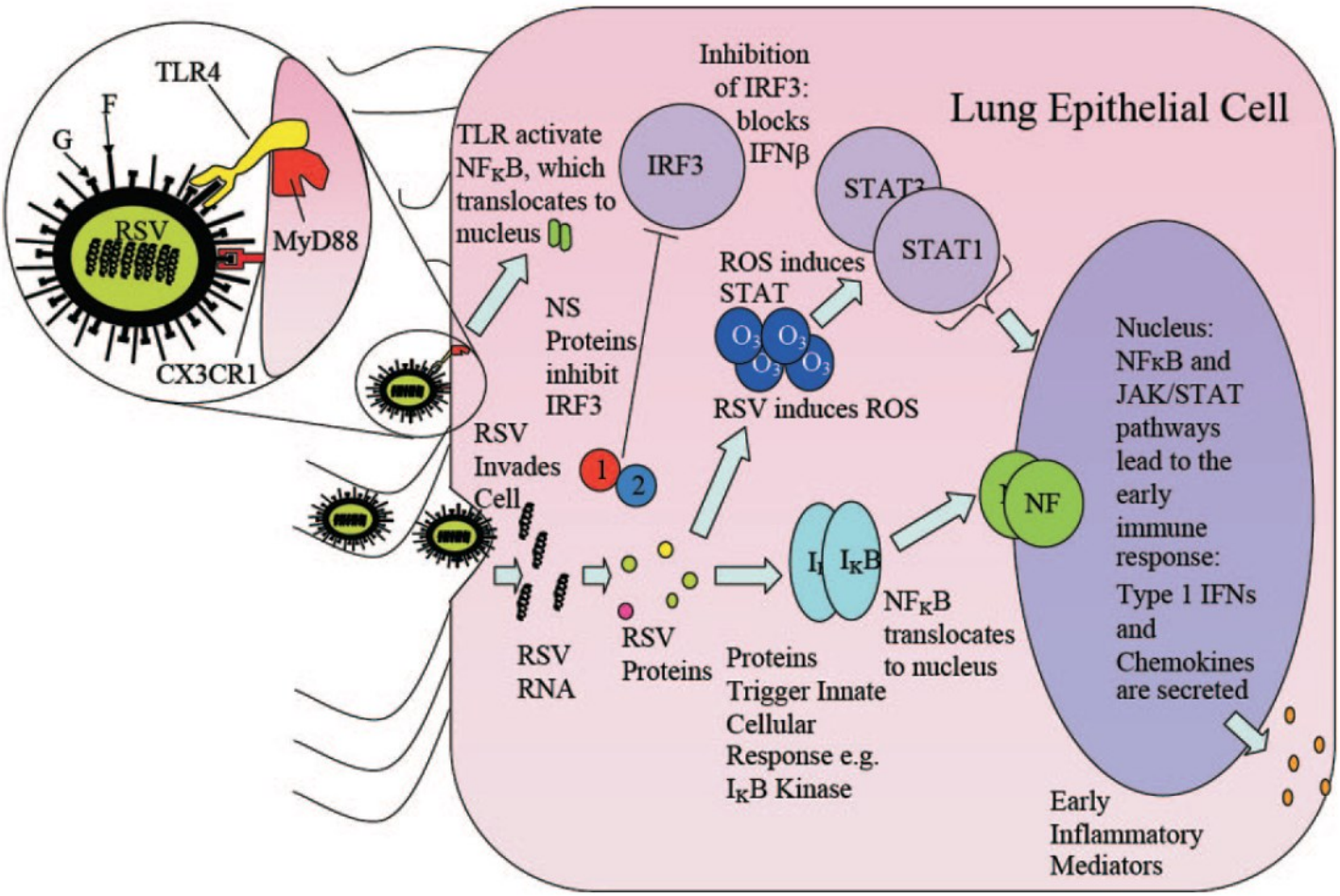

Figure 9. RSV binding, replication, and triggering of innate immunity. RSV binds to the surface of the mucociliary epithelium via multiple receptors including TLR4 and glycosaminoglycans. Upon binding and entry the virus replicates, generating reactive oxygen species that activate NF-κB-mediated pathways. These include production of type I interferons and several chemokines that create an anti-viral state and recruit innate cells that produce downstream mediators (taken from Openshaw PJ et al. Clin Microbiol Rev 2005 18 541).

mediator of many innate genes that drive the anti-viral response including the interferons and many chemokines (658,659). These chemokines recruit innate cells to the lung tissue and airways and the production of more pro-inflammatory mediators. For example, IL-8 drives neutrophilia (660). Several of these chemokines [e.g. MIP-1α (661), CCL11 (662), and CCL5 (663)] have been shown to be central mediators of severe disease as their depletion or blockade reduces disease severity. CCL5 may be particularly important because genetic polymorphisms that regulate chemokine signalling correlate with disease severity in humans (664). IL-9 is also of interest in RSV bronchiolitis as it is crucial to the development of Th2 responses (573) and asthma (566,665), and neutrophils have recently been identified as a significant source (666).

Newly-formed virions bud from the luminal surface of infected cells and spread to neighbouring cells by cilial motion and by syncytia formation (650).

3.1.6. The immune response to RSV infection

An overview of the immune response to RSV infection is shown [Fig.10; (657)]. The innate response against RSV infection begins prior to the first round of viral replication. Upon viral entry, surfactant proteins act to bind virions and prevent viral entry (667). They also label the neutralised virions for destruction by professional phagocytes such as macrophages and neutrophils. Following RSV infection, macrophages produce a range of pro-inflammatory mediators [e.g. type I IFN (668)] that recruit other innate cells (102). They are also important for clearing cellular debris that may contain infectious virus (669). One such cell type recruited early after RSV challenge is the NK cell that infiltrates the airway and lung tissue.

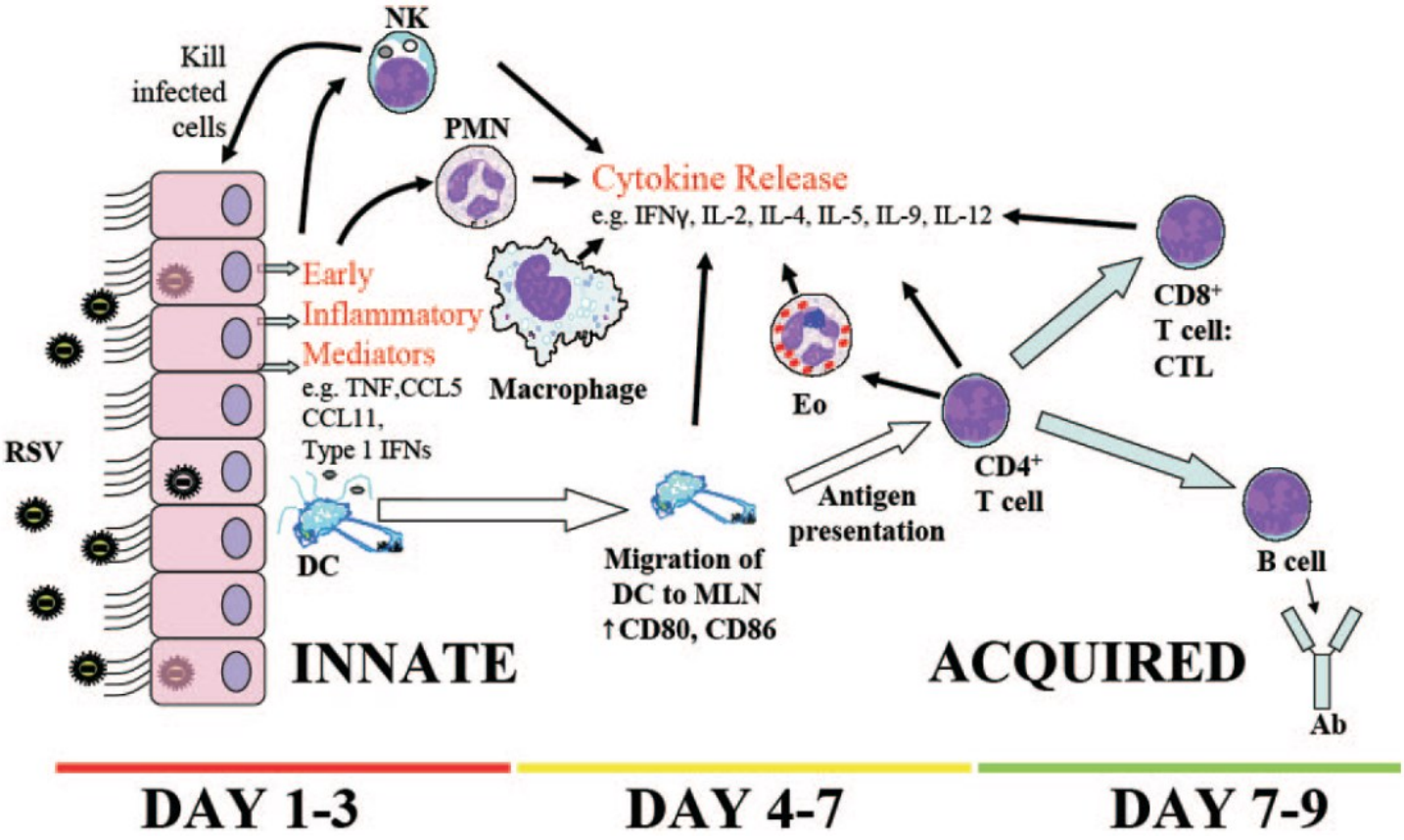

Figure 10. An overview of innate and adaptive immunity to RSV. RSV infects the mucociliary epithelium initiating soluble mediator release (e.g. TNF, CCL5 CCL11) that recruits innate cells. Such cells include NK cells, neutrophils, and macrophages. At this time DCs ingest viral antigens and migrate to the draining lymph nodes where they present processed antigens to naïve T cells. Activated T cells proliferate and migrate to the lung tissue and airways where they release more soluble mediators and kill infected cells. CD4 T cells induce B cell activation, infiltration, and antibody production(taken from Openshaw PJ et al. Clin Microbiol Rev 2005 18 541).

These cells are crucial to optimal protection as they produce significant amounts of IFN-γ that promotes Th1 differentiation of T cells to drive more effective anti-viral immunity, and are directly cytotoxic to virally-infected cells, reducing viral replication (670). Neutrophils are also recruited early after challenge and as professional phagocytes may also be important for clearing debris from the airways, reducing inflammation (671). Although fewer in number eosinophils may also aid in viral clearance and tissue repair (672). Finally, DCs are an important early source of IL-12 that aids development of a Th1 response, and ingest viral antigen for presentation to naïve T cells in the draining lymph nodes, initiating the adaptive immune response (101).

Although antibodies are considered essential for long-term protection from infection, T cells are thought to be more important for viral clearance upon challenge. This is clear from studies in T-cell-depleted mice (332), and in humans deficient in T cells, or with defective T cell function (673). Viral replication continues in these hosts significantly longer than in T cell competent individuals. CD8 T cells are considered to be the central mediators of viral clearance because of their cytotoxic effector function (674). However even with CD4 T cell help (believed to be mediated by IL-21 secretion) the memory is short-lived (675). The host may also actively down regulate the cytotoxic activity of CD8 T cells in order to minimise lung damage (676).

The most extensive studies of adaptive immune responses during RSV infection have been performed in rodent models of augmented disease. In particular, the Formalin-Inactivated-RSV (FI-RSV) model is the best studied as it accurately replicates what occurred in a now infamous vaccine trial conducted in 1966-7 (677). In this trial, FI-RSV was administered to infants and children between two months and nine years of age, vaccine doses were

separated by 1-3 months to ensure adequate boosting of the immunological memory. Control children were given a parainfluenza vaccine. Upon exposure to RSV, the rate of viral infection was similar between the two groups, suggesting that there was no antibody-mediated protection of the vaccines. However, whereas only 5% of the control vaccines required hospitalisation, 80% of the FI-RSV-vaccinated children required hospitalisation. These children presented with severe symptoms of bronchiolitis, bronchitis, rhinitis, and pneumonia. Tragically, two of these patients died (678). Postmortem analysis showed a significant cellular infiltrate in the lungs of these two children, comprising a mixture of lymphocytes, neutrophils, and eosinophils. The fact that there was no increase in viral replication indicated in these children suggests that it was the immunopathological response rather than direct effects of the virus that killed them (679). Serum analysis indicated that while high titres of anti-F- and anti-G-specific antibodies were produced by all the FI-RSV vaccines, they were non-neutralising and therefore not protective (680).

Subsequent animal studies have confirmed that T cells (and not antibodies) are the cause of the pathology as their depletion ablates disease severity (681). Moreover, further analysis revealed that the immune response was Th2 in phenotype as there was a significant increase in production of IL-4, IL-5, and IL-13, and was mediated by CD4 T cells (682).

More recent prime-challenge studies, using vaccinia vectors expressing individual RSV antigens to prime mice, have better elucidated the immune responses induced by each RSV antigen. A particularly interesting result from these studies is that priming with the RSV G protein promotes a CD4-T-cell-mediated Th2-biased memory. Upon RSV challenge, these primed mice develop a Th2 CD4 T cell response with a pulmonary eosinophilia, as observed in FI-RSV studies (683). This is the only protein known to elicit this response as RSV F protein

drives a Th1 memory comprising both CD4 and CD8 T cells (684), and RSV M2 protein drives a Tc1 CD8 T cell memory (685). Further studies on the immunological properties of RSV G protein demonstrated that priming with the secreted form of G protein only elicited a stronger Th2 response and pulmonary eosinophilia than the membrane-bound form (686). Moreover, using deletion mutants of RSV G protein, a 13-amino acid sequence of the receptor binding site has been identified as responsible for the Th2 response and pulmonary eosinophilia (687). Interestingly, this region is essential for the attachment function of RSV G protein as the sequence is heavily conserved between RSV strains. Therefore, based on these studies it is believed that the secreted form of RSV G protein and its receptor binding site are fundamental to any causative properties RSV may possess in predisposing individuals to asthma and allergy.

While Th2-biased CD4 T cells promote pulmonary eosinophilia, adoptive transfer of Tc1 CD8 T cells inhibits it (688). Moreover, depletion of CD8 T cells promotes pulmonary eosinophilia in mouse strains normally resistant to eosinophilic responses (689). This suggests that the CD4:CD8 T cell balance plays a crucial role on multiple aspects of the immune response to RSV including the level of disease severity, viral clearance, cytokine production, and pulmonary eosinophilia.

There is strong evidence that long term protection against rechallenge with antigenically-syngeneic RSV strains is mediated by neutralising antibodies against the surface fusion and attachment proteins of RSV (675). To confirm this, it was demonstrated that passive transfer of serum from previously infected animals into naïve recipients protects them against RSV infection (690). This fact has been utilised clinically by the prophylactic use of Palivizumab to protect high-risk infants from RSV challenge (649). Mucosal IgA is also thought protect

against rechallenge but local levels decrease with time and serum antibodies are considered a more reliable marker of protection. However, serum antibodies enter the respiratory tract by passive transudation, an inefficient process that requires a high serum antibody concentration before significant protection is achieved (691). Therefore, long-term protection may depend upon a combination of locally-derived virus-specific IgA 'topped up' by passive transudation of virus-specific serum IgG1 and IgG2a antibodies.

3.1.7. Immunological challenges to vaccine design

In the half-century since the discovery of RSV many vaccine candidates have been designed, including killed or attenuated virus, purified RSV proteins, viral nanoparticles, virus-like particles (VLPs; particles that mimic virion structure but lack genetic material), virosomes (vesicles carrying virus-derived proteins but lacking genetic material), and replication-competent and -incompetent vectors carrying RSV genes. While many of these have shown promise in small animal models this has not translated into larger animal models or humans. This disappointment has led to a certain hesitation when interpreting data from small animal models. There are obvious limitations testing RSV vaccines in rodent models, given their genetic, structural, and immunological differences. Therefore, larger animal models such as non-human primates are desirable but costly, lengthy, and ethically complex. Moreover, while human trials are ultimately essential only those in healthy adults and the elderly are undertaken with any frequency. This is because trials in babies and infants are avoided given the terrible consequences of the 'Lot 100' FI-RSV trial in the 1960s. Therefore, vaccine candidates are only tested in this group when all other possibilities have been exhausted. However, interpretation of trial results from previously-infected adults with

fully-developed immune systems may not translate into infants with immature immune systems (692).

There is general agreement that a successful RSV vaccine must elicit memory from CD4 & CD8 T cells as well as B cells in order to maximally protect the host from re-infection. Some vaccine studies, where only one of these populations has been activated, would support this idea. However, there is a clear correlation between the 'amount' of lymphocytic memory generated and the development of pathology. This pathology is multi-factorial: antibodies from B cells generate immune complexes that activate NK cells, phagocytes, and fix complement; while CD4 T cells secrete pro-inflammatory cytokines such as IFN-γ and TNF, and CD8 T cells kill host cells via cytotoxic molecules such as perforin, granzymes, TRAIL, and TNF. Despite knowing this, it is proving incredibly difficult to tease apart the immune system components necessary for long-lived, effective protection from those that cause pathology. This is made more complex by the presence of maternal antibodies in infants, the clinically most significant population. It may be that protection and pathology are intrinsically-linked, in which case it will be necessary to reduce pathology while maintaining effective protection (692).

4. Background to the project

The most significant clinical burden caused by RSV disease is infection of the very young or very old. In infants, where the immune system is still relatively immature, RSV infection causes mucus plugging of the airways, destruction of the airway and bronchial epithelium, and exacerbation of pre-existing asthmatic conditions. In the elderly where there is stronger

cellular immunity, particularly lymphocytic memory, there is increased immune complex deposition, cellular influx, and extensive destruction of the airway and bronchial epithelium. Both these responses lead to significantly reduced lung function, and compromised gas exchange, leading to breathing difficulties, and possible need for hospitalisation. Therefore, there is an urgent need to limit the destruction of the epithelium, and prevention of breathing problems, without compromising viral clearance. Activated, cytotoxic CD8 T cells are primarily responsible for destruction of the epithelium and are therefore the natural target for therapeutic intervention. However, CD8 T cells are crucial to viral clearance and so their depletion is ill-advised. Therefore, limiting CD8 T cell activation and effector function may inhibit epithelial cell destruction without significantly compromising viral clearance. Multiple factors affect CD8 T cell activation and effector function (e.g. IL-2, IL-15, IFN-γ) but are considered too important to the development of an effective anti-viral response to be ablated.

Another γc chain cytokine family member expressed during respiratory viral infections is IL-21. Increased expression has also been observed in emphysema patients and correlates with T cell influx into the airway, suggesting they are the major source. The IL-21R protein is expressed by epithelial cells, macrophages, DCs, and numerous lymphocytes and is increased in various inflammatory conditions. However, most importantly, CD8 T cell activity, particularly cytotoxic functions are enhanced by IL-21 exposure. This has been demonstrated in intestinal disease, including IBD, but it has never been tested if IL-21 can be targeted to reduce lung CD8 T cell activity and effector function. This is a particularly attractive possibility as CD8 T cell proliferation is driven mainly by IL-2 and IL-15, therefore sufficient CD8 T cell activity may remain after IL-21 depletion to effectively clear the virus

without leading to excessive epithelial cell damage. Therefore, the major aim of this study was to determine if IL-21 modulation could alleviate RSV disease without compromising viral clearance.

5. Hypothesis

IL-21 enhances the cytotoxic effector functions of infiltrating CD8 T cells responding to RSV challenge, leading to detrimental epithelial cell damage and increased RSV disease.

6. Study aims and objectives

1. To determine the effect of IL-21 depletion on the primary CD8 T cell response to RSV challenge.

2. To determine the effect of IL-21 depletion on memory CD8 T cell responses to RSV challenge.

3. To determine the effect of IL-21 over expression on primary and memory CD8 T cell responses to RSV challenge.

G. Materials and Methods

1. Hep-2 cells

Hep-2 cells (American Type Culture Collection; LGC Standards, Middlesex, UK) is a larynx epidermoid carcinoma derived from adult human tissue. When healthy, it propagates adherently. However, recent studies have indicated that it may contain a contaminant: Hela (Henrietta Lacks: the patient that these cervical cells were isolated from) cells, a cervical adenocarcinoma that is indistinguishable from Hep-2 cells (693,694).

2. Viruses

Respiratory Syncytial Virus (RSV) A2 strain was used in this study. The original stocks were donated by E.J.Stott (Institute for Research on Animal Diseases, Newbury, UK), and propagated thereafter in-house. The Recombinant RSV strain that encoded the murine *il21* gene was donated by Peter Collins (National Institute of Allergy and Infectious Disease, Bethesda, USA).

Recombinant vaccinia viruses (rVV; Western Reserve strain) were used to prime memory T cell populations in BALB/c mice against individual RSV proteins. rVV-βgal (recombinant vaccinia virus expressing β-galactosidase) was donated by SmithKline Beecham Biologicals. rVV-G and rVV-M2 were donated by Professor Gail Wertz (University of Alabama, Birmingham, USA) and stored at -80°C. All virus preparations were propagated in-house, as well as tested and confirmed to be free of mycoplasma (Gen-Probe, San Diego, CA).

3. Growth of virus stocks

Hep-2 cells (5×10^6/flask) were propagated in $175cm^2$ flasks in 25ml supplemented RPMI 1640 medium [Roswell Park Memorial Institute 1640 medium (Invitrogen, Paisley, UK) supplemented with heat-inactivated foetal bovine serum (FBS; 10% v/v; Invitrogen, Paisley, UK), L-glutamine (2nM; Invitrogen, Paisley, UK), penicillin G (100U/ml; Invitrogen, Paisley, UK), streptomycin sulphate (100µg/ml; Invitrogen, Paisley, UK)]. The cells were monitored daily until ~75% confluent. The media was removed and the cells washed by exposure to 25ml FBS-free-RPMI 1640 twice to remove remaining FBS which interferes with viral binding to cells. FBS-free media was removed and the cells exposed to 5ml FBS-free-RPMI 1640 containing RSV [Multiplicity of Infection (MOI): 0.01]. The flasks were incubated at 37°C/5% CO_2 for 90mins and rotated every 15mins by 90° to ensure equal exposure of all cells to virions. A control flask was also used in which cells were exposed to serum-free RPMI 1640 medium only. After 90mins, 15ml of the original media was re-added back to each flask to ensure survival of the cells. All flasks were incubated at 37°C/5% CO_2 and monitored daily for signs of viral growth (i.e. CPE: cytoplasmic effect, cells 'bleb' and detach from the surface of the flask as the virus replicates and buds from the cells). The cells were harvested when ~75% show signs of CPE. To harvest the virus, first remaining adherent cells are detached from the flask by scraping them with a cell scraper. 1ml medium aliquots are then immediately transferred into 1.8ml cryovial tubes and snap-frozen in liquid nitrogen. The vials are then transferred to liquid nitrogen tanks for long-term storage.

Propagation of vaccinia virus stocks was performed in an identical manner except the medium is transferred into 200µl eppendorf tubes in 100µl aliquots and stored at -80°C.

4. Titration of virus stocks

For titration of both RSV and vaccinia virus stocks, Hep-2 cells (5×10^5/well) were seeded into 6-well plates in 5ml supplemented RPMI 1640 medium and incubated at 37°C/5% CO_2 overnight. The media was then removed and the cells washed with 3ml serum-free RPMI 1640 twice. Test virus stocks were diluted in a ten-fold dilution series in serum-free RPMI 1640 medium from 10^{-1} to 10^{-7}. A viral stock of known viral titre was used as a positive control, and serum-free RPMI-1640 medium as a negative control. 1ml of each virus dilution is added to a well and each dilution is tested in duplicate wells. The plates are incubated at 37°C/5% CO_2 for 90mins, during which the plates are rotated every 15mins by 90° to ensure equal exposure of the cells to virions.

For titration of RSV stocks, after 90mins 2ml of the original media is re-added to the wells to ensure cell survival. The plates are then monitored every 24hrs for signs of syncytia formation (fusing of plasma cell membranes to form 'giant' multi-nucleated cells), typically up to a maximum of 48hrs. Once syncytia formation is observed, the media is removed, the cells washed with Phosphate-buffered-saline [PBS: NaCl (137mmol/l), KCl (2.7mmol/l), $Na_2HPO_4\bullet2\ H_2O$ (10mmol/l), KH_2PO_4 (2mmol/l), pH 7.4] twice to remove residual serum, and the cells fixed in 1ml methanol containing H_2O_2 (2% v/v) for 20mins. The methanol was removed and the cells washed twice in 3ml PBS/BSA (1% w/v). Cells were stained for RSV antigen expression by exposing them to 1ml biotinylated goat anti-RSV antibody (AbD Serotec, Kidlington, UK) diluted 1:200 (20µg/ml) in PBS/BSA (1% w/v). The cells were incubated for 1hr at room temperature. The cells were washed twice with PBS/BSA (1% w/v), and then stained with 1ml Extravidin-HRP (Sigma-Aldrich, Gillingham, UK) diluted 1:500 in PBS/BSA (1% w/v). The cells were incubated for 30mins at room temperature. The

cells were washed twice with PBS/BSA (1% w/v), and then stained with 1ml AEC substrate [3-amino-9-ethylcarbozole (3.3mg/ml in DMSO), H_2O_2 (2µl/ml in citrate phosphate buffer); Sigma-Aldrich, Gillingham, UK]. The cells were incubated in the dark and monitored for the development of insoluble red staining on infected cells expressing RSV antigens. Once the negative control cells showed signs of colour development (via non-specific staining of extravidin-HRP) the cells were washed with PBS to remove the substrate and plaques counted in each well. The pfu/ml was calculated as follows: number of plaques × virus dilution factor.

For titration of vaccinia virus stocks, after 90mins the cells were washed with PBS and cells were overlayed with 1ml low-boiling point agar solution (0.75% w/v; Sigma-Aldrich, Gillingham, UK). The cells were incubated at 37°C/5% CO_2 for a minimum of 48hrs. After this time, the wells are monitored for the presence of plaques (holes in the monolayer where cells have lysed due to viral replication and budding). If none are present the wells are re-incubated under the same conditions and monitored daily until plaques appear. Once plaques are present the agar is carefully removed and the cells fixed with 1ml methanol containing H_2O_2 (2% v/v) for 20mins at room temperature. The cells are washed twice with PBS and the cells stained for 5mins with 1ml crystal violet solution (1mg/ml; Sigma-Aldrich, Gillingham, UK). The cells are washed once in PBS to remove excess crystal violet and the plaques counted in each well using a light microscope. The pfu/ml was calculated as follows: number of plaques × virus dilution factor.

5. Testing of virus stocks for mycoplasma contamination

Prior to use, sample vials of all virus stocks were tested for mycoplasma contamination, a minimum of three randomly-chosen vials per stock were tested. An enzyme immunoassay was used for the detection of four serologically-distinct strains of mycoplasma (*M.arginini*, *M.hyorhinis*, *A.laidlawaii*, and *M.orale*) according to the manufacturer's instructions.

Virus stocks to be tested were thawed and 2ml of each diluted with 0.5ml sample buffer. 200µl of the coating antibodies for the four mycoplasma strains was coated into duplicate wells on a microplate. The plate was sealed with cling film and incubated for 2hrs at room temperature. The coating antibody solution was removed by banging the plate dry onto paper towel and the wells blocked by adding 200µl of blocking buffer [PBS/BSA (1% w/v)]. The plates were sealed with cling film and incubated for 2hrs at room temperature. The wells were washed three times with wash buffer [PBS/Tween20 (0.05% v/v)] and dried on paper towel. 200µl of each sample plus negative and positive controls was then pipetted into duplicate wells. The plate was then sealed with cling film and incubated overnight at 4°C. Wells were washed three times with wash buffer and dried on paper towel. 200µl of the relevant biotinylated detection antibodies were added into the relevant wells, the plates sealed with cling film, and then incubated for 2hrs at room temperature. The plates were washed three times with wash buffer and dried on paper towel. 200µl of streptavidin-alkaline phosphatase was added to all wells, the plates sealed with cling film, and incubated for 1hr at room temperature. The plates were washed four times with wash buffer and dried on paper towel. 200µl of substrate solution was added to all wells, the plates sealed with cling film and incubated at room temperature in the dark. The plates were monitored for colour development and once colour was detected in the negative wells the plates were

read on a microplate reader (Molecular Devices, Wokingham, UK) using a reference wavelength of 405nm. Readings could be accurately determined down to and optical density (OD) value of 0.2.

6. Mice

All mice used in this study were eight-week-old female BALB/c (BALB/cAnNCrl strain; H-2^d haplotype) mice purchased from Harlan Olac (Bicester, U.K.). They were kept in specific pathogen-free conditions, housed in individually-ventilated cages, according to institutional and UK Home Office guidelines. All protocols used in this study were reviewed and approved by local ethics, safety, and regulatory committees and licensed by the UK Home Office.

7. Mouse infection and treatment

For cutaneous infection with recombinant vaccinia virus (rVV), mice were first anaesthetised using an isofluorane chamber and their rumps shaved with clippers to expose the skin. The skin was then decornified using an emery board until there was a visible abrasion, and rVV-βgal, -G or -M2 [10μl, 10^6 pfu; (695)] applied to the decornified area with a pipette. The viral inoculum was rubbed into the skin to ensure optimal exposure. The mice were replaced into the cages and left to recover from the anaesthetic. They were then left for 10 days for the lesion to develop, and were monitored daily during this time to ensure no abnormalities with regard to lesion development or mouse health occurred. By 14 days post infection the lesions were healed and the mice were challenged with RSV.

For RSV challenge, mice were first anaesthetised using an isofluorane chamber. The mice were then held upright by clamping the neck between the thumb and fore-finger such that the nose of the mouse was upright. RSV inoculum (100µl; 10^6 pfu) was then slowly pipetted onto the nose sealing the nostrils. Once the mouse had inhaled the drop it was replaced with another and the process continued until the inoculum had been fully inhaled. The mouse was then placed carefully in the cage and left to recover from the procedure.

8. Antibody administration

Some mice were treated with rabbit anti-mouse-IL-21 polyclonal antibody (Novo Nordisk, Copenhagen, Denmark) to deplete endogenous IL-21. This treatment was performed one day prior and one day post cutaneous infection with vaccinia virus. To do this stock anti-IL-21 antibody (15mg/ml) was diluted to the required concentration (typically 1mg/ml, though for *in vivo* antibody titration experiments this was 0.02, 0.04, 0.1, 0.2, 0.4, or 1mg/ml) in PBS. Mice were restrained via the neck, back, and tail in one hand and 0.5ml of anti-IL-21 antibody injected, using a 2.5ml syringe (Becton Dickinson, Oxford, UK) with a 27G needle (Becton Dickinson, Oxford, UK) attached, into the peritoneum. Once the antibody had been administered, the mice were replaced in the cage.

9. Mouse weighing

Mice were weighed daily after RSV challenge. Mouse cages are only opened, and mice are only ever handled, inside a microbiological safety cabinet. Mice were therefore weighed by placing them individually into a plastic container which was sealed with a lid. Weight is

expressed as a percentage of the day 0 weight for each mouse. This removes any inherent bias due to some mice being inherently larger than others, and normalises the data for all mice.

10. Mouse euthanasia

In order to harvest samples from mice they must be euthanized in such a way as to minimise distress of the animal without affecting the organs of interest. To do this, mice were injected with pentobarbital (3mg/mouse; i.p). The mice were then placed in a sealed box for the drug to take effect. Once the animal is unconscious it is pinned to a cork board via its legs and swabbed with ethanol (70% v/v). The skin is then cut away to reveal the femoral arteries. These arteries are then severed to ensure terminal blood loss such that the animal cannot recover.

11. Tissue recovery

Once mice are euthanized, the skin is cut from the genitals to the neck. The skin is then pinned down at the neck to the cork board to allow it to be peeled back. The mouse is swabbed with ethanol (70% v/v) and the peritoneal lining cut away to expose the digestive organs, spleen and diaphragm. The spleen can be carefully cut from surrounding connective tissue and placed into a macerator tube (Miltenyi Biotec, Bisley, UK) containing 5ml supplemented RPMI 1640 medium and placed on ice.

The diaphragm is then carefully punctured at the point where the heart is visible. This

ensures that the lungs are not pierced which is important for acquiring bronchoalveolar.

The diaphragm is then carefully punctured at the point where the heart is visible. This ensures that the lungs are not pierced which is important for acquiring bronchoalveolar lavage fluid (BALF) samples. The diaphragm is then carefully cut away which will allow the lungs to inflate during the lavage process.

The tissue covering the trachea is then carefully removed without piercing the trachea itself. A horizontal incision is then made with a scalpel blade in the trachea itself between two cartilage rings approximately half-way up the trachea. 1.5ml of lavage buffer [PBS/BSA (1% w/v)/EDTA (2mM)] is then administered into the lungs through the incision using a 2.5ml syringe, with a 27G needle covered by 2cm of 3mm diameter plastic tubing. The fluid is withdrawn and the process repeated. Once the fluid has been recovered a second time, it is placed into a 15ml conical tube and placed on ice.

Once the BALF has been recovered the lungs can be removed. The rib cage is cut away either side of the lung tissue. The lungs are then teased away from the heart and mediastinal lymph nodes using tweezers and carefully cut away using scissors. The tissue is placed into a macerator tube (Miltenyi Biotec, Bisley, UK) containing 5ml supplemented RPMI 1640 medium and placed on ice.

12. Organ processing and cell recovery

Lavage cells are recovered by centrifuging BALF at 770g for 2mins and decanting the BALF into 2ml eppendorf tubes. These tubes are sealed and stored in plastic bags at -80°C for

further analysis. The pelleted cells are resuspended in 1.3ml of lavage buffer and stored on ice for staining for flow cytometry.

Lung cells and spleen cells are recovered by first macerating them using a GentleMacs™ dissociator (Miltenyi Biotec, Bisley, UK). Lungs were macerated for 45s, spleens for 20s. The cell suspension was then poured through a 100µm filter (Sartorius, Epsom, UK) into a 50ml conical tube. Tissue pieces were pressed through the filter using the plunger from a 2.5ml syringe. Remaining cells in the macerator tube were washed out with 5ml supplemented RPMI 1640 medium through the filter into the 50ml tube. This was repeated for all samples. The filtered samples were closed and centrifuged at 770g for 2mins and the supernatant decanted into waste. Red blood cells (RBCs) were now lysed by resuspending the remaining cells in 5ml RBC lysis buffer (eBioscience, Hatfield, UK). The cells were vortexed, left to stand for 2mins, and then vortexed again to ensure equal cell distribution. After 2mins, 5ml supplemented RPMI 1640 medium was added to each sample and vortexed again. The samples were centrifuged again at 770g for 2mins, the supernatant decanted, and remaining cells resuspended in 20ml supplemented RPMI 1640 medium. The cells were then counted by Trypan blue exclusion assay.

13. Trypan blue exclusion assay

To count cells by trypan blue exclusion, 50µl trypan blue solution (0.4% v/v; Invitrogen, Paisley, UK) was pipetted into wells of a 96-well, v-bottomed plate. 50µl of each cell sample were added to individual wells and mixed to ensure equal exposure of cells to trypan blue. 10µl of each sample was immediately pipetted in-turn into a Countess™ cell counting slide

chamber (Invitrogen, Paisley, UK). The chamber was then inserted into a digital Countess™ machine and counted. Duplicate counts were taken for each sample and an average taken.

14. Staining and flow cytometric analysis of surface antigens

Cellular phenotyping was performed on BALF, lung, and spleen cells by flow cytometry as described (695). Cells (1×10^6/well) were transferred into 96-well, v-bottomed plates and washed in FACS staining buffer [PBS/BSA (1% w/v)/EDTA (2mM)/NaN_3 (0.1% w/v)]. Cells were then stained with 50µl αCD16/32 antibodies (10µg/ml; F_c block; Becton Dickinson, Oxford, UK) diluted in FACS staining buffer. Cells were incubated at 4°C for 20mins. Cells were then stained against cell surface proteins that identify cell subsets and other markers of interest (e.g. activation). A list of all proteins stained for by specific monoclonal antibodies and the relevant isotype control antibodies used are listed (Table 2). All antibodies were titrated prior to use within a dilution range of 1:100-1:12000 using spleen cells from naïve mice. Typically, a dilution of 1:200 was found to be optimal for a significant distinction between negative (MFI: $<10^1$) and positive (MFI: $>10^3$) populations. The relevant antibodies were diluted together in FACS staining buffer and 50µl of each cocktail was added to relevant wells without washing them. The cells were shaken on a plate-shaker to ensure mixing of cells and antibodies, and then they were incubated at 4°C for 45mins. The plates were centrifuged at 770g for 2mins and the supernatants removed using a multi-channel pipette. The cells were washed in 200µl PBS to remove any remaining BSA and centrifuged again at 770g for 2mins. The supernatants were removed using a multi-channel pipette and the cells fixed by adding 100µl PBS/paraformaldehyde (4% v/v). The cells were resuspended on a plate-shaker and incubated at 4°C for 20mins. The plates were then

centrifuged at 770g for 2mins and the supernatants removed using a multi-channel pipette. The cells were then washed with 200µl FACS staining buffer, centrifuged at 770g for 2mins, and the supernatants removed using a multi-channel pipette. The cells were finally resuspended in 200µl FACS staining buffer and taken for analysis. For all samples, at least 50×10^3 cells were collected on a Dako Cyan flow cytometer. Analysis was performed using FlowJo v3.1.2.

15. Staining and flow cytometric analysis of intracellular antigens

Cytokine production by individual lung and spleen cell types was determined by intracellular staining of cells stimulated with a polyclonal stimulus. To achieve this, cells (2×10^6/sample) were stimulated with either nothing or αCD3/28-expressing beads [50µl/well (2×10^6 beads); Invitrogen, Paisley, UK] in a final volume of 1ml supplemented RPMI 1640 medium in a 48-well flat-bottomed plate. The cells were incubated at 37°C/5% CO_2 for 24hrs, and then the supernatants carefully transferred into an empty 48-well plate without disturbing the cells. The supernatants are then stored at -80°C for further analysis.

The remaining cells are resuspended in 0.5ml of supplemented RPMI 1640 medium containing PMA (100ng/well; Sigma-Aldrich, Gillingham, UK), Ionomycin (1µg/well; Sigma-Aldrich, Gillingham, UK) and brefeldin A (1µl/well; Becton Dickinson, Oxford, UK). PMA is a phorbol ester and Ionomycin a calcium ionophore that stimulate cells in a polyclonal manner. Brefeldin A is a lactone antibiotic produced by *Eupenicillium brefeldianum*. It inhibits protein transport from the endoplasmic reticulum to Golgi apparatus, thus preventing release of any cytokines produced by stimulated cells. The cells are incubated for

a further 4hrs, the cells resuspended, and transferred into a 96-well, v-bottomed plate. Typically for staining for intracellular cytokines, each sample was split into two wells: one for assaying cytokine production by CD4 T cells and one for CD8 T cells. The cells were then centrifuged at 770g for 2mins and the supernatant removed using a multi-channel pipette. The cells were washed with FACS staining buffer, centrifuged at 770g for 2mins, the supernatant removed using a multi-channel pipette, and cells stained for surface antigens of interest as described in section 14. After fixation and washing of the samples, the cells were permeabilised by resuspending them in 50µl FACS staining buffer containing permeabilisation solution (1:10 dilution; Becton Dickinson, Oxford, UK). The permeabilisation solution contains saponin (a plant-derived amphipathic glycoside) that allows macromolecules (in this case antibodies) to pass through plasma membranes. The cells were shaken on a plate-shaker and incubated at 4°C for 20mins. Cells were then stained for the cytokine or transcription factor of interest. A list of all proteins stained for by specific monoclonal antibodies and the relevant isotype control antibodies used are listed (Table 2). All antibodies were titrated prior to use as described in section 2.14. The relevant antibodies were diluted together in FACS staining buffer containing permeabilisation solution and 50µl of each cocktail was added to relevant wells without washing them. The cells were shaken on a plate-shaker to ensure mixing of cells and antibodies, and then they were incubated at 4°C for 45mins. The plates were centrifuged at 770g for 2mins and the supernatants removed using a multi-channel pipette. The cells were washed in 200µl FACS staining buffer, the cells centrifuged at 770g for 2mins, and the supernatant removed using a multi-channel pipette. The cells were finally resuspended in 200µl FACS staining buffer and taken for analysis. For all samples, at least 50×10^3 cells were collected on a Dako Cyan flow cytometer. Analysis was performed using FlowJo v3.1.2.

Table 3. Details of fluorochrome-conjugated antibodies and isotype controls utilised in this study

Antibody specificity	Clone	Isotype	Fluorochrome	Company
B220	RA3-6B2	Rat IgG2a	PE-Cy5.5	Biolegend
CCR3	83101	Rat IgG2a	PerCP	R&D Systems
CD4	GK1.5	Rat IgG2b	Pacific Blue	Biolegend
CD8	53-6.7	Rat IgG2a	Pacific Blue	Becton Dickinson
CD11b	M1/70	Rat IgG2b	Pacific Blue	Biolegend
CD11c	N418	Armenian Hamster IgG λ2	APC	Becton Dickinson
CD19	6D5	Rat IgG2a	FITC	Biolegend
CD27	LG.3A10	Armenian Hamster IgG	PE	Biolegend
CD40	3.23	Rat IgG2a	APC	Biolegend
CD69	310106	Rat IgG2b	FITC	R&D Systems
F4/80	BM8	Rat IgG2a	PE-Cy5.5	Biolegend
ICOS	C398.4A	Armenian Hamster IgG	PE-Cy5.5	Biolegend
ICOSL	HK5.3	Rat IgG2a	PE	Biolegend
MHCII	M5/114.15.2	Rat IgG2b	Pacific Blue	Biolegend
NKp46	29A1.4	Rat IgG2a	PE-Cy5.5	Biolegend
OX40	Polyclonal	Goat IgG	PE	R&D Systems
PD-1	29F.1A12	Rat IgG2a	FITC	Biolegend
TCRβ	H57-597	Armenian Hamster IgG	PE-Cy5.5	Biolegend
IFN-γ	37895	Rat IgG2a	APC	R&D Systems
IL-17	Polyclonal	Goat IgG	PE	R&D Systems
FoxP3	FJK-16s	Rat IgG2a	PE	eBioscience
RORγt	B2D	Rat IgG1	PE	eBioscience
T-bet	4B10	Mouse IgG1	PE	eBioscience

Isotype controls: specificity	Clone	Isotype	Flurochrome	Company
KLH	RTK2758	Rat IgG2a	Multiple	Biolegend
KLH	54447	Rat IgG2a	PerCP	R&D Systems
Trinitrophenol + KLH	RTK4530	Rat IgG2b	Multiple	Biolegend
Mouse pooled immunoglobulin	R35-95	Rat IgG2a	Pacific Blue	Becton Dickinson
Trinitrophenol + KLH	G235-2356	Armenian Hamster IgG λ1	APC	Becton Dickinson
Trinitrophenol + KLH	HTK888	Armenian Hamster IgG	Multiple	Biolegend
KLH	141945	Rat IgG2b	FITC	R&D Systems
Polyspecific	Polyclonal	Goat IgG	PE	R&D Systems
Unknown	Unknown	Rat IgG1	PE	eBioscience
Unknown	P3.6.2.8.1	Mouse IgG1	PE	eBioscience

16. *In vitro* cytokine production by lung and spleen T cells

Lungs and spleens were harvested and processed as described in section 12, and cells were counted using trypan blue exclusion assay as described in section 13. Lung and spleen cells (2×10^6well) were set up in 48-well plates, and set up in culture with media, RSV (MOI 2.0; 6×10^6pfu/well), or αCD3/αCD28-coated beads [Invitrogen, Paisley, UK; pre-titrated to 50µl/well (2×10^6 beads)] in a final volume of 1ml supplemented RPMI 1640 medium. Cells were incubated at 37°C/5% CO_2 for 72hrs. After incubation, supernatants were carefully removed without disturbing the cells, and stored at -80°C for further analysis.

In some experiments, cells were stimulated with media or αCD3/αCD28-coated beads [50µl/well (2×10^6 beads)] in a final volume of 1ml supplemented RPMI 1640 medium. Cells were incubated at 37°C/5% CO_2 for 24hrs. After incubation, supernatants were carefully removed without disturbing the cells, and stored at -80°C for further analysis.

17. *In vitro* cytokine production from sorted lung DCs and CD4 T cells

Lung cells from control, or IL-21-depleted, rVVG-primed mice were collected as described in section 11, processed as described in section 12, and counted as described in section 13.

CD4 T cells from both groups were MACS-sorted using a positive isolation kit (Miltenyi Biotech, Bisley, UK). Lung cells were centrifuged at 770g for 2mins in a 50ml conical tube, and the supernatant decanted. The cells were resuspended in 100µl of lavage buffer per 10^7 cells, containing αCD16/32 antibodies [1:200 dilution (10µg/ml); F_c block; Becton Dickinson, Oxford, UK]. The cells were vortexed to ensure equal exposure of cells to the antibody, and were incubated at 4°C for 20mins. The cells were centrifuged at 770g for 2mins, the

supernatant decanted, and the cells resuspended in 90µl of lavage buffer per 10^7 cells, and then 10µl of CD4 microbeads per 10^7 cells added. The cells were vortexed to ensure equal exposure of cells to the microbeads, and incubated on ice for 20mins. The cells were centrifuged at 770g for 2mins, the supernatant removed and the cells washed in 2ml lavage buffer per 10^7 cells. The cells were centrifuged at 770g for 2mins, the supernatant decanted, and the cells resuspended in 0.5ml lavage buffer per 10^8 cells. The cells were then sorted on an autoMACS™ machine on a positive selection programme. A CD4 T cell purity of >90% was routinely obtained, as determined by flow cytometry.

Dendritic cells were FACS-sorted from the remaining lung cells. Cells were centrifuged at 770g for 2mins and the supernatant decanted. The cells were then resuspended in 100µl of lavage buffer per 10^7 cells, containing FITC-conjugated αCD11c and PE-conjugated αCD11b antibodies (1:200 dilution; Becton Dickinson, Oxford, UK). The cells were vortexed to ensure equal exposure of the cells to the antibody, and the cells incubated on ice for 30mins. The cells were centrifuged at 770g for 2mins, the supernatant decanted, and the cells resuspended in 1ml lavage buffer per 10^7 cells, vortexed, and taken for sorting. Cells staining positively for both CD11b and CD11c were collected on a FACS Diva sorter into 5ml FBS to ensure maximum cell viability. DC purity of >93% was obtained. Collected DCs were centrifuged at 770g for 2mins, the supernatant decanted, and the cells washed in 5ml supplemented RPMI 1640 medium. The cells were centrifuged at 770g for 2mins, resuspended in 5ml supplemented RPMI 1640 medium, and counted by trypan blue exclusion as described in section 2.13. Once counted, DCs were set up in culture in round-bottomed 96-well plates (4×10^4/well), centrifuged at 770g for 2mins, and the supernatant removed using a multi-channel pipette. The cells were then resuspended in supplemented

RPMI 1640 medium (100µl/well) containing either a non-specific RSV G negative control peptide (p11: G64-78; Cambridge Research Biochemicals, Cleveland, UK) or antigen-specific peptide (p31: G184-198; ProImmune, Oxford, UK; both at 10µg/ml). The cells were incubated at 37°C/5% CO_2 for 1hr. Sorted CD4 T cells (100µl; 4×10^5 cells/well) were then immediately added to each well and the cells incubated at 37°C/5% CO_2 for 72hrs. After incubation, the supernatants were carefully removed without disturbing the cells, transferred into empty round-bottomed 96-well plates, and stored at -80°C for further analysis.

18. Cytokine sandwich ELISA

Cytokine levels in supernatants were quantified using sets of paired monoclonal antibodies (Duosets; R&D Systems, Abingdon, UK) using the following protocol. Immunosorp™ ELISA plates (VWR, Lutterworth, UK) were coated with 100µl/well of the relevant capture antibody (1:200 dilution of stock in PBS), sealed with cling film and incubated at 4°C overnight. The supernatants were decanted and the wells dried on paper towel. The wells were blocked with 200µl of blocking buffer, sealed with cling film, and incubated at room temperature for 2hrs. After incubation the wells were washed three times with wash buffer. Samples were thawed and vortexed, as well as doubling dilution standard curves prepared in ELISA buffer [PBS/BSA (1% w/v)/Tween 20 (0.05% v/v)]. Samples and standards (100µl/well) were added to relevant duplicate and triplicate wells respectively, the plates sealed with cling film, and incubated at 4°C overnight. A list of standard curve ranges in pg/ml is listed (Table 3). The wells were washed four times with wash buffer and relevant

Table 3. Cytokine standard curves used for sandwich ELISA

Cytokine ELISA	Lower limit of standard curve (pg/ml)	Upper Limit of Standard Curve (pg/ml)
6kine (CCL21)	15.63	2000
Granzyme B	7.32	15000
IFN-γ	29.3	60000
IL-4	2.44	5000
IL-10	39.06	5000
IL-17	78.13	10000
IL-21	4.88	10000
IP-10 (CXCL10)	15.63	2000
MIP-1α (CCL3)	15.63	2000
MIP-2α (CXCL2)	15.63	2000
MIP-3α (CCL20)	15.63	2000
MIP-3β (CCL19)	15.63	2000
RANTES (CCL5)	4.88	10000
TNF	4.88	10000

detection (biotinylated) antibodies were diluted in ELISA buffer (1:200 dilution of stock) and added to relevant wells (100µl/well). The plates were sealed with cling film and incubated at room temperature for 1hr. After incubation, the wells were washed four times with wash buffer, and avidin-horseradish peroxidase conjugate (Becton Dickinson, Oxford, UK) was diluted in ELISA buffer (1:1000 dilution) and added to all wells (100µl/well). The plates were sealed with cling film and incubated at room temperature for 30mins. The wells were washed five times with wash buffer and then OPD substrate (*O*-Phenylenediamine; Sigma-Aldrich, Gillingham, UK) was added (100µl/well; 1mg/ml) and incubated in the dark. The wells were monitored for colour development, and once the full range of the standard curve could be observed then the reaction was terminated by adding sulphuric acid (2M; 50µl/well). The plates were read on a microplate reader (Molecular Devices, Wokingham, UK) using a reference wavelength of 490nm. The standard curves were used to calculate sample concentrations.

19. RSV-specific antibody ELISA

Levels of RSV-specific IgA, IgE, IgG1, and IgG2a were determined as follows. Immunosorp™ ELISA plates (VWR, Lutterworth, UK) were coated with 200µl/well of RSV antigen or Hep-2 control lysate (1:500 dilution in PBS), sealed with cling film and incubated at 4°C overnight. The supernatants were decanted and the wells dried on paper towel. The wells were blocked with 200µl of blocking buffer, sealed with cling film, and incubated at room temperature for 2hrs. After incubation the wells were washed three times with wash buffer, the supernatants decanted, and the wells dried on paper towel. Two-fold dilution series of mouse serum (starting at 1:100 dilution) and BALF (starting at 1:4 dilution) samples were

prepared in ELISA buffer. A two-fold dilution series of normal mouse serum was also prepared and used as a negative control. Negative control samples, serum samples, and BAL samples were added to the relevant wells (100µl/well) on the plate, the plates sealed with cling film, and incubated at 4°C overnight. All samples were tested in duplicate wells. The wells were washed four times with wash buffer and biotinylated mouse IgA, IgE, IgG1, and IgG2a-specific antibodies (Becton Dickinson, Oxford, UK) were diluted in ELISA buffer (1:500 dilution; 1µg/ml) and added to relevant wells (100µl/well). The plates were sealed with cling film and incubated at room temperature for 1hr. After incubation, the wells were washed four times with wash buffer, and avidin-horseradish peroxidase conjugate (Becton Dickinson, Oxford, UK) was diluted in ELISA buffer (1:1000 dilution) and added to all wells (100µl/well). The plates were sealed with cling film and incubated at room temperature for 30mins. The wells were washed five times with wash buffer and then OPD substrate (Sigma-Aldrich, Gillingham, UK) was added (100µl/well) and incubated in the dark. The wells were monitored for colour development, and once colour began to develop at the lowest concentration of the samples then the reaction was terminated by adding sulphuric acid (2M; 50µl/well). The plates were read on a microplate reader (Molecular Devices, Wokingham, UK) using a reference wavelength of 490nm.

20. MACS sorting and adoptive transfer of splenic CD4 T cells

Mice were immunised with rVV-G and RSV-challenged as described in section 2.7. as well as IL-21-depleted (or not) as described in section 2.8. The mice were left for 28d to allow memory development and spleens harvested as described in section 2.12. CD4 T cells from both control and IL-21-depleted groups were MACS-sorted using a positive isolation kit

(Miltenyi Biotech, Bisley, UK). Spleen cells were centrifuged at 770g for 2mins in a 50ml conical tube, and the supernatant decanted. The cells were resuspended in 100µl of MACS buffer (formulation as for lavage buffer) per 10^7 cells, containing αCD16/32 antibodies [1:200 dilution (10µg) F_c block; Becton Dickinson, Oxford, UK]. The cells were vortexed to ensure equal exposure of cells to the antibody, and were incubated at 4°C for 20mins. The cells were centrifuged at 770g for 2mins, the supernatant decanted, and the cells resuspended in 90µl of MACS buffer per 10^7 cells, and then 10µl of CD4 microbeads per 10^7 cells added. The cells were vortexed to ensure equal exposure of cells to the microbeads, and incubated on ice for 20mins. The cells were centrifuged at 770g for 2mins, the supernatant removed and the cells washed in 2ml lavage buffer per 10^7 cells. The cells were centrifuged at 770g for 2mins, the supernatant decanted, and the cells resuspended in 0.5ml lavage buffer per 10^8 cells. The cells were then sorted on an autoMACS^{TM} machine on a positive selection programme. A CD4 T cell purity of >90% was routinely obtained, as determined by flow cytometry. Mice were injected with relevant splenic CD4 T cells (2.5×10^6/mouse in 0.5ml PBS; i.p) 24h before RSV challenge (1×10^6pfu/mouse; i.n).

21. Quantification of viral replication and transcription factor gene expression

To determine viral replication and gene expression in the pulmonary compartment, lungs were harvested as described in section 2.11. However, the lungs were placed into a 1.8ml cryovial (VWR, Lutterworth, UK) and snap frozen in liquid nitrogen. For processing, lungs were transferred into a 7ml bijou (VWR, Lutterworth, UK) containing 1ml RLT buffer (Qiagen, Crawley, UK). Lung tissue was then homogenised using a motorised homogeniser until no tissue remains. 700µl of each sample was pipetteded into RNeasy columns (Qiagen,

Crawley, UK) then centrifuged at 9600g at room temperature for 1min. Flow-through in the collector tube was discarded. This step was repeated until all of the sample had been passed through the RNeasy column. 700μl of RW1 buffer (Qiagen, Crawley, UK) was added to the column, followed by centrifugation at 9600g at room temperature for 1min. Flow-through was discarded. 500μl RPE buffer was added to the columns, followed by centrifugation at 9600g at room temperature for 1min. Flow-through was discarded. This step was repeated. The RNeasy columns were transferred to 2ml collection tubes and centrifuged at 9600g at room temperature for 1min. The previous collection tubes were discarded. The RNeasy columns were transferred to 1.5ml collection tubes and the previous collection tubes were discarded. 30μl of nuclease-free water was added to the RNeasy columns followed by centrifugation at 9600g at room temperature for 1min. This step was repeated resulting in 60μl of RNA in nuclease-free water in the collection tube. The Rneasy columns were discarded and the concentration of the eluted RNA was determined using spectrophotometry and measured as a concentration of μg/ml.

Before conversion to cDNA, the RNA samples were diluted with nuclease-free water to the concentration of the sample with the lowest concentration. Following these dilutions, 60μl of each RNA sample was added to 8.6μl 10x buffer (Qiagen, Crawley, UK), 8.6μl dNTPs (Qiagen, Crawley, UK), 4.3μl random hexamers (Promega, Southampton, UK), and 4.3μl reverse trascriptase (Qiagen, Crawley, UK) in 0.2ml eppendorf tubes on ice. The samples were then placed in a thermal block at 37C° for 1 hour to generate cDNA, and then stored at -20°C.

Viral replication was measured as the number of L gene copies expressed in the lung. They were measured against standard plasmids (10^7 to 10^1 copies) and a non-template control.

100ng cDNA was used per reaction in 96-well FAST reaction plates (Applied Biosystems, Paisley, UK) with the following reagents: 2× mastermix (Qiagen, Crawley, UK), Forward L gene primer (900nM; Invitrogen, Paisley, UK), Reverse L gene primer (300nM; Invitrogen, Paisley, UK), and L gene probe (100nM; Invitrogen, Paisley, UK) in a 20μl total volume. The same machine and conditions were used to measure L gene expression as described above for transcription factor gene expression. The data were analysed and a L gene copy number was determined using the standard curve that was run in parallel by comparing C_t values.

22. Statistical Analysis

In order to increase the probability that data followed a Gaussian distribution and were normally distributed all experiments were performed at least twice with a minimum of five mice per group, however many were performed several times. Prior to statistical analysis all data were analysed in individual columns using a D'Agostino-Pearson normality test to confirm a high P value (i.e. that the data follow a Gaussian distribution) and that parametric statistical test were valid. A *Student* t-test was used to analyse statistical differences between two groups. One-way analysis of variance (ANOVA) was employed to analyze differences between more than two groups and significance was assumed at $p<0.05$. A Tukey post-test was used to identify differences between specific groups, with a significance threshold of $p<0.05$.

H. Endogenous IL-21 regulates pathogenic mucosal CD4 T cell responses during primary RSV challenge in mice

1. Introduction

Given the broad, pleiotropic actions of IL-21, affecting the activation and effector functions of several cell types, it is unsurprising that previous studies modulating its expression in different disease models have dramatically different effects, leading to conflicting conclusions. For example, IL-21 is considered a pathological factor in several diseases of the gastrointestinal tract [e.g. colitis (696), Crohn's disease (697), and IBD (698)] and RA (699), and its depletion is considered a promising candidate for therapy in these settings. In contrast, IL-21 is considered beneficial for immune responses against HBV (700) and HIV (701), and for inhibiting hypersensitivity reactions (702) and rhinitis (703). The conflicting data from these studies may be in part due to the effects of IL-21 on cytokine production. Separate studies have conclusively shown that IL-21 is a Th1-biasing factor by boosting IFN-γ production by NK cells (589), a Th2-biasing factor by boosting STAT6 and inhibiting STAT4 expression in naïve CD4 T cells (592), and a Th17-biasing factor by stabilising IL-23R and RORγt expression in activated CD4 T cells (386,446,583). More recently, other studies have demonstrated that IL-21 can act as an anti-inflammatory factor similar to IL-10 (704). Therefore, the action of IL-21 is likely to be very different in different disease states.

Previous studies investigating the actions of IL-21 in different diseases have focussed on autoimmune conditions of the gastrointestinal tract and joints as described above. There have also been many studies in different cancer models (705-707), but limited studies in

other diseases [e.g. Listeria (708), Leishmaniasis (709), HBV infection (700), HIV infection (710), and immediate hypersensitivity (702)]. In contrast, there have been almost no studies investigating the role of IL-21 in respiratory disease, and those that have are focussed on allergy [e.g. rhinitis (703)]. Specifically, there has been no comprehensive study of the role of IL-21 in immune responses to respiratory viral infection. This is particularly important as these infections are ubiquitous, occur throughout life, are the most common cause of asthma exacerbations, and therefore have a prominent clinical and economic burden. Given the known effects of IL-21 on CD8 T cell activation and effector function it possesses considerable therapeutic potential in respiratory disease that to date has not been investigated. This study addresses this gap in our knowledge in a murine model of Respiratory Syncytial Virus (RSV). I have studied the effect of endogenous IL-21 on disease severity and viral replication during primary RSV infection via antibody-mediated depletion. Further, I show the effects of IL-21 depletion on recruitment and activity of immune cells and production of soluble immune factors over the course of primary challenge.

2. Titration of the IL-21-depleting antibody

Prior to studying the effect of IL-21 depletion on immune responses to primary RSV challenge, I determined the optimal antibody dose for *in vivo* use by titration. Groups of mice were injected intraperitoneally (i.p) to systemically deplete IL-21 with doses ranging from 0-500µg of anti-IL-21 or isotype control antibody. Previous studies have demonstrated that IL-21 plays a crucial role in optimal B cell activation and antibody production (625,711,712). Therefore, as well as serum IL-21, antibody levels were determined seven days later. Serum IL-21 was undetectable by sandwich ELISA and therefore could not be

used a read out (Fig.1.1a), however serum IgA (Fig.1.1b), IgE (Fig.1.1c), IgG1 (Fig.1.1d) and IgG2a (Fig.1.1e) were detectable. I observed no effect on serum antibody levels by isotype control antibody. Moreover, no effect on serum antibody levels was detected when 0-50µg doses of anti-IL-21 were used. Using 100-500µg antibody doses, I observed a dose-dependent reduction on serum IgG1 (Fig.1.1d) and IgG2a (Fig.1.1e), but not IgA (Fig.1.1b) or IgE (Fig.1.1c), antibody levels. This reduction peaked at the 500µg dose, therefore this dose was used for further studies.

To confirm this dose was depleting IL-21, I injected rmIL-21 (10ng/mouse; i.p) every 48hrs for six days. rmIL-21 significantly boosted serum IgG1 (compare Fig.1.2a and Fig.1.1d) but not IgG2a (compare Fig.1.2b and Fig.1.1e) levels. Co-injection of pre-titrated anti-IL-21 antibody (0.5mg/mouse; i.p) on day -1 and +1 relative to the first rmIL-21 dose (on d0) ablated the effects of rmIL-21 and significantly decreased serum IgG1 (Fig.1.2a) and IgG2a (Fig.1.2b) compared to baseline levels. In contrast, co-injection of isotype control antibody had no effect.

3. Titration of the Respiratory syncytial virus stock *in vitro* and *in vivo*

Prior to use, RSV stocks have to be titrated both *in vitro* (to determine the titre in pfu/ml) and *in vivo*. I generated an RSV stock and determined the titre *in vitro* by plaque assay. Viral stock was tested at three dilutions (10^{-3}, 10^{-5}, and 10^{-7}) that are known to enable reliable counting of plaques to determine an accurate viral titre. No viral antigens were detected on the surface of uninfected Hep2 cells (Fig.1.3a), however large plaques were visible when Hep2 cells were infected with RSV (Fig.1.3b). As expected, the greatest numbers of plaques

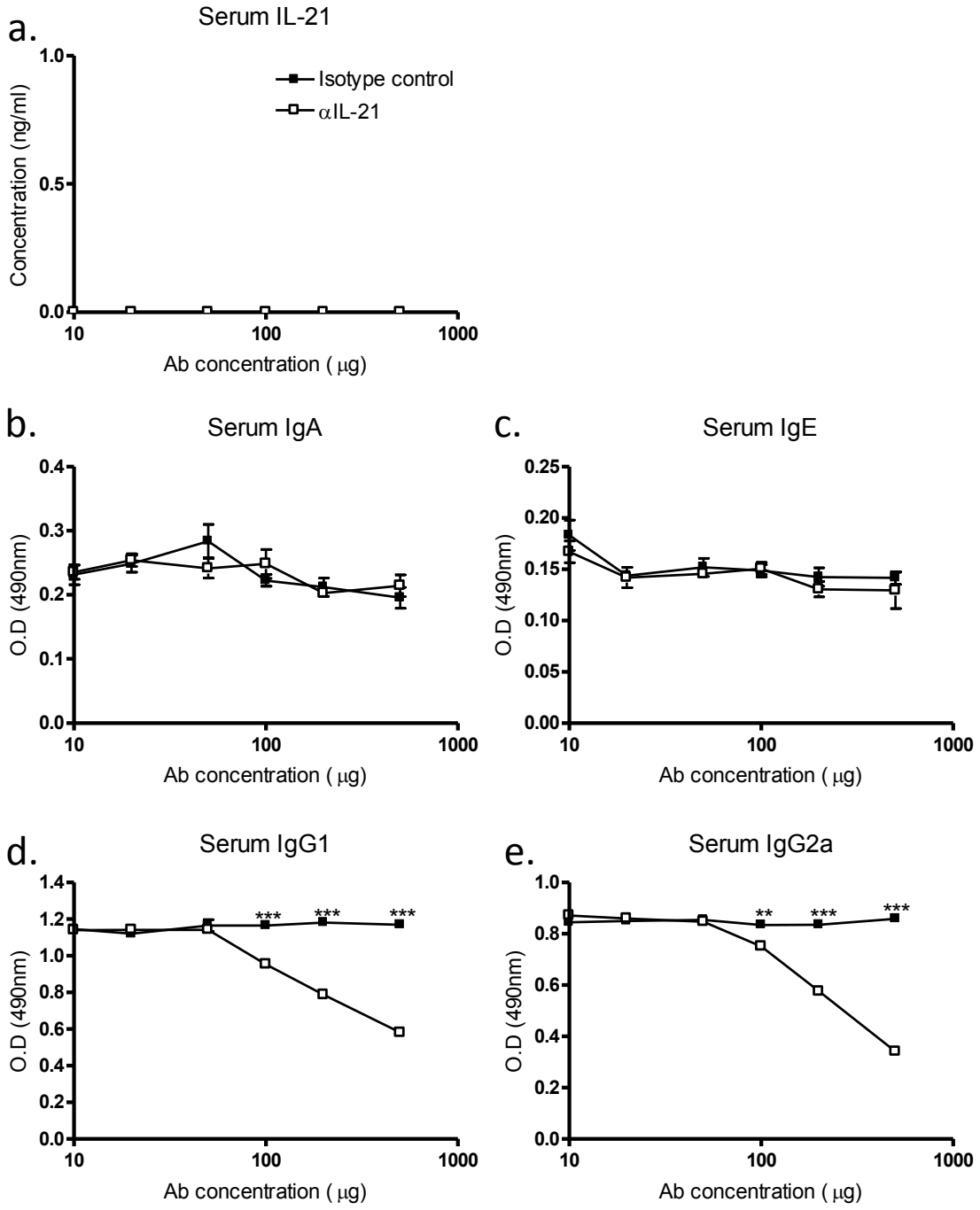

Figure 1.1. Titration of the IL-21-depleting antibody. Mice were injected (i.p) with 0, 10, 20, 50, 100, 200, or 500μg of anti-IL-21 or isotype control antibody. Serum IL-21 (a), IgA (b), IgE (c), IgG1 (d), and IgG2a (e) levels were determined seven days later by ELISA. Data is expressed as mean±SEM. The graphs are representative of two independent experiments of five mice per group. Student *t* test result **: $p<0.01$, ***: $p<0.001$.

were observed when the virus was least diluted (10^{-3}). Plaques were counted and a consistent titre of 2.2×10^{7}pfu/ml was calculated. The RSV dose used to infect mice varies between different laboratories and different studies because each stock used contains a different number of replication-competent, and defective-interfering (DI), particles. Therefore, it is important to determine the effective RSV dose for *in vivo* studies prior to use. I challenged multiple groups of mice with between 0, 1×10^{5}, 2×10^{5}, 5×10^{5}, 1×10^{6}, or 2×10^{6}pfu/mouse, and weighed them for 14 days. In this model weight loss is a reliable marker of disease severity (102). Weight loss also correlates with T cell recruitment and activation and a productive RSV infection is known to elicit a T cell response that causes disease severity. Weight loss of 5-10% of baseline weight is considered acceptable because disease severity caused by RSV infection is mild. Moreover, changes in disease severity by IL-21 depletion can be easily monitored by weighing the mice.

Weight loss was not detectable in uninfected mice (vehicle only) or mice infected with low doses of RSV ($<5\times10^{5}$pfu; Fig.1.3c). Significantly greater weight loss was observed in mice challenged with higher doses and peaked at the highest dose (Fig.1.3c). On the basis of weight loss, a dose of 1×10^{6}pfu/mouse was chosen for future studies. To confirm that the virus was fit and replicating *in vivo*, I determined the number of viral L gene copies in the lungs of infected mice at several time points post challenge by quantitative PCR (qPCR; protocol described in *Materials and Methods*). The L gene is measured to determine viral replication as it is the most 3' gene on the polysome. Therefore, detection of viral RNA encoding this gene ensures that transcription of the whole polysome, and productive viral

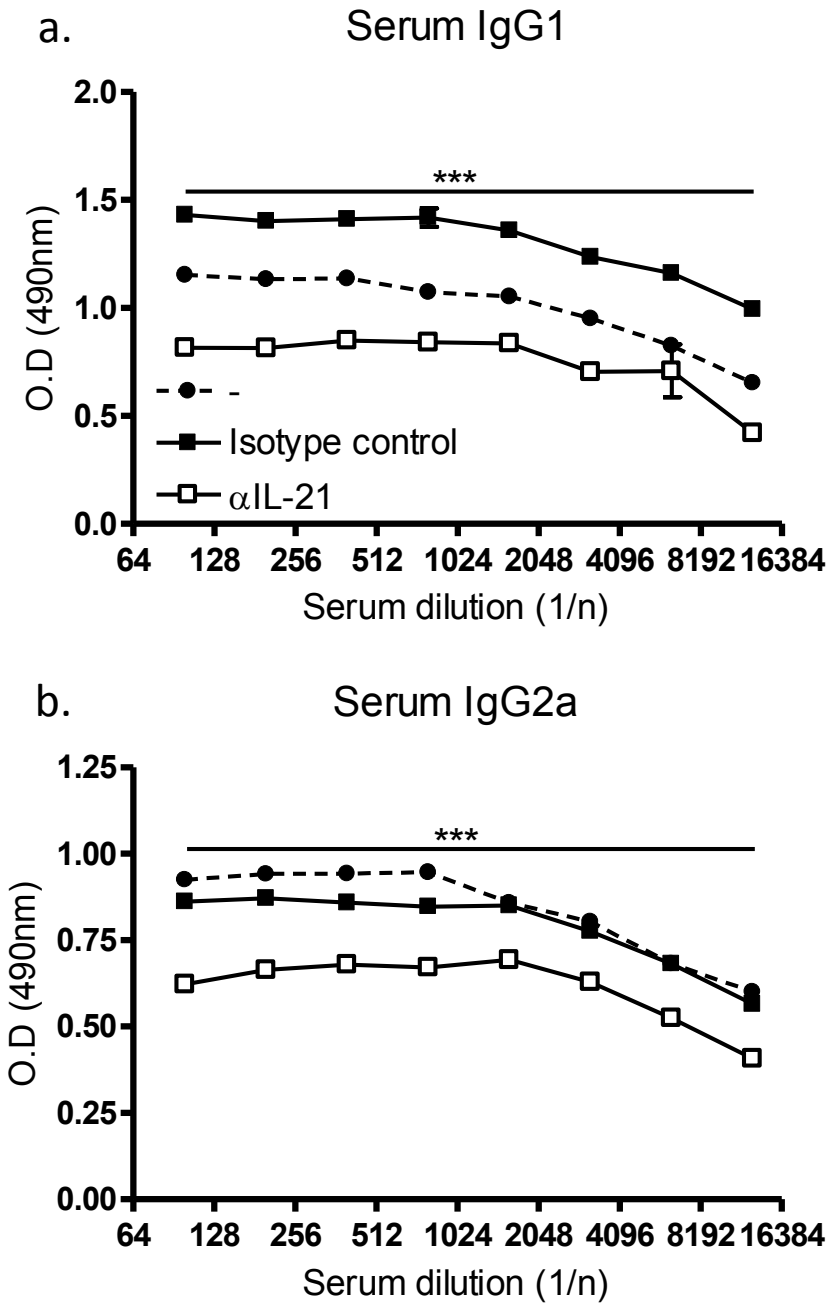

Figure 1.2. Testing of the IL-21-depleting antibody. Mice were injected with rmIL-21 (10ng/mouse; i.p) every 48hrs for six days. Anti-IL-21 or isotype control antibody (0.5mg/mouse; i.p) was injected one day prior and one day after the first injection of rmIL-21. Serum IgG1 (a), and IgG2a (b) levels were determined by ELISA. Data is expressed as mean±SEM. The graphs are representative of two independent experiments of five mice per group. Student *t* test ***: $p<0.001$.

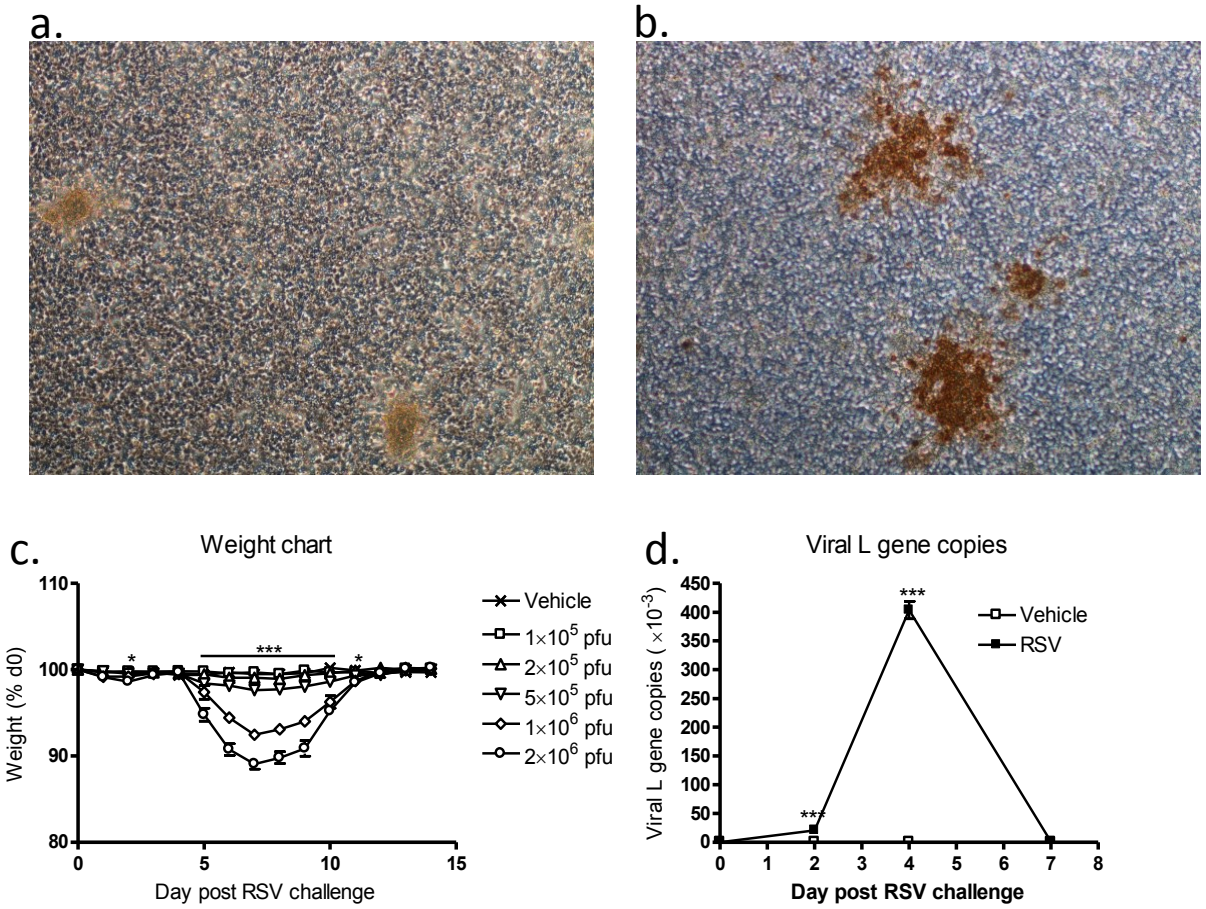

Figure 1.3. Titration of the Respiratory syncytial virus stock *in vitro* and *in vivo*. RSV stock was generated and the titre determined *in vitro* by plaque assay (as described in *Materials and Methods*). Viral stock was tested at three dilutions (10^{-3}, 10^{-5}, and 10^{-7}) and the number of plaques counted. Representative images of plaques from uninfected cells (a) and infected cells (b) are shown (10x magnification). Mice were challenged with RSV (0-2×10^6 pfu/mouse; i.n) and weighed them for 14 days. Weight is shown as a percentage of baseline weight (c). The number of viral L gene copies in the lungs of infected mice was determined on d0, 2, 4, and 7 post challenge by quantitative PCR (qPCR; protocol described in *Materials and Methods*; d). Data is expressed as mean±SEM. The graphs are representative of two independent experiments of five mice per group. ANOVA or student *t* test result *: $p<0.05$, ***: $p<0.001$.

replication has occurred.

Viral L gene was undetectable in lung tissue from uninfected mice but was detectable at day two p.c, peaking at day four p.c before returning to baseline at day seven (Fig.1.3d).

4. IL-21 depletion increases disease severity after primary RSV challenge

To determine the effect of IL-21 depletion on the response to RSV, I administered 0.5mg anti-IL-21 antibody or isotype control (i.p) on d-1 and +1 relative to RSV challenge (on d0). The mice were weighed from challenge to determine the level of disease severity. Mice treated with control antibody exhibited weight loss typical of a primary infection (Fig.1.4): no significant weight loss occurred between d0-4 p.c, followed by a period of weight loss that peaked on d7 p.c. after which mice regained weight with all mice reaching their starting weight by d10 p.c. IL-21-depleted mice exhibited the same phenotype as control mice from d0-4 (Fig.1.4). However, from d4-7 p.c this group lost significantly more weight than the control group, peaking on the same day prior to recovery thereafter.

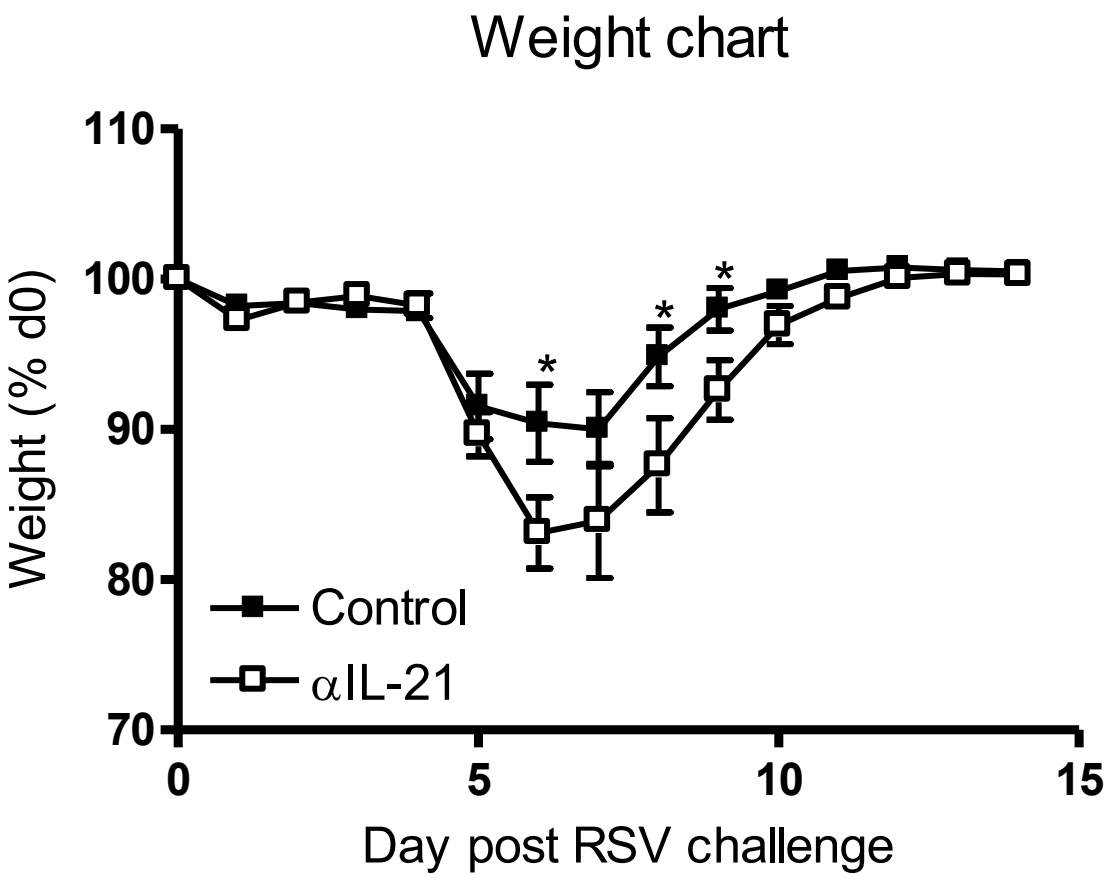

Figure 1.4. IL-21 depletion increases disease severity after primary RSV challenge. Mice were challenged with RSV (1×10^6 pfu/mouse; i.n) on d0. Anti-IL-21 antibody or isotype control (0.5mg/mouse; i.p) was administered one day prior and one day after RSV challenge. Mice were weighed daily for 14 days. Weight is shown as a percentage of baseline weight. Data is expressed as mean±SEM. The graphs are representative of at least six independent experiments of five mice per group. Student *t* test result *: $p<0.05$.

5. IL-21 depletion increases viral clearance after primary RSV challenge

Next, I determined the effect of IL-21 depletion on viral replication and clearance. Viral replication was measured by detection of the number of viral L gene copies in lung tissue by qPCR at several time points post RSV challenge. The virus replicated similarly in both groups of mice: L gene copies were first detected at d2 p.c, peaked at d4 p.c, and cleared by d7 p.c (Fig.1.5). IL-21 depletion did not affect the kinetics of viral replication and clearance but there was a significant reduction in L gene copy number at d4 p.c (Fig.1.5).

6. IL-21 depletion increases CD4 T cell recruitment after primary RSV challenge

Viral clearance is associated with the initiation of an anti-viral T cell response and recruitment of T cells to the lung tissue and airway. As the number of L gene copies was significantly reduced I hypothesised this was due to an increase in T cell numbers and activity in the pulmonary compartment. Therefore, I determined the number of immune cells in the airway and lung tissue at several time points post challenge. Cellular recruitment into the airway (Fig.1.6a) and lung tissue (Fig.1.6b) increased after RSV challenge, peaking at d7 p.c. IL-21 depletion did not significantly alter cellular recruitment into either compartment up to d4 p.c. However, by d7 p.c I observed a significant increase in both compartments in IL-21-depleted mice.

To determine if the cellular phenotype of the anti-viral response had been altered by IL-21 depletion, I characterised the cell types recruited by flow cytometry. At d2-4 p.c there was a significant influx of neutrophils (identified as $CD3^{-}B220^{-}CD11b^{+}CCR3^{-}$; BAL: Fig.1.6c; Lung:

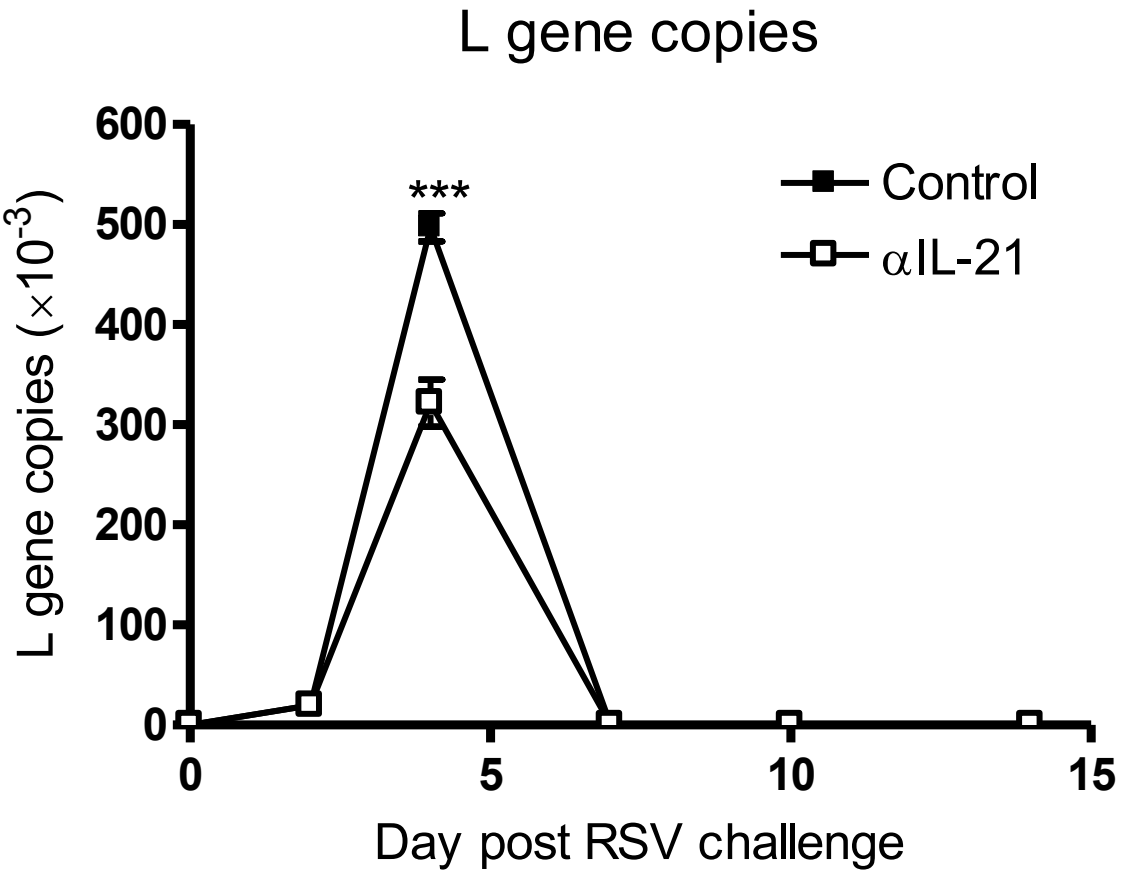

Figure 1.5. IL-21 depletion increases viral clearance after primary RSV challenge. Mice were challenged with RSV on d0. The number of viral L gene copies in the lungs of infected mice was determined on d0, 2, 4, and 7 post challenge by quantitative PCR. Data is expressed as mean±SEM. The graphs are representative of three independent experiments of five mice per group. Student *t* test result ***: $p<0.001$.

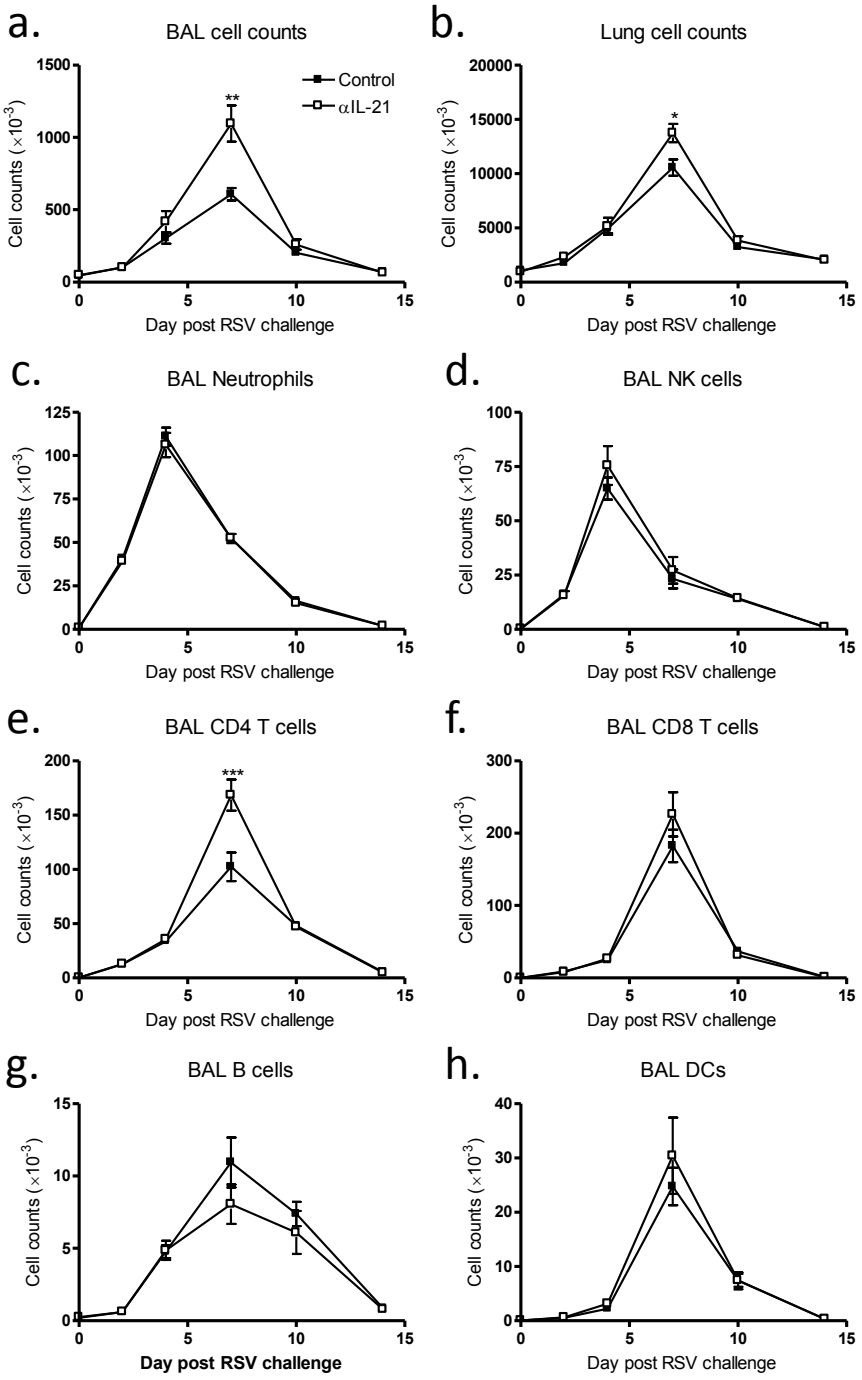

Figure 1.6. IL-21 depletion increases CD4 T cell recruitment after primary RSV challenge. Mice were challenged with RSV on d0. BALF (a) and lung tissue (b) were harvested at d0, 2, 4, 7, 10, and 14 post challenge. BALF cells were phenotyped by flow cytometry and neutrophil (c), NK cell (d), CD4 T cell (e), CD8 T cell (f), B cell (g), and DC (h) cell counts determined. At least 50×10^3 cells/sample were collected. Data is expressed as mean±SEM. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: p<0.05, **: p<0.01, ***: p<0.001.

Appendix 1.1a) and NK cells (identified as $TCR\beta^{-}NKp46^{+}$; BAL: Fig.1.6d; Lung: Appendix 1.1b). IL-21 depletion did not significantly alter the number of either cell type at this time, though there was a trend for increased NK cell numbers. By d7 p.c, the number of these cells decreased and both CD4 (Fig.1.6e) and CD8 (Fig.1.6f) T cell recruitment peaked. IL-21 depletion did not significantly affect the number of CD8 T cells recruited to either compartment, though increased numbers were detected (BAL: Fig.1.6f; Lung: Appendix 1.1d). However, I did observe a significant increase in CD4 T cell recruitment in both airway (Fig.1.6e) and lung tissue (Appendix 1.1c).

The recruitment of other cell types was also determined. I observed no significant changes in the numbers of B cells ($B220^{hi}CD19^{+}$; BAL: Fig.1.6g; Lung: Appendix 1.1e), dendritic cells (DCs; identified as $CD11c^{+}MHCII^{hi}$; BAL: Fig.1.6h; Lung: Appendix 1.1f), eosinophils (identified as $CD3^{-}B220^{-}MHCII^{-}CD11b^{+}CCR3^{+}$; BAL: Appendix 1.2a; Lung: Appendix 1.2b), or macrophages (identified as $MHCII^{int}CD11b^{+}CD11c^{+}F4/80^{+}$; BAL: Appendix 1.2c; Lung: Appendix 1.2d) between the two groups in either compartment at any time post challenge. However, I did observe a trend for increased numbers of DCs and a reduction in B cells in the airway of IL-21-depleted mice.

7. IL-21 depletion increases NK cell and CD4 T cell activity after primary RSV challenge

After RSV challenge, disease severity positively correlates with T cell recruitment to the pulmonary compartment. Moreover, it also correlates with increases in T cell activity (e.g. expression of co-stimulatory molecules and cytokine production). To determine if recruited T cells were active I first determined their level of co-stimulatory molecule expression by flow cytometry. ICOS and CD69 are two reliable surface markers of cell activity, particularly

lymphocytes. CD69, but not ICOS, expression was detected on recruited NK cells to BAL (Fig.1.7a) and lung (Fig.1.7b), peaking at d4 p.c, and IL-21 depletion slightly increased expression of CD69, but not ICOS, on this cell type. CD69 and ICOS was also detected on both BAL (Fig.1.7c) and Lung (Fig.1.7d) CD4 T cells, and BAL (1.7e) and Lung (Fig.1.7f) CD8 T cells after RSV challenge, peaking at d7 p.c. However, while IL-21 depletion slightly increased expression of ICOS (but not CD69) on CD4 T cells, it had no significant effect on either marker on CD8 T cells.

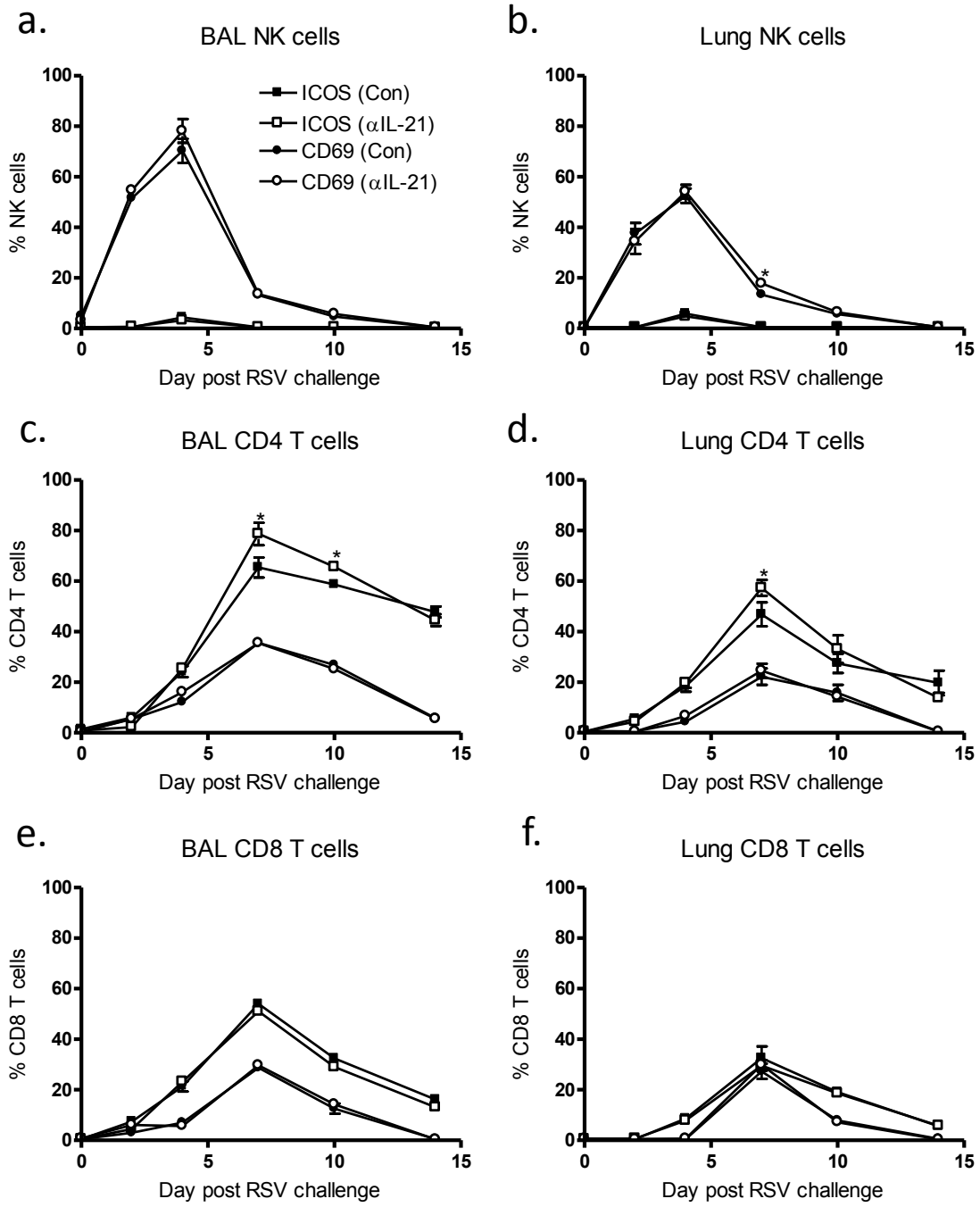

Figure 1.7. IL-21 depletion increases NK cell and CD4 T cell activity after primary RSV challenge. Mice were challenged with RSV on d0. BALF and lung cells were harvested at d0, 2, 4, 7, 10, and 14 post challenge and phenotyped by flow cytometry. Activity of BAL (a, c, e) and lung (b, d, f) NK cells (a-b), CD4 T cells (c-d), and CD8 T cells (e-f) were determined by measuring the percentage of cells positive for ICOS or CD69 expression by flow cytometry. At least 50×10^3 cells/sample were collected. Data is expressed as mean±SEM. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: $p<0.05$.

8. IL-21 depletion increases pro-inflammatory cytokine and chemokine production after primary RSV challenge

The cytokine response to RSV challenge is characterised by significant production of many cytokines and chemokines. Pro-inflammatory cytokines such as IFN-γ and TNF as well as the cytolytic protein granzyme B are associated with type I responses and significant T cell influx and effector function. This type of response elicits effective viral clearance. Chemokines such as RANTES are responsible for recruitment of T cells to the pulmonary compartment and the anti-inflammatory cytokine IL-10 is produced upon their activation as a means of self-regulation. Finally, IL-4 is a Th2 cytokine that has been found to be up regulated in cases of RSV bronchiolitis. To assess whether the increased activation state of infiltrating T cells resulted in changes in the pulmonary environment I measured production of these soluble factors into the bronchoalveolar lavage fluid (BALF) and by polyclonal stimulation of lung cells. Levels of IFN-γ, IL-4, TNF, IL-10, RANTES, and granzyme B were determined by sandwich ELISA. RSV challenge elicits an anti-viral response that is type I in phenotype, characterised by increased BAL IFN-γ (Fig.1.8a), granzyme B (Fig.1.8b), TNF (Fig.1.8c), RANTES (Fig.1.8d), and negligible IL-4 (Fig.1.8e) production. Production of IFN-γ, granzyme B, and TNF peaked at d7 p.c, consistent with the peak of T cell recruitment, the primary source in this model. In contrast, IL-10 peaked at d4 p.c, though it was remained detectable at d7 p.c (Fig.1.8f). IL-21 depletion had no effect on IL-4 production (Fig.1.8e). Depletion also had no significant effect on granzyme B (Fig.1.8b) or TNF (Fig.1.8c), though there were increased levels detected in the BAL. In contrast, IL-21 depletion significantly increased production of IFN-γ (Fig.1.8a) at d7 p.c, IL-10 (Fig.1.8f) at d4 p.c, and significantly increased RANTES (Fig.1.8d) production at d2 and d7 p.c.

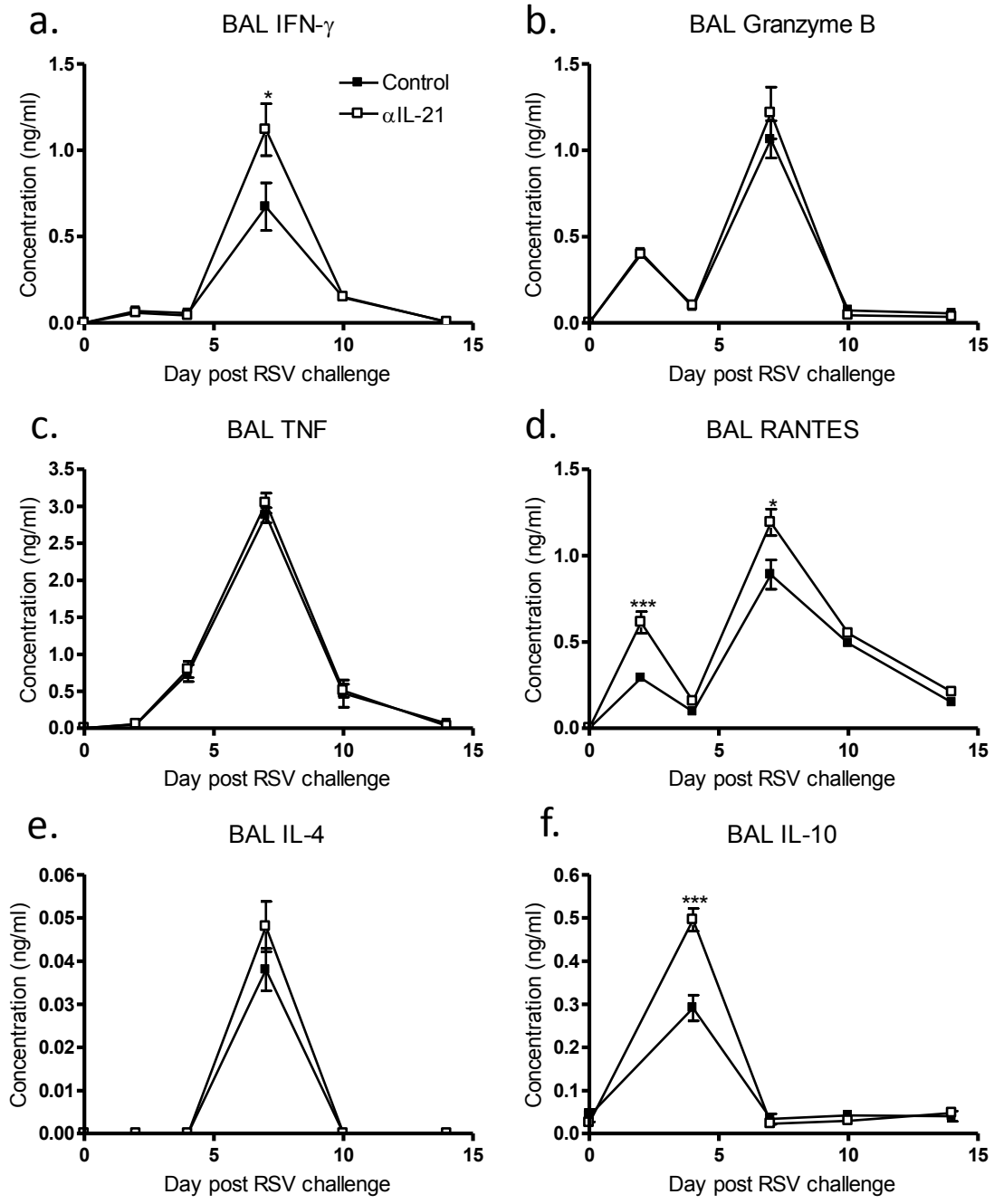

Figure 1.8. IL-21 depletion increases pro-inflammatory cytokine and chemokine production after primary RSV challenge. Mice were challenged with RSV on d0. BALF was harvested on d0,2, 4, 7, 10, and 14 post challenge and IFN-γ (a), Granzyme B (b), TNF (c), RANTES (d), IL-4 (e), and IL-10 (f) were determined by sandwich ELISA. Data is expressed as mean±SEM. The graphs are representative of three independent experiments of five mice per group. Student *t* test result *: $p<0.05$, ***: $p<0.001$.

To assess the cytokine profile of T cells in the lung tissue I stimulated lung cells with αCD3 /αCD28-antibody-expressing beads. Peak production of IFN-γ (Fig.1.9a), granzyme B (Fig.1.9b), TNF (Fig.1.9c), RANTES (Fig.1.9d), IL-4 (Fig.1.9e), and IL-10 (Fig.1.9f) was detected in the supernatants of stimulated lung cells from RSV-challenged mice at d7 p.c. Lung cells from IL-21-depleted mice exhibited a similar phenotype to that observed in the BALF at this time, with increased production of IFN-γ, granzyme B, TNF, RANTES, and also IL-10. There was no effect on IL-4 production.

9. IL-21 depletion increases IFN-γ production by CD4 T cells after primary RSV challenge

IL-21 depletion increased cytokine production into the BALF and by polyclonally-stimulated lung T cells. To confirm these cytokines were T-cell-derived I performed intracellular staining of stimulated lung cells to phenotype cytokine production at the single cell level. As IFN-γ is the signature cytokine of the type I response elicited by RSV challenge and was significantly increased by IL-21 depletion, I assayed its production by different cell types at several time points post challenge. IFN-γ was not produced by macrophages, neutrophils (Fig.1.10a), DCs, or B cells (Fig.1.10b) at any time point tested. IFN-γ production was detected by NK cells at d4 p.c, though IL-21 depletion had no effect on the percentage of IFN-γ-producing-NK-cells

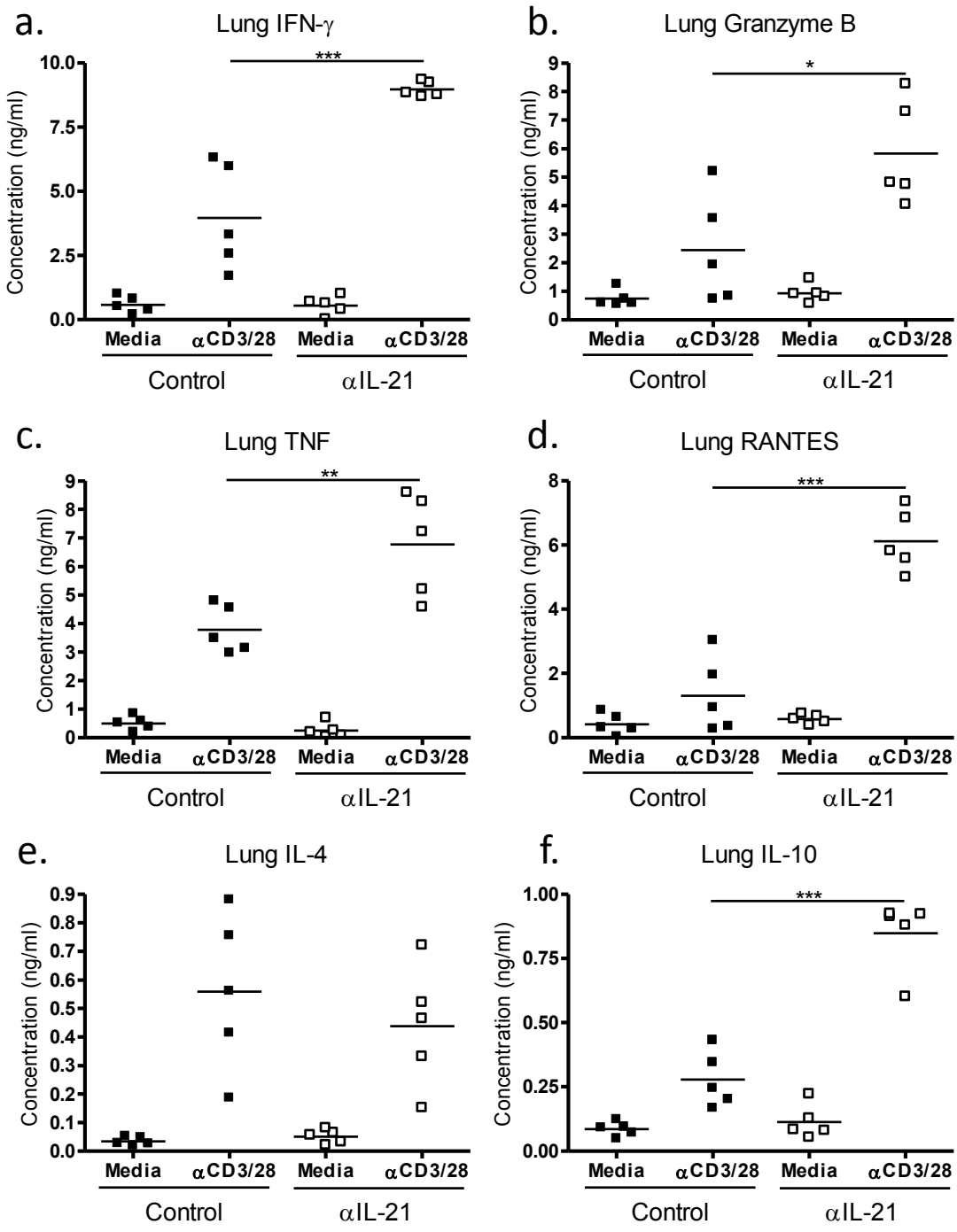

Figure 1.9. IL-21 depletion increases cytokine and chemokine production by T cells after primary RSV challenge. Mice were challenged with RSV on d0. Lung cells were harvested seven days post challenge, RBCs lysed, and single cell suspensions counted by trypan blue exclusion assay. Lung cells (2×10^6 cells/well) were stimulated with either media alone or αCD3/28-expressing beads (50µl/well) for 24hrs. Supernatants were harvested and IFN-γ (a), Granzyme B (b), TNF (c), RANTES (d), IL-4 (e), and IL-10 (f) were determined by sandwich ELISA. Data are expressed as mean values. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

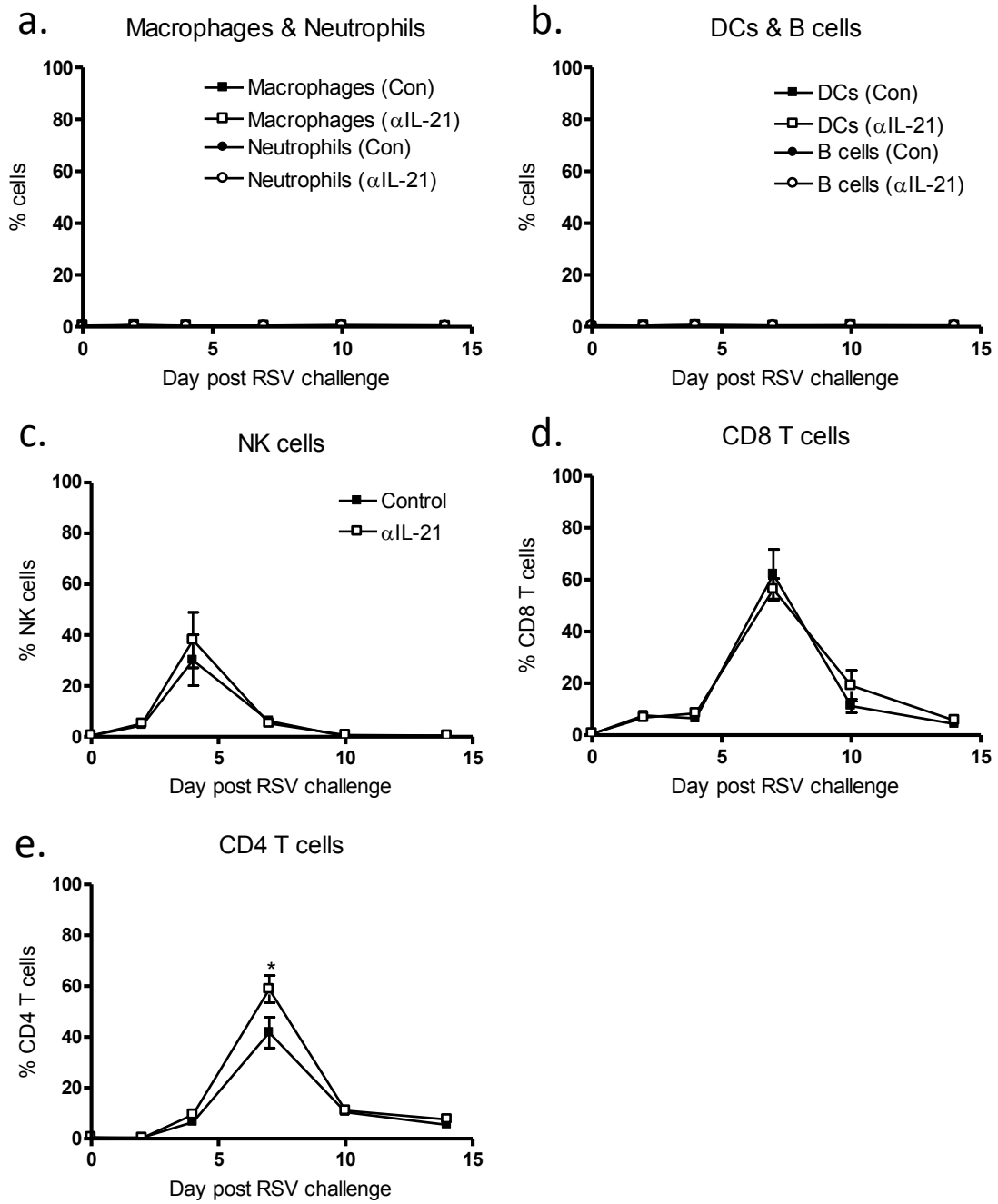

Figure 1.10. IL-21 depletion increases IFN-γ production by CD4 T cells after primary RSV challenge. Mice were challenged with RSV on d0. Lung cells were harvested on d0, 2, 4, 7, 10, and 14 post challenge lungs and stimulated overnight with media or αCD3/28-expressing beads (10µl/10^6 cells). Macrophages and neutrophils (a), DCs and B cells (b), NK cells (c), CD8 T cells (d), and CD4 T cells (e) were stained for IFN-γ using specific catch and detection reagents (10µl/10^6 cells). The percentage of each cell type staining positive for IFN-γ was determined by flow cytometry and is shown. At least 50×10^3 cells/sample were collected. Data is expressed as mean±SEM. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: $p<0.05$.

(Fig.1.10c). At d7 p.c, the majority of IFN-γ-producing cells were T cells (Fig.1.10d&e). CD8 T cells were the most common source of IFN-γ in RSV-challenged mice, and IL-21 depletion did not alter the percentage of cells (Fig.1.10d). However, in IL-21-depleted mice the primary source of IFN-γ was the CD4 T cell as depletion significantly increased the percentage of CD4 T cells producing this cytokine (Fig.1.10e).

10. IL-21 depletion reduces virus-specific antibody production after primary RSV challenge

Previous studies have demonstrated that pathogen-specific antibodies are essential for optimal long-term protection against re-infection with an antigenically-syngeneic virus. Moreover, IL-21 is known to boost B cell activation and memory development as well as increasing antibody isotype class switching and production. Therefore, I determined the effect of IL-21 depletion on RSV-specific antibody production. Serum was harvested from mice 14 days post RSV challenge and RSV-specific IgA, IgE, IgG1, and IgG2a levels were determined by ELISA.

No virus-specific serum IgA (Fig.1.11a) or IgE (Fig.1.11b) could be detected in either group. However, virus-specific serum IgG1 (Fig.1.11c) and IgG2a (Fig.1.11d) could be detected from control mice. Levels of both isotypes were significantly reduced in IL-21-depleted mice.

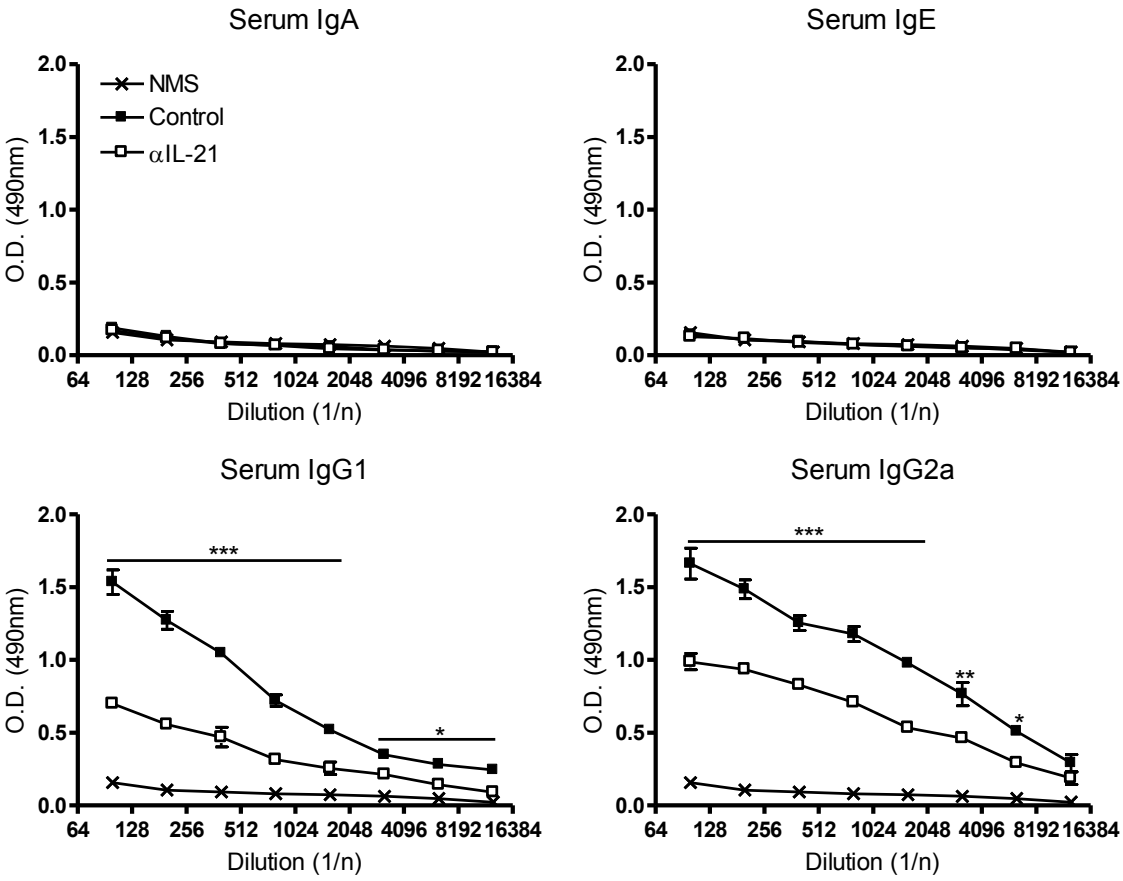

Figure 1.11. IL-21 depletion reduces virus-specific antibody production after primary RSV challenge. Mice were challenged with RSV on d0. Serum was harvested 14 days later and virus-specific IgA (a), IgE (b), IgG1 (c), and IgG2a (d) levels were determined by ELISA. Data is expressed as mean±SEM. The graphs are representative of two independent experiments of five mice per group. ANOVA result *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

11. Discussion

We hypothesised that IL-21 increased CD8 T cell activation and effector function driving lung tissue damage, and reducing lung function upon RSV challenge. However, the results of this chapter indicate that IL-21 primarily acts to limit CD4 T cell activation, possibly by inhibiting DC-mediated activation in the draining lymph nodes. There was little or no effect on responding CD8 T cells. Consequently, disease severity was reduced but viral replication was enhanced. The reasons for the change in viral replication are unknown but likely reflect an as yet unobserved effect on innate immune components. Therefore, IL-21 is acting as an anti-inflammatory factor, as demonstrated in rhinitis studies, rather than pro-inflammatory, as demonstrated in autoimmunity and cancer studies.

One of the first cell types that may be affected by IL-21 depletion are epithelial cells. As bronchial and alveolar epithelium are the primary targets of RSV infection any effects that IL-21 has on viral replication kinetics or the epithelial cell response to infection could have significant downstream effects on the immune response. It is unknown if lung epithelium expresses IL-21R, though it has been shown on intestinal and gastric epithelium (713,714). Moreover, IL-21 was shown to increase CCL20 and matrix metalloproteinase production from these cells in IBD patients, suggesting a pathogenic role (713). My data do not support the hypothesis that IL-21 is pro-inflammatory as disease severity was increased upon IL-21 depletion. However, more direct studies on epithelial cells would be required to ascertain any effect of IL-21. There is also no evidence that IL-21 affects viral replication kinetics as this was unaltered between control and depleted mice. However, changes on days that were untested cannot be ruled out and need to be confirmed. As there was a reduction in viral replication at d4 p.c it is possible that IL-21 increases viral productivity, independent of

any effects on the immune system. Studies of acute influenza and vaccinia infection concluded that IL-21 had no effect on viral clearance, which does not support a direct anti-viral role for IL-21 (608). It is not clear what clears RSV in this study; it is likely to be a combination of unidentified innate cells and T cells. Graham *et al* elucidated the effect of T cells on viral replication (332). In their study they demonstrated that depletion of either T cell subset prolonged viral replication in the lung, and depletion of both CD4 and CD8 T cells further extended viral replicative capacity. However, this does not account for viral clearance from d4 p.c when T cells are largely absent from the airway. Depletion of individual innate populations would help understand which are important for early RSV clearance.

Alveolar macrophages are also responsive to IL-21 and their phagocytic and cytokine/chemokine-producing activity could be modulated by IL-21 depletion. These cells line the alveolar sacs and are susceptible to infection by RSV, though to a much more limited extent than epithelium. Previous studies have shown that IL-21 does not increase macrophage proliferation but supports their survival by increasing expression of $p21^{waf1}$ and $p27^{Kip1}$ anti-apoptotic factors (715). Moreover, this study demonstrated that IL-21 increased antigen uptake, processing, and presentation to CD4 T cells (but not CD8 T cells) by macrophages. Therefore, this supports a pro-inflammatory role for IL-21 on macrophages via CD4 T cell activation. As disease was increased upon IL-21 depletion in my study, it is unlikely that these changes occurred, though an effect on macrophages by changes in other innate factors cannot be ruled out. However, as macrophages were not studied in more detail further experimentation would be required to confirm this.

Dendritic cells (DCs) are known to be central to naïve T cell activation in the lymph nodes upon antigen capture, processing, and presentation via MHC molecules. Therefore, changes in T cell recruitment, activity, and effector function could be the result of changes in DC function. Previous studies have shown that IL-21 prevents DC maturation, antigen presentation by DCs, and costimulatory molecule expression (622,716). Consequently, T cell activation is reduced. This mechanism is supported by my data as IL-21 depletion increased CD4 T cell recruitment, activation, and IFN-γ production. However, DC recruitment to the BALF was not altered by IL-21 depletion at the time points tested and migration to the draining, mediastinal lymph nodes was not assessed. To determine if IL-21 depletion altered T cell activation by DCs in the draining lymph nodes, expression of costimulatory molecules by migrating DCs and activation markers on T cells would need to be measured. Therefore, the data generated in this chapter do not clarify whether IL-21 effects on DC-mediated, rather than macrophage-mediated, T cell activation are responsible in this disease model.

IL-21 depletion has known effects on other cells of the innate immune system. Previous studies have shown that IL-21 inhibits NK cell proliferation and increases death by apoptosis, but the cytotoxicity of these same cells is enhanced by IL-21 in the presence of IL-2 or IL-15 (626,630). NK cells are important in the early response to RSV as they kill infected cells and provide an early source of IFN-γ (717). As IFN-γ levels increased with IL-21 depletion it is possible that NK cells contributed to this. However, there was no evidence that IL-21 depletion significantly affected NK cell responses in terms of recruitment, expression of activation markers, or production of IFN-γ. Moreover, BALF granzyme B and TNF levels were unchanged by IL-21 depletion, and as these are all major products of activated NK cells adds support that they are unaffected by IL-21 depletion in this setting.

Other innate factors may also have been affected by IL-21 depletion but were not studied. These include Natural Killer (NK) T cells, γδ T cells, and innate lymphoid cells (ILCs). NKT cells are known to produce IL-21 upon activation (582); in this model this is most likely to be cytokine-driven as NKT cells recognise glycolipid antigens that are not expressed by RSV. However, they may respond to lipid antigens released by dead and dying cells (718). There is little information regarding the effect of IL-21 depletion on NKT cells, they are a potent early source of a wide array of soluble factors and any changes in their expression may be due to effects on this cell type. A 'Tfh-like' NKT cell population has recently been identified that increases antibody production (719). Therefore, the observed reduction in virus-specific antibody levels may be due to effects on this population, but more detailed studies of these possibilities are required. γδ T cells and ILCs are also potent early cytokine sources but there is insufficient data generated in this study to conclusively support an effect of IL-21 depletion on these effector functions. Previous studies of γδ T cell responses to RSV suggested that this cell type was important in optimal memory CD8 T cell responses, but had little effect on primary responses (123). As CD8 T cell responses were unchanged by IL-21 depletion there is no evidence that γδ T cells are affected. However, the same study also identified γδ T cell as a potent RANTES source and as this cytokine was significantly upregulated by IL-21 depletion. This indicates that IL-21 may regulate cytokine production by these cells. There is no information about the effects of IL-21 on ILCs and specific studies of this cell type would be required to address this question.

Granulocytes, particularly neutrophils, are recruited early after RSV challenge (657). Basophil, mast cell, and eosinophil recruitment were not measured in this study and neutrophil recruitment was unaffected by IL-21 depletion, suggesting no effect. This cannot

be correlated with IL-17 or CCL20 production as levels were not assayed in this study. As there was no increase in IL-4 production but a significant increase in IFN-γ production, there is no evidence that type 2 responses had been induced by IL-21 depletion, a type 1 response remains dominant.

Given the significant changes in disease severity as well as different aspects of the anti-RSV immune response at d7 p.c, there is strong evidence that the adaptive immune response has been affected by IL-21 depletion. My hypothesis was that CD8 T cells would be most affected by IL-21 depletion given its known effects on CD8 T cell activation, proliferation, cytotoxic functions, and memory development (720). However, there is little data to support this hypothesis in this study. No changes in CD8 T cell recruitment, activity, or effector function were observed. This may reflect the relative lack of effect on primary CD8 T cell responses as compared to memory (720). Although this was not addressed here, secondary RSV challenge of depleted vs control mice would help to answer this question. Nevertheless, Zeng *et al* showed that IL-21 potently synergised with IL-15 to significantly increase proliferation and IFN-γ production by both naïve and memory CD8 T cells (721). They also demonstrated that CD8 T cell activation and effector function was impaired in IL-21R-deficient mice. Assaying IL-15 levels after RSV challenge (including after IL-21 depletion) may help elucidate the importance of IL-15 in the anti-RSV CD8 T cell response. More recently, Spolski *et al* have studied pneumovirus infection in IL-21R-deficient mice (722). They demonstrated that both CD4 and CD8 T cell responses were inhibited in IL-21R-deficient mice with diminished IL-6 and CXCL1 production, and reduced neutrophilia. Consequently disease severity and mortality was reduced in these mice. This study is particularly interesting as RSV and pneumovirus belong to the same *pneumovirinae*

subfamily, have similar genome structure, and cause similar clinical disease. Why then does IL-21 have different effects on the anti-viral responses? The reasons for this are unclear currently, it is possible that IL-21R-deficient mice have homeostatic alterations that affect responses to respiratory challenge, and studying responses to RSV in these mice would answer this. Performing antibody depletion studies in pneumovirus-challenged mice would be the most direct way of comparing the effects on the two pathogens. CD8 T cell cytotoxic function was also not specifically measured so changes cannot be ruled out. For example, IL-21 depletion significantly increased granzyme B levels from stimulated lung cells which could be derived from several sources [e.g. CD4 T cells, γδ T cells, NKT cells, or NK cells indirectly (723)]. The same group have also suggested that the balance of GM-CSF and IL-21 determines whether DCs apoptose or survive to stimulate T cells (724). Therefore, the observed differences in IL-21 effects between PVM and RSV disease models may be due to differences in this balance. As neither study has measured both factors further work will be required to ascertain if this is a possibility.

In contrast, there is stronger evidence that the CD4 T cell response is affected by IL-21 depletion. The importance of T cells in the development of RSV disease severity was first identified by Graham *et al* (332), and their potential to augment disease severity was confirmed by Alwan *et al* (683,688). Graham *et al* also elucidated the effect of T cells on viral replication (332). RSV-specific T cells are activated in the mediastinal lymph nodes by dendritic cells from the airway and lung tissue and proliferate rapidly. They then migrate to the pulmonary compartment starting at d4 p.c and peaking at d7 p.c. Peak recruitment of CD4 and CD8 T cells is observed at this time, but IL-21 depletion does not appear to affect timing, only the magnitude of the CD4 T cell influx. Recruitment is likely chemokine-driven

and I detected increased RANTES [a potent T cell chemokine; (725)] levels in the BALF at d2 p.c preceding T cell recruitment. Other chemokines not measured here may also contribute and should be measured. However, cytokine production is affected at d7 p.c, particularly IFN-γ which is elevated. This correlates with enhanced recruitment of CD4 T cells to the BALF and together with the increased production of this cytokine by these cells, indicates that IL-21 depletion has increased IFN-γ production by responding CD4 T cells. As polyclonal stimuli have been used to activate these cells I cannot determine that this is production by antigen-specific CD4 T cells, but as antigen-specific T cells dominate this phase of the acute response [bystander-activated T cells are recruited earlier and are declining by this time (726)] it is likely that the observed IFN-γ is produced in an antigen-specific manner.

There is little evidence here that IL-21 depletion affected T cell differentiation and the gross immunological phenotype of the response. As RSV is an obligate intracellular pathogen, a type 1 response is typically generated, dominated by IFN-γ production, T cell recruitment, and IgG2a production by activated B cells (657). There is negligible production of other signature cytokines such as IL-4 (type 2), though IL-9 (Th9), IL-17 (Th17), and IL-22 (Th22) production were not determined here. In this study, the cytokine response was dominated by IFN-γ, granzyme B, TNF, and RANTES. IL-17 was not tested and would need to be measured to confirm any effect, though neutrophilia was unchanged by IL-21 depletion which may indicate the type 17 response is little altered. Although IL-21 depletion increased IFN-γ and RANTES production in the BALF and IFN-γ, granzyme B, TNF, and RANTES by stimulated lung cells, there is no evidence that IL-21 depletion changed the overall phenotype of the response, it remained predominantly type 1.

The observed increase in disease severity and CD4 T cell activity could be explained by a reduced immunoregulatory response, confirmed by a reduction in regulatory T cell recruitment and/or activity. Regulatory T cells (Tregs) are known to play a crucial role in protecting against the development of several autoimmune diseases, and previous studies have demonstrated their importance in microbial infections (727). Unfortunately, they were not measured in this study and it would be important to do so to ascertain whether IL-21 depletion affected them. No previous studies have observed a direct effect of IL-21 on regulatory T cells, but IL-21 can increase 'resistance' of effector T cells to Treg-induced suppression (728). However, this does not fit with my observations as IL-21 depletion should make T cells prone to immunosuppression but this has not been observed. IL-21 may be required for optimal Treg influx into the pulmonary compartment and further work should test this possibility. One method that Tregs use to inhibit cellular responses is production of anti-inflammatory factors such as IL-10 and TGF-β. TGF-β was not measured in this study but a significant increase in IL-10 production was observed in both BALF and lung. This could be interpreted as an increase in Treg recruitment or activity but as activated effector T cells also produce IL-10 as a method of self-regulation it is just as likely that the latter is the source.

An alternative mechanism is that IL-21 is directly anti-inflammatory. IL-21, like IL-10, is produced by all pro-inflammatory T cell lineages indicating it may have crucial anti-inflammatory functions (like IL-10) by inhibiting T cell activation. For example, IL-21 has been shown to restrict primary and secondary CD8 T responses against melanomas, carcinomas, and lymphomas (729). Furthermore, Spolski *et al* demonstrated that IL-10 levels were elevated in a mouse model of SLE, and that IL-21 was necessary for optimal IL-10

induction (704). Moreover, they showed that Th1 priming in these mice in the presence of IL-21 induced a subpopulation of T cells with immunosuppressive capacity. Therefore, IL-21 may act to limit immune responses by inducing IL-10 production. IL-21 has also been shown to inhibit immediate hypersensitivity reactions in the skin (702). It boosts IL-10 production in visceral leishmaniasis (709), by human naïve CD4 T cells (730), Tr1 cells (731), and NK cells (732). A lack of IL-21 may lead to dysregulated responses against Hepatitis B virus in the young (700), and boost IL-17 production by CD4 T cells in *L.monocytogenes* infection (708). Increased IL-21 expression by CD4 T cells was associated with control of HIV replication, but this may simply reflect greater T cell activity (701,733). Its increased production has also been positively-correlated in several diseases, but this again may reflect self-regulation by activated T cells (734,735). In this model of RSV infection, IL-10 peaked at d4 p.c when T cell activation in the draining mediastinal lymph nodes and recruitment to the pulmonary compartment have been initiated but not peaked. Therefore, these cells may contribute but are unlikely to be the major IL-10 source. One alternative are regulatory T cells that are known to be a potent source of IL-10 and other anti-inflammatory cytokines (736,737). Intracellular staining for IL-10 of recruited cells would help clarify the IL-10 source.

Anti-viral antibodies provide long-term protection against viral rechallenge and are considered a crucial component of successful vaccination (657). Moreover, recent studies have demonstrated a central role for IL-21 in optimal B cell activation and antibody production by supporting Bcl-6 expression and follicular T helper cell differentiation [Tfh; (511,600)], as well as acting directly on B cells (625). Therefore, IL-21 depletion would be expected to reduce antibody production and this was observed in this study. Virus-specific IgG antibody levels were significantly reduced in depleted mice, suggesting a crucial role in

induction of humoral immunity. This agrees with early studies that demonstrated IL-21 was crucial for development of antibody-secreting plasma cells (738). IL-21 depletion may inhibit Tfh differentiation and/or B cell activation to induce the observed phenotype. Other studies demonstrated a crucial role for Tfh-derived-IL-21 for antibody production (739), and more recent studies have demonstrated an essential role for IL-21 in Bcl-6-mediated Tfh cell development and function (600,740). These effects of IL-21 on Tfh and B cell activity play a critical role on germinal centre formation and reactions within (712,741). Markers for Tfh cells were not included measured in this study but further analysis including these markers (CXCR5, PD-1, ICOS, and Bcl-6), as well as those for B cell activation (CD19/21), would answer these questions.

The results of this chapter indicate a novel aspect of IL-21 function: that it acts as an anti-inflammatory factor in RSV immunity, important for limiting responding CD4 T cell activity during primary immune responses to viral challenge. Surprisingly, it has no effect on CD8 T cell function. Several mechanisms have been discussed that may explain the effects of IL-21 on primary responses to RSV challenge, but the effect on memory T cell responses has not been addressed. It is possible that IL-21 has a more prominent role in recall T cell responses to viral challenge, therefore in the next chapter I address the role of IL-21 on memory CD4 & CD8 T cell responses to RSV challenge.

I. Endogenous IL-21 regulates pathogenic mucosal CD4 T cell responses during enhanced RSV disease in mice.

1. Introduction

In the previous chapter I demonstrated that IL-21 was important for limiting pathology associated with primary RSV challenge. This correlated with IL-21 controlling the magnitude of the cellular response, the cytokine response, and other effector functions (e.g. granzyme B production) to RSV challenge. In particular, IL-21 was crucial for controlling the primary CD4 T cell response rather than the CD8 T cell response, suggesting its action on CD4 T cells was more important in the context of primary RSV infection. However, it is unknown if the same effects would be observed in memory T cell responses against RSV. Clinically, this is relevant for two reasons. First, it is unclear if exacerbated RSV disease is caused by primary or memory T cell responses so both must be studied. Second, RSV vaccination enhances T cell memory so the effects of IL-21 depletion are potentially important in this setting.

To determine if memory responses were affected I utilised a model of augmented RSV disease involving priming BALB/c mice with a vaccinia virus vector (rVV) encoding individual RSV genes in place of the *thymidine kinase* (*tk*) gene (742). The advantage of this model is that priming mice with these vectors leads to activation and memory development of a restricted subset of T cells specific for the expressed RSV protein that will immediately respond upon RSV challenge. For example, priming BALB/c mice with rVV-G (a vaccinia virus expressing RSV G protein) activates G184-198-specific CD4 T cells with an oligoclonal TCR repertoire and a type II cytokine phenotype (i.e. increased IL-4 production) (743). No RSV-

specific CD8 T cells are primed. Upon RSV challenge, the CD4 T cells are recalled to the pulmonary compartment immediately where they produce increased amounts of IL-4, IL-5, and IL-13, induce a pulmonary eosinophilia, with increased and more rapid pathology compared to primary RSV challenge. RSV-specific CD8 T cells are also activated but are recruited with kinetics observed after primary RSV challenge. This model replicates some of the clinical and immunological features of RSV bronchiolitis observed in children (744). In contrast, mice primed with rVV-M2 develop an oligoclonal, M2 82-90-specific CD8 T cell memory with a type I cytokine phenotype (i.e. increased IFN-γ and TNF production) (685). No RSV-specific CD4 T cells are primed. Upon RSV challenge, the CD8 T cells are immediately recalled to the pulmonary compartment where they produce increased amounts of IFN-γ, TNF, and granzyme B, with increased and more rapid pathology compared to primary RSV challenge (685). RSV-specific CD4 T cells are also recruited after RSV challenge but with kinetics observed after primary RSV challenge. This model replicates many of the clinical and immunological features of 'shock lung' (acute respiratory distress syndrome) (11). Using these two vectors I can study the effect of IL-21 depletion on memory CD4 and CD8 T cell responses to RSV exclusively. This will allow me to more accurately determine the effects of IL-21 depletion on CD4 and CD8 T cell responses to RSV.

2. Assessment of the effect of IL-21 depletion on immune responses to vaccinia virus immunisation

Prior to studying the effects of IL-21 depletion on CD4 or CD8 T cell recall responses to RSV, I wanted to confirm that IL-21 depletion had no effect on cutaneous vaccinia virus infection. This is important as changes to the amount of RSV antigen induced by IL-21 depletion may

affect the development of RSV-specific T cell memory indirectly, giving a false impression of the effects of IL-21 on T cell responses to RSV. Monitoring on-going cutaneous vaccinia virus replication is technically difficult because the levels of virus shedding are hard to detect without sacrificing the animal. However, upon cutaneous infection a lesion develops on the surface of the skin that increases in size as the virus replicates and disappears once the virus is cleared by the host (745). The diameter of this lesion can be easily and accurately measured without affecting the welfare of the animal. Therefore, lesion sizes were measured on mice cutaneously-infected with rVV-βgal (β-galactosidase: used as a negative control; Fig.2.1a), rVV-G (Fig.2.1b), or rVV-M2 (Fig.2.1c) over 14 days. All mice were infected with the same dose of vaccinia virus and consequently lesion sizes were very similar between groups. These mice were treated with control antibody (i.p) one day prior and one day post cutaneous infection, and were compared to mice treated with anti-IL-21 antibody (i.p) infected with the same dose of vaccinia viruses (Fig.2.1). Lesion sizes between mice treated with anti-IL-21 antibody were very similar to those treated with control antibody, suggesting that vaccinia virus replication (and therefore the level of RSV antigen expressed) had not been affected by IL-21 depletion.

3. IL-21 depletion during priming increases cytokine production by RSV-G-specific CD4 T cells but not RSV-M2-specific CD8 T cells

Before studying the effects of IL-21 depletion on T cell responses to RSV challenge, I first determined the effects on primary T cell responses to rVV priming. Spleen cells from primed mice were isolated; then CD4 T cells sorted from rVV-G ('G')-primed mice and CD8 T cells sorted from rVV-M2 ('M2')-primed mice. Equal numbers of T cells were then stimulated for

72hrs with either media, specific peptide (CD4 T cells recognise RSV G 184-198 in the context of I-E^d, CD8 T cells recognise RSV M2 82-90 in the context of K^d) presented by irradiated splenic APC, or αCD3/αCD28-expressing beads. Cytokine and granzyme B production into the supernatants was assayed by ELISA. As expected, splenic cells from mice immunised with control vector (rVV-βgal) mice did not produce cytokines to RSV peptide stimulation, only to polyclonal stimulation (Fig.2.2&2.3). I did not observe any effect of IL-21 depletion on these cells. Unstimulated cells did not produce cytokines (Fig.2.2&2.3), though granzyme B could be detected in CD8 T cell cultures (Fig.2.3b).

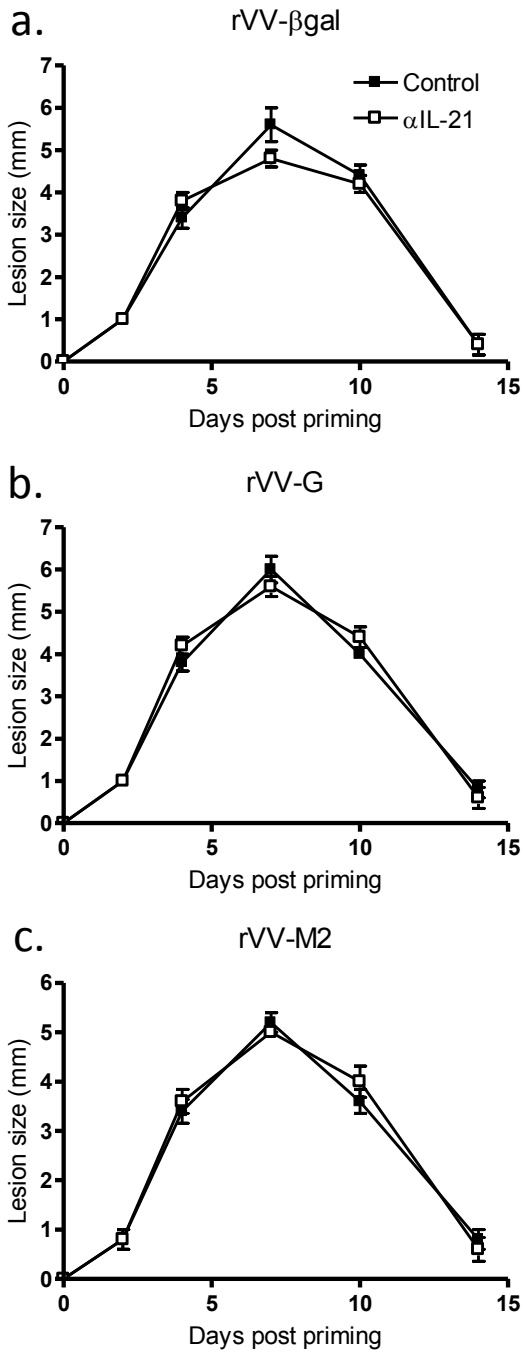

Figure 2.1. IL-21 depletion prior to cutaneous vaccinia virus infection has no effect on lesion size**.** BALB/c mice were immunised by cutaneous scarification with rVV-βgal, rVV-G, or rVV-M2 (1×10^6 pfu/mouse; scar) vaccinia virus vectors. Anti-IL-21 antibody or isotype control (0.5mg/mouse; i.p) was administered one day prior and one day after priming. On d2, 4, 7, 10, and 14 post challenge lesion sizes at the scarification site were measured. Data is expressed as mean±SEM. The graph is representative of two independent experiments of five mice per group.

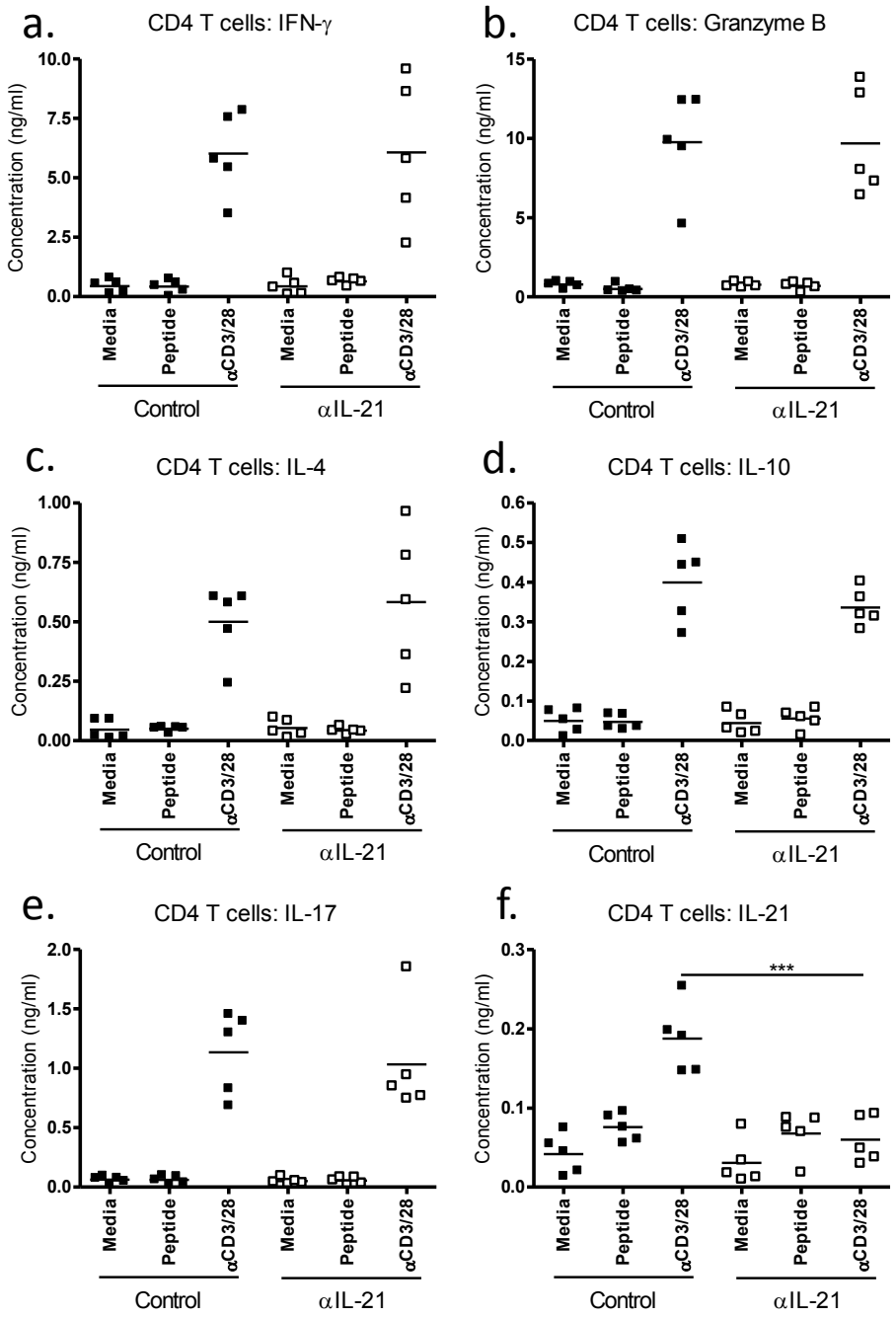

Figure 2.2. IL-21 depletion during priming with rVV-βgal reduces IL-21 production by CD4 T cells**.** Mice were immunised with rVV-βgal and spleens were harvested 14 days post priming, RBCs lysed, and single cell suspensions counted by trypan blue exclusion assay. CD4 T cells were sorted by MACS and purity confirmed by flow cytometry. CD4 T cells (2×10^6 cells/well) were stimulated with either media alone, irradiated APC (2×10^5 cells/well) pulsed with specific peptide (G 184-198), or αCD3/28-expressing beads (50µl/well) for 72hrs. Supernatants were harvested and IFN-γ (a), Granzyme B (b), IL-4 (c), IL-10 (d), IL-17 (e), and IL-21 (f) were determined by sandwich ELISA. The graphs are representative of two independent experiments of five mice per group. Student *t* test result ***: $p<0.001$.

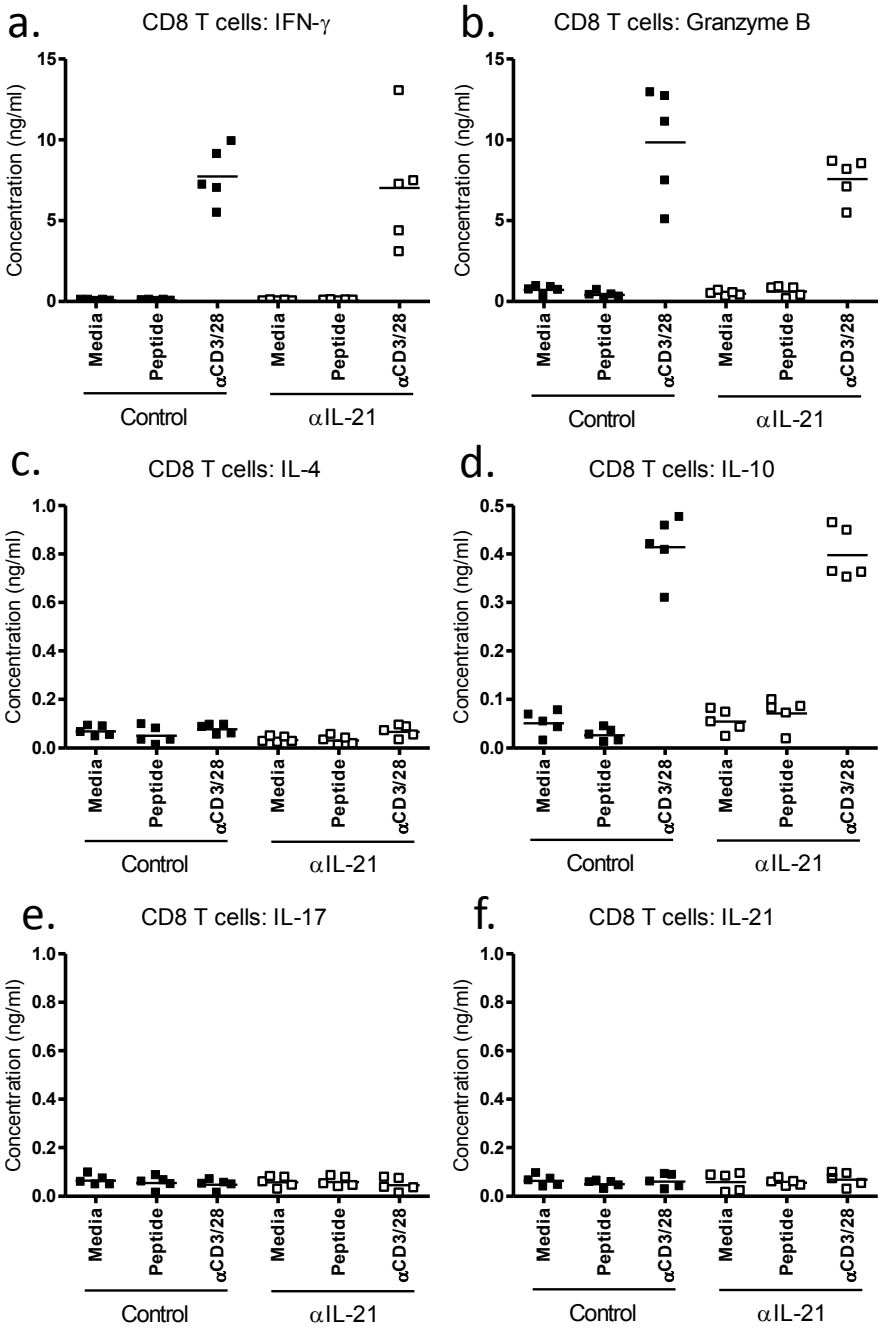

Figure 2.3. IL-21 depletion during rVV-βgal priming has no effect on cytokine production by CD8 T cells**.** Mice were immunised with rVV-βgal and spleens were harvested 14 days post priming, RBCs lysed, and single cell suspensions counted by trypan blue exclusion assay. CD8 T cells were sorted by MACS and purity confirmed by flow cytometry. CD8 T cells (2×10^6 cells/well) were stimulated with either media alone, irradiated APC (2×10^5 cells/well) pulsed with specific peptide (M2 82-90), or αCD3/28-expressing beads (50µl/well) for 72hrs. Supernatants were harvested and IFN-γ (a), Granzyme B (b), IL-4 (c), IL-10 (d), IL-17 (e), and IL-21 (f) were determined by sandwich ELISA. The graphs are representative of two independent experiments of five mice per group.

CD4 T cells from G-primed, non-depleted mice produced detectable amounts of IFN-γ (Fig.2.4a), IL-4 (Fig.2.4c), IL-10 (Fig.2.4d), IL-17 (Fig.2.4e), but not IL-21 (Fig.2.4f) after stimulation with specific peptide. These levels were all increased after polyclonal stimulation and all cytokines assayed could be detected. IL-21 depletion significantly increased IFN-γ (Fig.2.4a) and IL-10 (Fig.2.4d) production by peptide-stimulated cells. In contrast, it reduced IL-4 production (Fig.2.4c). No other changes were observed under these conditions. IL-21 depletion significantly increased production of all cytokines by CD4 T cells stimulated with αCD3/CD28-expressing beads, except IL-4 (Fig.2.4c) and IL-21 (Fig.2.4f) which was ablated. In contrast, granzyme B production was unaffected. As expected, CD8 T cells isolated from G-primed mice did not respond to G peptide stimulation but did when stimulated with the polyclonal stimulus (Fig.2.5). IL-21 depletion had no effect on their cytokine production.

Likewise, CD4 T cells isolated from M2-primed mice did not respond to M2 peptide stimulation but did when stimulated with polyclonal stimulus (Fig.2.6). IL-21 depletion had no effect, other than significantly reduce IL-21 production by these cells when stimulated with the polyclonal stimulus (Fig.2.6f). CD8 T cells from non-depleted, M2-primed mice produced significant amounts of IFN-γ (Fig.2.7a), granzyme B (Fig.2.7b), and IL-10 (Fig.2.7d) but IL-4, IL-17, and IL-21 were not detected. IL-21 depletion had no significant effect on cytokine or granzyme B production.

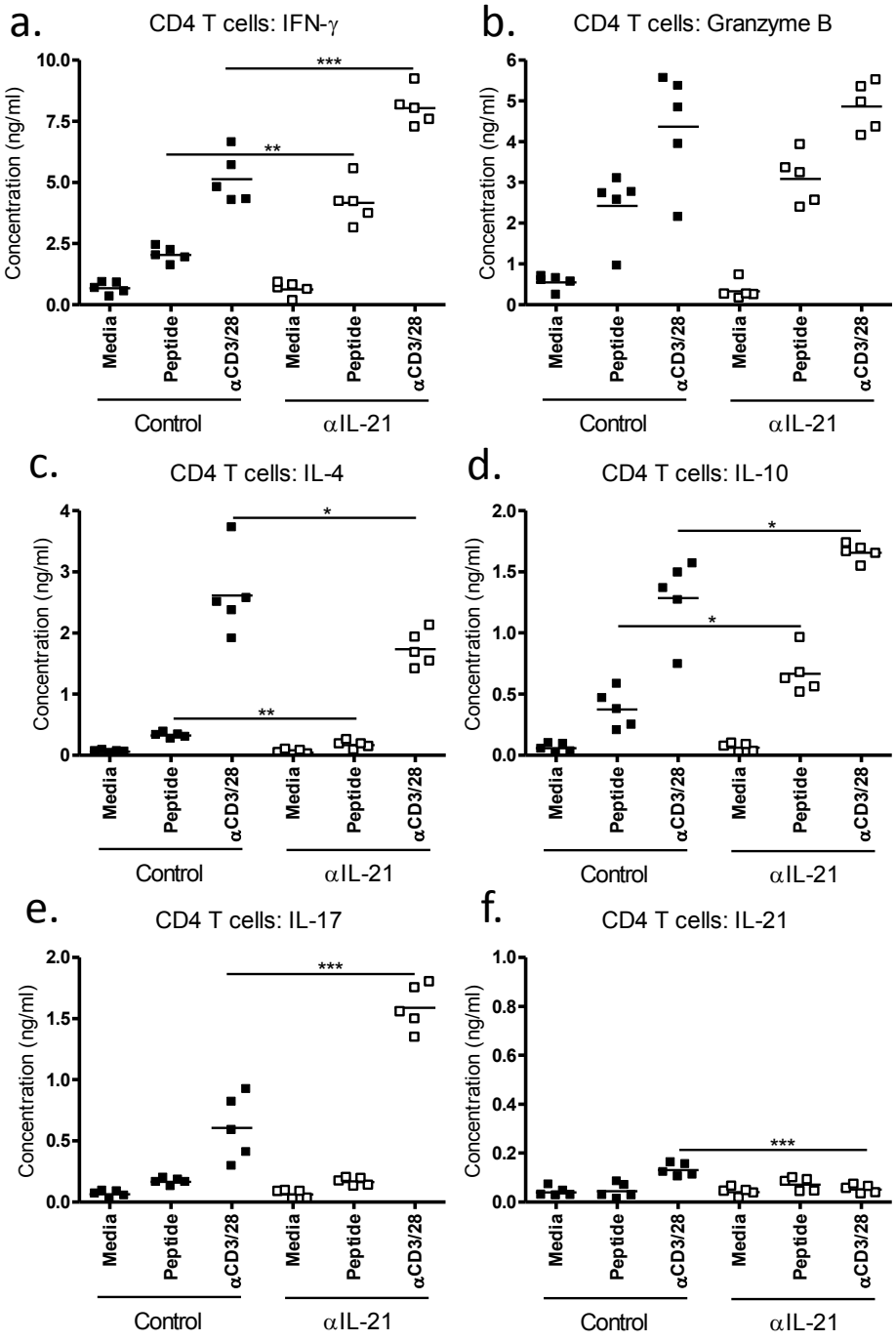

Figure 2.4. IL-21 depletion during rVV-G priming increases IFN-γ, IL-10, and reduces IL-4 production by RSV-G-specific CD4 T cells**.** Mice were immunised with rVV-G and spleens were harvested 14 days post priming, RBCs lysed, and single cell suspensions counted by trypan blue exclusion assay. CD4 T cells were sorted by MACS and purity confirmed by flow cytometry. CD4 T cells (2×10^6 cells/well) were stimulated with either media alone, irradiated APC (2×10^5 cells/well) pulsed with specific peptide (G 184-198), or αCD3/28-expressing beads (50µl/well) for 72hrs. Supernatants were harvested and IFN-γ (a), Granzyme B (b), IL-4 (c), IL-10 (d), IL-17 (e), and IL-21 (f) were determined by sandwich ELISA. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

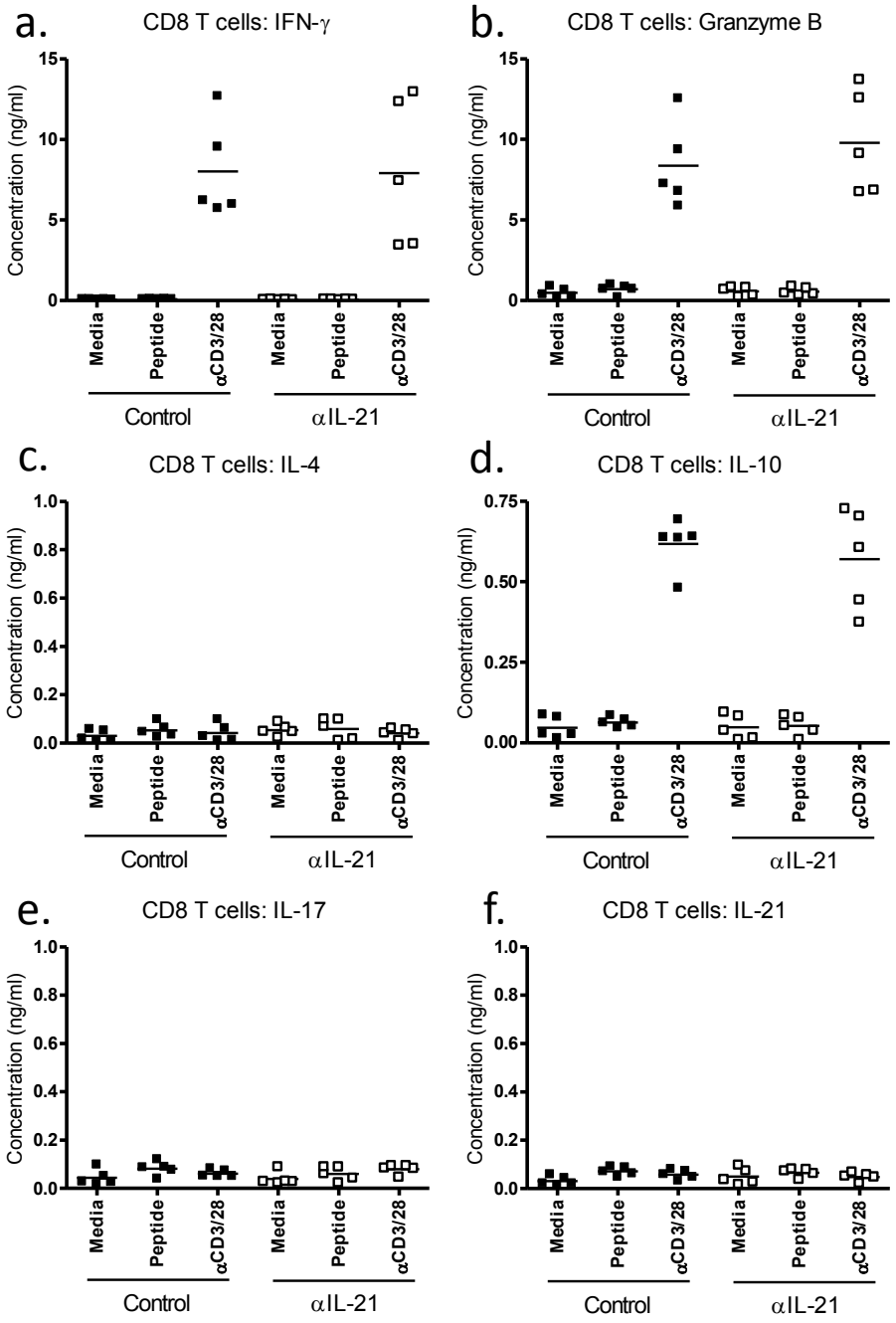

Figure 2.5. IL-21 depletion during rVV-G priming does not affect cytokine production by CD8 T cells**.** Mice were immunised with rVV-G and spleens were harvested 14 days post priming, RBCs lysed, and single cell suspensions counted by trypan blue exclusion assay. CD8 T cells were sorted by MACS and purity confirmed by flow cytometry. CD8 T cells (2×10^6 cells/well) were stimulated with either media alone, irradiated APC (2×10^5 cells/well) pulsed with specific peptide (G 184-198), or αCD3/28-expressing beads (50µl/well) for 72hrs. Supernatants were harvested and IFN-γ (a), Granzyme B (b), IL-4 (c), IL-10 (d), IL-17 (e), and IL-21 (f) were determined by sandwich ELISA. The graphs are representative of two independent experiments of five mice per group.

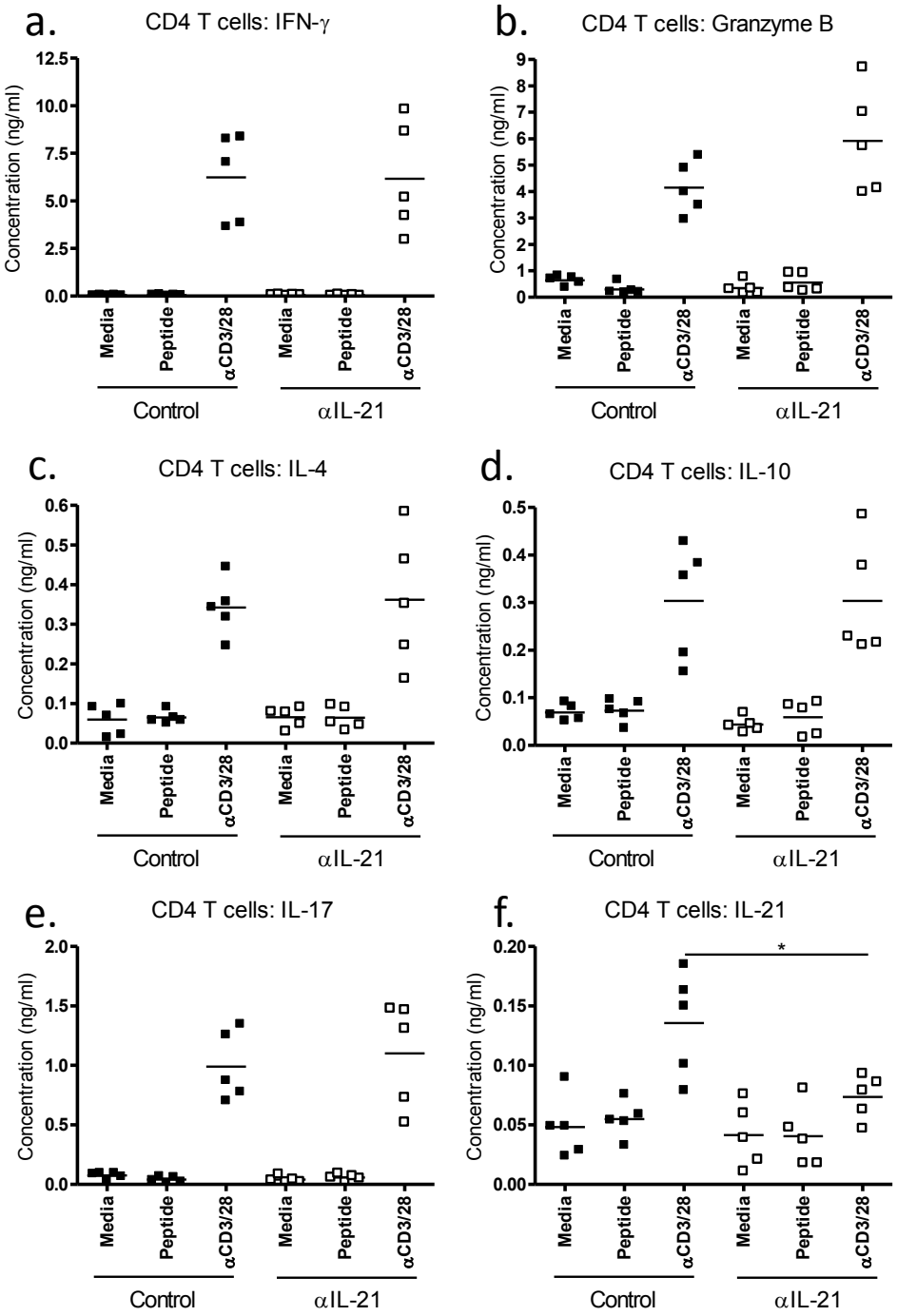

Figure 2.6. IL-21 depletion during rVV-M2 priming inhibits IL-21 production by CD4 T cells**.** Mice were immunised with rVV-M2 and spleens were harvested 14 days post priming, RBCs lysed, and single cell suspensions counted by trypan blue exclusion assay. CD4 T cells were sorted by MACS and purity confirmed by flow cytometry. CD4 T cells (2×10^6 cells/well) were stimulated with either media alone, irradiated APC (2×10^5 cells/well) pulsed with specific peptide (M2 82-90), or αCD3/28-expressing beads (50µl/well) for 72hrs. Supernatants were harvested and IFN-γ (a), Granzyme B (b), IL-4 (c), IL-10 (d), IL-17 (e), and IL-21 (f) were determined by sandwich ELISA. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: $p<0.05$.

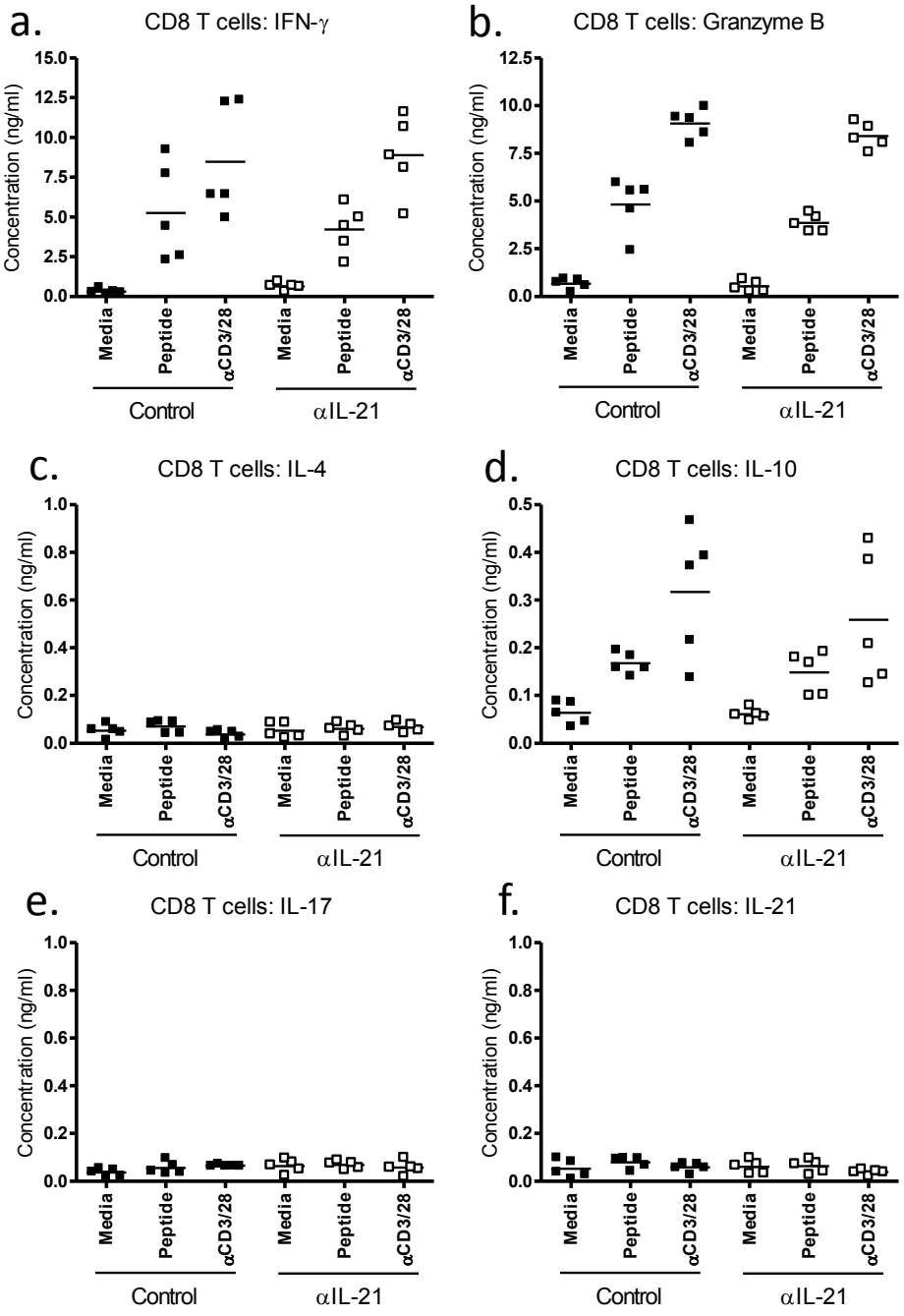

Figure 2.7. IL-21 depletion during rVV-M2 priming has no effect on cytokine production by CD8 T cells**.** Mice were immunised with rVV-M2 and spleens were harvested 14 days post priming, RBCs lysed, and single cell suspensions counted by trypan blue exclusion assay. CD8 T cells were sorted by MACS and purity confirmed by flow cytometry. CD8 T cells (2×10^6 cells/well) were stimulated with either media alone, irradiated APC (2×10^5 cells/well) pulsed with specific peptide (M2 82-90), or αCD3/28-expressing beads (50µl/well) for 72hrs. Supernatants were harvested and IFN-γ (a), Granzyme B (b), IL-4 (c), IL-10 (d), IL-17 (e), and IL-21 (f) were determined by sandwich ELISA. The graphs are representative of two independent experiments of five mice per group.

4. IL-21 depletion during priming with rVV-G exacerbates pathology after RSV challenge significantly more than during priming with rVV-M2

Next, I wanted to determine whether the enhanced cytokine production from memory CD4 T cells, but not CD8 T cells, altered the recall response in immunised mice after RSV challenge. Therefore, mice were challenged with RSV 14 days after priming with the vaccinia virus vectors. They were weighed daily and observed for visible signs of pathology. Primed mice develop a more rapid and aggressive response after RSV challenge. Consequently, disease severity (as measured by weight loss) is much more severe than observed in unimmunised mice. Primed mice began to lose weight immediately after RSV challenge and at a greater rate than observed in unimmunised mice (Fig.2.8). Weight loss peaked at d5-6 p.c in primed mice, two days earlier than unimmunised animals. At this time primed mice were exhibiting signs of severe disease (e.g. piloerection, hunched form, no/slower movement) not observed in unimmunised animals. Weight loss was more severe in M2-primed mice (Fig.2.8c) than G-primed mice (Fig.2.8b), demonstrating that CD8 T cells are more important for the observed pathology in this setting. Finally, primed mice recover more slowly than unimmunised animals; some mice do not recover their baseline weight by experiment termination.

IL-21 depletion increased weight loss in primed mice. In G-primed animals, IL-21 depletion significantly enhanced weight loss from d5 p.c until d12 p.c (Fig.2.8b). Moreover, whereas non-depleted mice recovered their baseline weights by d8 p.c, IL-21-depleted mice had not and were still exhibiting visible signs of illness. In M2-primed animals, IL-21 depletion had a similar but less marked effect. Pathology was enhanced, but the difference from non-depleted mice was reduced compared to that observed in G-primed mice (d7 p.c: G-primed

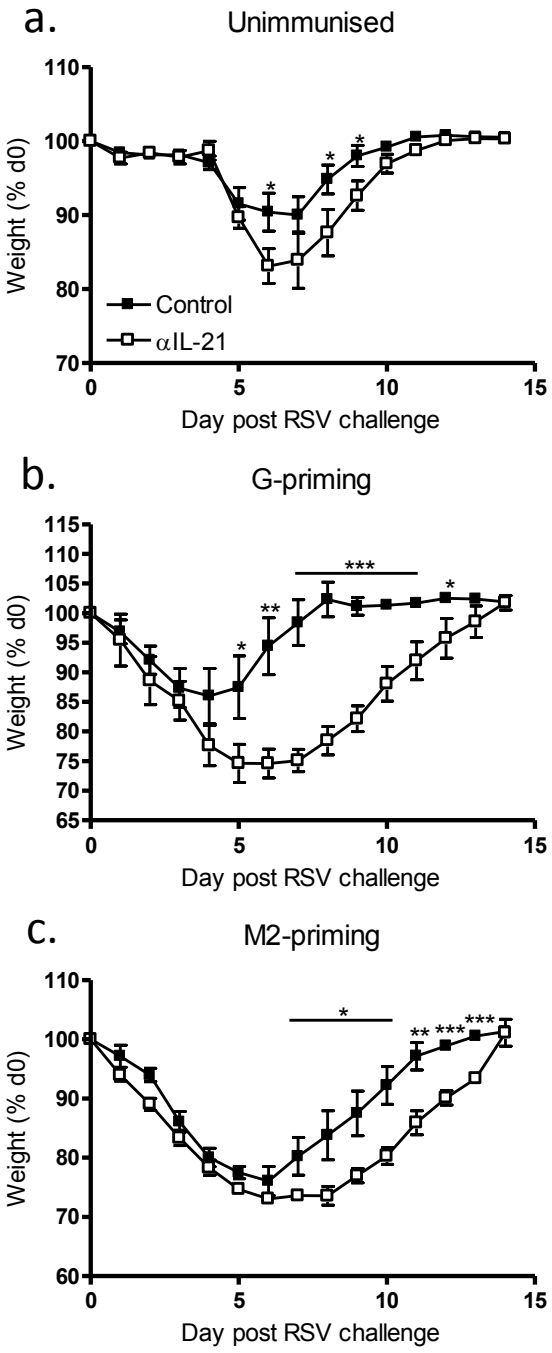

Figure 2.8. IL-21 depletion during priming with rVV-G exacerbates pathology after RSV challenge significantly more than during priming with rVV-M2. Mice were immunised with rVV-βgal, -G, or –M2 as described in Fig.2.1. 14 days later mice were challenged with RSV (1×10^6 pfu/mouse; i.n). Mice were weighed daily for 14 days. Weight is shown as a percentage of baseline weight prior to RSV challenge. Error bars represent SEM. The graphs are representative of at least six independent experiments of five mice per group. Student *t* test result *: p<0.05, **: p<0.01, ***: p<0.001.

mice, 24.2%; M2-primed mice, 6.8%). The difference became statistically significant from d7 p.c until d14 p.c (Fig.2.8c). Both groups of mice exhibited similar visible signs of disease, yet recovered with the same kinetic as control animals.

5. IL-21 depletion significantly increases T cell recruitment in rVV-G-, but not rVV-M2-, primed mice after RSV challenge

Pathology in RSV disease positively correlates with cell recruitment to the pulmonary compartment. I therefore, determined the effect of IL-21 depletion on this in augmented disease. Total live cell counts from BAL and lung tissue were determined by trypan blue assay. As expected, total cell counts were greatest when pathology was most severe, and were significantly higher in primed (Fig.2.9c-f) than unprimed (Fig.2.9a&b) mice at all time points tested. In G-primed mice, IL-21 depletion significantly enhanced cell recruitment to the BAL (Fig.2.9c) and lung (Fig.2.9d). In contrast, in M2-primed mice IL-21 depletion significantly enhanced cell recruitment to the BAL (Fig.2.9e) but not the lung (Fig.2.9f). As described in the previous chapter, IL-21 depletion in primary RSV challenge significantly increases CD4 T cell recruitment to the BAL (Fig.1.6e) and lung tissue (Appendix 1.1e) by d7 p.c. I therefore determined recruitment of memory CD4 and CD8 T cells in augmented disease. The same effect was observed in rVV-βgal-immunised mice for BAL (Fig.2.10a) and lung (Appendix 2.1a) CD4 T cells as was observed in primary RSV challenge. Further, there was no effect on CD8 T cell recruitment (Fig.2.10b & Appendix 2.1b). The numbers of CD4 and CD8 T cells recruited to the airway in G- (Fig.2.10c) and M2-primed (Fig.2.10f) mice respectively were significantly enhanced compared to rVV-βgal-immunised mice.

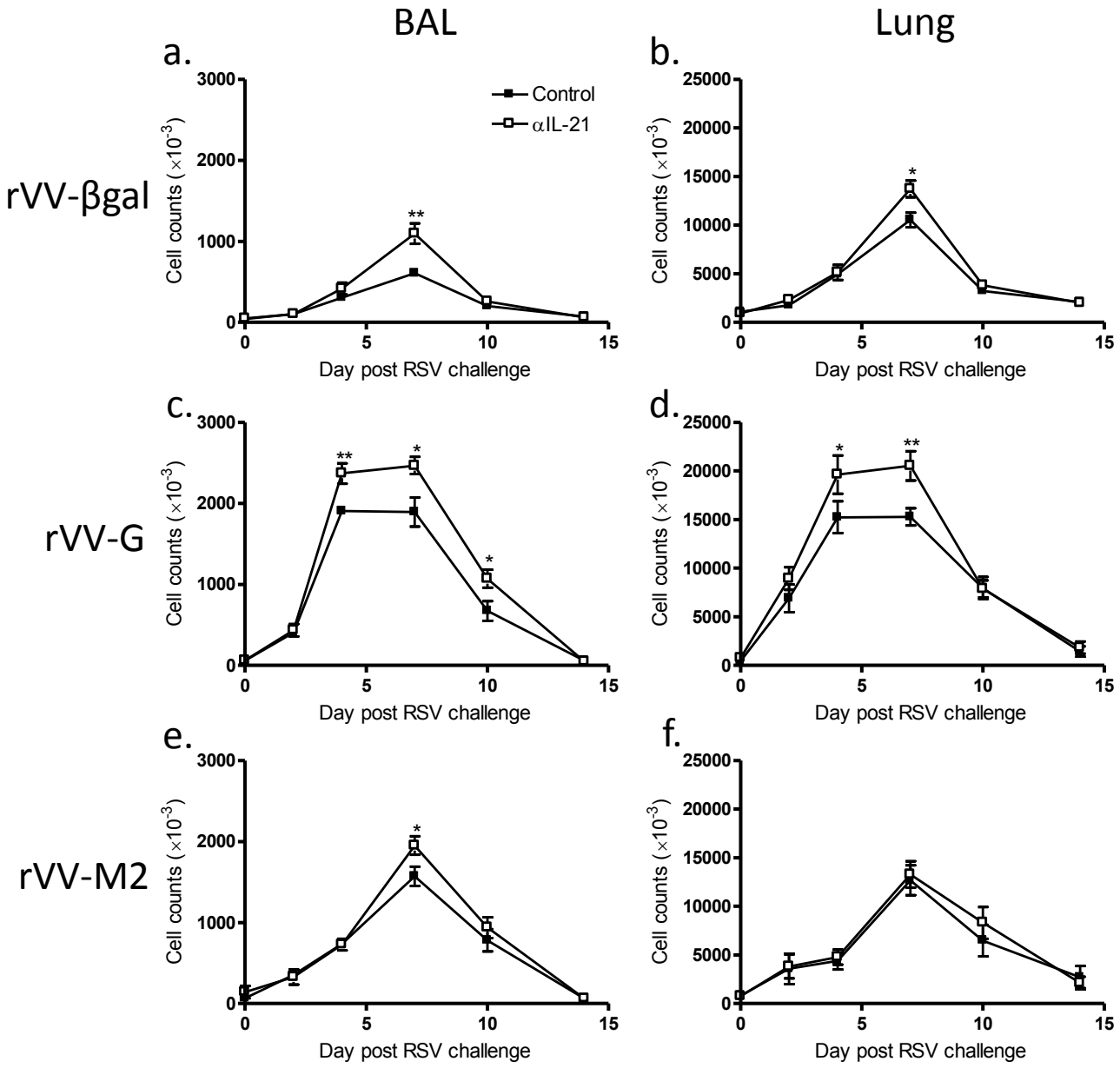

Figure 2.9. IL-21 depletion significantly increases cell recruitment in G-, but not M2-, primed mice after RSV challenge. Mice were immunised and challenged as described in Fig.2.8. BALF (a, c, e) and lung tissue (b, d, f) were harvested at several time points post challenge. Samples were processed, the RBCs lysed, and live cells counted by trypan blue exclusion assay. Error bars represent SEM. The graphs are representative of three independent experiments of five mice per group. Student *t* test result *: $p<0.05$, **: $p<0.01$.

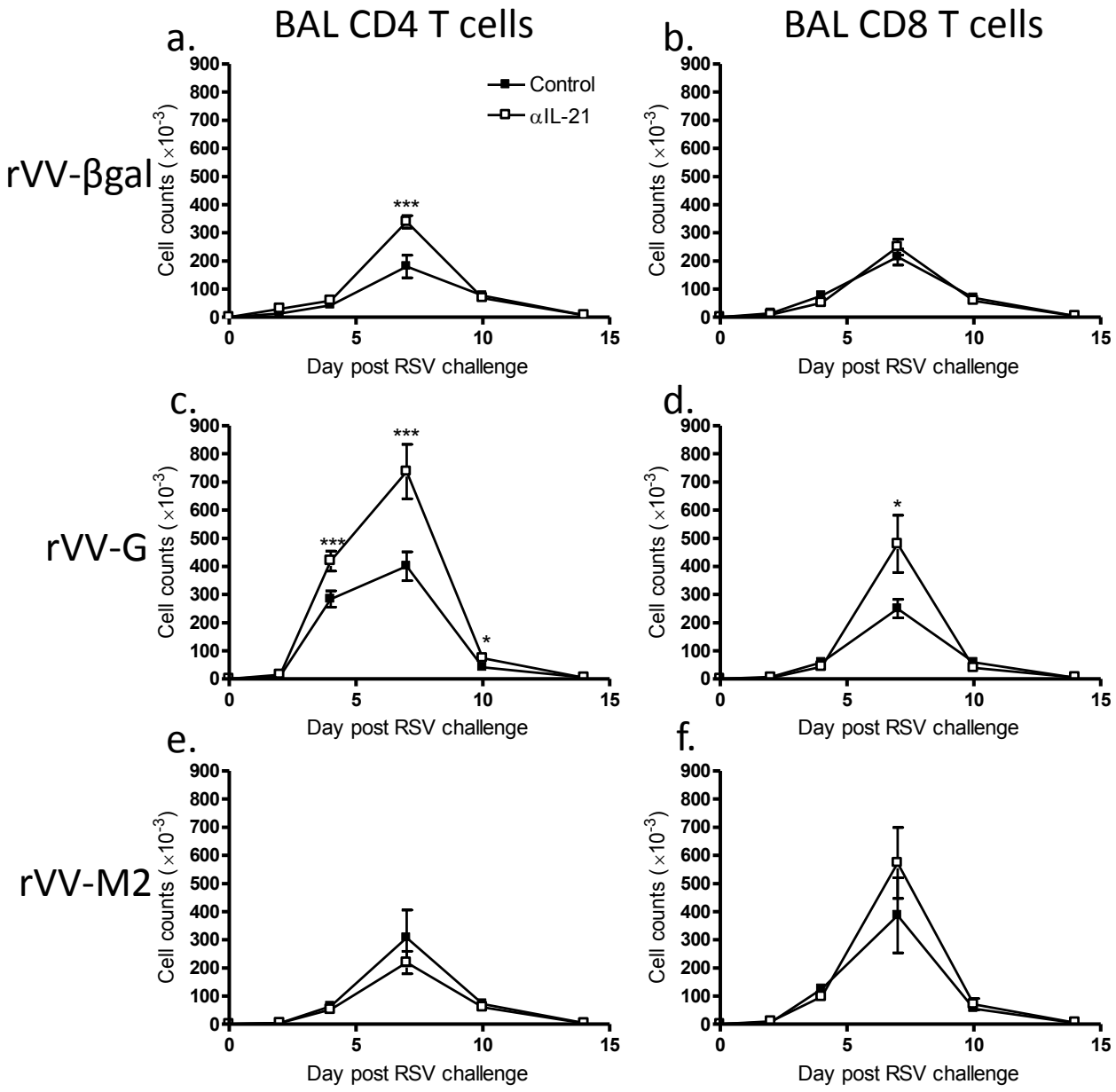

Figure 2.10. IL-21 depletion significantly increases T cell recruitment in G-, but not M2-, primed mice after RSV challenge. Mice were immunised and challenged as described in Fig.2.8. BALF cells were harvested at several time points post challenge. Samples were processed and live cells counted by trypan blue exclusion assay. BALF CD4 (a, c, e) and CD8 (b, d, f) T cells in rVV-βgal (a-b), rVV-G (c-d), and rVV-M2 (e-f) were phenotyped by flow cytometry and cell counts determined. At least 50×10^3 cells/sample were collected. Data are expressed as mean±SEM. The graphs are representative of three independent experiments of five mice per group. Student *t* test result *: $p<0.05$, ***: $p<0.001$.

In G-primed mice, IL-21 depletion significantly increased CD4 T cell recruitment from d4-10 p.c in both BAL (Fig.2.10c) and lung tissue (Appendix 2.1c). Moreover, the number of CD8 T cells was also significantly increased in the BAL but only at d7 p.c (Fig.2.10d). No increase was observed in the lung tissue (Appendix 2.1d). In M2-primed mice, IL-21 depletion did not alter CD4 T cell recruitment to the BAL (Fig.2.10e) or lung tissue (Appendix 2.1e), and did not significantly increase CD8 T cell recruitment (Fig.2.10f and Appendix 2.1f).

6. IL-21 depletion during priming increases cytokine production in BAL and lung after RSV challenge

As IL-21 depletion increased recall CD4, but not CD8, T cell responses after RSV challenge I wanted to know if this affected cytokine production in the pulmonary compartment. Therefore, I measured cytokines in the BALF, and supernatants from lung cells stimulated with αCD3/αCD28-expressing beads, at several time points post RSV challenge. In unimmunised mice, BAL IFN-γ levels did not increase until d4 p.c, peaked at d7, and returned to baseline levels by d10 p.c (Fig.2.11a). As described for primary RSV challenge, IL-21 depletion significantly increased BAL IFN-γ levels but only at d7 p.c, the peak of pathology. There was negligible IL-4 production in rVV-βgal-immunised mice and IL-21 depletion had no effect on the levels produced (Fig.2.11c). I also measured BAL IL-10 (Fig.2.11d), IL-17 (Fig.2.11e), granzyme B (Fig.2.11b), and IL-21 (Fig.2.11f) levels. There was little IL-17 and no IL-21 could be detected.

BAL granzyme B was detectable from d2 p.c, peaked at d7 p.c, and became undetectable at d10 p.c (Fig.2.11b). IL-21 depletion had no effect on the levels produced. Finally, BAL IL-10 peaked at d4 p.c, and was undetectable by d10 p.c (Fig.2.11d). IL-21 depletion significantly increased levels at d4 p.c but had no effect at d7 p.c, as observed during primary RSV challenge.

In G-primed mice, peak IFN-γ levels were 3-fold higher than in unimmunised mice. IFN-γ was detectable at d2 p.c, peaked at d4-7 p.c, and returned to baseline at d14 p.c (Fig.2.12a). IL-21 depletion significantly increased production at d4&7 p.c. BAL IL-4 levels were also significantly higher than in unimmunised mice peaking at d4 p.c and became undetectable at d10 p.c (Fig.2.12c). IL-21 depletion reduced production which was reached statistical significant at d4&7 p.c. BAL IL-21 (Fig.2.12f) was undetectable in G-primed mice. Granzyme B was detectable in the BAL and peaked at d4-7p.c but IL-21 depletion had no significant effect on its production (Fig.2.12b). Finally, BAL IL-10 (Fig.2.12d) and IL-17 (Fig.2.12e) levels peaked at d4 p.c and IL-21 depletion significantly increased levels at both d4&7 p.c.

In M2-primed mice, peak BAL IFN-γ levels were >2-fold higher than in G-primed mice (Fig.2.13a). The kinetics of production was similar to G-primed mice but IL-21 depletion had no significant effect on production. No IL-4 was detectable at any time and IL-21 depletion had no effect (Fig.2.13c). Likewise BAL IL-17 (Fig.2.13e), and IL-21 (Fig.2.13f) were undetectable in M2-primed mice and IL-21 depletion had no effect on production. BAL IL-10 was detectable on d4-7 p.c but at lower levels than those observed in G-primed mice

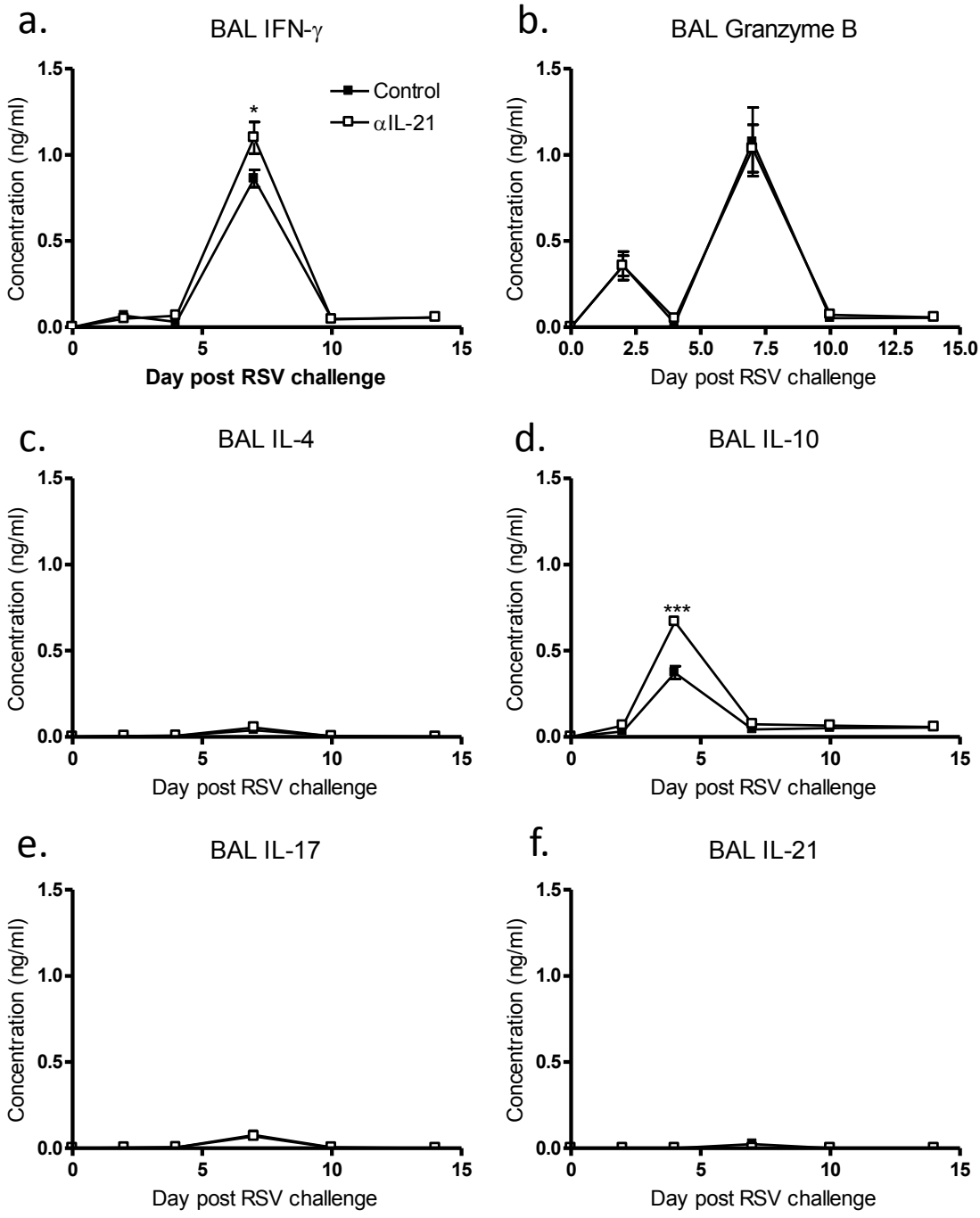

Figure 2.11. IL-21 depletion during rVV-βgal priming increases BAL IFN-γ and IL-10 levels after RSV challenge. Mice were immunised with rVV-βgal and challenged as described in Fig.2.8. BALF was harvested at several time points post challenge and IFN-γ (a), Granzyme B (b), IL-4 (c), IL-10 (d), IL-17 (e), and IL-21 (f) were determined by sandwich ELISA. Error bars represent SEM. The graphs are representative of three independent experiments of five mice per group. Student *t* test result *: $p<0.05$, ***: $p<0.001$.

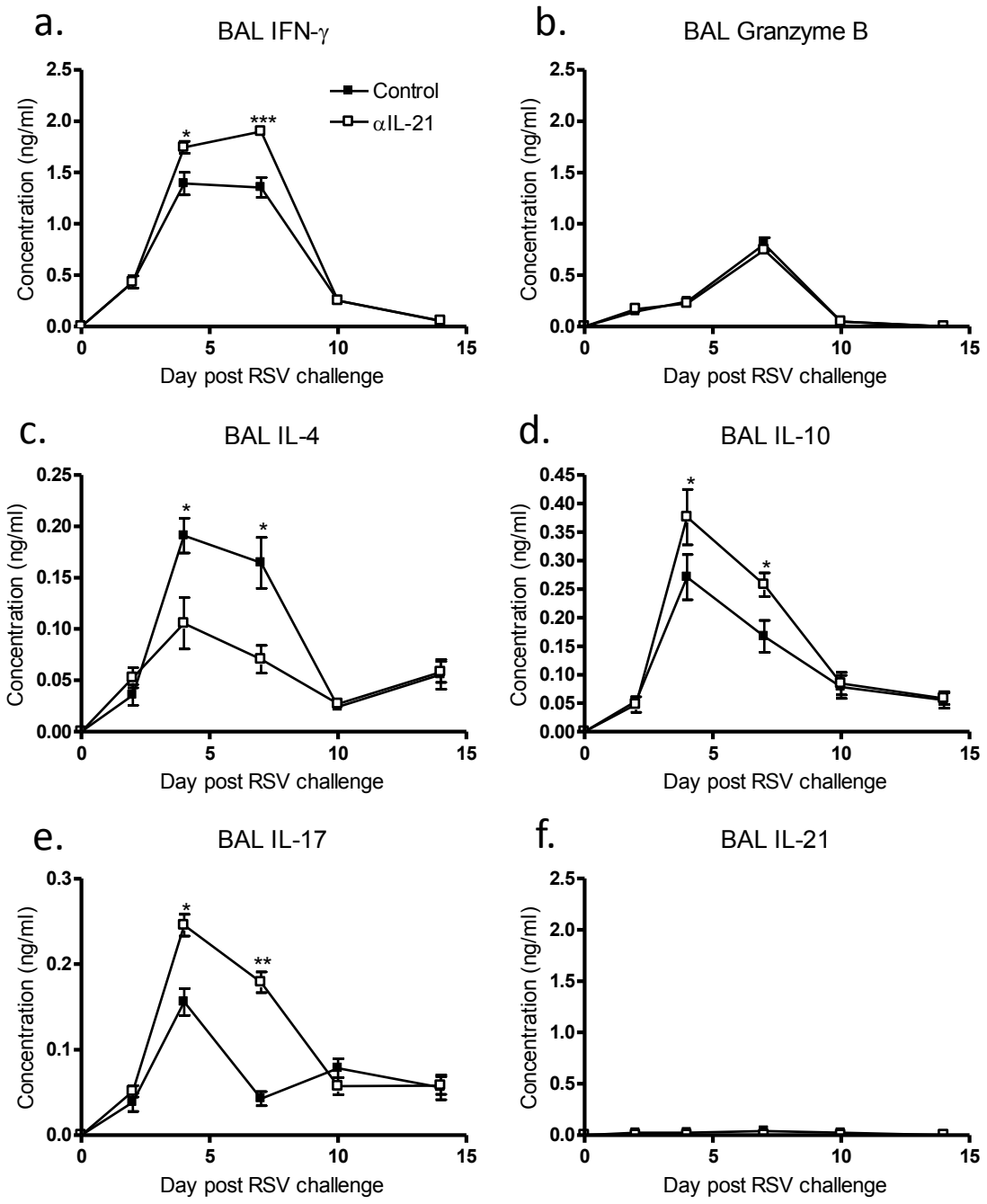

Figure 2.12. IL-21 depletion during rVV-G priming increases BAL IFN-γ, IL-10, and IL-17 and reduces IL-4 levels after RSV challenge. Mice were immunised with rVV-G and challenged as described in Fig.2.8. BALF was harvested at several time points post challenge and IFN-γ (a), Granzyme B (b), IL-4 (c), IL-10 (d), IL-17 (e), and IL-21 (f) were determined by sandwich ELISA. Error bars represent SEM. The graphs are representative of three independent experiments of five mice per group. Student *t* test result *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

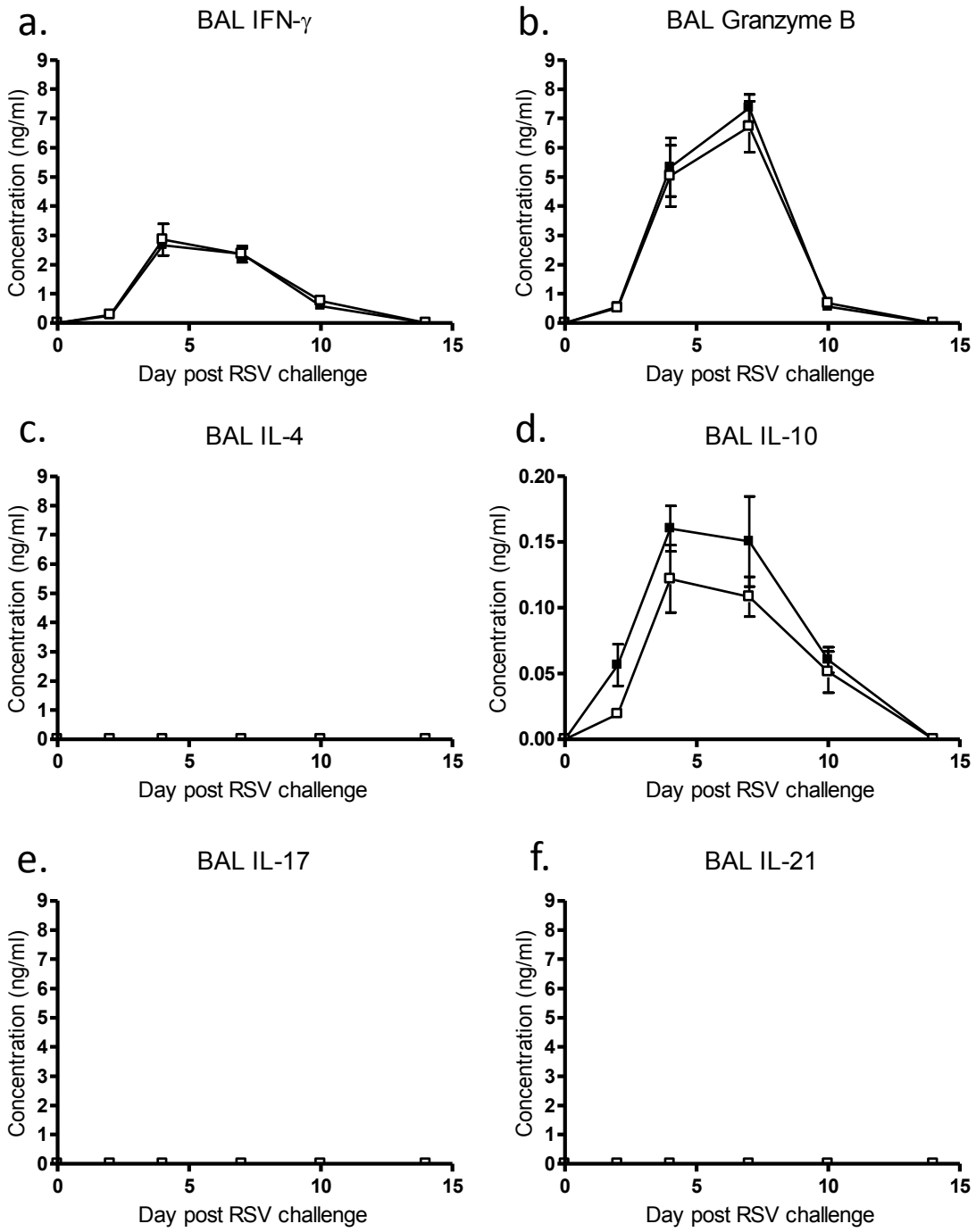

Figure 2.13. IL-21 depletion during rVV-M2 priming has no effect on BAL cytokine levels after RSV challenge. Mice were immunised with rVV-M2 and challenged as described in Fig.2.8. BALF was harvested at several time points post challenge and IFN-γ (a), Granzyme B (b), IL-4 (c), IL-10 (d), IL-17 (e), and IL-21 (f) were determined by sandwich ELISA. Error bars represent SEM. The graphs are representative of three independent experiments of five mice per group.

(Fig.2.13d). Again, IL-21 depletion had no effect on IL-10 production. BAL granzyme B was detectable at d2 p.c , peaked at d7 p.c, and was undetectable at d14 p.c (Fig.2.13b). However, IL-21 depletion had no significant effects on production.

Cytokine production by lung cells from rVV-βgal-immunised (Appendix 2.2), G-primed (Appendix 2.3), and M2-primed (Appendix 2.4) mice stimulated with a polyclonal stimulus showed a very similar phenotype to that described for the BAL. However, IL-21 production was detected in G-primed mice, suggesting that memory CD4 T cells were a potent source of this factor.

These data from primed animals confirm that IL-21 plays a much more significant role in controlling the recruitment, activation and effector functions of CD4 T cells and CD4-T-cell-driven immune responses during RSV infection. Therefore, I focussed subsequent studies on G-primed animals and CD4-T-cell-mediated immune responses to RSV challenge.

7. IL-21 depletion during priming increases cell recruitment to the pulmonary compartment after RSV challenge

Having shown that IL-21 depletion increased both CD4 and CD8 T cell recruitment to the pulmonary compartment in G-primed mice after RSV challenge, next I determined if recruitment of other immune cells were also affected. To determine this BALF and lung tissue were harvested from mice at the peak of pathology (d5 p.c) as this time correlates with the greatest changes in total cell recruitment, CD4 T cell recruitment, and cytokine production. Recruitment of several different cell types was determined by flow cytometry. In non-depleted mice, I detected recruitment of lymphocytes [CD4 T cells ($TCR\beta^{+}CD4^{+}$), CD8

T cells ($TCR\beta^+CD8^+$), B cells ($B220^+CD19^+$), and NK cells ($TCR\beta^-DX5^+$)], granulocytes [neutrophils ($CD3^-B220^-CD11b^+CCR3^-$) and eosinophils ($CD3^-B220^-CD11b^+CCR3^+$)], and APCs ($MHCII^+CD11b^+CD11c^+$) to the BAL. IL-21 depletion increased recruitment of CD4 T cells and CD8 T cells (as described previously), and NK cells (Fig.2.14a). However, B cell recruitment was significantly decreased. IL-21 depletion also significantly increased neutrophil recruitment to the airway, but eosinophilia remained unchanged (Fig. 2.14c). Recruitment of antigen-presenting cells (APCs; DCs) was also significantly increased by IL-21 depletion (Fig.2.14e). Only a minority of these cells were $F4/80^+$ indicating that these were mostly DCs and not macrophages.

A similar effect of IL-21 depletion was also observed on cell recruitment to the lung tissue. Lymphocytosis (Fig.2.14b), granulocytosis (Fig.2.14d), and DC recruitment (Fig.2.14f) were all enhanced in IL-21-depleted mice.

8. IL-21 depletion during priming compromises viral clearance after RSV challenge

In RSV disease, enhanced cellular recruitment positively correlates with enhanced viral clearance. To determine the effect of IL-21 depletion on viral clearance, I measured the number of RSV L gene copies in lung tissue at several time points post RSV challenge by qPCR. mRNA was purified from lung tissue, converted to cDNA, and the number of L gene copies determined using L-gene-specific primers and probe. In unimmunised mice, viral L gene copies were first detected at d2 p.c, peak at d4 p.c, and return to baseline levels by d7 p.c (Fig.2.15). In contrast, viral L gene remains undetectable in immunised, non-depleted mice as they produce a much more rapid and vigorous anti-viral response. However, viral L

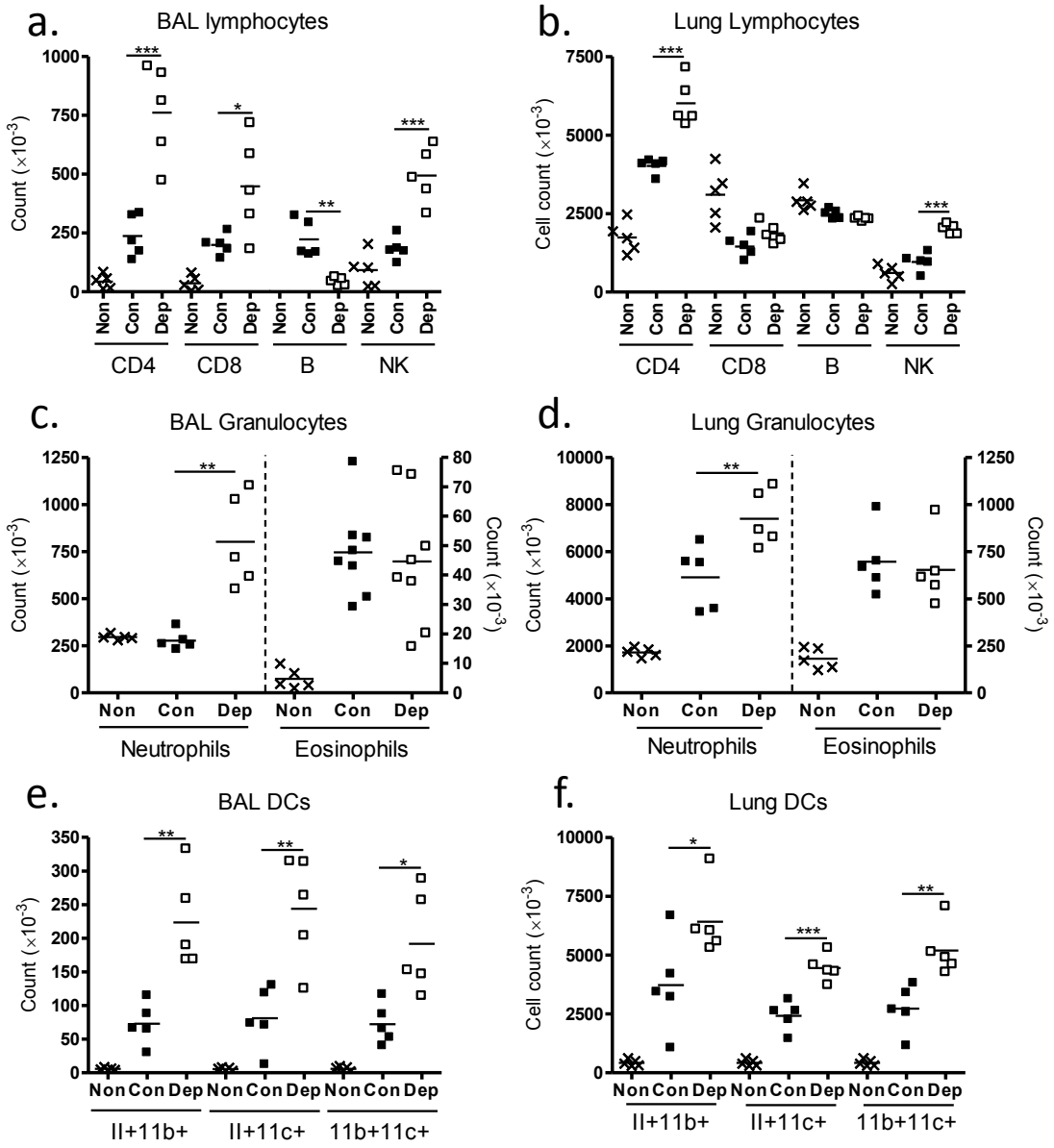

Figure 2.14. IL-21 depletion during rVV-G priming increases cell recruitment to the pulmonary compartment after RSV challenge. Mice were immunised with rVV-βgal ('non') or rVV-G ('Con' and 'Dep') and challenged as described in Fig.2.8. BALF (a, c, e) and lung (b, d, f) samples were harvested at d5 post challenge. Samples were processed, RBCs lysed, and live cells counted by trypan blue exclusion assay. CD4 T cells, CD8 T cells, B cells and NK cells (a-b); neutrophils and eosinophils (c-d); and DCs (e-f) were phenotyped by flow cytometry and cell counts determined. At least 50×10^3 cells/sample were collected. Error bars represent SEM. The graphs are representative of at least five independent experiments of five mice per group. Student *t* test result *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

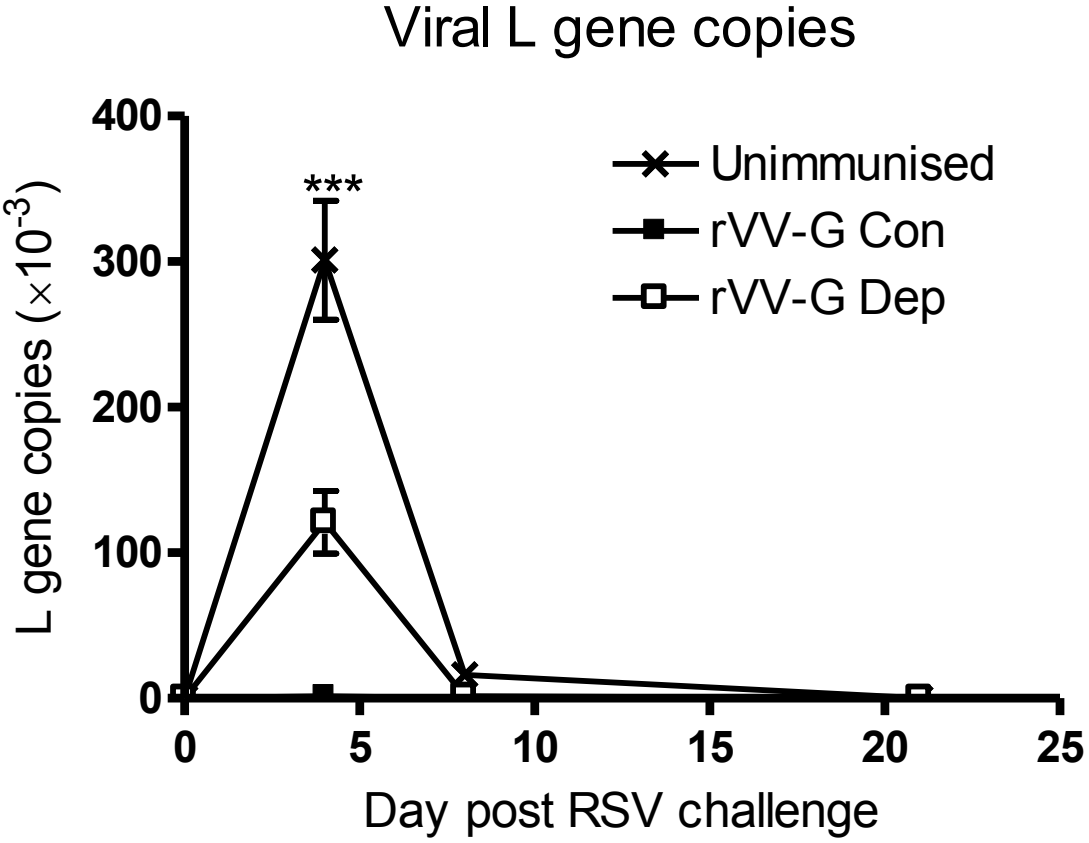

Figure 2.15. IL-21 depletion during rVV-G priming compromises viral clearance after RSV challenge. Mice were immunised with rVV-βgal ('Unimmunised') or rVV-G ('Con' and 'Dep') and challenged as described in Fig.2.8. Lungs were harvested at several time points post challenge. The number of viral L gene copies in the lungs of infected mice was determined at several time points post challenge by quantitative PCR (qPCR; protocol described in *Materials and Methods*). Error bars represent SEM. The graphs are representative of three independent experiments of five mice per group. ANOVA (Tukey post test) ***: $p<0.001$.

gene was detectable at d4 p.c in IL-21-depleted mice, though the L gene copy number was 3-fold lower than unimmunised animals (Fig.2.15).

9. IL-21 depletion at priming boosts the number of RORγt$^+$ and T-bet$^+$ pulmonary CD4 T cells after RSV challenge

Changes in cytokine production may reflect alterations in CD4 T cell differentiation. To examine how IL-21 depletion affects CD4 T cell differentiation, I determined FoxP3, RORγt, and T-bet expression in splenic CD4 T cells prior to RSV challenge and lung CD4 T cells at d5 p.c (the peak of disease severity). IL-21 depletion did not alter FoxP3 (Fig.2.16a), RORγt (Fig.2.16b), or T-bet (Fig.2.16c) expression by splenic CD4 T cells (Fig.2.16d), nor did it alter cell numbers (Fig.2.16e). However, at d5 p.c, a significantly reduced proportion of BAL CD4 T cells from IL-21-depleted mice expressed FoxP3 (Fig.2.17a&d), while the proportion expressing RORγt (Fig.2.17b&d) or T-bet (Fig.2.17c&d) was similar. Consequently, there was an increase in total RORγt$^+$ and T-bet$^+$ BAL CD4 T cell numbers as a result of IL-21-depletion (Fig.2.17e). This trend also occurred in lung tissue (Fig.2.18), but there were no significant differences in the cells of the mediastinal lymph nodes (Fig.2.19).

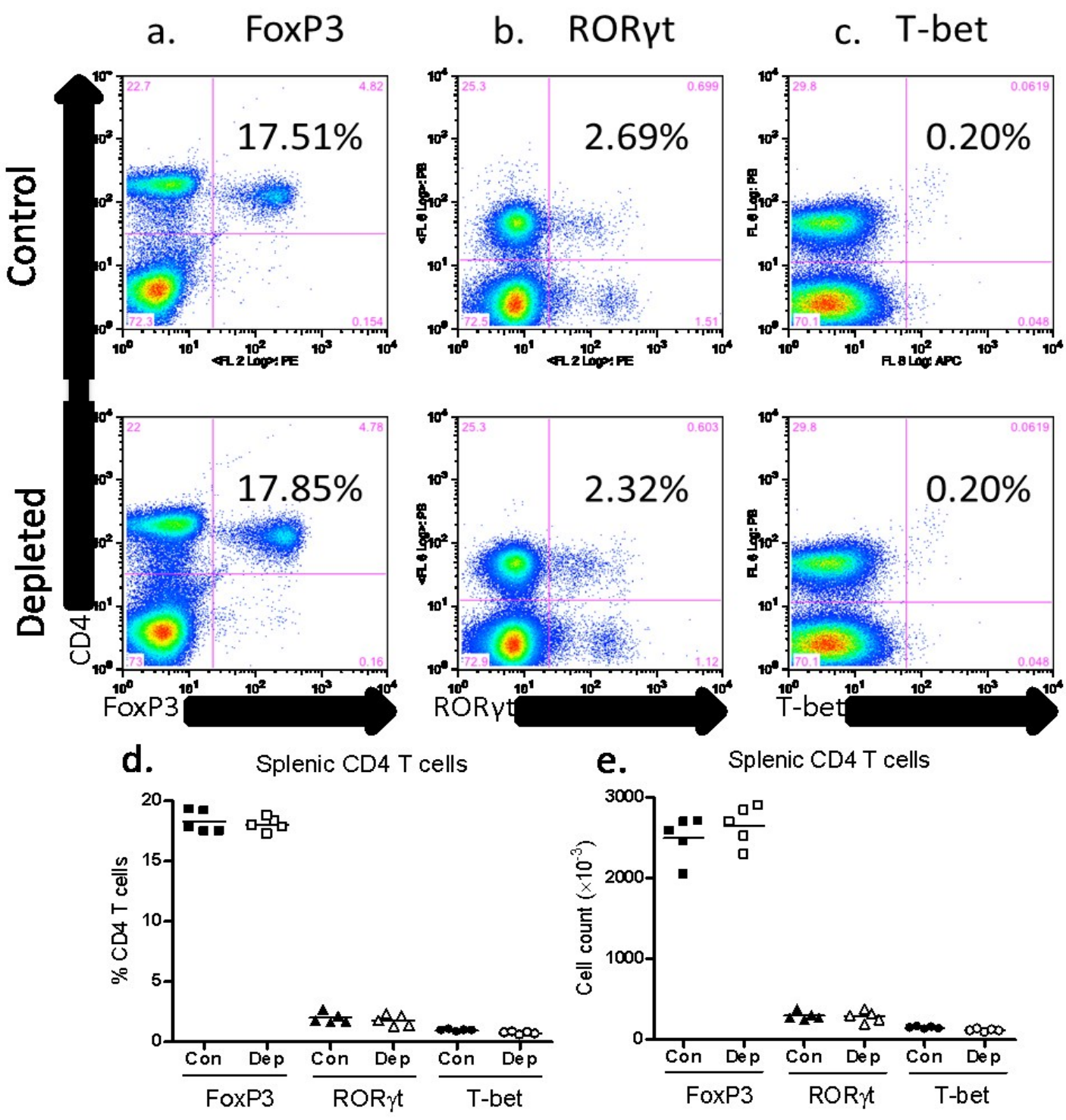

Figure 2.16. IL-21 depletion has no effect on FoxP3, RORγt, and T-bet expression by splenic CD4 T cells after priming with recombinant vaccinia virus. Mice were immunised with rVV-G as described in Fig.2.8. Fourteen days post priming spleens were harvested. CD4 T cells were stained for FoxP3 (a), RORγt (b), or T-bet (c) according to the manufacturer's instructions. The percentage of CD4 T cells expressing each transcription factor was determined by flow cytometry and is shown in each dotplot. Grouped data for percentage (d) and total number (e) is also shown. The graph is representative of two independent experiments of five mice per group.

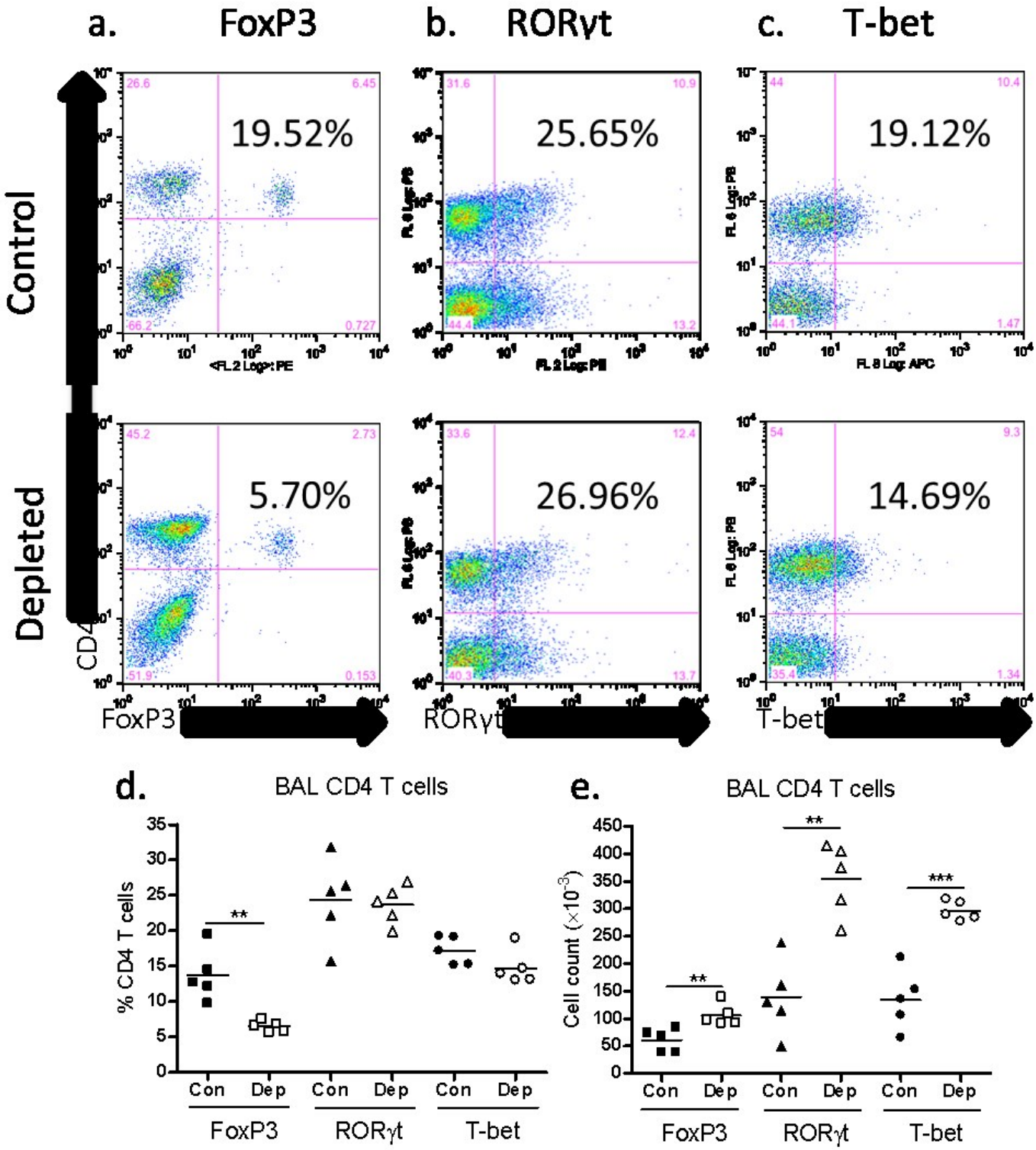

Figure 2.17. IL-21 depletion in primed mice reduces FoxP3 expression by BAL CD4 T cells after RSV challenge. Mice were immunised with rVV-G and challenged as described in Fig.2.8. Five days post challenge BALF was harvested. CD4 T cells were stained for FoxP3 (a), RORγt (b), or T-bet (c) according to the manufacturer's instructions. The percentage of CD4 T cells expressing each transcription factor was determined by flow cytometry and is shown in each dotplot. Grouped data for percentage (d) and total number (e) is also shown. The graph is representative of two independent experiments of five mice per group. Student t-test result **: $p<0.01$, ***: $p<0.001$.

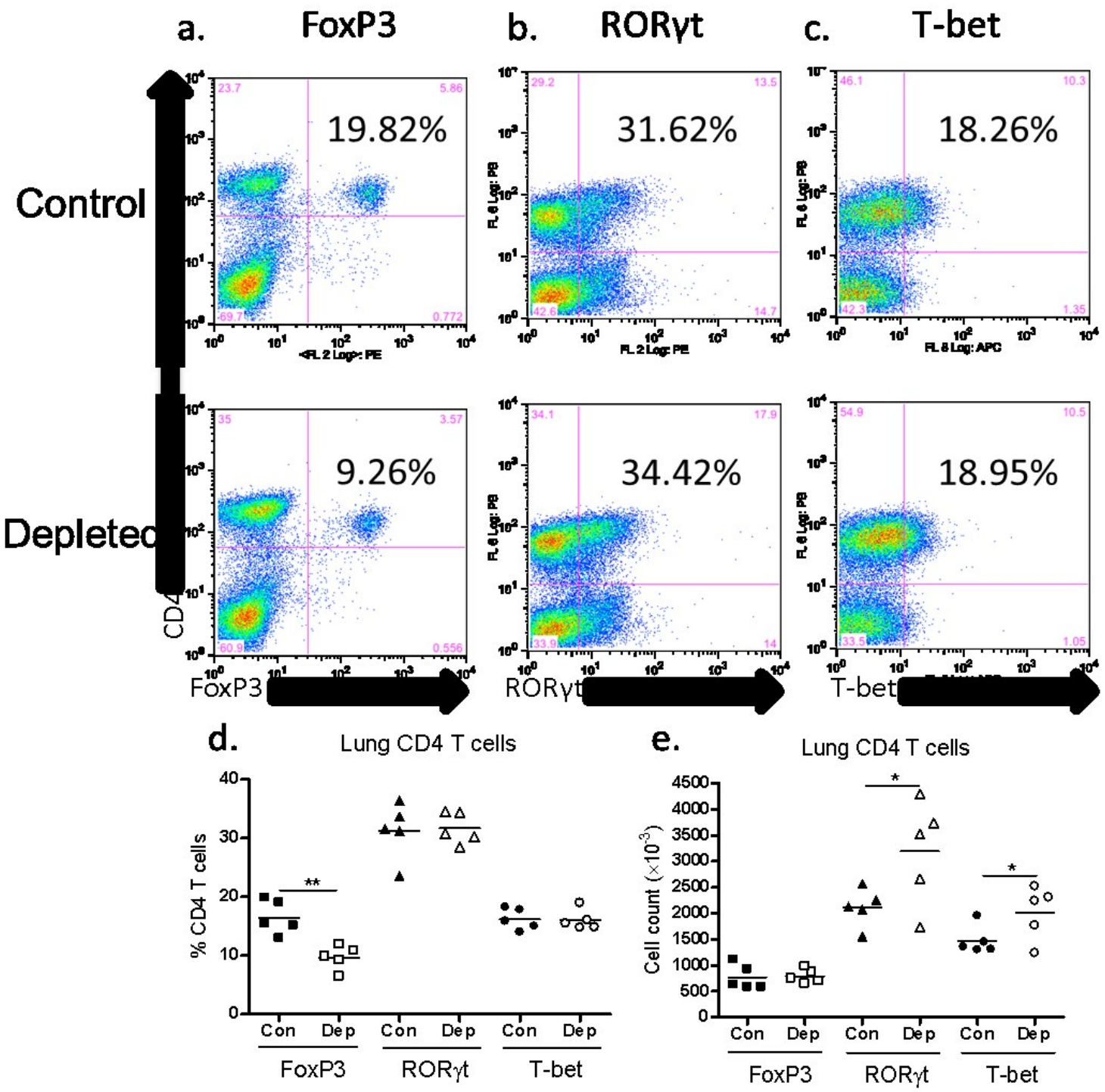

Figure 2.18. IL-21 depletion in primed mice reduces FoxP3 expression by lung CD4 T cells after RSV challenge. Mice were immunised with rVV-G and challenged as described in Fig.2.8. Five days post challenge lungs were harvested. CD4 T cells were stained for FoxP3 (a), RORγt (b), or T-bet (c) according to the manufacturer's instructions. The percentage of CD4 T cells expressing each transcription factor was determined by flow cytometry and is shown in each dotplot. Grouped data for percentage (d) and total number (e) is also shown. The graph is representative of two independent experiments of five mice per group. Student t-test result *: $p<0.05$.

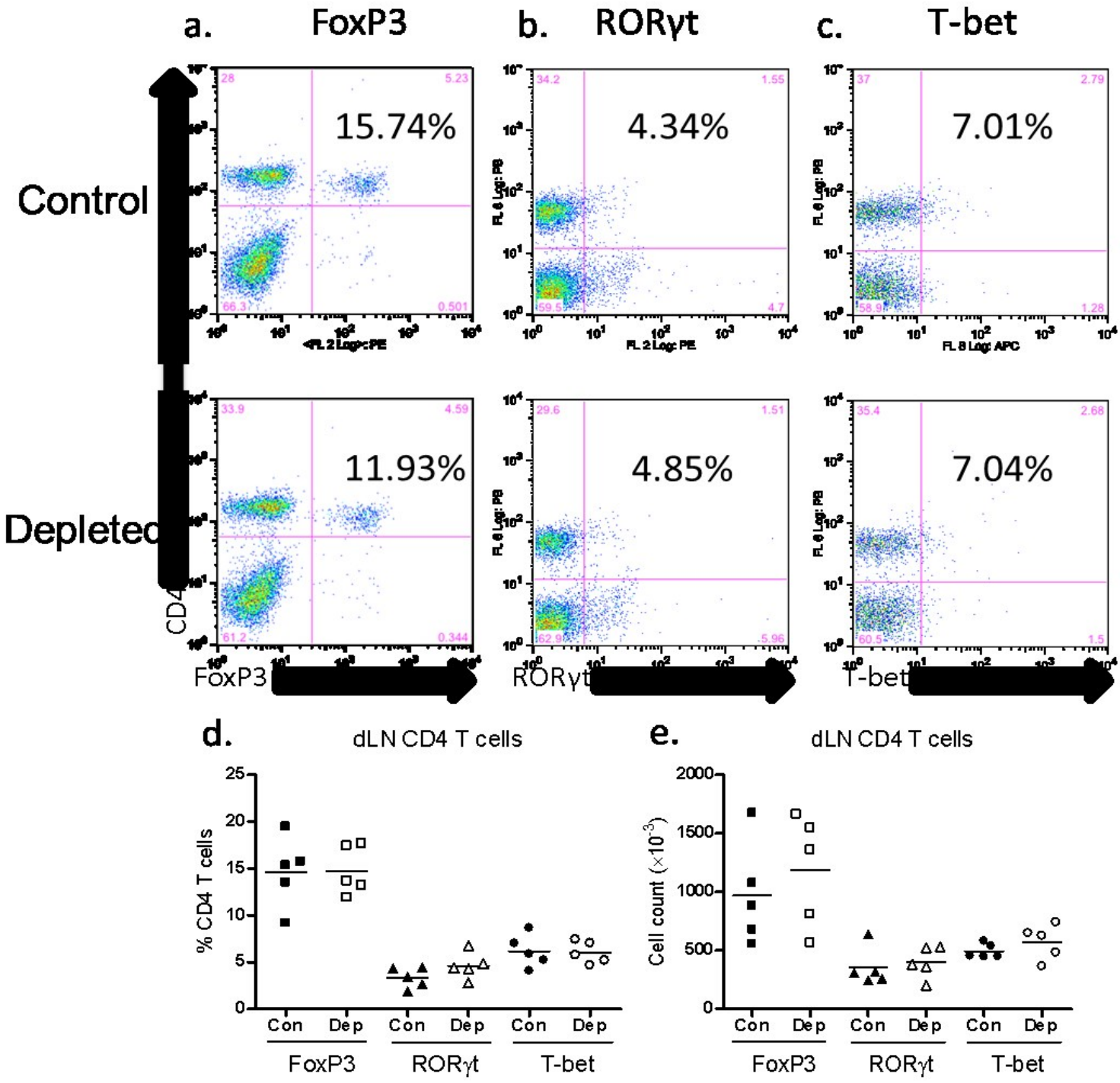

Figure 2.19. IL-21 depletion in primed mice has no effect on FoxP3, RORγt, and T-bet expression by dLN CD4 T cells after RSV challenge. Mice were immunised with rVV-G and challenged as described in Fig.2.8. Five days post challenge draining lymph nodes were harvested. CD4 T cells were stained for FoxP3 (a), RORγt (b), or T-bet (c) according to the manufacturer's instructions. The percentage of CD4 T cells expressing each transcription factor was determined by flow cytometry and is shown in each dotplot. Grouped data for percentage (d) and total number (e) is also shown. The graph is representative of two independent experiments of five mice per group. Student t-test result *: $p<0.05$.

10. IL-21 depletion during priming increases IFN-γ and IL-17 production by CD4 T cells after RSV challenge

Analysis of transcription factor expression by pulmonary CD4 T cells indicated that IL-21 depletion reduced FoxP3 and increased RORγt and T-bet expression. Therefore, next I determined whether these increased signals for the Th1 (T-bet) and Th17 (RORγt) signature transcription factors resulted in greater expression of IFN-γ and IL-17 by these cells. To achieve this, spleens were processed from control and IL-21-depleted mice 14 days post cutaneous vaccinia virus infection and CD4 T cells sorted using MACS technology.

These cells were then stimulated with media or αCD3/αCD28-expressng beads. Cytokine secretion was blocked using Golgiplug and IFN-γ and IL-17 production measured by CD4 T cells at the single-cell level by flow cytometry.

~90% of the stimulated cells in culture were CD4 T cells (Fig.2.20a). No cytokine production was detected by CD4 T cells from either group after stimulation with media (Fig.2.20b). However, after polyclonal stimulation ~80-85% of CD4 T cells from both groups produced IFN-γ (Fig.2.20c), and there was no significant difference between CD4 T cells from control or IL-21-depleted mice (Fig.2.20d). However, while no IL-17 production was detected by CD4 T cells from control mice, there was a small (1.74%) but visible population of CD4 T cells from IL-21-depleted mice producing IL-17 (Fig.2.20c). 93% of the CD4 T cells producing IL-17 co-produced IFN-γ, and the difference in IL-17 production between the two groups was significant (Fig.2.20d).

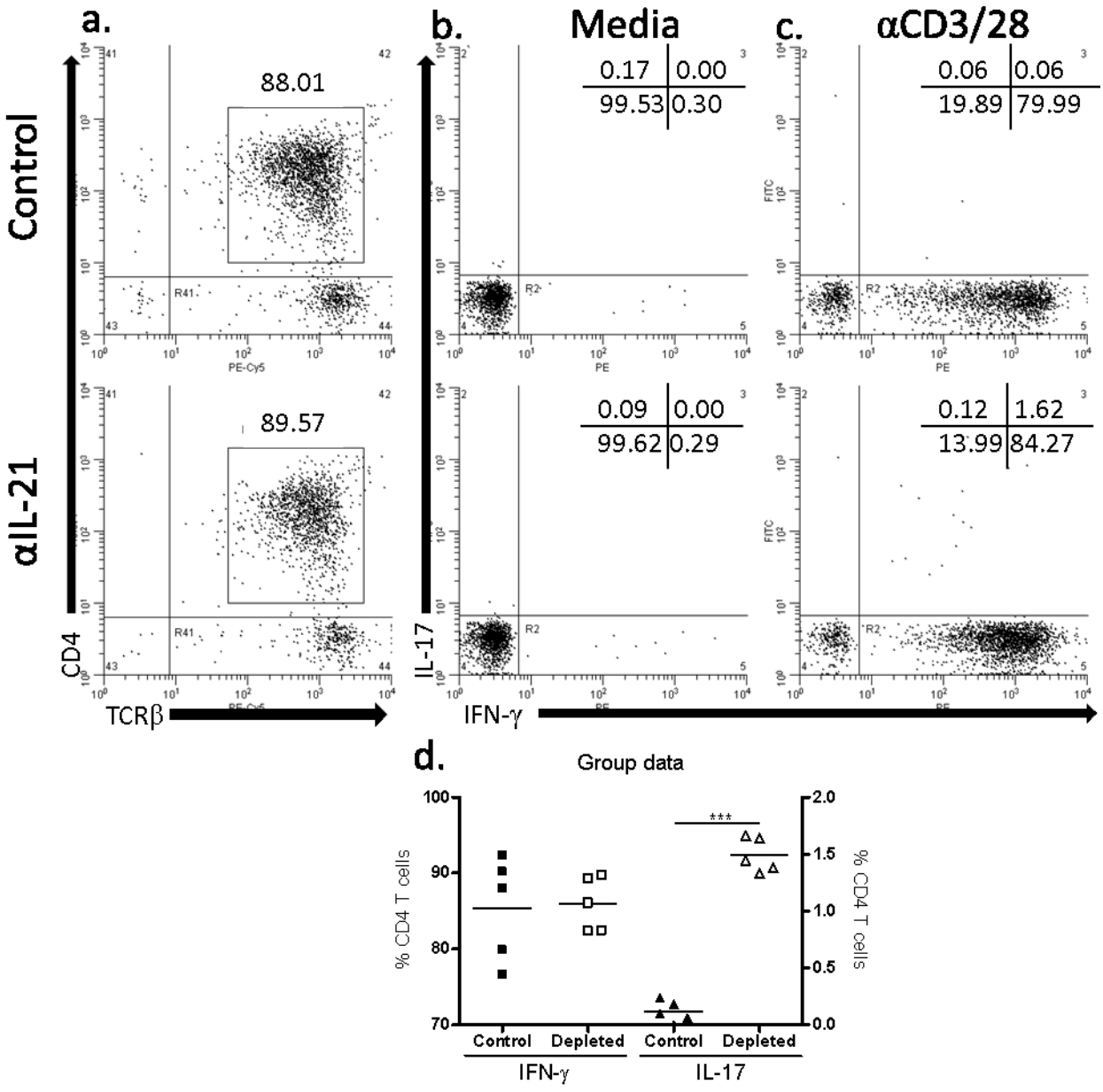

Figure 2.20. IL-21 depletion during rVV-G priming increases IL-17 production by CD4 T cells. Mice were immunised rVV-G and 14 days post priming spleens were harvested. CD4 T cells were sorted by MACS and stimulated overnight with media (b) or αCD3/28 beads (10µl/10^6 cells; c). CD4 T cells (a) were stained for IFN-γ and IL-17 using specific catch and detection reagents (10µl/10^6 cells). The percentage of CD4 T cells secreting each cytokine was determined by flow cytometry and is shown in each dotplot. Grouped data is also shown (d). The graphs are representative of two independent experiments of five mice per group. Student t-test result ***: $p<0.001$.

Next, I determined whether this difference in cytokine production was present post RSV challenge. Therefore, lungs were processed from control and IL-21-depleted mice at d5 p.c and stimulated as above. CD4 T cells were identified as above and comprised 23-28% of lung lymphocytes (Fig.2.21a). ~12% of CD4 T cells produced IFN-γ when cultured in media alone, but IL-17 was undetectable (Fig.2.21b). Polyclonal stimulation of lung CD4 T cells increased the percentage producing IFN-γ in both groups (control: 12.6%; IL-21-depleted: 19.5%) but significantly more CD4 T cells from IL-21-depleted mice produced IFN-γ compared to control cells (Fig.2.21c&d). Moreover, significantly more lung CD4 T cells from IL-21-depleted mice produced IL-17 compared to those from control mice (control: 2.9%; IL-21-depleted: 9.1%). However, in contrast to splenic CD4 T cells post priming, IL-17^{+} CD4 T cells were split into IFN-γ^{-} (~60%) and IFN-γ^{+} (40%) subsets.

11. IL-21 depletion during priming increases antigen-specific cytokine production by CD4 T cells after RSV challenge

Previous data indicate that IL-21-depleted-CD4-T-cells produce increased amounts of IFN-γ and IL-17 upon polyclonal stimulation compared to non-depleted controls. However, it was unclear if this phenotype occurred if the CD4 T cells were stimulated with cognate antigen. To determine this, I MACS-sorted lung CD4 T cells and FACS-sorted lung DCs from control and IL-21-depleted mice at d5 p.c. Cell purity was >90%. DCs were pulsed with either specific (G 184-198) peptide or a control (G 64-78) peptide and co-cultured with CD4 T cells for 72hrs. Supernatants were harvested and cytokines assayed by sandwich ELISA.

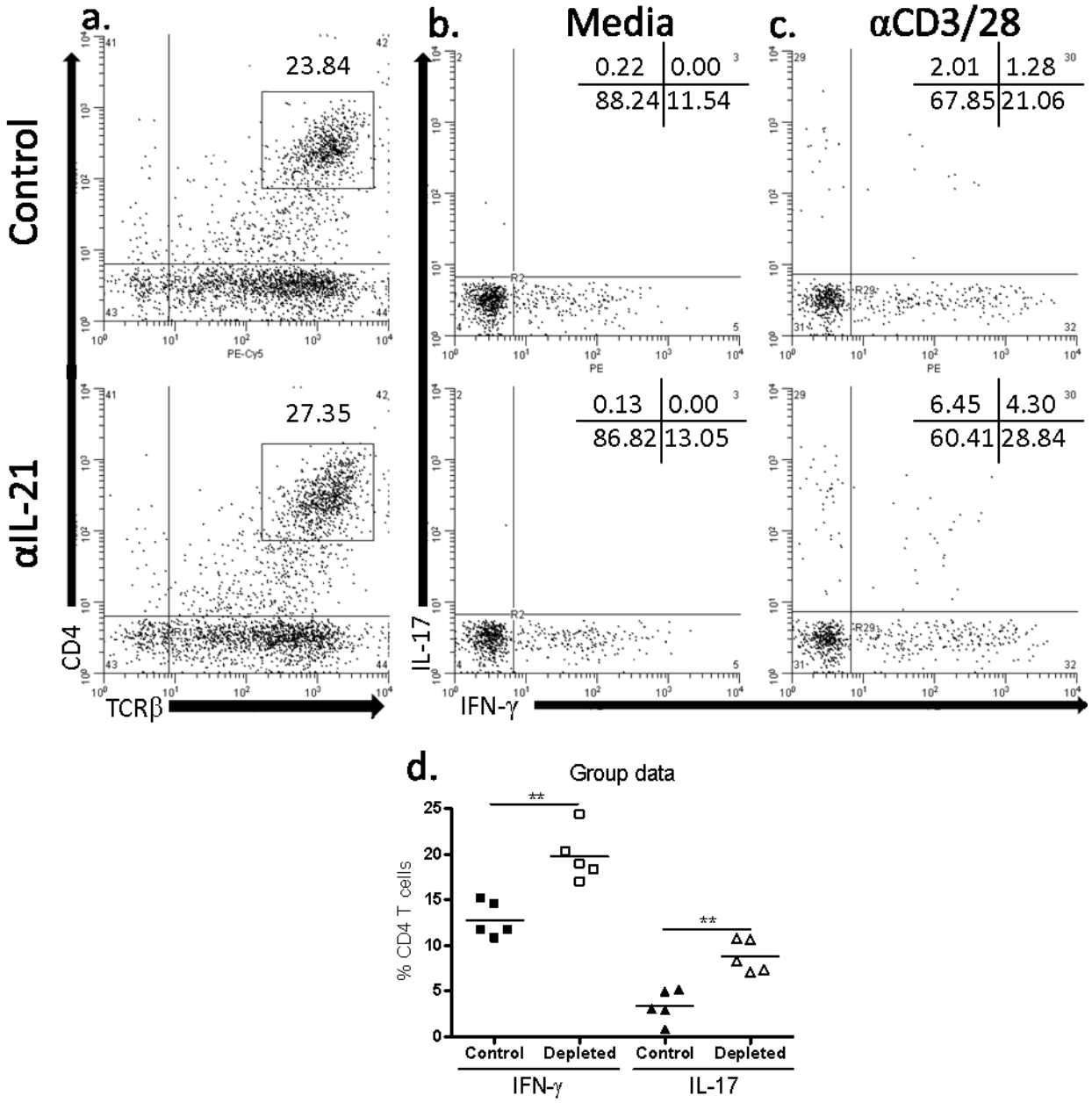

Figure 2.21. IL-21 depletion during rVV-G priming increases IFN-γ and IL-17 production by CD4 T cells. Mice were immunised rVV-G and challenged as described in Fig.2.8. Five days post challenge lungs were harvested. CD4 T cells were sorted by MACS and stimulated overnight with media (b) or αCD3/28 beads (10µl/10^6 cells; c). CD4 T cells (a) were stained for IFN-γ and IL-17 using specific catch and detection reagents (10µl/10^6 cells). The percentage of CD4 T cells secreting each cytokine was determined by flow cytometry and is shown in each dotplot. Grouped data is also shown (d). The graphs are representative of two independent experiments of five mice per group. Student t-test result **: $p<0.01$.

No cytokines were detected when CD4 T cells were stimulated with control peptide (Fig.2.22). CD4 T cells from non-depleted mice produced significant amounts of IFN-γ (Fig.2.22a) and IL-21 (Fig.2.22e), and small but detectable amounts of IL-10 (Fig.2.22c), IL-4 (Fig.2.22b), and IL-17 (Fig.2.22d). However, CD4 T cells from IL-21-depleted mice produced significantly increased levels of all cytokines assayed except IL-21 whose production was ablated (Fig.2.22e). IFN-γ (Fig.2.22a) and IL-17 (Fig.2.22d) production were particularly enhanced compared to the increase in IL-4 (Fig.2.22b) and IL-10 (Fig.2.22c).

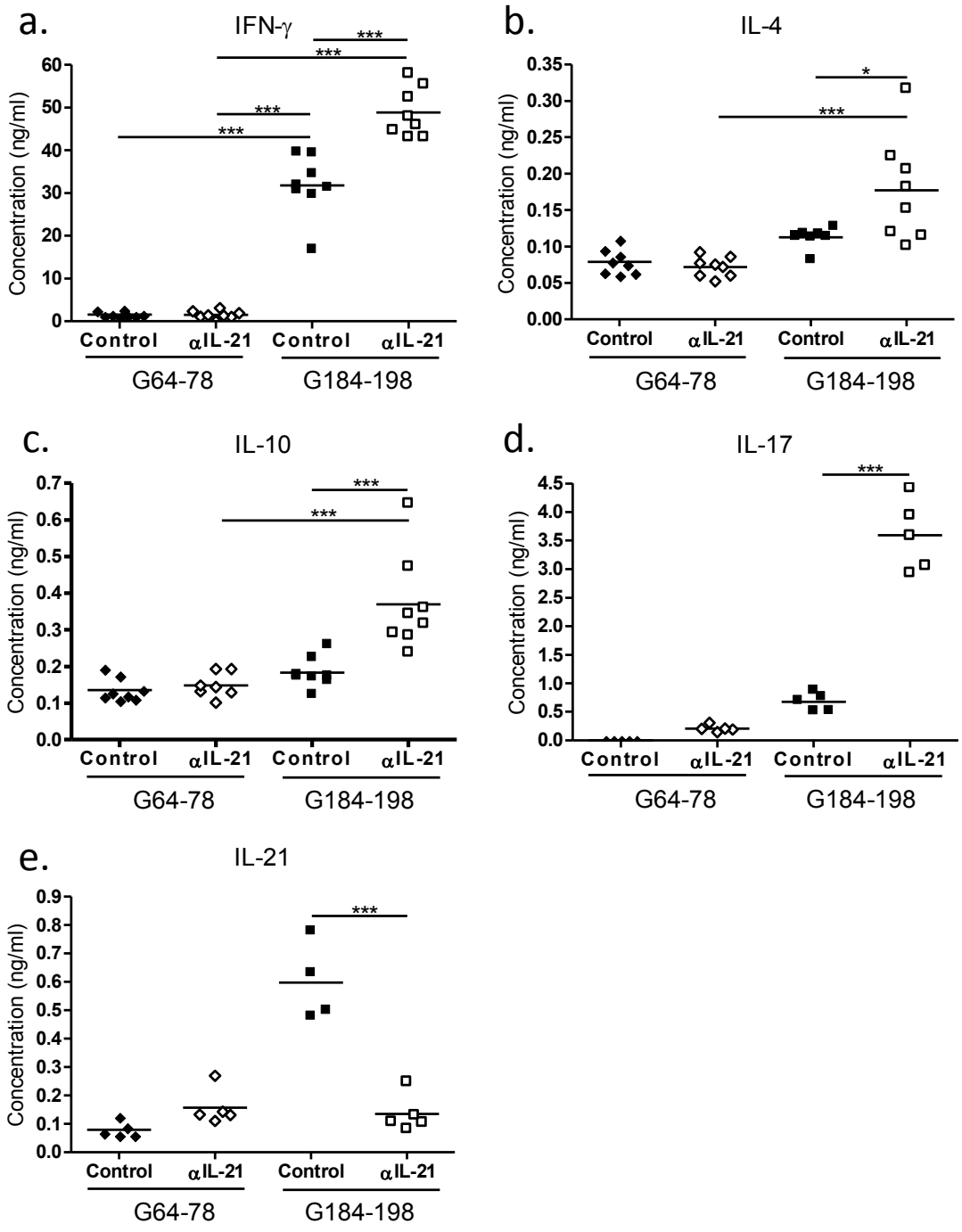

Figure 2.22. IL-21 depletion during rVV-G priming increases antigen-specific cytokine production by CD4 T cells after RSV challenge. Mice were immunised rVV-G and challenged as described in Fig.2.8. Five days post challenge lungs were harvested. CD4 T cells were sorted by MACS and DCs sorted by FACS. DCs (4×10^4 cells/well) were pulsed with specific (G184-198) peptide or control (G64-78) peptide (10μg/ml) for 1hr prior to co-culture with CD4 T cells (4×10^5 cells/well). Cells were incubated for 72hrs, the supernatants were harvested, and IFN-γ (a), IL-4 (b), IL-10 (c), IL-17 (d), and IL-21 (e) levels determined by sandwich ELISA. The graphs are representative of three independent experiments of five mice per group. Student t-test result *: $p<0.05$, ***: $p<0.001$.

12. Adoptive transfer of CD4 T cells from rVV-G-primed, IL-21-depleted, RSV-challenged mice exacerbates immunopathology in recipient mice after RSV challenge

IL-21 depletion increases CD4 T cell recruitment and cytokine production to enhance pathology after RSV challenge. However, it is possible that other cells apart from CD4 T cells are responsible for the observed increase in pathology. To determine how important CD4 T cells were to pathology, I adoptively-transferred memory CD4 T cells into naïve recipients prior to RSV challenge. Post RSV challenge, memory T cells exist in several tissues, including BALF, lung, the mediastinal lymph nodes, and spleen. The memory populations comprise distinct subsets of memory T cells that elicit different effects on disease (746). However, as the spleen was the site of memory T cell development post priming, and was where most memory T cells resided (in numerical terms) I used this site as the memory T cell source. I therefore phenotyped the splenic CD4 T cells 28 days post RSV challenge to determine the effect of IL-21 depletion on the transferred cells. Since there is no direct method by which to isolate anti-RSV-G-protein-specific memory CD4 T cells, I determined the cytokine secretion profile of G-specific cells taken 28 days post RSV challenge, after stimulation with specific peptide (G184-198). There was no cytokine production by naïve spleen cells after peptide stimulation (Fig.2.23). No IL-4 (Fig.2.23b), IL-17 (Fig.2.23d), or IL-21 (Fig.2.23f) production was detected from G-specific spleen cells from control or IL-21-depleted mice; there was weak but detectable IL-10 production (Fig.2.23c), but no significant difference resulting from IL-21 depletion. In contrast, there was significant IFN-γ (Fig.2.23a) and granzyme B (Fig.2.23e) production by spleen cells, which was increased by IL-21 depletion *in vivo*.

The percentage of splenic CD4 T cells expressing FoxP3 (Fig.2.24a), RORγt (Fig.2.24b), T-bet (Fig.2.24c) did not differ significantly between groups (Fig.2.24d), but priming and RSV

challenge increased splenic CD4 T cell numbers so that FoxP3^{+}, RORγt^{+}, and T-bet^{+} CD4 T cells increased in total number. Importantly, there were significantly more FoxP3^{+} splenic CD4 T cells from control mice compared to IL-21-depleted (Fig.2.24e).

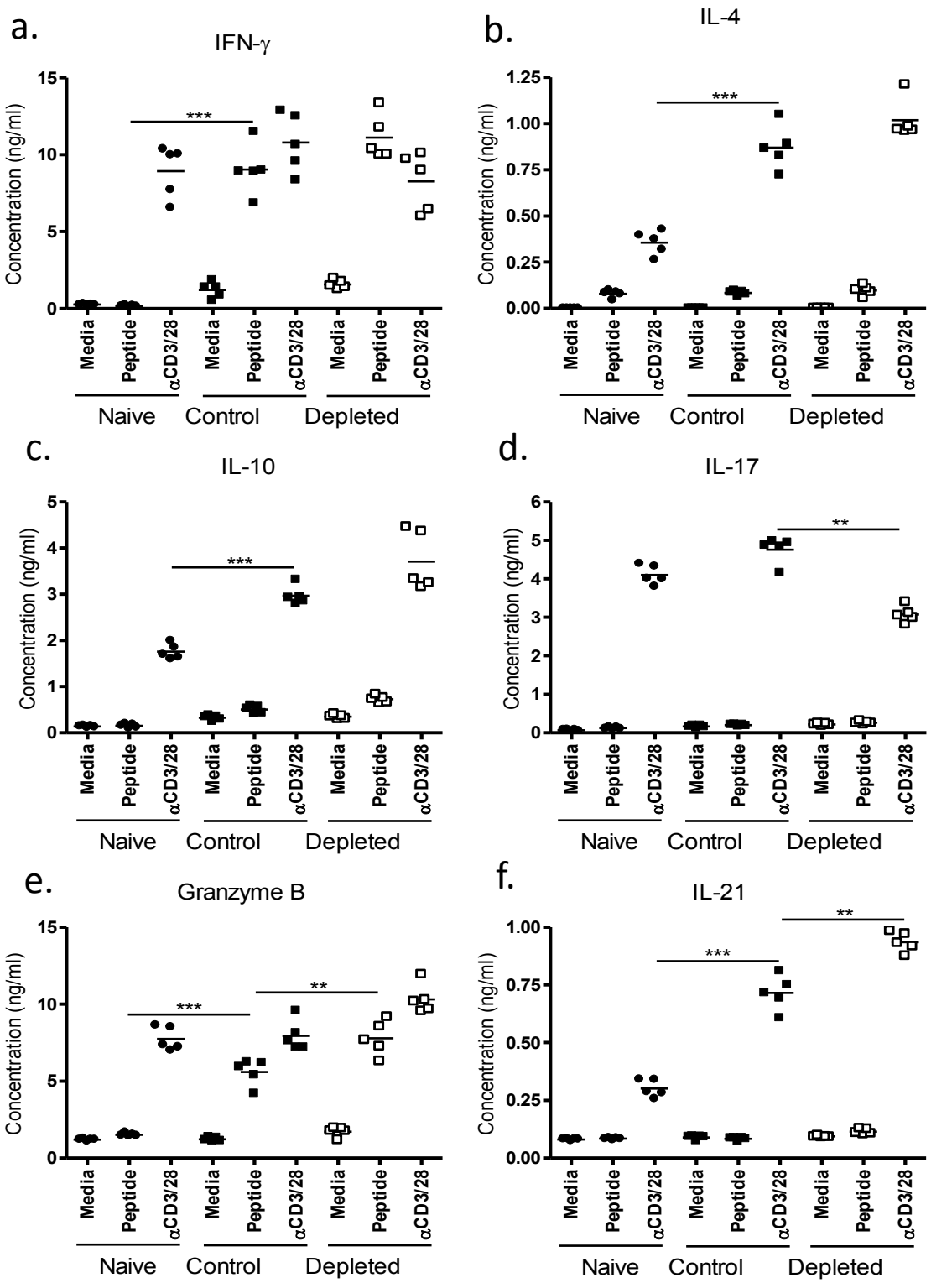

Figure 2.23. IL-21 depletion at priming increases IFN-γ and granzyme B production by splenic CD4 T cells 28 days post RSV challenge. Mice were immunised with rVV-G and challenged as described in Fig.2.8. 28 days post challenge, spleen cells from both groups plus naïve mice were harvested and processed. Spleen cells (2×10^6 cells/well) were stimulated with media, specific G peptide (10µg/ml), or αCD3/28-expressing beads (50µl/well) for 72hrs. The supernatants were harvested, and IFN-g (a), IL-4 (b), IL-10 (c), IL-17 (d), granzyme B (e) and IL-21 (f) levels determined by sandwich ELISA. The graphs are representative of two independent experiments of five mice per group. Student t-test result **: $p<0.01$, ***: $p<0.001$.

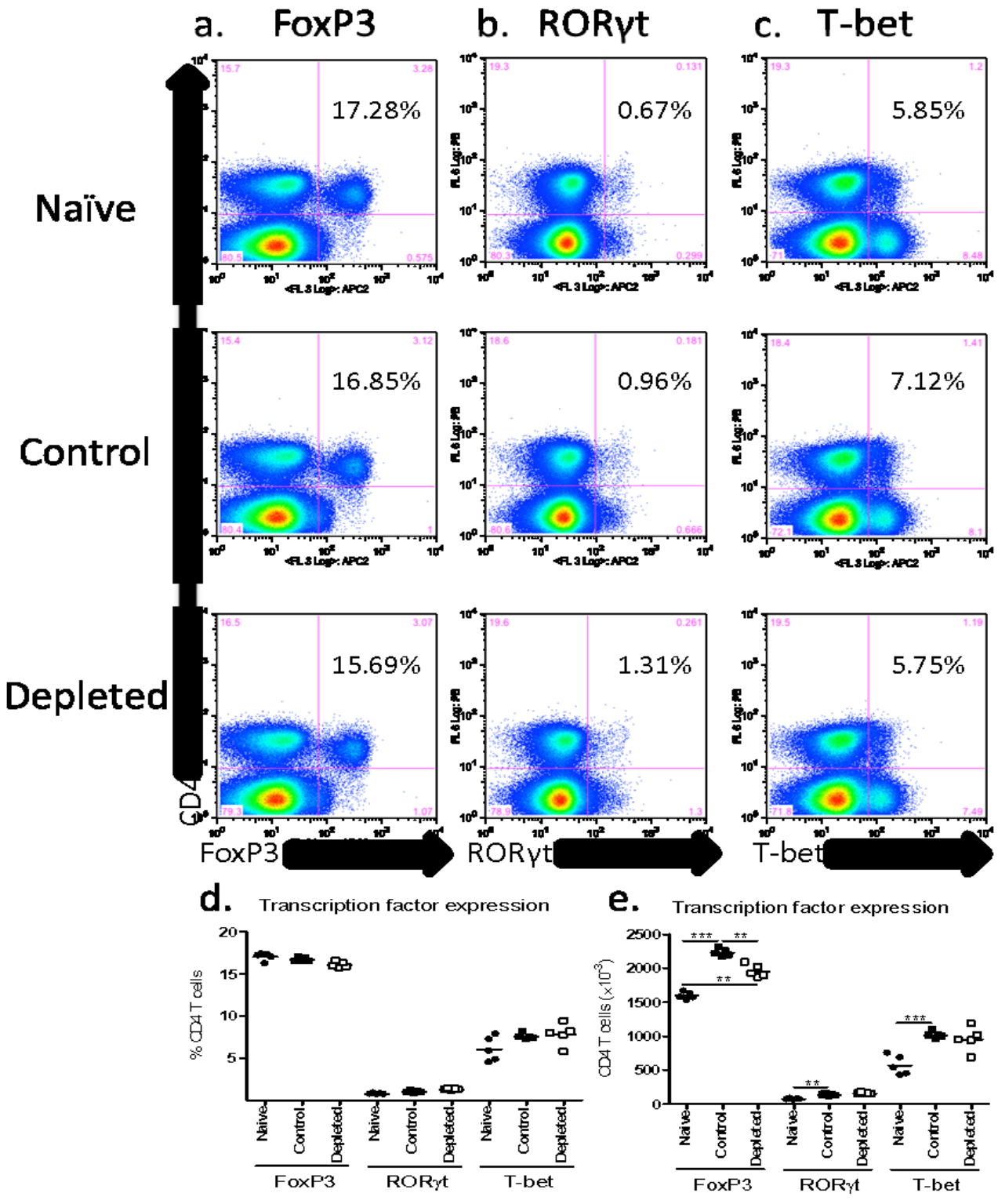

Figure 2.24. IL-21 depletion at priming significantly alters the number FoxP3$^+$, RORγt$^+$, and T-bet$^+$ CD4 T cells 28 days post RSV challenge. Mice were immunised with rVV-G and challenged as described in Fig.2.8. 28 days post challenge, spleen cells from both groups plus naïve mice were harvested and processed. CD4 T cells were stained for FoxP3 (a), RORγt (b), or T-bet (c) according to the manufacturer's instructions. The percentage of CD4 T cells expressing each transcription factor was determined by flow cytometry and is shown in each dotplot. Grouped data for percentage (d) and total number (e) is also shown. The graph is representative of two independent experiments of five mice per group. Student t-test result **: $p<0.01$, ***: $p<0.001$.

Splenic CD4 T cells from naïve, control or IL-21-depleted primed and RSV challenged mice were MACS-sorted and adoptively-transferred (i.p) into naïve recipient BALB/c mice 24hrs before i.n. challenge with RSV. Organs were harvested on d7 (p.c). Transfer of naïve CD4 T cells did not protect against disease, whereas CD4 T cells from primed, non-depleted, mice significantly reduced weight loss. In contrast, CD4 T cells from primed, IL-21-depleted, mice increased the magnitude of weight loss and did not protect against disease (Fig.2.25a). However, addition of CD4 T cells from G-primed mice did significantly reduce viral replication compared to naive (Fig.2.26). Increased weight loss was associated with enhanced T cell recruitment to the airway (Fig.2.25d&e). Of the CD4 T cells recruited to the BAL (Fig.2.25b) and lung (Fig.2.25c) most were T-bet^{+}, and there were significantly more when CD4 T cells from IL-21-depleted mice were administered. There was also an increase in FoxP3^{+} and RORγt^{+} BAL CD4 T cells in these mice. Significantly more CD4 (Fig.2.25d) and recipient CD8 (Fig.2.25e) T cells expressed an activated phenotype (CD69^{+}, OX40^{+}, and ICOS^{+}) when CD4 T cells were administered from IL-21-depleted mice. CD4 T cells from IL-21-depleted mice also recruited more BAL recipient NK cells, though there activity (as measured by CD69 expression) was identical (Fig2.25f). This increase in T-bet^{+} CD4 T cells, CD8 T cells, and NK cells increased BAL IFN-γ (Fig.2.25g) but not IL-4 levels (Fig.2.25h); in contrast there was no increase in BAL IFN-γ, and a significant increase in IL-4 when control CD4 T cells were administered. BAL IL-17 levels were unaltered (Fig.2.25i).

To confirm these effects of IL-21 depletion were restricted to CD4 T cells I performed parallel experiments in mice primed with RSV M2 protein (rVV-M2; Fig.2.27). Priming with this protein elicits a CD8 T cell memory that is recalled to the pulmonary compartment upon

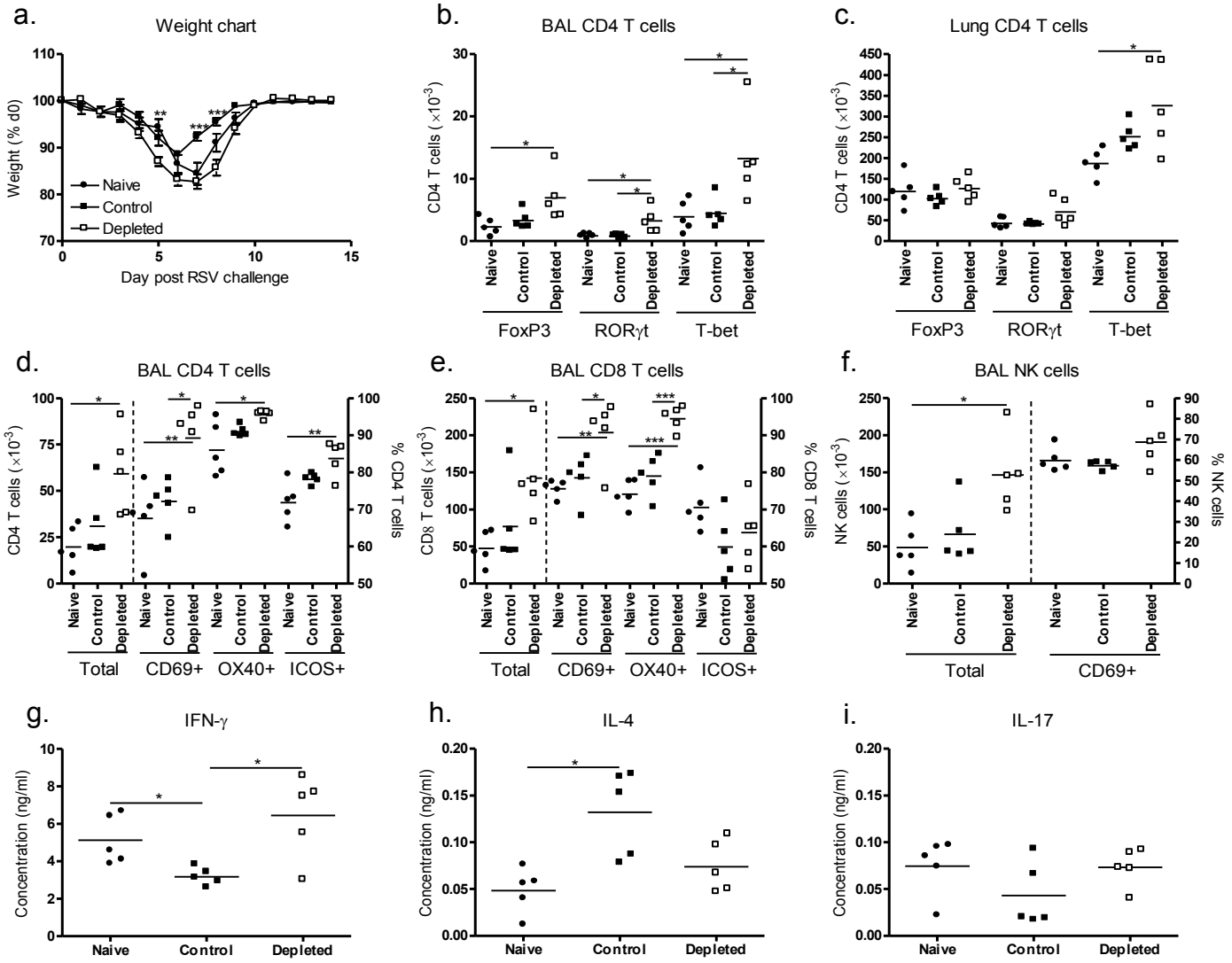

Figure 2.25. Adoptive transfer of CD4 T cells from IL-21-depleted mice exacerbates pathology in recipient mice upon RSV challenge. Mice were immunised with rVV-G and challenged as described in Fig.2.8. 28 days post challenge, splenic CD4 T cells were MACS-sorted and 2×10^{6} cells/mouse were transferred (i.p) into naïve recipients one day prior to RSV infection. Naïve T cells were sorted and transferred into a third group as a control. Weights were measured daily for fourteen days (a). Seven days post challenge BAL fluid and lungs were harvested. The number of FoxP3^{+}, RORγt^{+}, and T-bet^{+} CD4 T cells in BAL (b) and lung (c) were determined by flow cytometry. Recruitment and activity of CD4 T cells (d), CD8 T cells (e), and NK cells (f) to the BAL was determined by flow cytometry using specific markers. IFN-γ (g) and IL-4 (h) and IL-17 (i) levels in BAL fluid were determined by sandwich ELISA. Error bars represent SEM. The graphs are representative of three independent experiments of five mice per group. ANOVA (Tukey post test) result *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

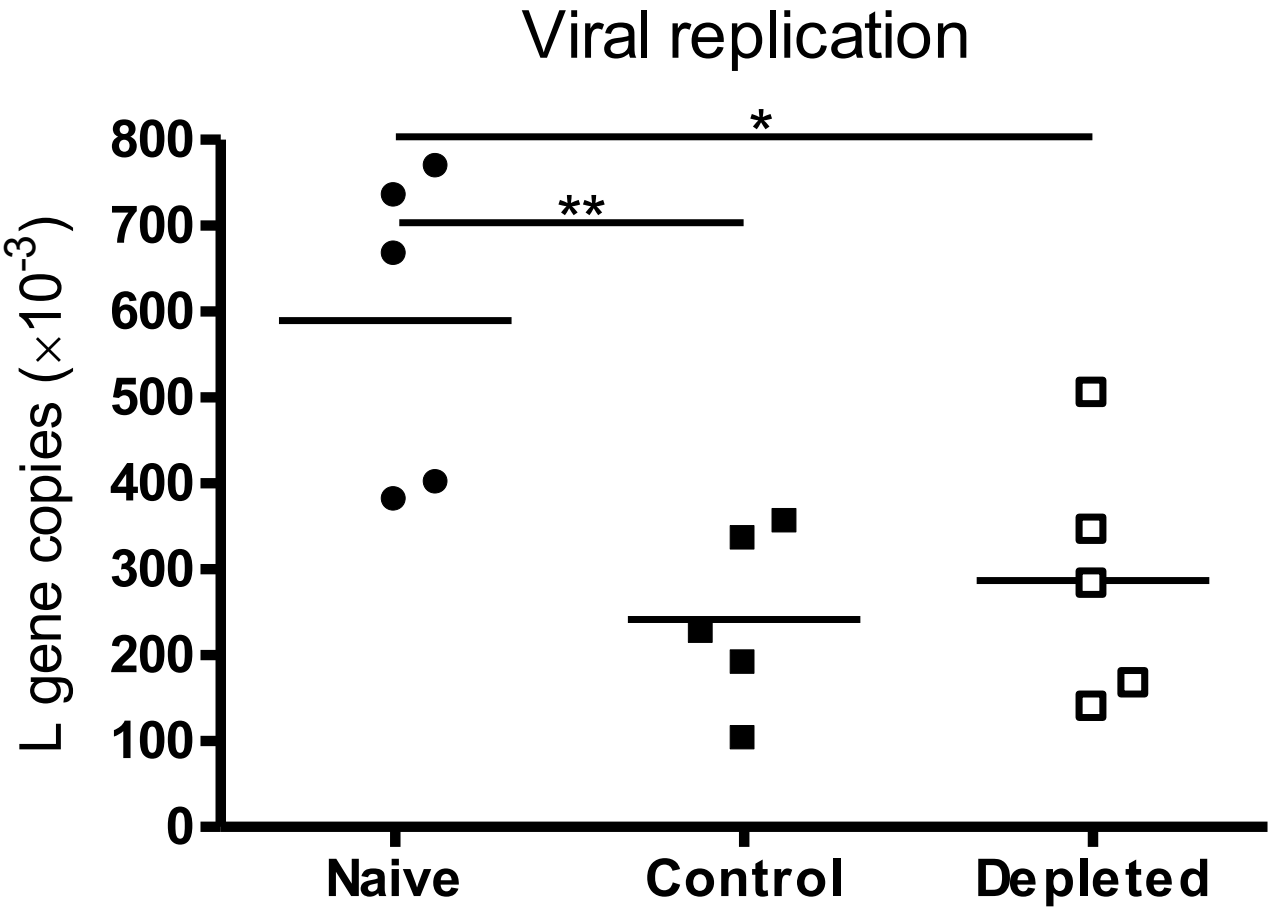

Figure 2.26. Adoptive transfer of CD4 T cells from primed and challenged mice reduces viral replication in recipient mice upon RSV challenge. Mice were treated as in Fig.2.23. Four days post challenge lungs were harvested into liquid nitrogen. Lungs were processed, and RNA extracted as described in *Materials & Methods*. cDNA was produced by RT-PCR and copies of the RSV L gene were determined by qPCR (Taqman). Plasmids encoding the L gene were used as standards to quantitate L gene copies. Results are expressed as the number of L gene copies. The graphs are representative of two independent experiments of five mice per group. ANOVA (Tukey post test) result *: $p<0.05$, **: $p<0.01$.

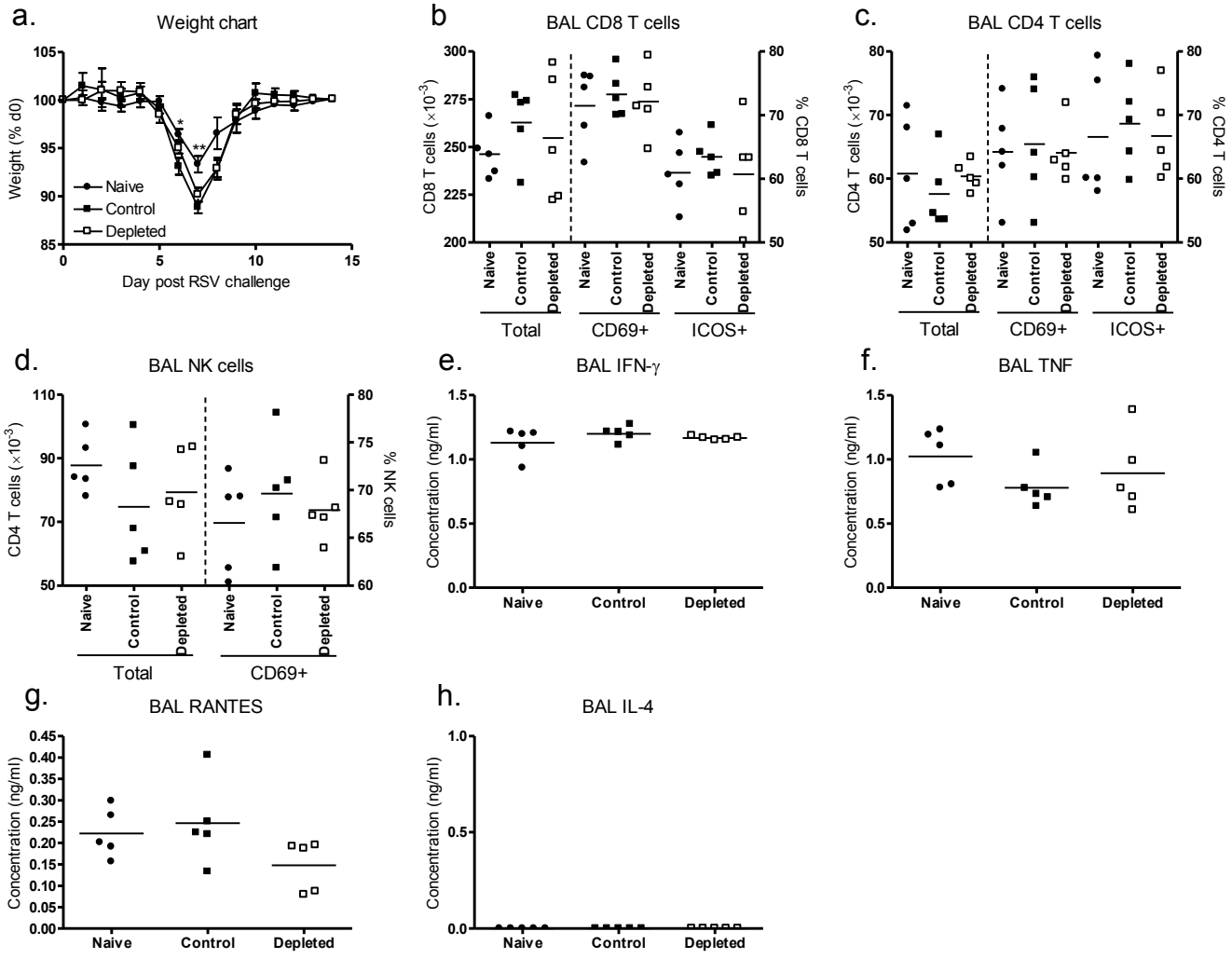

Figure 2.27. Adoptive transfer of CD8 T cells from IL-21-depleted mice does not alter pathology in recipient mice upon RSV challenge. Mice were immunised with rVV-M2 and challenged as described in Fig.2.8. 28 days post challenge, splenic CD8 T cells were MACS-sorted and 2×10^{6} cells/mouse were transferred (i.p) into naïve recipients one day prior to RSV infection. Naïve T cells were sorted and transferred into a third group as a control. Weights were measured daily for fourteen days (a). Seven days post challenge BAL fluid was harvested. The number of CD8 T cells (b), CD4 T cells (c), and NK cells (d) and activity as determined by CD69 and ICOS expression were determined by flow cytometry. IFN-γ (e), TNF (f), IL-17 (g), and IL-4 (h) levels in BAL fluid were determined by sandwich ELISA. Error bars represent SEM. The graphs are representative of three independent experiments of five mice per group. ANOVA (Tukey post test) result *: $p<0.05$, **: $p<0.01$.

RSV challenge. Upon CD8 T cell adoptive transfer and RSV challenge there were no significant changes in disease severity (Fig.2.27a) between recipients receiving control CD8 T cells or CD8 T cells from IL-21-depleted mice. This correlated with unaltered recruitment and activity of CD8 T cells (Fig.2.27b), CD4 T cells (Fig.2.27c), or NK cells (Fig.2.27d) into the BALF at the peak of disease (d7 p.c). Moreover, BAL IFN-γ (Fig.2.27e), TNF (Fig.2.27f), RANTES (Fig.2.27g), and IL-4 (Fig.2.27h) levels were unchanged, confirming that the observed effects described in this study are limited to CD4 T cells.

13. IL-21 depletion during priming reduces antibody production after RSV challenge

In the previous chapter, I observed that IL-21 depletion significantly reduced RSV-specific serum IgG1 and IgG2a levels compared to control animals. To determine if the same effect was observed in G-primed animals, serum was harvested 14 days post cutaneous vaccinia virus infection, prior to RSV challenge, and 14 days post RSV challenge. RSV-specific IgA, IgE, IgG1, and IgG2a were determined by ELISA.

No RSV-specific serum IgA (Fig.2.28a) or IgE (Fig.2.28b) were detected in either group prior to RSV challenge. RSV-specific serum IgG1 was detected in non-depleted mice, and this was significantly reduced in IL-21-depleted mice (Fig.2.28c). Very low levels of RSV-specific serum IgG2a were detected in control mice and IL-21 depletion had no significant effect on its production (Fig.2.28d).

No RSV-specific serum IgA (Fig.2.29a) or IgE (Fig.2.29b) could be detected from either group in serum samples taken 14 days post RSV challenge. RSV-specific serum IgG1 was detectable in both groups and was significantly reduced in IL-21-depleted mice (Fig.2.29c). Moreover,

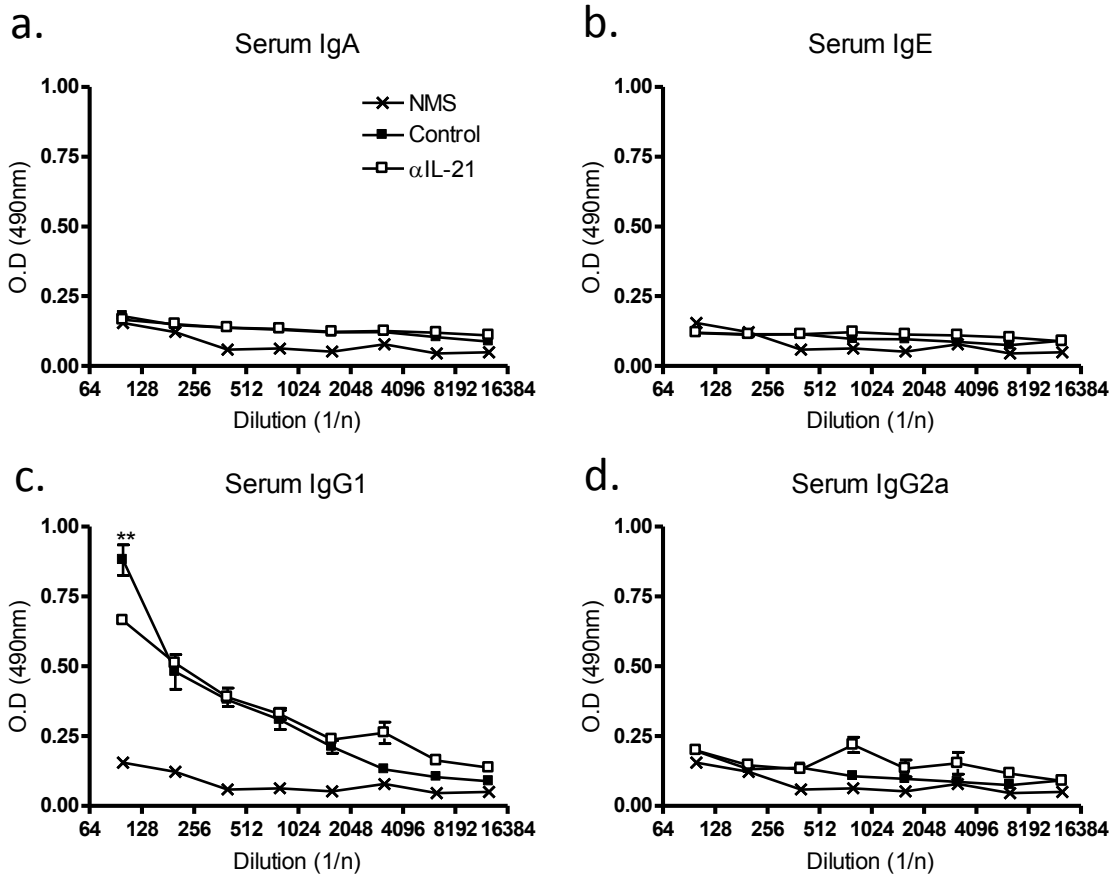

Figure 2.28. IL-21 depletion during rVV-G priming reduces antibody production. Mice were immunised with rVV-G and serum was harvested 14 days later and virus-specific IgA (a), IgE (b), IgG1 (c), and IgG2a (d) levels were determined by ELISA. Error bars represent SEM. The graphs are representative of three independent experiments of five mice per group. ANOVA (Tukey post test) result **: $p<0.01$.

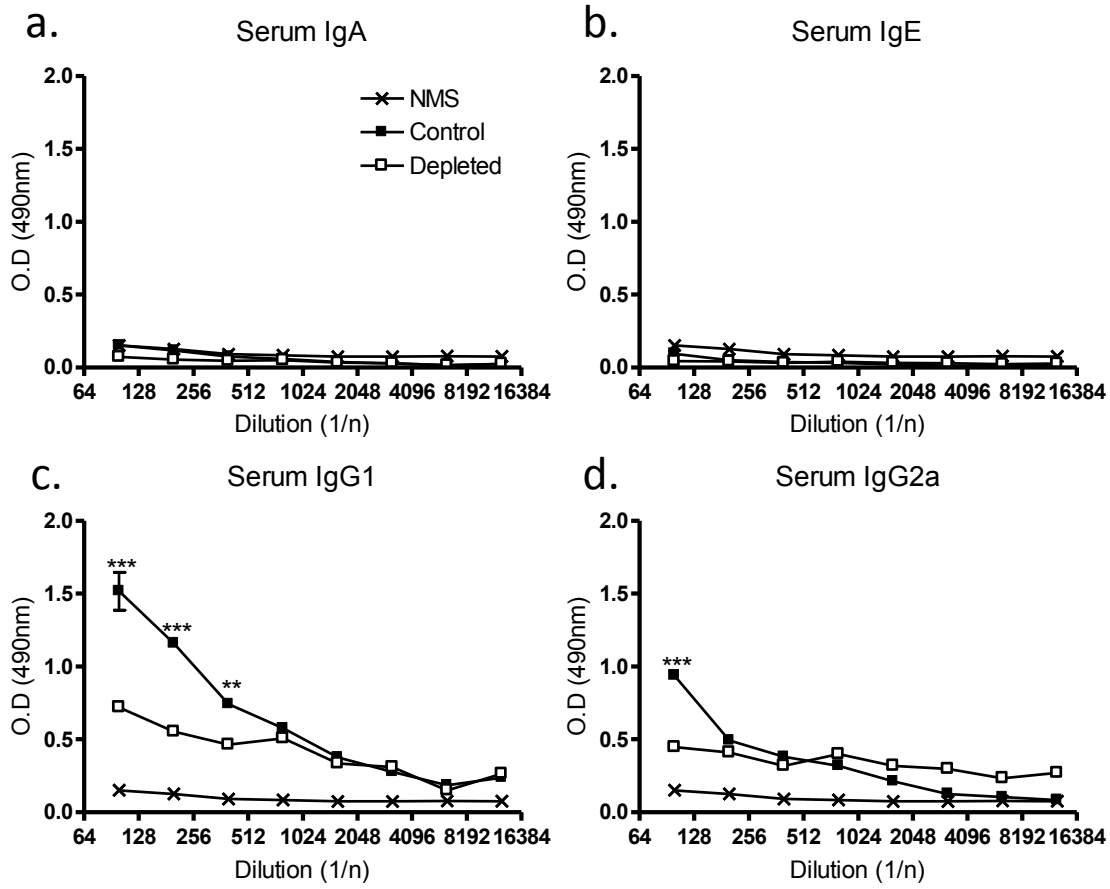

Figure 2.29. IL-21 depletion during rVV-G priming reduces antibody production after RSV challenge. Mice were immunised rVV-G and challenged as described in Fig.2.8. Serum was harvested 14 days later and virus-specific IgA (a), IgE (b), IgG1 (c), and IgG2a (d) levels were determined by ELISA. Error bars represent SEM. The graphs are representative of three independent experiments of five mice per group. ANOVA (Tukey post test) result **: $p<0.01$, ***: $p<0.001$.

RSV-specific serum IgG2a was also detectable in both groups but again was significantly reduced in IL-21-depleted mice (Fig.2.29d).

14. Discussion

The results here confirm and extend the previous finding that IL-21 is a critical factor in controlling CD4 T cell responses to RSV challenge. Compared to the effects of IL-21 depletion on primary RSV infection, the effect on memory CD4 T cell responses is more striking, in terms of changes in disease severity, cellular influx, T cell differentiation, and cytokine production. Conversely, the effect on memory CD8 T cell responses is more limited.

As systemic IL-21 depletion occurred at the time of vaccinia virus priming, most changes in lymphocyte memory development are likely associated with changes in cell behaviour at this time and not after RSV challenge. There was no effect of IL-21 depletion on lesion size at the vaccinia virus infection site, indicating that IL-21 did not affect vaccinia virus infection or replication. However, viral replication was not directly measured as viral genome and/or protein is difficult to detect in whole skin samples, therefore lesion size was used as an indirect measure. Using this, the observed changes in T cell priming were not due to changes in vaccinia virus RSV antigen production.

Upon scarification, lymphocytes are primed by cognate antigens captured by Langerhans cells and transported to the local draining pancreaticolienal (splenic) lymph nodes and spleen (747). Therefore, the effects of IL-21 depletion likely occur at these sites. The two most direct effects of IL-21 depletion may occur on the APCs presenting antigen (Langerhans cells and/or splenic DCs), and/or the antigen-specific T cells themselves. The data from G-primed mice suggest that T cell activation was increased by IL-21 depletion, as cytokine production by αCD3/28-stimulated splenic T cells was increased, though proliferation was not measured. As this is APC-independent it suggests that the T cells are affected by IL-21

depletion, however it does not rule out an effect of APCs. Upon antigen-specific cytokine production with G or M2 peptide, increased cytokine production was also observed upon IL-21 depletion. This does not confirm an effect of IL-21 on APC as T cell effects may explain the phenotype. It will be necessary to stimulate an unrelated transgenic T cell population with FACS-sorted APC from the spleen or dLN from control and IL-21-depleted mice to confirm if the APC have been affected.

Previous studies have demonstrated that by 14 days post priming memory splenic T cell populations have developed, as transfer of splenic T cells into naïve recipients transfers the disease phenotype (688). Therefore it is these memory T cells that respond to RSV challenge. Post RSV challenge T cell memory is also located in the lungs and draining, mediastinal lymph node, as well as spleen. However, the spleen retains the numerically-largest memory T cell pool.

There is also an antibody response upon priming, which is much stronger after G-priming compared to M2-priming. This is likely due to the increased T cell help provided by G-specific CD4 T cells rather than M2-specific CD8 T cells (687,748). IL-21 depletion reduced RSV-specific IgG1 production; however it was only significant at a high serum concentration suggesting the effect is minimal. Moreover, there was no increased production of other isotypes which supports the idea that IL-21 depletion had not significantly altered class switching. Several studies have demonstrated a critical role for IL-21 in antibody production but not necessarily isotype switching. IL-21RKO mice have reduced IgG1 and increased IgE which suggests a regulatory role in production of these isotypes, but not in boosting isotype classes associated with a particular response type (625). Some effects on antibody production may be due to changes in follicular helper T cell activity, a possibility not

investigated in this study. Analysis of Bcl-6^{+}, CXCR5^{+}, ICOS^{+}, and PD-1^{+} CD4 T cell numbers in the spleen and draining lymph nodes would begin to address this. Several studies have shown an important role for IL-21 in CD4 T cell differentiation into Tfh cells (511,600), and the changes in antibody production observed here support an effect on Tfh differentiation.

No changes in transcription factor expression were observed in splenic CD4 T cells isolated from depleted mice post priming, suggesting that IL-21 does not alter CD4 T cell differentiation after primary activation under these conditions. However, there was a small increase in IL-17, but not IFN-γ, production upon CD4 T cell stimulation *in vitro* indicating an effect on responding CD4 T cells. Previous studies suggest that IL-21 can influence Th1, Th2, and Th17 lineage development (386,589,592). Although IL-21 is acknowledged to be an important factor for CD4 T cell differentiation into Th17 cells (386,446,583), there are other studies that have observed an increase in IL-17 production in IL-21-deficient conditions. Ertelt *et al* observed that IL-21 deficiency resulted in enhanced IL-17 (but not IFN-γ) production by L.monocytogenes-specific CD4 T cells (708). Coquet *et al* observed that IL-21 deficiency did not affect Th17 differentiation in a model of EAE, yet disease severity was increased in IL-21- and IL-21R-deficient mice, suggesting that IL-21 was an anti-inflammatory cytokine in this model of autoimmunity (749). No changes in splenic CD8 T cell cytokine production were observed in M2-primed mice after IL-21 depletion, indicating the effects were restricted to CD4 T cells. These data correlate with the previous observation in the primary challenge model that only primary CD4 T cell responses, and not CD8, were altered by depletion.

The effects of IL-21 depletion on CD4 T cells could be indirect by acting other cell types (e.g. B cells). For example, IL-21 depletion may reduce regulatory B cell subsets that results in

increased CD4 T cell activation. Such a $CD24^{+}$ $CD38^{+}$ regulatory population was recently described by Blair *et al* in SLE patients (750) and analysis should be extended to this model at both the post-priming and post RSV challenge stages. Other cells may also be involved such as DCs and macrophages that are also present in the spleen. There is little evidence that macrophage activation is altered by IL-21 depletion, but there is for DC activity and studies by Brandt *et al* support the idea (622). Selective depletion of these various cell types would help determine if they were crucial for the observed changes in CD4 T cell activation upon IL-21 depletion.

In the 14 days between priming and RSV challenge T cell memory is formed. The strength of the recall response is affected by the size of the memory pool, which may be increased by IL-21 depletion. My observed increase in IFN-γ, IL-10, and IL-17 production after both polyclonal stimulation and specific peptide antigen support the concept of an increased T cell memory. The concomitant reduction in IL-4 production suggests that CD4 T cell differentiation may be altered at this time though no changes in T-bet and RORγt were observed and GATA-3 was not measured. Currently, it is unfeasible to determine the size of the memory CD4 T cell pool as there are no G-specific I-E^{d} tetramers available to measure G184-198-specific CD4 T cells in G-primed mice. However, M2 82-90 H-$2K^{d}$ tetramers are commercially available to detect antigen-specific CD8 T cells in M2-primed mice to confirm any effect of IL-21 depletion in M2-primed mice.

The consequences of IL-21 depletion during priming were striking after RSV challenge. Disease severity in this model, in contrast to the primary RSV challenge model, is much more rapid and severe because of the presence of T cell memory elicited by priming. As observed in the previous chapter IL-21 depletion significantly increased disease severity

after RSV challenge. The disease kinetic was very similar though IL-21-depleted mice required more time to recover compared to control mice. These observations were also made after both G- and M2-priming, indicating that IL-21 depletion does affect memory CD8 T cell responses, despite no changes in CD8 T cell activation or cytokine production being observed post priming or using the primary challenge model. Indeed, IL-21 depletion in M2-primed mice had no effect on the response type as measured by cytokine production, remaining dominated by IFN-γ and granzyme B with no IL-4 or IL-17 production. This suggests that IL-21 depletion in this model had no effect on other cell types that are known sources of these cytokines (e.g. IL-4 from CD4 T cells, NKT cells, or basophils; IL-17 from CD4 T cells, γδ T cells, or NKT cells). Critically however, the effect in G-primed mice was much greater than M2-primed mice, supporting the previous conclusion that IL-21 depletion has a far greater effect on CD4 T cells than CD8.

Could the increase in disease severity in primed animals be caused by a common mechanism that is independent of T cell activation? For example, could the observed changes be due to changes in innate immune components (particularly those in the pulmonary compartment)? This is possible as the depletion was systemic and could affect immune cells in the lung. As discussed in the previous chapter IL-21 has wide-ranging effects on several components of innate immunity, though many of these effects do not predict the observations on T cell activity and effector function that I have made in this study. For example, IL-21 has been shown to increase macrophage survival and their capacity to activate CD4 T cells. Therefore, IL-21 depletion should have the opposite effects that were not observed here. Could the effects of IL-21 depletion on macrophages be specific to regulatory T cell (Treg) influx and activity as this CD4 T cell subset were specifically reduced

after RSV challenge? This could apply to all innate components: does IL-21 depletion affect the ability of the innate immune system to recruit and/or activate Tregs? Does IL-21 depletion affect naturally-occurring Tregs more than induced Tregs? Individual Treg populations were not identified in this study, but my data clearly show that loss of IL-21 reduces the proportion of the responding T cells comprising Tregs as opposed to pro-inflammatory effector T cell populations. It is possible that the conventional T cell response may have been boosted directly by depletion of IL-21 (the effect on Tregs being insignificant or consequential rather than causal). However, given what is known about the effect of the size of the Treg response on pulmonary immune responses, it is very likely that IL-21 at least partly acts via modulating Tregs. If increased pathology was maintained in IL-21-depleted mice in the presence of increased numbers of Tregs then this would indicate that IL-21 has immunological effects beyond those on Tregs.

There are conflicting data from other studies on the effects of IL-21 on regulatory T cells. Piao *et al* showed in a model of EAE that IL-21 blockade directly reduced regulatory T cell activity, thereby increasing proteolipid peptide (PLP) 139-151-autoreactive CD4 T cell influx into the CNS and disease severity (751). However, two further studies (one using human CD4 T cells, the other a mouse model of spontaneous diabetes) demonstrated that IL-21 blocks the suppressive effects of regulatory T cells on effector T cells, without affecting regulatory T cell activity (752,753). The effect of IL-21 on regulatory T cells was further complicated by the finding that these cells can produce pro-inflammatory cytokines such as IL-17, and differentiate into Th17 cells, in the presence of IL-21 (754). Therefore, the effect of IL-21 on regulatory T cell activity and pro-inflammatory effector T cells is clearly

dependent on the disease conditions. My data does not clarify this ambiguity and it warrants further, more targeted studies of the effects of IL-21 on individual cell types.

In the previous chapter a central role of IL-21 in DC-mediated T cell activation was highlighted, and is further supported here. Although the increase in BAL DC numbers was low compared to CD4 T cells after RSV challenge, they have the potential to significantly increase T cell activity and cytokine production. As for T cells, IL-21 may have affected DC activity at priming that is further enhanced after RSV challenge. It is unknown if IL-21 affects different DC subsets at different anatomical locations, and whether these subsets are affected equally. My analyses were restricted to MHCII, CD11c, and CD11b protein expression which only marks for $CD11b^{+}$ inflammatory DCs. Extension of these studies into other DC subset markers shown to be dominant in type 1 or type 2 responses [e.g. OX40, PDL1, PDL2 (755)] would help determine whether IL-21 had specific effects on different DC subsets. IL-21 has been shown to inhibit antigen presentation by DCs, their maturation, and costimulatory molecule expression. Consequently this reduces their ability to stimulate T cells (622). While my T cell:DC co-culture data do not highlight differing effects of IL-21 depletion on specific DC subsets (as individual lung DC subsets were not sorted), they do support the hypothesis that IL-21 depletion increases their T-cell-activating capacity.

In vivo, CD4 T cells interact with DCs in tertiary lymphoid organs in the lung, particularly in the inducible bronchus-associated lymphoid tissue (iBALT). Recent studies in influenza infection have found that $CD11c^{hi}$ DCs are essential for the maintenance of iBALT via production of lymphotoxin beta (LTβ), and the homeostatic chemokines CXCL12, CXCL13, CCL19, and CCL21 (756). Depletion of DCs or LTβ leads to ablation of iBALT and lymphocyte activation, and IL-21 reduces MHCII and costimulatory molecule expression on DCs, thereby

reducing their ability to activate CD4 T cells (622). Therefore, if IL-21 is central to DC activity then further studies should be performed to determine whether IL-21 is important in iBALT formation and T cell activation during RSV disease.

The increase in IFN-γ and IL-17 production suggests that IL-21 is an anti-inflammatory cytokine and not necessarily subset-biasing. This may correlate with the co-stimulatory molecule repertoire expressed by the DCs, as OX40L and PDL2 have recently been shown to bias T cell cytokine production towards Th1 and Th2 respectively (755). Further studies will likely extend this to Th17 cytokines. However, as IL-21 has been shown to affect several types of immune response (type 1, 2, and 17) its action may be site- and disease-specific. For example, during primary RSV challenge IL-21 may act as a type 1 factor boosting IFN-γ production, therefore its depletion should reduce type 1 immunity. However, its effects on DC activation, antigen presentation and costimulatory molecule expression may mask this and the resulting phenotype is a slight increase in the T-cell-driven-type-1 response. In the G-priming and challenge model, IL-21 may act in a different manner, acting as a type 2 factor that results in increased IFN-γ and reduced IL-4 production upon depletion. B cells may be a source of IL-4 (757), and their reduction both in this study and in previous RSV studies where their recruitment is attenuated also indicates them as a source (695). NKT cells are also a potent early source of IL-4 and IL-21 may also affect this immune cell type. The observed IL-10 increase is likely effector T-cell-derived as there were significantly fewer pulmonary regulatory CD4 T cells expressed Foxp3 after RSV challenge. However, my data do not rule out other lung cell sources of these cytokines after RSV challenge and intracellular staining of other cell types (e.g. B cells, macrophages, DCs, ILCs, γδ T cells,

epithelial cells, mast cells, basophils, and granulocytes) would help clarify whether these cytokines are not just T-cell-derived.

Upon RSV challenge, increases in BAL CD4 T cells and neutrophils were greatest, followed by CD8 T cells and NK cells, and finally DCs ($MHCII^+/CD11b^+/CD11c^+$ cells). As G-priming activates CD4 T cells, it is unsurprising that (upon recall) their numbers would increase greatly in the event of increased activation and expansion caused by a lack of immunoregulatory IL-21. In contrast the 3-fold increase in neutrophilia likely reflects an increase in chemokine levels (e.g. KC) caused by a more pro-inflammatory environment (758). A similar mechanism may be responsible for the increase in NK cell numbers (102,759). CD8 T cells respond to RSV challenge in an antigen-specific manner, but it is likely that increased chemokine levels and greater CD4 T cell help also contribute to their increased recruitment (760,761). Viral clearance is associated with strong cellular immune responses; however in this study I observed a compromised anti-viral response. This is likely due to the reduction in anti-viral serum antibody levels, indicative of reduced B cell activity. The reduced B cell recruitment to the airway supports this. Given that IL-21 depletion occurred prior to RSV challenge, it is highly unlikely that the changes in viral L gene copies are due to direct effects of IL-21 on viral replication. Several studies have demonstrated a crucial role for IL-21 on both B cell activation (625) and cells crucial for their activation [e.g. Follicular T helper (Tfh) cells (511,600)] as discussed in the previous chapter. These data, including those from these further studies, demonstrate that IL-21 is crucial for optimal antibody production and its depletion is detrimental to short- and long-term protection against viral challenge.

In conclusion, my findings show that IL-21 plays a key role in limiting the magnitude and regulating the phenotype of virus-specific CD4 T and B cell responses and in anti-viral immunity. These new insights extend our understanding of the role of IL-21, which has not previously been shown to be involved in defence against respiratory infection. Moreover, my findings highlight the therapeutic potential of IL-21 in limiting inflammation while enhancing pathogen clearance. IL-21 co-administration with RSV vaccine antigens might be particularly effective in infancy, when IL-21 responses are impaired, and warrant further study (700). On the basis of these data, I hypothesise that in the context of RSV challenge, IL-21 administration would reduce cellular influx into the pulmonary compartment, reduce pro-inflammatory cytokine production (possible via the induction of regulatory T cells), and increase virus-specific antibody production, thereby boosting protection. Therefore in the next chapter I address whether IL-21 has the anticipated effects as described above by comparing responses against WT RSV and a novel IL-21-expressing RSV construct.

J. IL-21 expression during RSV challenge differentially regulates both primary and secondary CD4 T cell responses in mice.

1. Introduction

My previous studies have shown that IL-21 is an important factor for the development of optimal immunity against RSV challenge. IL-21 is crucial for short-term recovery from viral challenge as it regulates CD4 T cell recruitment, activation, and pro-inflammatory cytokine production. These effects aid viral clearance and limit disease severity. Moreover, IL-21 is essential for long-term protective immunity against viral rechallenge by increasing virus-specific antibody production by B cells. Therefore, while IL-21 depletion is not desirable in this disease setting, I hypothesised that IL-21 would be beneficial for the host by limiting CD4 T cell responses to RSV challenge, and boosting virus-specific antibody production.

In order to test this hypothesis I have delivered murine IL-21 into the lung during RSV challenge. To do this I have utilised a novel RSV strain that encodes the murine *il21* gene between the F and G genes in the viral genome. Upon viral infection of epithelial cells murine IL-21 is produced as the virus replicates, which is secreted from infected cells. This method has been previously used to study the effects of cytokine administration on immune responses to RSV (762-764). This method of delivery is highly desirable because cytokines have a very high turnover rate, as they bind to their cognate receptors and are internalised or enter the kidneys from the circulation for degradation (765). As such, recombinant cytokines have to be injected on a daily basis to maintain biologically relevant levels in the serum and target tissues. This is not only distressing for the animal, triggering stress

hormones and other factors which may inadvertently affect immune responses, but the act of injection may introduce artefact by altering the on-going immune response. This is particularly applicable for intranasal injections into the pulmonary compartment. These problems are circumvented by the use of these cytokine-expressing RSV strains. Typically, the cytokine is produced and is detectable only while the virus replicates (763), however, the effects on the immune response are long-lasting and easily assayed. More importantly, these cytokine-expressing RSV strains are identical to wild-type RSV at the protein level as all mutations of the genome are within introns and are silent post-transcription (763). Therefore, antigen-specific immune responses are unaffected. There are mutations at the nucleotide level within the *sh* gene but these do not affect detection of viral replication by qPCR, which detects copies of the viral L gene (763).

Therefore, our collaborators (Dr U Bucholz and P Collins, NIAID, USA) constructed, and I have grown, an IL-21-expressing RSV stock (RSV-IL-21). I titred the virus and confirmed IL-21 expression. I have then challenged BALB/c mice with either wild-type RSV or RSV-IL-21 and compared the immune responses to primary challenge, T cell memory development, antibody production, and protection against rechallenge with wild-type RSV.

2. Titration of the IL-21-expressing Respiratory syncytial virus (RSV-IL-21) stock *in vitro*

Prior to use *in vivo* the RSV-IL-21 stock was titred by plaque assay and the IL-21 concentration in the inoculum determined by ELISA. As RSV-IL-21 is the only virus that encodes the murine *il21* gene, it should be the only stock that contains murine IL-21. Two stocks were grown, 'p3' and 'p4'. The RSV-IL-21 titres were calculated to be: p3

1×10^7pfu/ml, p4: 8.5×10^6pfu/ml. The IL-21 concentration in the inoculum was found to be very high in both stocks (~10ng/ml). A wild-type RSV stock (titre: 2.2×10^7pfu/ml) was used as a negative control and no IL-21 was detected (Fig.3.1). Further, an IL-7-expressing RSV stock (3.3×10^7pfu/ml) was also tested. This strain has the murine *il7* gene encoded in the same place as the *il21* gene using the same methodology, and controls for the presence of a gene in the viral genome. No IL-21 was detected in this inoculum either (Fig.3.1). Therefore, only RSV-IL-21 was capable of producing murine IL-21. The RSV-IL-21 'p3' stock was used for further *in vivo* experiments.

3. IL-21 expression ablates disease severity upon primary RSV challenge

To determine the effect of IL-21 expression on the response to RSV, mice were challenged with RSV or RSV-IL-21 (1×10^6pfu/mouse; i.n) on d0. Mice were weighed from challenge to determine the level of disease severity. Mice challenged with wild-type RSV exhibited weight loss typical of a primary infection with peak weight loss on d7 p.c (Fig.3.2). Strikingly, mice challenged RSV-IL-21 exhibited no weight loss upon challenge (Fig.3.2).

4. IL-21 expression significantly inhibits cell recruitment after primary RSV challenge

To determine why mice challenged with RSV-IL-21 lost no weight I determined cell recruitment to the airway and lung tissue on day seven post challenge, the peak of pathology in mice challenged with wild-type RSV. Cell recruitment to the airway (Fig.3.3a) and lung tissue (Fig.3.3b) was significantly reduced in mice challenged with RSV-IL-21

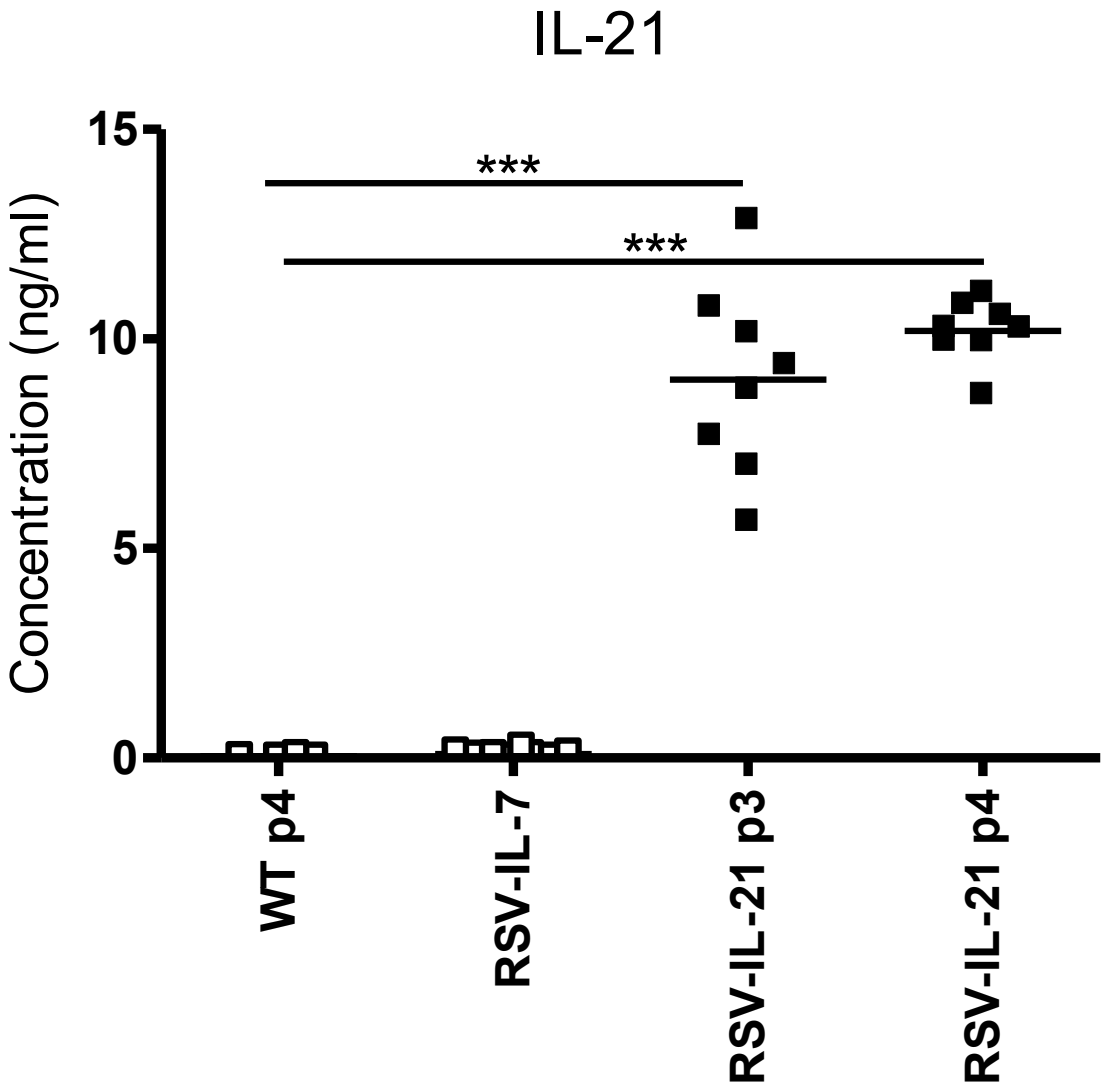

Figure 3.1. Titration of the Respiratory syncytial virus stock *in vitro*. An IL-21-expressing RSV (RSV-IL-21) stock (p3 and p4) was generated and the titre determined *in vitro* by plaque assay (as described in *Materials and Methods*). The concentration of IL-21 in the viral inoculum was determined by sandwich ELISA. WT RSV and an IL-7-expressing RSV (RSV-IL-7) stock were tested as negative controls. Data is expressed as mean values. The graphs are representative of two independent experiments of five mice per group. Student *t* test result ***: $p<0.001$.

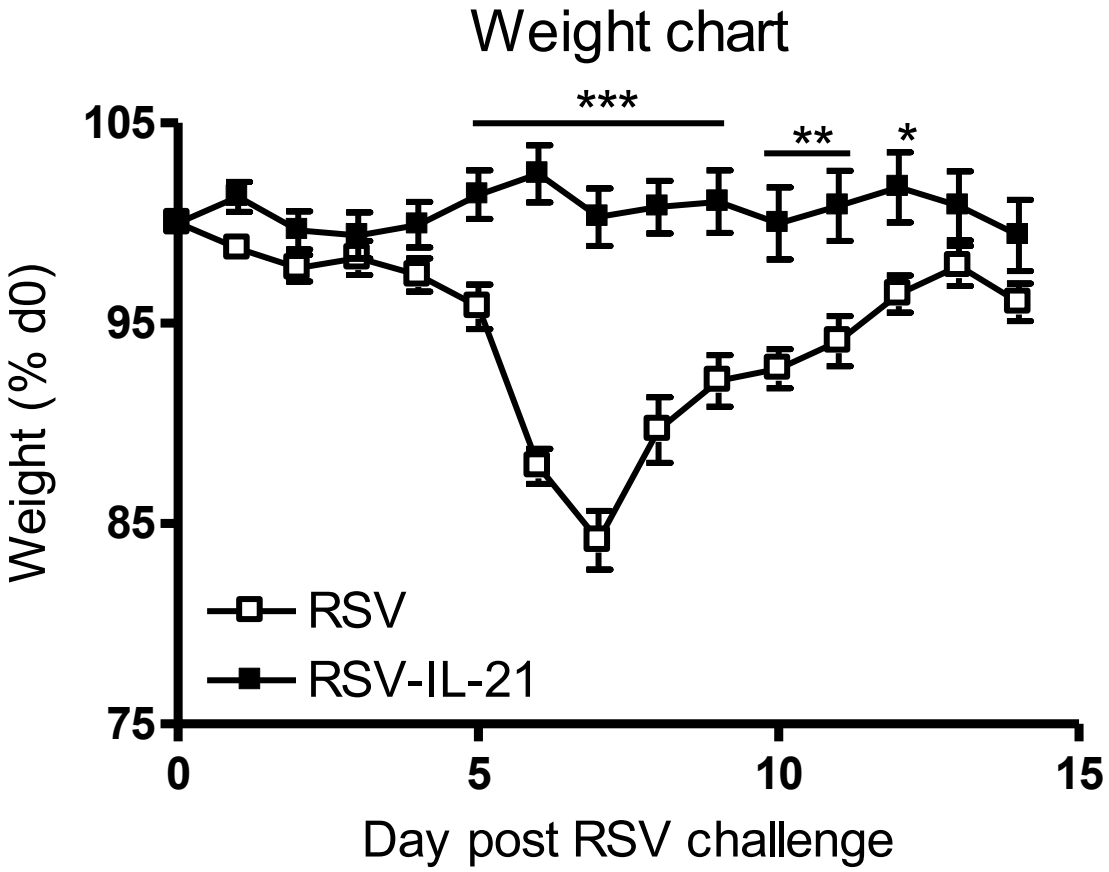

Figure 3.2. IL-21 expression ablates disease severity upon primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^6 pfu/mouse; i.n) on d0. Mice were weighed daily for 14 days. Weight is shown as a percentage of baseline weight. Data is expressed as mean±SEM. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

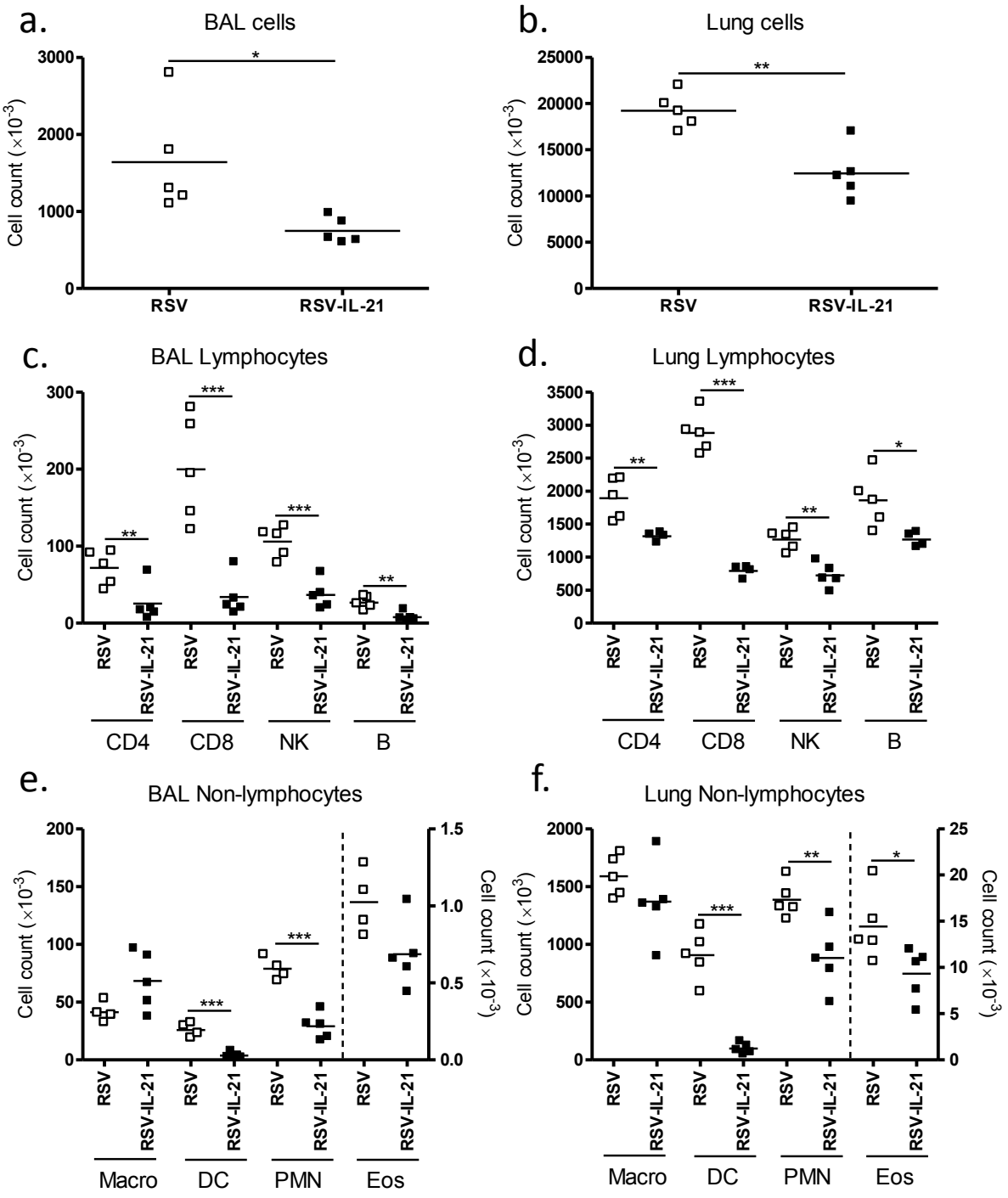

Figure 3.3. IL-21 expression significantly inhibits cell recruitment after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^6 pfu/mouse; i.n) on d0. BALF cells and lung tissue were harvested seven days post challenge. Samples were processed and total cell counts in the BALF (a) and lung tissue (b) determined. CD4 T cells, CD8 T cells, NK cells, and B cells in BALF (c) and lung tissue (d) were phenotyped by flow cytometry and cell counts determined. Macrophages, DCs, neutrophils (PMN), and eosinophils in BALF (e) and lung tissue (f) were determined using the same method. At least 50×10^3 cells/sample were collected. Data are expressed as mean values. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

compared to RSV WT. To determine which cell recruitment was reduced I characterised cell recruitment by flow cytometry. I observed a significant reduction in recruitment of CD4 T cells, CD8 T cells, NK cells, and B cells into the airway (Fig.3.3c) and lung tissue (Fig.3.3d). The reduced lymphocytosis was evident on forward/side scatter plots by flow cytometry (Appendix 3.1). Moreover, non-lymphocytic cells were also affected: there were significant reductions in recruitment of DCs, neutrophils into the airway (Fig.3.3e) and lung tissue (Fig.3.3f). Eosinophils were reduced in the lung tissue only. Interestingly, macrophage numbers in both compartments were unaltered.

5. IL-21 expression has little effect on viral clearance after primary RSV challenge

As described in the previous results chapter (section 2.8), cell recruitment to the pulmonary compartment negatively correlates with viral clearance. Therefore, I wanted to know if viral clearance had been affected by the reduction in cell recruitment to the airway and lung tissue. To achieve this I measured the number of RSV L gene copies in lung tissue at several time points post RSV challenge by qPCR. Total RNA was purified from lung tissue, converted to cDNA, and the number of L gene copies determined using L-gene-specific primers and probe. In mice challenged with RSV WT, viral L gene copies were first detected at d2 p.c, peak at d4 p.c, and return to baseline levels by d7 p.c (Fig.3.4). There were no significant differences in viral L gene copies detected in mice challenged with RSV-IL-21 from d0-4 p.c. However, viral L gene copies remained detectable in the RSV-IL-21-challenged mice at d7 p.c whereas virus had been cleared in RSV-challenged mice by this time (Fig.3.4). By d10 p.c, the number of viral L gene copies returned to baseline levels.

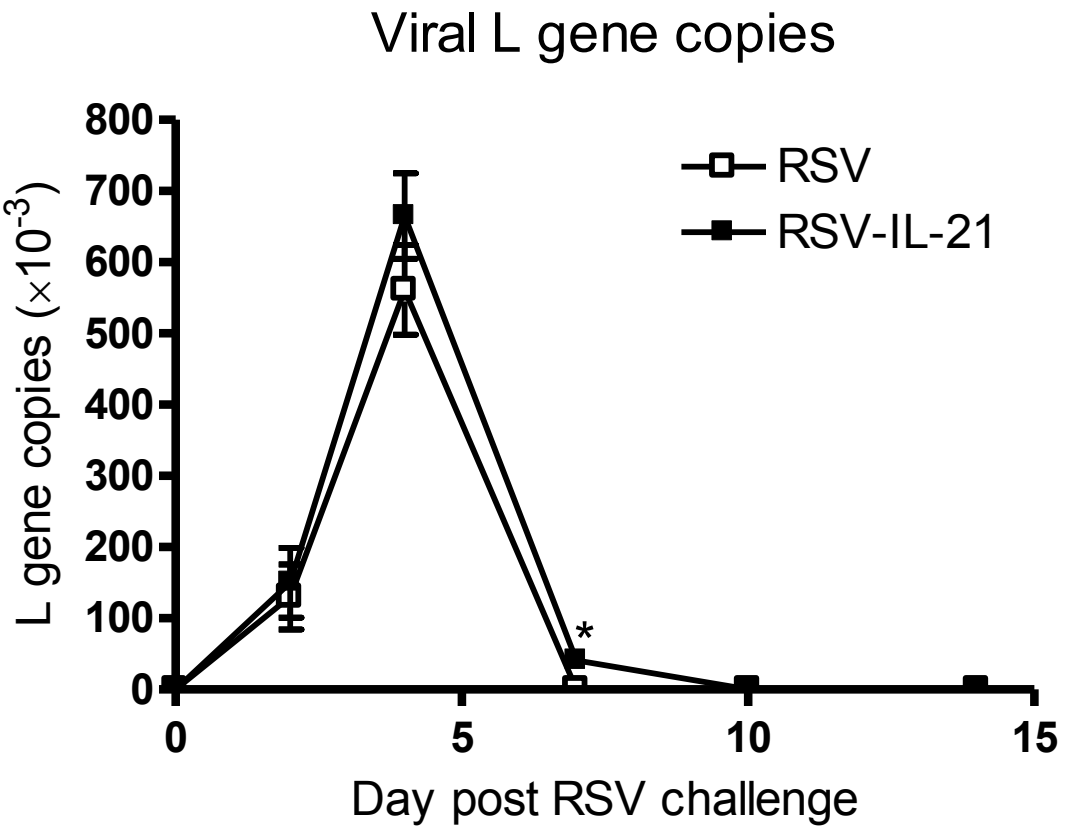

Figure 3.4. IL-21 over expression has little effect on viral clearance after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^{6} pfu/mouse; i.n) on d0. Lungs were harvested at several time points post challenge. The number of viral L gene copies in the lungs of infected mice was determined at several time points post challenge by quantitative PCR (qPCR; protocol described in *Materials and Methods*). Data is expressed as mean±SEM. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: $p<0.05$.

6. IL-21 expression inhibits T cell activation after primary RSV challenge

As T cells are central to the weight loss observed in this model, and their recruitment was severely inhibited, I determined if their activity was also affected by measuring ICOS expression on T cells in the airway and lung tissue. At the peak of weight loss, the majority of CD4 and CD8 T cells recruited to the airway are activated and express ICOS. However, there was a significant reduction in ICOS expression on both BALF CD4 and CD8 T cells from RSV-IL-21-challenged mice (Fig.3.5a). The same difference was observed in the lung tissue, though the overall percentage of ICOS-positive T cells was further reduced. Representative dot plots for CD4 T cells (Fig.3.5b) and CD8 T cells (Fig.3.5c) are shown.

7. IL-21 expression increases ICOSL expression on macrophages and DCs after primary RSV challenge

As ICOS expression was reduced on T cells in RSV-IL-21-challenged mice compared to RSV-challenged mice, I determined whether ICOSL expression was also altered on APCs. This is important because IL-21 is known to reduce DC activation and costimulatory molecule expression (622) but increase macrophage activity (715). Therefore alterations in APC activation by IL-21 may account for the reduction in T cell activation. ICOSL expression was measured on macrophages and DCs in the airway and lung tissue seven days post challenge. Approximately 50% of airway macrophages and DCs expressed ICOSL in RSV-challenged mice, ICOSL expression was slightly reduced on APCs from the lung tissue (Fig.3.6a). In RSV-IL-21-challenged mice ICOSL expression significantly increased on both macrophages and DCs, and on APCs from both airway and lung tissue (Fig.3.6a). Representative dot plots for

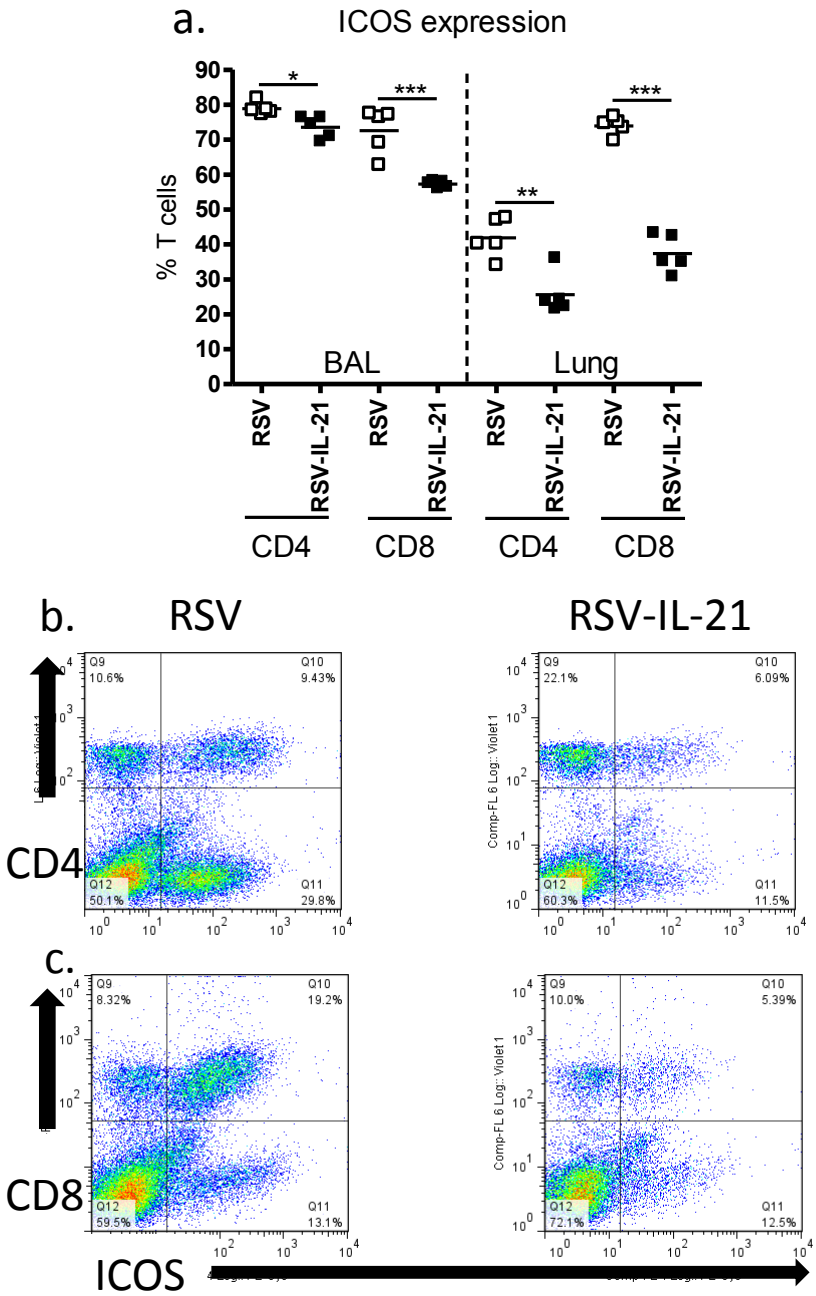

Figure 3.5. IL-21 expression inhibits T cell activation after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^6 pfu/mouse; i.n) on d0. BALF cells and lung tissue were harvested seven days post challenge. Samples were processed and ICOS expression on CD4 and CD8 T cells was phenotyped by flow cytometry (a). At least 50×10^3 cells/sample were collected. Data are expressed as mean values. Representative dot plots for CD4 T cells (b) and CD8 T cells (c) are shown. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

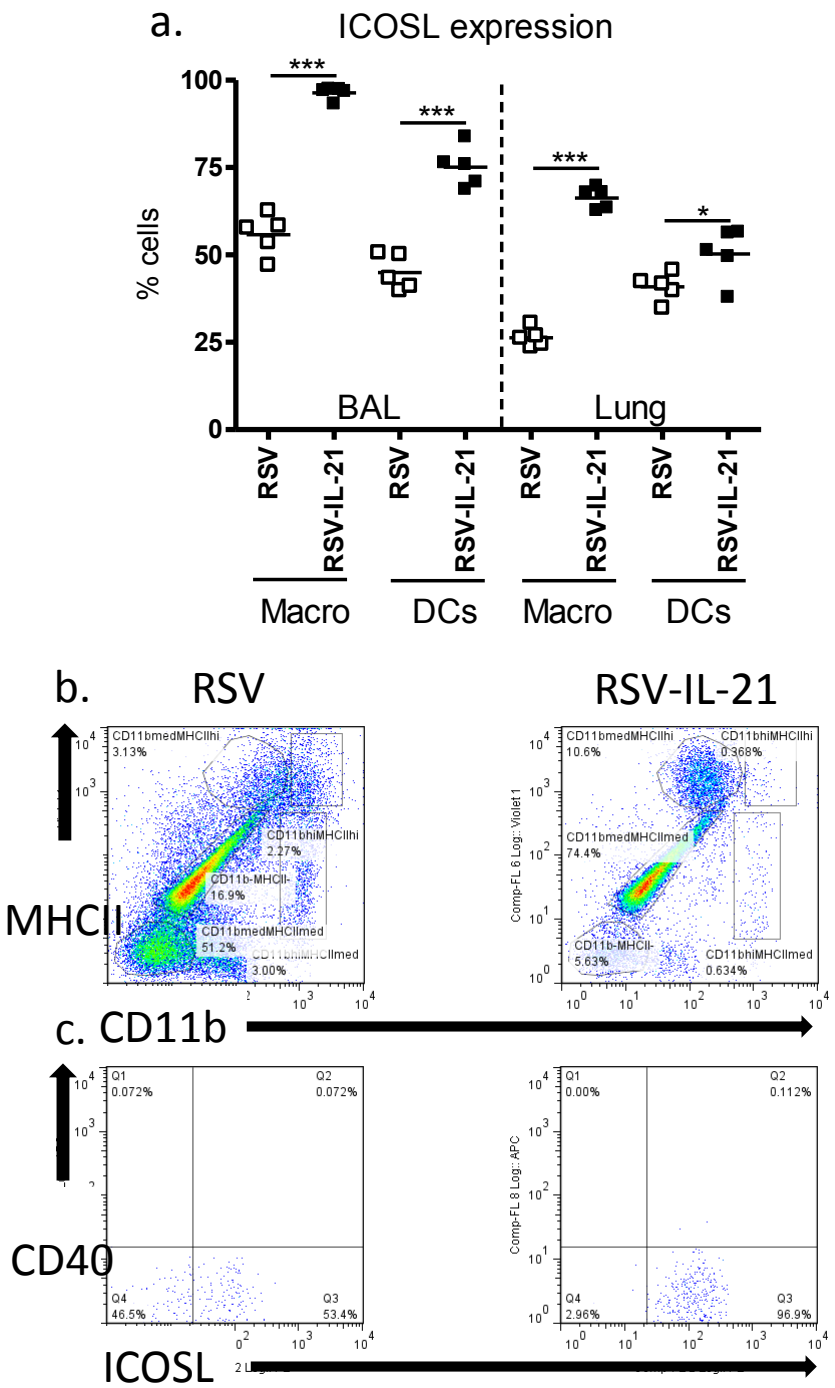

Figure 3.6. IL-21 expression increases ICOSL expression on macrophages and DCs after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^{6} pfu/mouse; i.n) on d0. BALF cells and lung tissue were harvested seven days post challenge. Samples were processed and ICOSL expression on macrophages and DCs was phenotyped by flow cytometry (a). At least 50×10^{3} cells/sample were collected. Data are expressed as mean values. Representative dot plots for ICOSL expression (c) on macrophages (b) are shown. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: $p<0.05$, ***: $p<0.001$.

ICOSL expression (Fig.3.6c) on macrophages (Fig.3.6b) from RSV-challenged and RSV-IL-21-challenged mice are shown.

8. IL-21 expression reduces T-bet$^+$ T cell recruitment to the pulmonary compartment after primary RSV challenge

As T cell recruitment and activation were reduced in RSV-IL-21-challenged mice I wanted to determine whether the remaining recruited T cells were regulatory T cells, explaining the lack of disease. To achieve this I determined FoxP3, RORγt, and T-bet expression in pulmonary CD4 T cells and CD4 T cells within the mediastinal (draining) lymph nodes at d7 p.c. IL-21 did not alter the percentage of BAL CD4 T cells expressing FoxP3 (Fig.3.7a&d) or RORγt (Fig.3.7b&d). However, it significantly reduced the percentage and number of BAL CD4 T cells expressing T-bet (Fig.3.7c&e respectively) as well as the number expressing RORγt (Fig.3.7e). This phenotype was largely replicated in lung tissue (Fig.3.8), however there was also a significant reduction in numbers of FoxP3$^+$ lung CD4 T cells (Fig.3.8e). In the draining lymph nodes there was a significant reduction in numbers of FoxP3$^+$ CD4 T cells (Fig.3.9e) but no alteration in RORγt (Fig.3.9b, d, e) or T-bet expression (Fig.3.9c, d, e). There was a similar phenotype observed by CD8 T cells: a significant reduction in the percentage (Appendix 3.2a, c, e) and number (Appendix 3.2b, d, f) of pulmonary CD8 T cells expressing Foxp3, RORγt, and particularly T-bet.

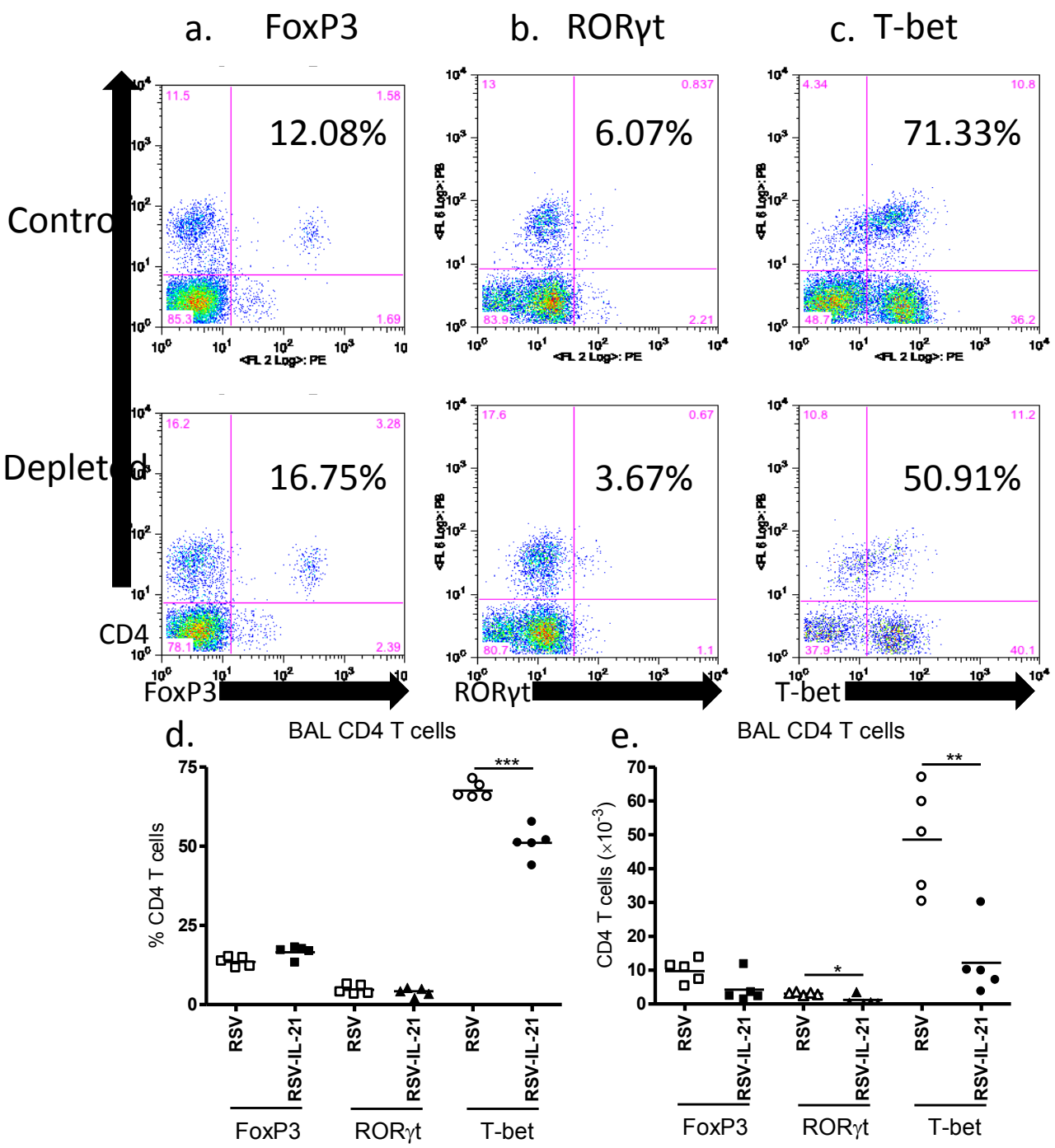

Figure 3.7. IL-21 expression significantly reduces T-bet expression by BAL CD4 T cells after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^6 pfu/mouse; i.n) on d0. BALF cells were harvested seven days post challenge. CD4 T cells were stained for FoxP3 (a), RORγt (b), or T-bet (c) according to the manufacturer's instructions. The percentage of CD4 T cells expressing each transcription factor was determined by flow cytometry and is shown in each dotplot. Grouped data for percentage (d) and total number (e) is also shown. The graph is representative of two independent experiments of five mice per group. Student t-test result *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

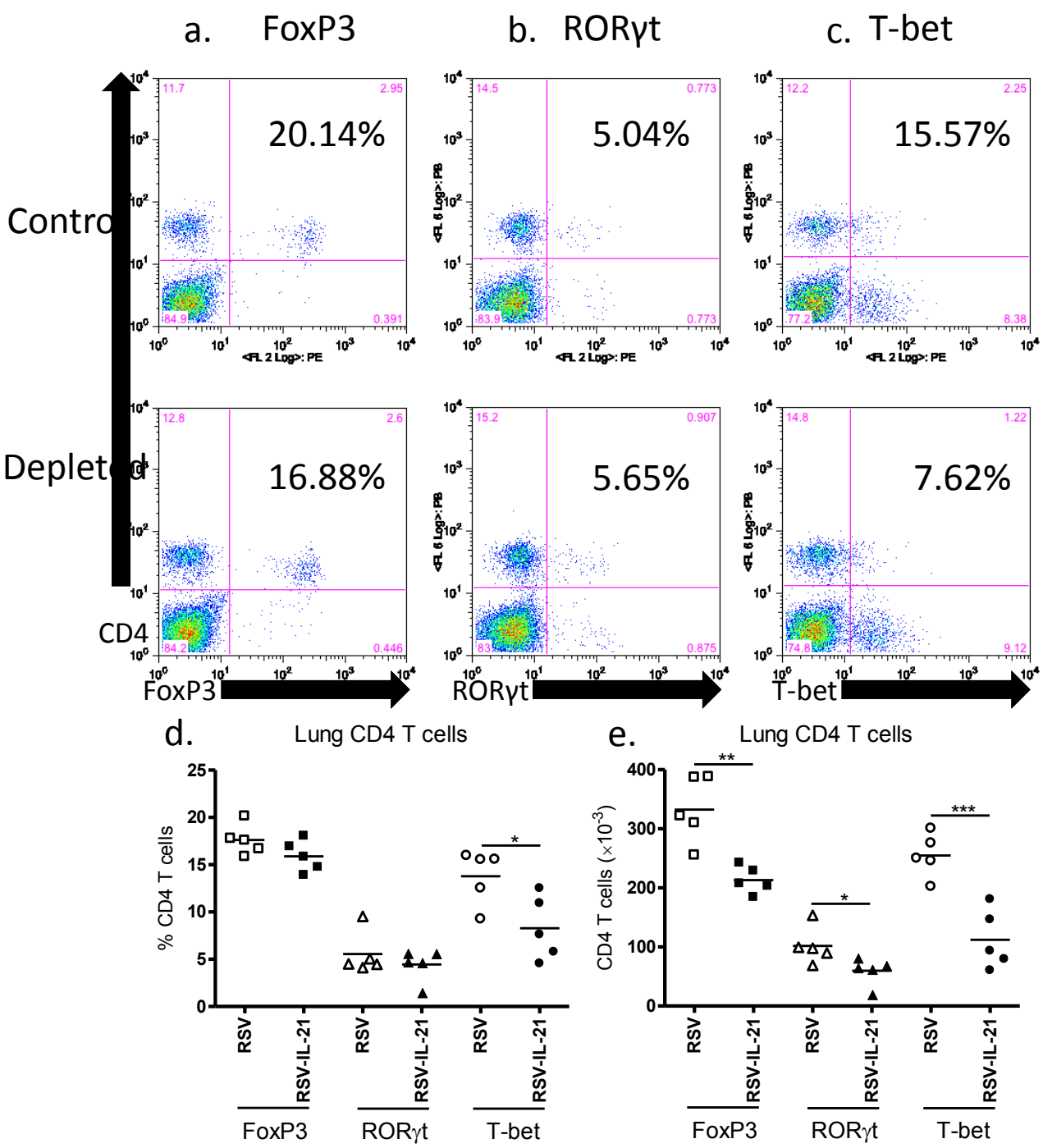

Figure 3.8. IL-21 expression significantly reduces T-bet expression by lung CD4 T cells after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^6 pfu/mouse; i.n) on d0. Lungs were harvested seven days post challenge and processed. CD4 T cells were stained for FoxP3 (a), RORγt (b), or T-bet (c) according to the manufacturer's instructions. The percentage of CD4 T cells expressing each transcription factor was determined by flow cytometry and is shown in each dotplot. Grouped data for percentage (d) and total number (e) is also shown. The graph is representative of two independent experiments of five mice per group. Student t-test result *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

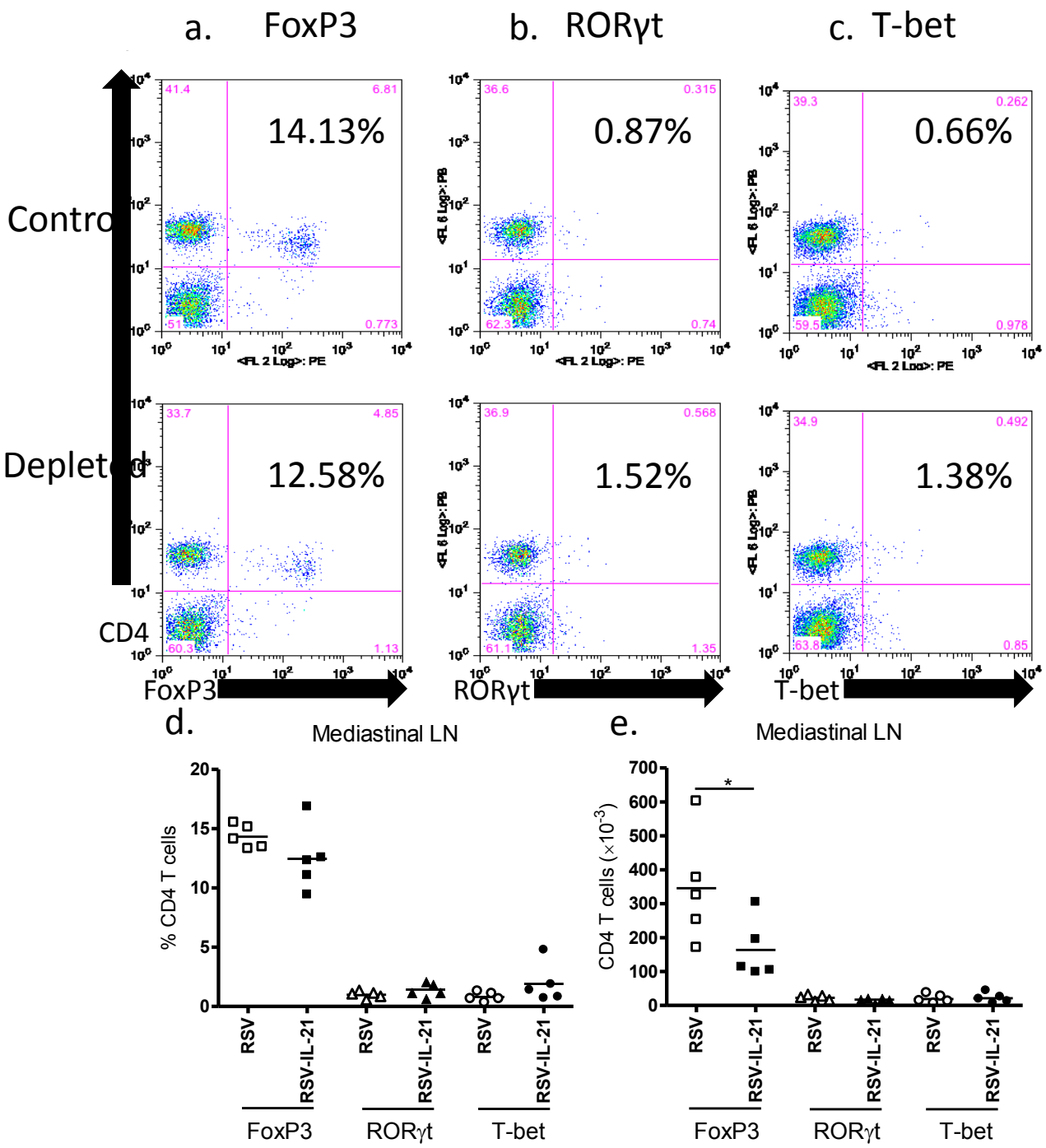

Figure 3.9. IL-21 expression significantly reduces the number of dLN FoxP3+ CD4 T cells after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^6 pfu/mouse; i.n) on d0. Mediastinal lymph nodes were harvested seven days post challenge and processed. CD4 T cells were stained for FoxP3 (a), RORγt (b), or T-bet (c) according to the manufacturer's instructions. The percentage of CD4 T cells expressing each transcription factor was determined by flow cytometry and is shown in each dotplot. Grouped data for percentage (d) and total number (e) is also shown. The graph is representative of two independent experiments of five mice per group. Student t-test result *: $p<0.05$.

9. IL-21 expression reduces BALF IFN-γ, granzyme B, and chemokine production after primary RSV challenge

As T cell recruitment and activation were reduced in RSV-IL-21-challenged mice I hypothesised that cytokine secretion into the airway would also be reduced. To determine this I measured pro-inflammatory cytokines described before (IFN-γ IL-4, IL-17), the cytolytic protein granzyme B, the anti-inflammatory cytokine IL-10, and IL-21 in the BALF seven days post RSV challenge. IL-21 was only detectable in the BALF of RSV-IL-21-challenged mice at this time (Fig.3.10a). Moreover, no changes in IL-4 (Fig.3.10d), IL-10 (Fig.3.10e), or IL-17 (Fig.3.10f) were detected between groups. However, I did detect a significant decrease in granzyme B production (Fig.3.10c), and IFN-γ production was ablated (Fig.3.10b), in the airway in RSV-IL-21-challenged mice.

T cell recruitment to the pulmonary compartment is mediated by chemokine gradients generated early after viral challenge between the draining lymph nodes and the pulmonary compartment. Therefore, I determined whether chemokine production had been reduced by measuring the lymphocyte chemoattractants MIP-3α (CCL20), MIP-3β (CCL19), 6kine (CCL21.) and IP-10 (CXCL10), as well as the granulocyte chemoattractants MIP-1α (CCL3), and MIP-2α (CXCL2) during the first 96hrs post challenge. There was production of MIP-1α (Fig.3.11a), MIP-2α (Fig.3.11b), MIP-3α (Fig.3.11c), and IP-10 (Fig.3.11f) 24hrs post RSV challenge, and apart from MIP-3α, rapidly dropped to baseline by 48hrs post challenge. MIP-3α remained detectable until 96hrs post challenge. MIP-2α levels did not alter after RSV-IL-21 challenge (Fig.3.11b), but MIP-1α (Fig.3.11a), MIP-3α (Fig.3.11c), and IP-10 (Fig.3.11f) levels were all significantly reduced at 24hrs post challenge. In contrast MIP-3β was only detectable after 48hrs post challenge at very low levels in RSV-challenge, but not RSV-IL-21

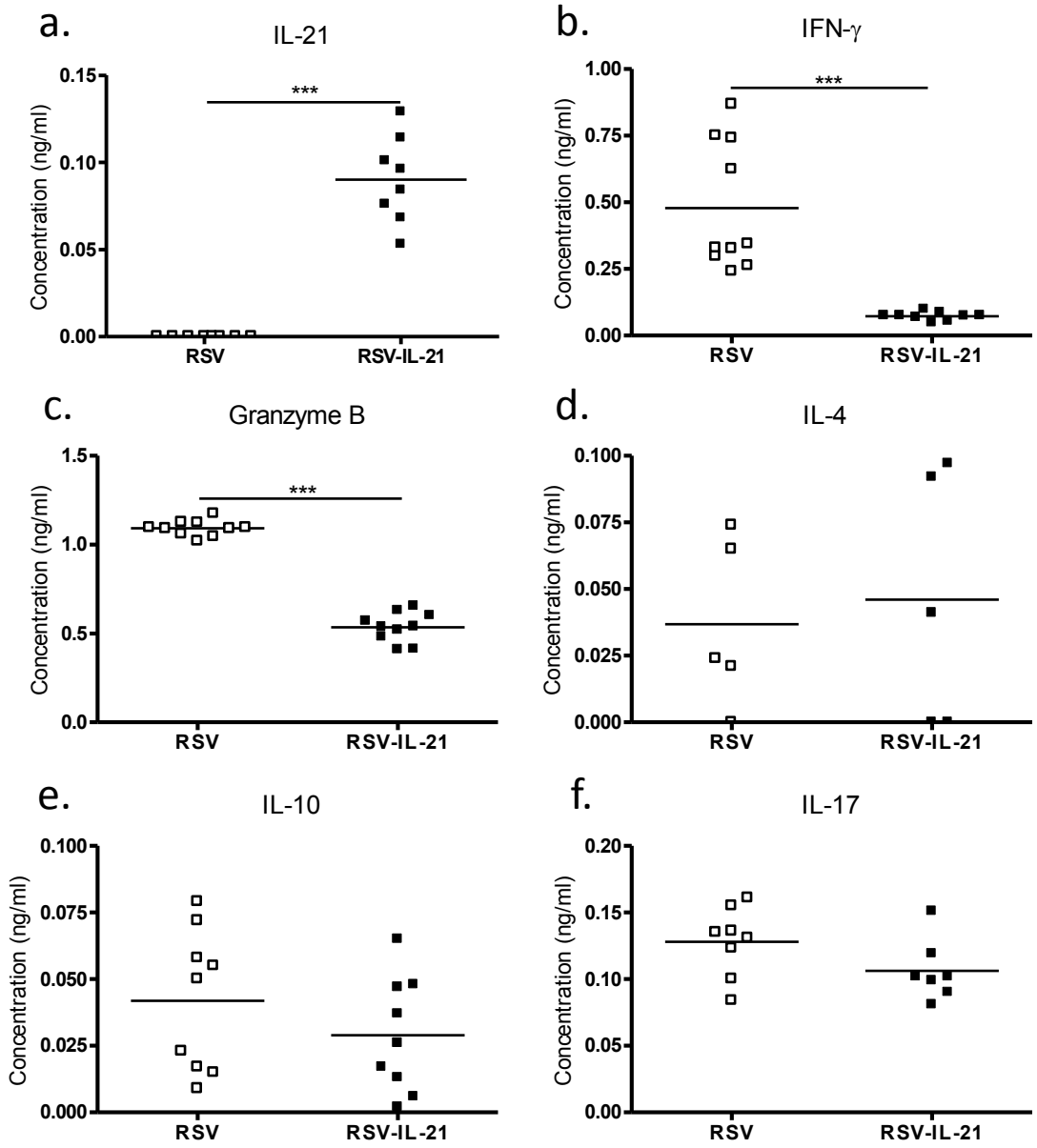

Figure 3.10. IL-21 expression reduces BALF IFN-γ and granzyme B production after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^6 pfu/mouse; i.n) on d0. BALF was harvested seven days post challenge and IL-21(a), IFN-γ (b), Granzyme B (c), IL-4 (d), IL-10 (e), and IL-17 (f) were determined by sandwich ELISA. Data are expressed as mean values. The graphs are representative of two independent experiments of five mice per group. Student *t* test result ***: $p<0.001$.

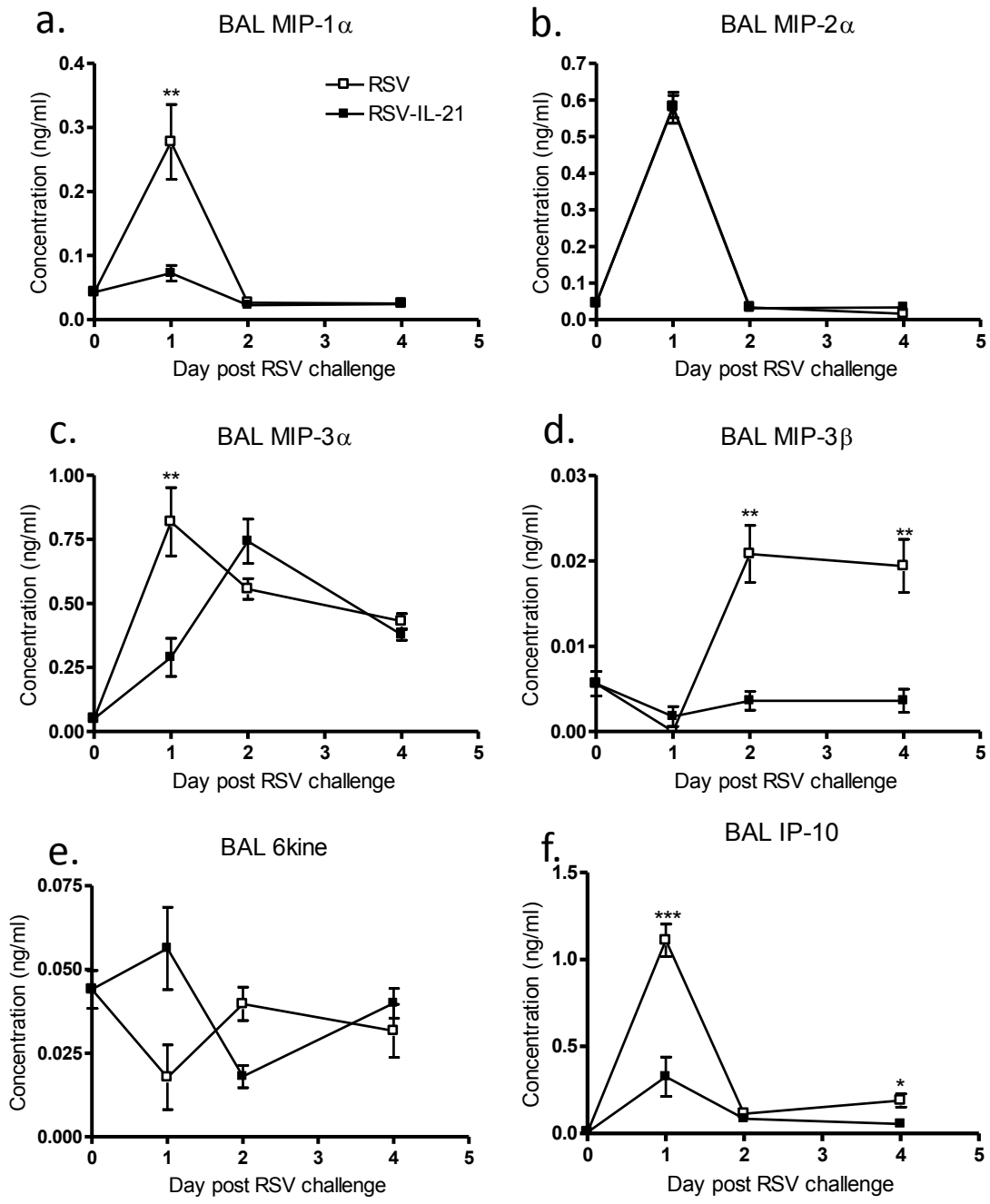

Figure 3.11. IL-21 expression reduces BALF chemokine production after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^6 pfu/mouse; i.n) on d0. BALF was harvested every 24hrs for four days post challenge and MIP-1α (a), MIP-2α (b), MIP-3α (c), MIP-3β (d), 6kine (e), and IP-10 (f) were determined by sandwich ELISA. Data are expressed as mean values. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

challenged mice (Fig.3.11d), and 6kine levels did not rise above baseline in either group at any time (Fig.3.11f).

10. IL-21 expression increases IFN-γ, IL-17 , and IL-21 production by lung T cells after primary RSV challenge

As pro-inflammatory cytokine secretion into the BALF was decreased in RSV-IL-21-challenged mice I next determined the effect of IL-21 expression on cytokine production by stimulated lung T cells seven days post challenge. Stimulated lung T cells from RSV-challenged mice produced significant amounts of IFN-γ (Fig.3.12b) and granzyme B (Fig.3.12c), and IL-4 (Fig.3.12d), IL-10 (Fig.3.12e), and IL-17 (Fig.3.12f) were detected at lower levels. However, no IL-21 was detected (Fig.3.12a). No changes were observed granzyme B, IL-4, and IL-10 production by stimulated lung T cells from RSV-IL-21-challenged mice. However, significant IL-21 (Fig.3.12a) production was detected and there were also significant increases in IFN-γ (Fig.3.12b) and IL-17 (Fig.3.12f) production by T cells from RSV-IL-21-challenged mice.

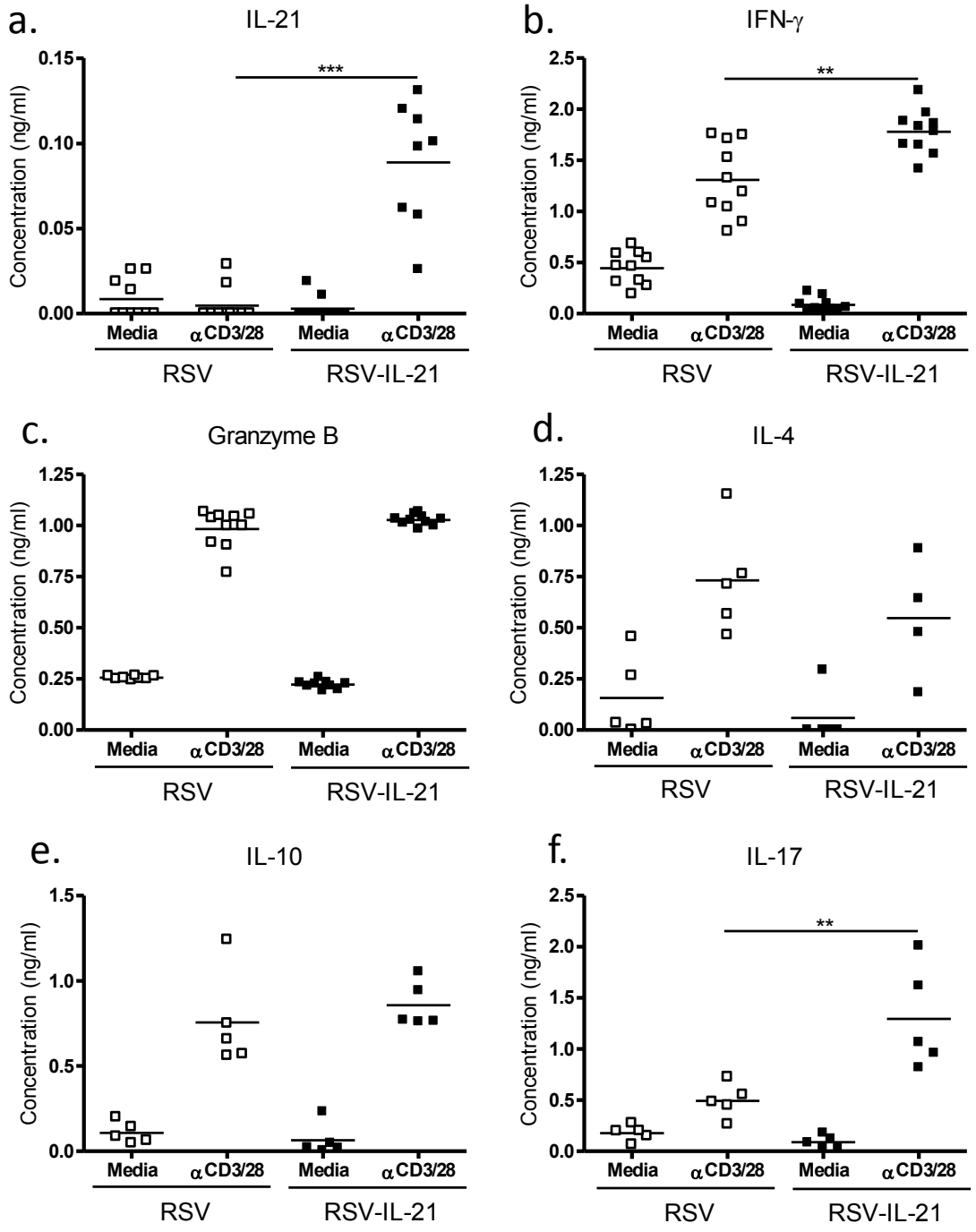

Figure 3.12. IL-21 expression increases IFN-γ, IL-17, and IL-21 production by lung T cells after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^6 pfu/mouse; i.n) on d0. Lung cells were harvested seven days post challenge and samples processed. Lung cells (2×10^6 cells/well) were stimulated with either media alone or αCD3/28-expressing beads (50µl/well) for 24hrs. Supernatants were harvested and IL-21 (a), IFN-γ (b), Granzyme B (c), IL-4 (d), IL-10 (e), and IL-17 (f) were determined by sandwich ELISA. Data are expressed as mean values. The graphs are representative of two independent experiments of five mice per group. Student *t* test result **: $p<0.01$, ***: $p<0.001$.

11. IL-21 expression inhibits T cell recruitment to the pulmonary compartment after primary RSV challenge

IL-21 expression during RSV challenge ablates weight loss by reducing chemokine production, T cell recruitment, activation, and pro-inflammatory cytokine production. I was therefore interested to determine what effect the reduced primary T cell response has on the development of memory T cell populations. To address this, mice were challenged with RSV or RSV-IL-21, weighed for 14 days, and then left for two weeks for memory T cell populations to develop. In this model of RSV disease, cell recruitment to the pulmonary compartment terminates 14 days post challenge, and by 28 days memory formation has completed (766). 28 days post challenge BALF, lung, and spleen cells were harvested and memory cell counts determined. Total cell counts in BALF (Fig.3.13a), lung (Fig.3.13c), and spleen (Fig.3.13e) were similar between groups. However, when lymphocyte populations were counted I observed an ablation in T cells and a severe reduction in B cell numbers in the BALF from RSV-IL-21-challenged mice (Fig.3.13b). Moreover, there were significant reductions in T cell numbers, particularly CD4 T cells, in the lung tissue from this group (Fig.3.13d). B cell numbers were unchanged. In contrast, T cell and B cell counts in the spleen were the same (Fig.3.13f).

12. IL-21 expression inhibits effector, but not central, memory T cell development in the lung tissue after primary RSV challenge

The development of lymphocyte memory is fundamental to long-term protection against rechallenge. As IL-21 expression had ablated memory T cell recruitment in the BALF, and

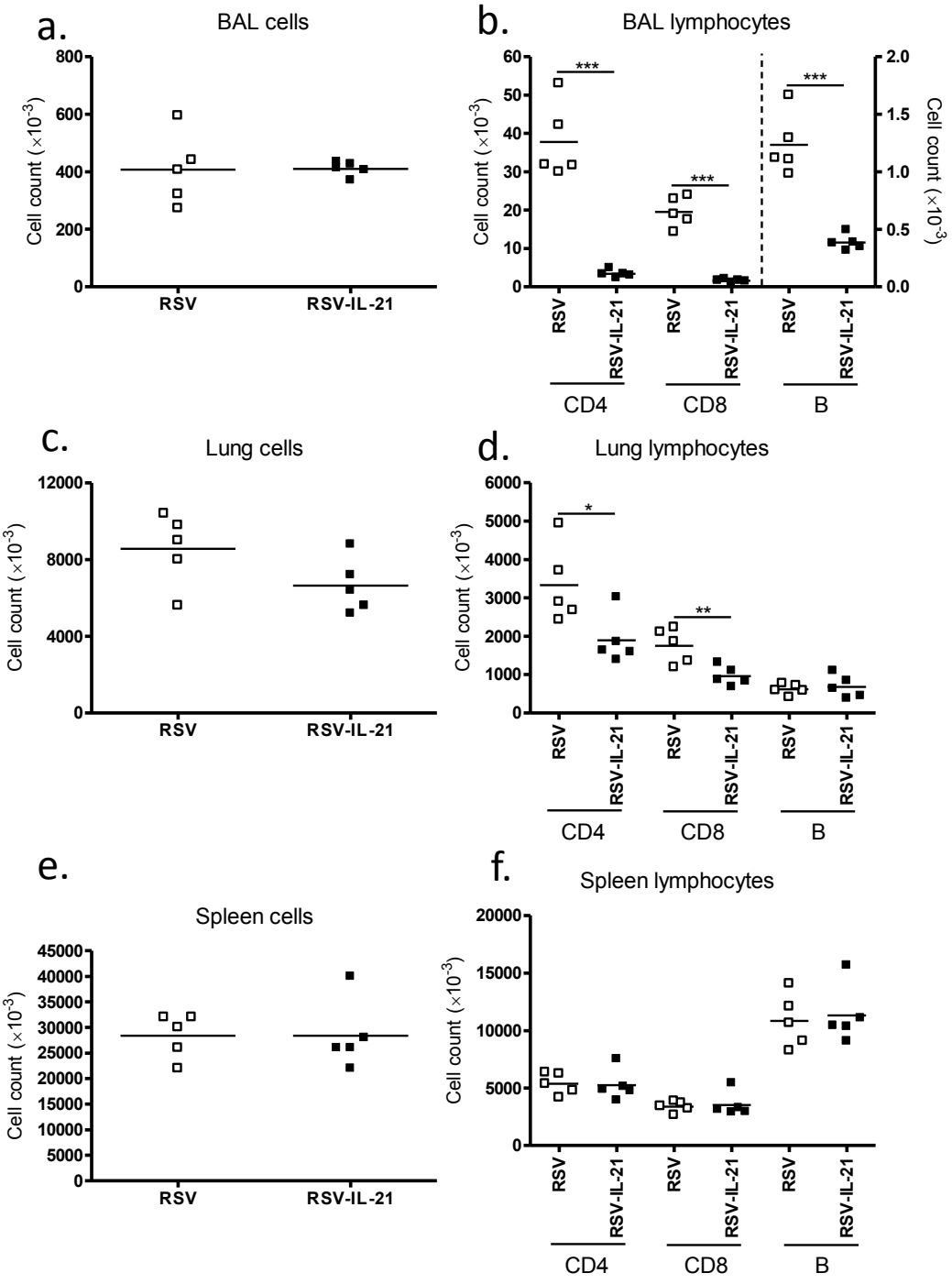

Figure 3.13. IL-21 expression inhibits memory T cell recruitment to the pulmonary compartment after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^{6} pfu/mouse; i.n) on d0. BALF, lung, and spleens were harvested 28 days post challenge and samples processed. Total cell counts in the BALF (a), lung (c), and spleen (e) were determined by trypan blue exclusion assay. CD4 T cells, CD8 T cells, and B cells were phenotyped in the BALF (b), lung (d), and spleen (e) by flow cytometry and counts determined. At least 50×10^{3} cells/sample were collected. Data are expressed as mean values. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

significantly reduced it in the lung, I wanted to know which memory subsets were reduced. Therefore, I determined T cell expression of CD44 and CD62L on CD4 and CD8 T cells in BALF, lung, and spleen from RSV-challenged- and RSV-IL-21-challenged mice. These two markers can be used to distinguish central memory T cells ($CD44^{hi}CD62L^{+}$) from effector memory T cells ($CD44^{hi}CD62L^{-}$) (767). Despite reducing memory T cell recruitment to the BALF, there was no change in the percentage of CD4 (Fig.3.14a), or CD8 (Fig.3.14b), T cells that were central or effector memory between the groups. However, in the lung tissue there was a significant reduction in the proportion of effector memory CD4 (Fig.3.14c), and CD8 (Fig.3.14d), T cells in RSV-IL-21-challenged mice. Representative dot plots are shown (Appendix 3.3). The proportion of T cells that were central memory remained similar. In the spleen there were more central memory T cells relative to effector memory T cells, and the proportion of CD4 (Fig.3.14e) and CD8 (Fig.3.14f) T cells comprising each subset remained similar between the groups.

13. IL-21 expression ablates cytokine production and significantly reduces granzyme B by antigen-specific lung T cells after primary RSV challenge

As IL-21 expression had affected the memory T cell composition in the lung tissue I wanted to know if it affected RSV-specific cytokine production of lung cells upon stimulation with RSV. Lung cells from challenged mice were stimulated *in vitro* with either media, RSV (MOI:2.0), or αCD3/28- expressing beads (to ensure T cells are functional) for 72hrs. Supernatants were then harvested and IFN-γ, IL-4, IL-10, IL-17, granzyme B, and IL-21 were measured. Lung cells from RSV-challenged mice produced significant amounts of IFN-γ (Fig.3.15a) and granzyme B (Fig.3.15e) upon exposure to RSV, with low but detectable

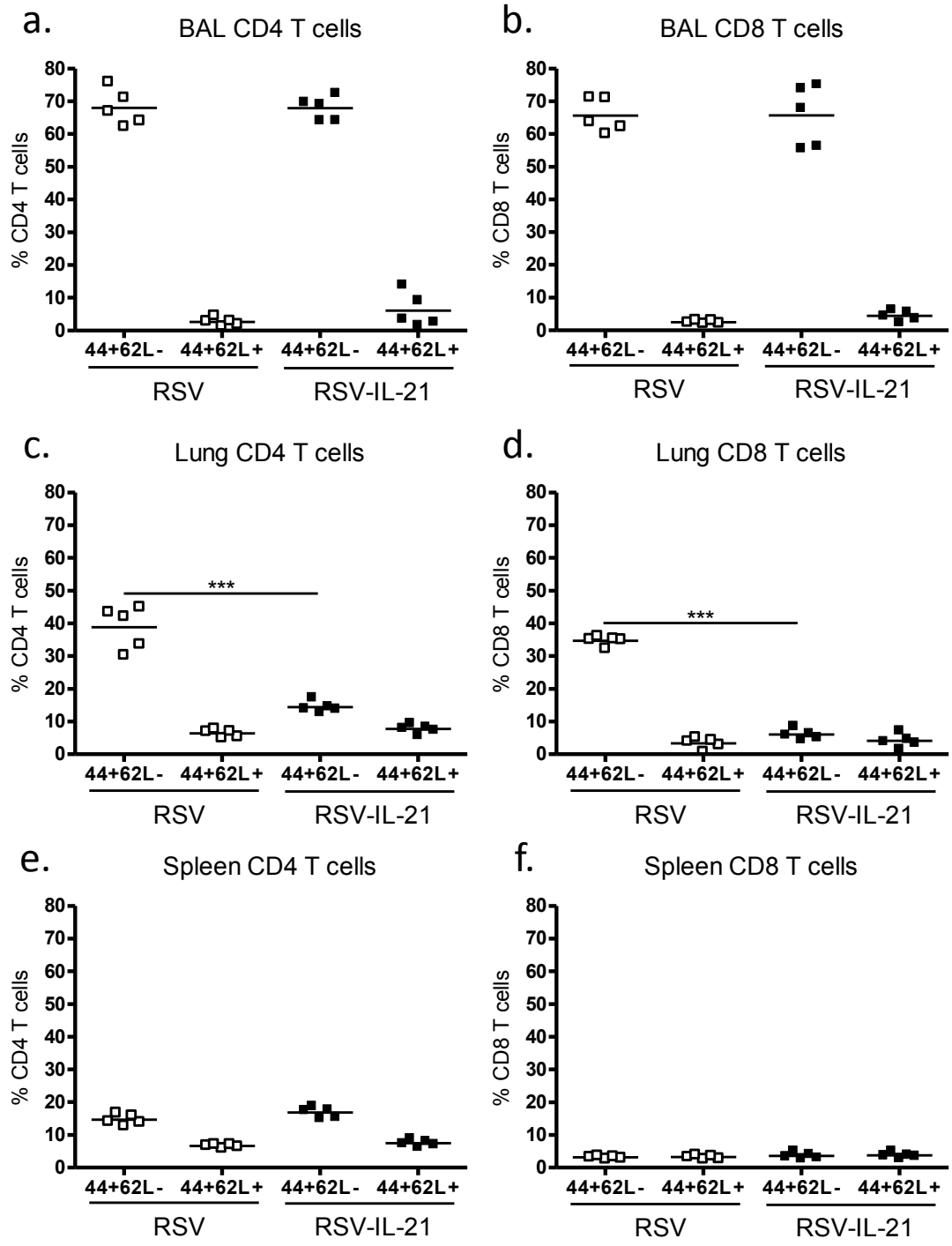

Figure 3.14. IL-21 expression inhibits effector, but not central, memory T cell development in the lung tissue after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^{6} pfu/mouse; i.n) on d0. BALF, lung, and spleens were harvested 28 days post challenge and samples processed. CD44 and CD62L expression on CD4 T cells (a, c, e) and CD8 T cells (b, d, f) were phenotyped in the BALF (a-b), lung (c-d), and spleen (e-f) by flow cytometry. At least 50×10^{3} cells/sample were collected. Data are expressed as mean values. The graphs are representative of two independent experiments of five mice per group. Student *t* test result ***: $p<0.001$.

levels of IL-10 (Fig.3.15c) and IL-17 (Fig.3.15d). No IL-4 (Fig.3.15b) or IL-21 (Fig.3.15f) was detected. Strikingly however, lung cells from RSV-IL-21-challenged mice produced no cytokines in response to RSV stimulation, except for a small but detectable level of granzyme B (Fig.3.15e). This ablation in cytokine production was not because T cells were unable to respond as lung cells from both groups produced significant levels of all cytokines measured after polyclonal stimulation with αCD3/28-expressing beads.

14. IL-21 expression reduces cytokine production by antigen-specific spleen T cells after primary RSV challenge

Next, I wanted to determine if IL-21 expression had ablated cytokine production by antigen-specific memory cells in the spleen as it had in the lung tissue. To determine this, I harvested spleens cells from the same mice as the lung cells, and stimulated them as for the lung cells. After 72hrs the same factors were measured in the supernatants. Spleen cells from RSV-challenged mice produced significant amounts of IFN-γ (Fig.3.16a), IL-10 (Fig.3.16c), and granzyme B (Fig.3.16e) upon exposure to RSV, a very similar phenotype to that observed for lung cells. There was small, but detectable production of IL-17 (Fig.3.16d) but no IL-4 (Fig.3.16b) or IL-21 (Fig.3.16f) production. Spleen cells from RSV-IL-21-challenged mice exhibited an identical cytokine phenotype albeit at a significantly reduced level. There was significant production of IFN-γ (Fig.3.16a), IL-10 (Fig.3.16c), and granzyme B (Fig.3.16e),

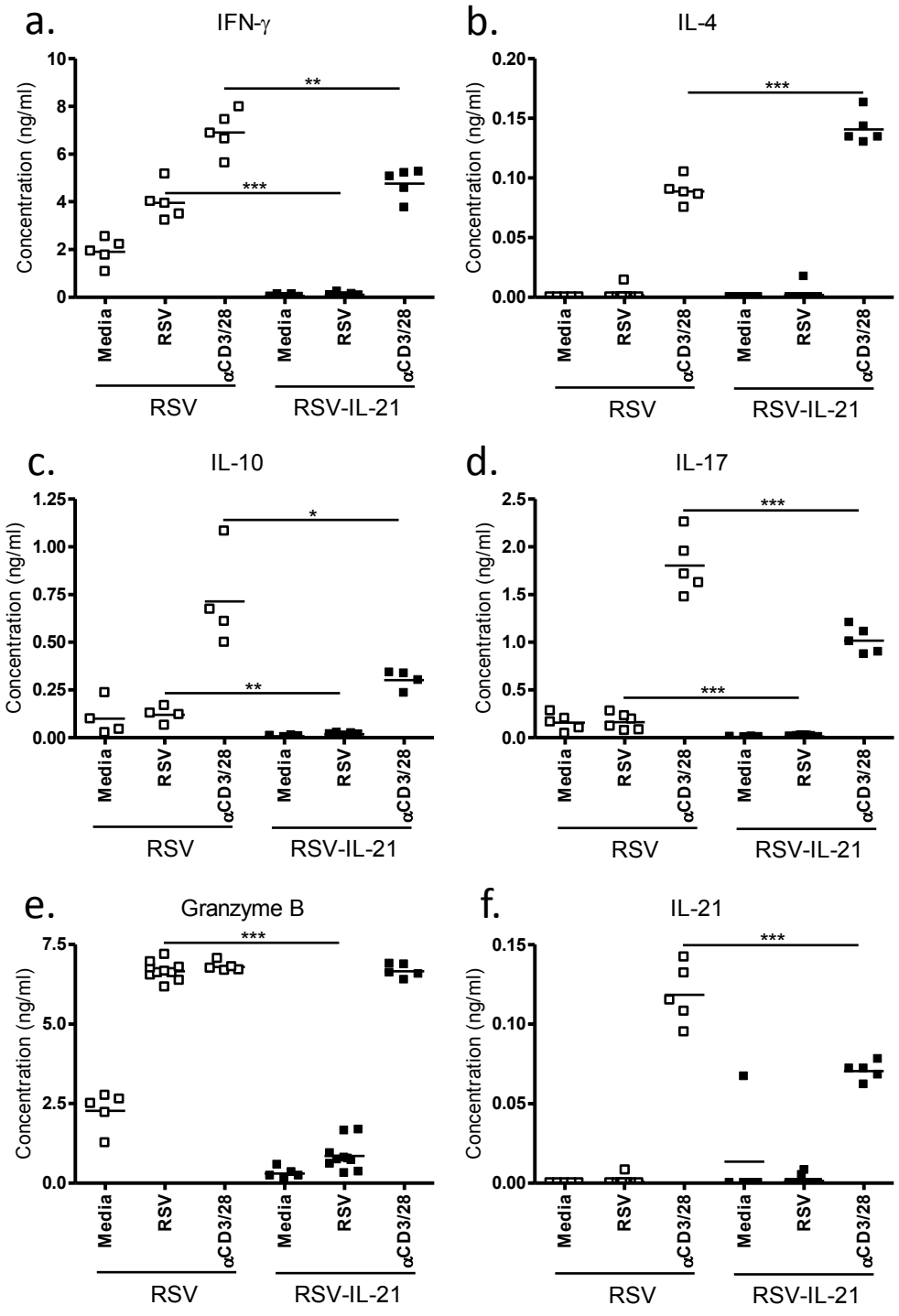

Figure 3.15. IL-21 expression ablates cytokine and granzyme B production by antigen-specific lung T cells after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^6 pfu/mouse; i.n) on d0. Lungs were harvested 28 days post challenge and samples processed. Lung cells (2×10^6 cells/well) were stimulated with either media alone, RSV (MOI: 2.0) or αCD3/28-expressing beads (50µl/well) for 72hrs. Supernatants were harvested and IFN-γ (a), IL-4 (b), IL-10 (c), IL-17 (d), Granzyme B (e), and IL-21 (f) were determined by sandwich ELISA. Data are expressed as mean values. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: p<0.05, **: p<0.01, ***: p<0.001.

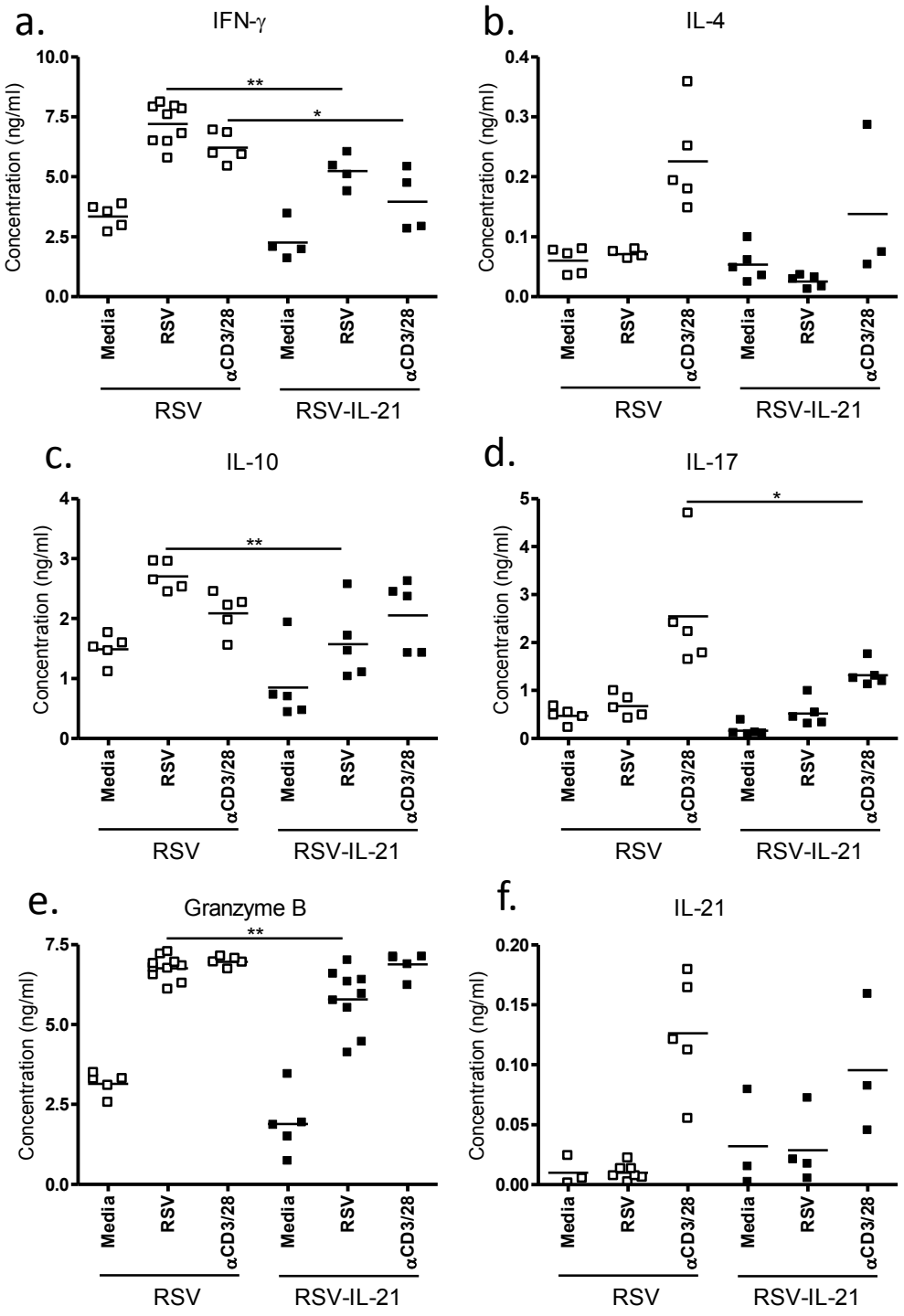

Figure 3.16. IL-21 expression reduces cytokine production by antigen-specific spleen T cells after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^6 pfu/mouse; i.n) on d0. Spleens were harvested 28 days post challenge and samples processed. Spleen cells (2×10^6 cells/well) were stimulated with either media alone, RSV (MOI: 2.0) or αCD3/28-expressing beads (50µl/well) for 72hrs. Supernatants were harvested and IFN-γ (a), IL-4 (b), IL-10 (c), IL-17 (d), Granzyme B (e), and IL-21 (f) were determined by sandwich ELISA. Data are expressed as mean values. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: p<0.05, **: p<0.01, ***: p<0.001.

though all were significantly reduced. IL-17 (Fig.3.16d) production was unchanged and there was no IL-4 (Fig.3.16b) or IL-21 (Fig.3.16f) production by spleen cells from these mice either.

15. IL-21 expression reduces virus-specific serum antibody production after primary RSV challenge

My previous studies have shown that IL-21 depletion in the context of primary RSV challenge, and after CD4 T cell priming and RSV challenge, significantly reduced virus-specific serum antibody production. Therefore, I had hypothesised that IL-21 expression would boost virus-specific antibody production. To determine if this was the case, I measured virus-specific serum antibody production in RSV- and RSV-IL-21-challenged mice 28 days post infection. No virus-specific serum IgA (Fig.3.17a) or IgE (Fig.3.17b) was detected in either group. However, virus-specific serum IgG1 (Fig.3.17c) and IgG2a (Fig.3.17d) were detected in RSV-challenged mice. These isotypes were also detected in RSV-IL-21-challenged mice but at a significantly reduced level.

16. IL-21 expression reduces virus-specific BAL antibody production after primary RSV challenge

Surprisingly, IL-21 expression reduced virus-specific serum antibody production. Therefore, I extended the analysis to virus-specific BALF antibody. As for serum, no virus-specific IgE (Fig.3.18b) was detected in either group, but virus-specific IgA (Fig.3.18a), IgG1 (Fig.3.18c) and IgG2a (Fig.3.18d) were detected in BALF from RSV-challenged mice. As observed in

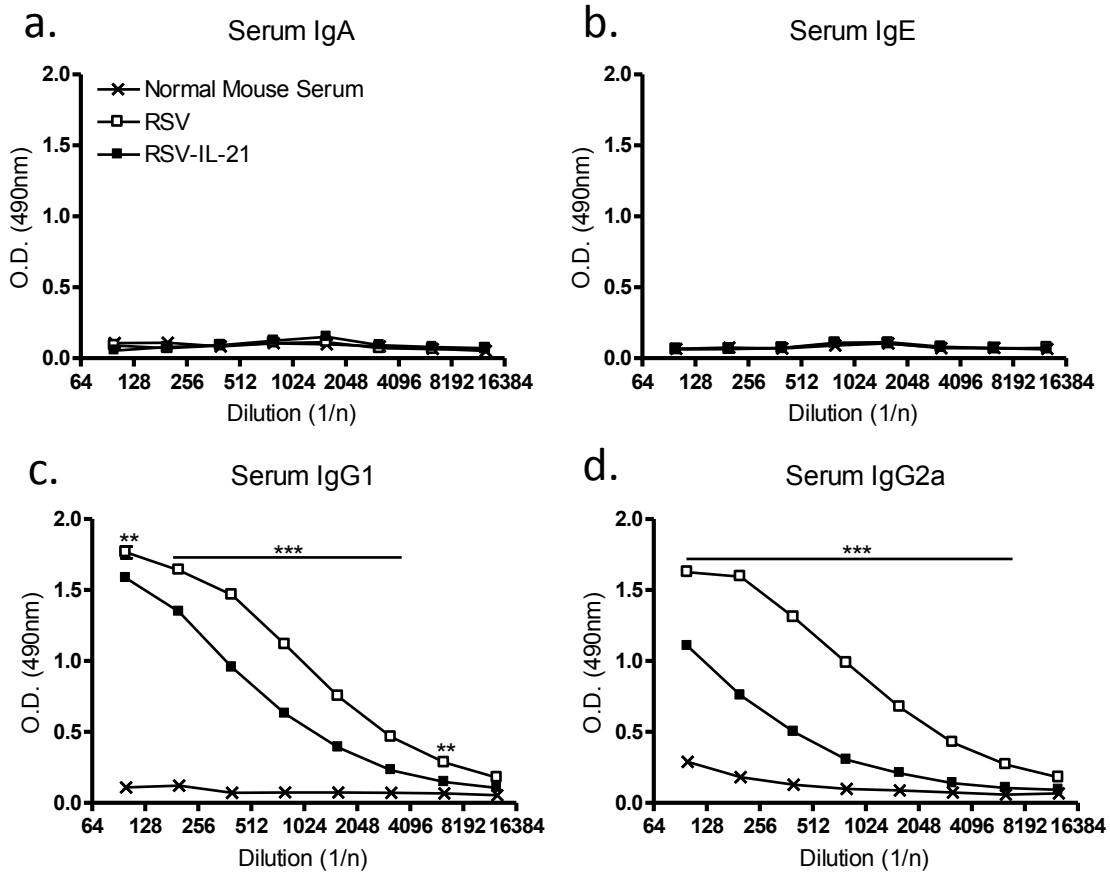

Figure 3.17. IL-21 expression reduces virus-specific serum antibody production after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^6 pfu/mouse; i.n) on d0. Serum was harvested 28 days later and virus-specific IgA (a), IgE (b), IgG1 (c), and IgG2a (d) levels were determined by ELISA. Data is expressed as mean±SEM. The graphs are representative of two independent experiments of five mice per group. ANOVA result ***: $p<0.001$.

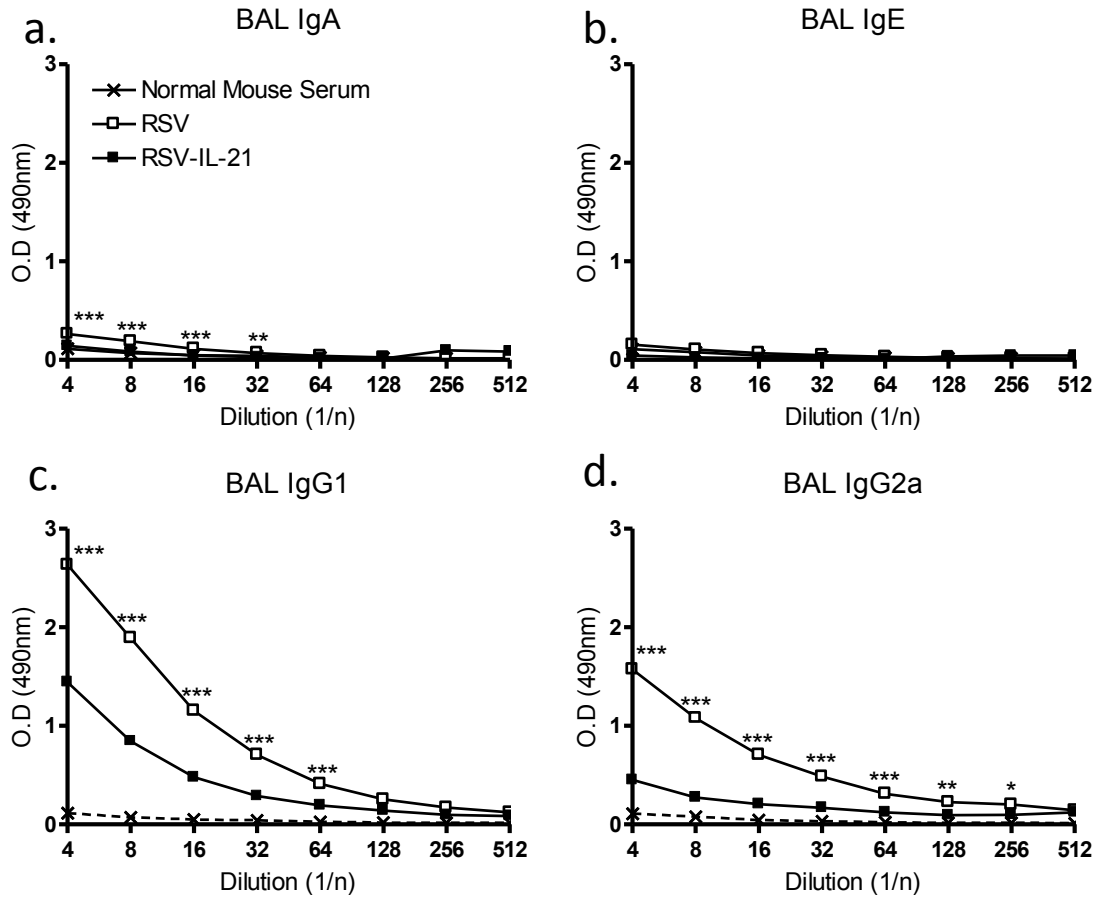

Figure 3.18. IL-21 expression reduces virus-specific BAL antibody production after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^{6} pfu/mouse; i.n) on d0. BALF was harvested 28 days later and virus-specific IgA (a), IgE (b), IgG1 (c), and IgG2a (d) levels were determined by ELISA. Data is expressed as mean±SEM. The graphs are representative of two independent experiments of five mice per group. ANOVA result *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

serum, BALF from RSV-IL-21-challenge mice contained significantly reduced levels of virus-specific IgA (Fig.3.18a), IgG1 (Fig.3.18c) and IgG2a (Fig.3.18d).

17. IL-21 expression during primary RSV challenge exacerbates weight loss upon secondary RSV challenge

My studies so far indicate that IL-21 expression ablates disease by significantly inhibiting the T and B cell response to RSV challenge, consequently reducing memory development in the lung against the virus. The only detectable memory is that present in the spleen, and RSV-specific antibody production was significantly reduced. Therefore, because of the lack of RSV-specific lymphocytic memory after primary challenge, protection against rechallenge should be reduced. To determine if that is the case, I challenged mice with RSV that were challenged four weeks previously with either RSV or RSV-IL-21. In my rechallenge studies, both groups of mice began to lose weight immediately after secondary challenge. Weight loss peaked in both groups on d2 post challenge, but the magnitude of weight loss in mice previously challenged with RSV-IL-21 was significantly greater than those previously challenged with RSV (Fig.3.19). Then kinetic of weight loss was similar however, with both groups almost recovered by d4 post challenge.

18. IL-21 expression during primary RSV challenge increases cell recruitment to lung tissue upon secondary RSV challenge

An increase in disease severity correlates with increases in cell recruitment to the pulmonary compartment. Therefore, I determined recruitment of both lymphocytic and

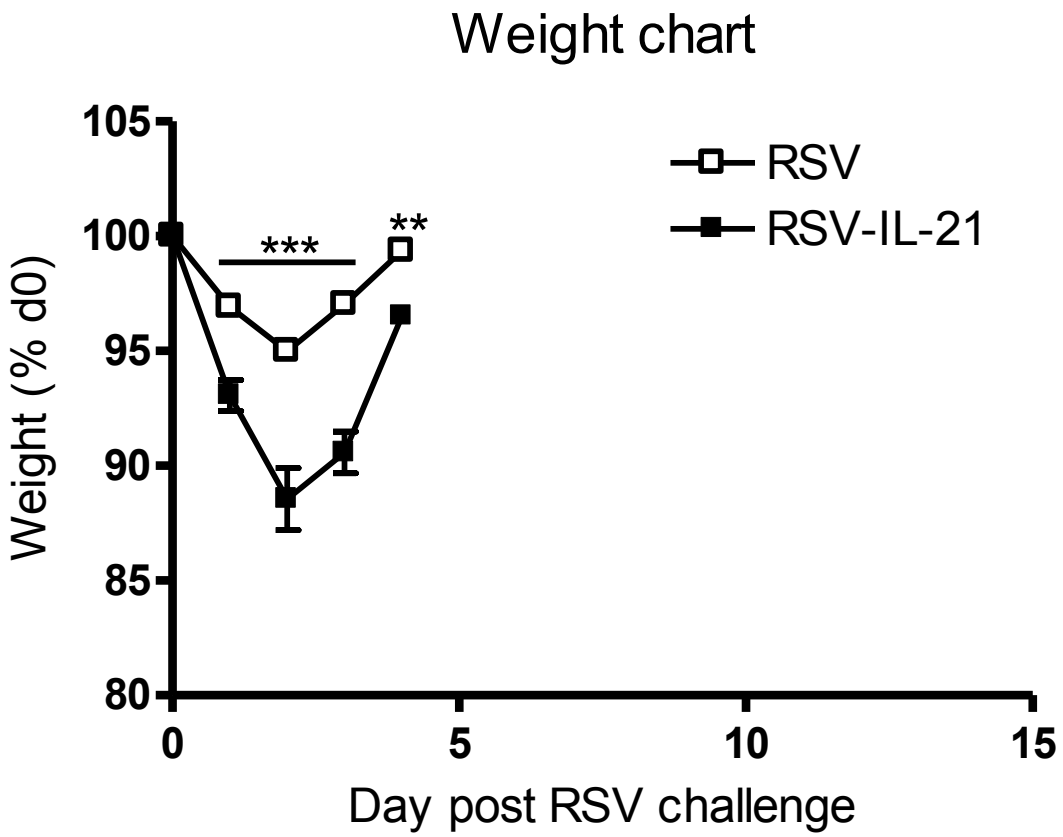

Figure 3.19. IL-21 expression during primary RSV challenge exacerbates weight loss upon secondary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^6 pfu/mouse; i.n) on d0. Mice were left for 28 days and then rechallenged with RSV (1×10^6 pfu/mouse; i.n). Mice were weighed daily for 4 days. Weight is shown as a percentage of baseline weight. Data is expressed as mean±SEM. The graphs are representative of two independent experiments of five mice per group. Student *t* test result **: $p<0.01$, ***: $p<0.001$.

non-lymphocytic populations after secondary RSV challenge. Total cell recruitment to the airway was unchanged (Fig.3.20a), though I did observe a significant increase in cell recruitment to lung tissue in mice previously challenged with RSV-IL-21 (Fig.3.20b). Recruitment of individual cell types was determined by flow cytometry. No changes in recruitment of lymphocytic (Fig.3.20c) or non-lymphocytic (Fig.3.20e) populations to the airway were observed. However, in the lung tissue I observed a significant increase in NK cells (Fig.3.20d) and neutrophils (Fig.3.20f), though no other changes were evident.

19. IL-21 over expression during primary RSV challenge has no effect on viral clearance upon secondary RSV challenge

An increase in cell recruitment negatively correlates with viral clearance. Therefore, I measured the number of RSV L gene copies in lung tissue at d0,2 and 4 post RSV challenge by qPCR (as described previously). Irrespective of IL-21 over expression during primary RSV challenge, viral L gene copies were below the limit of detection in lungs of mice rechallenged with RSV WT at the time points tested.

20. IL-21 expression during primary RSV challenge increases airway T cell activity upon secondary RSV challenge

Although there was no increase in T cell recruitment to the pulmonary compartment after viral rechallenge, I assessed whether there was an increase in T cell activity as this also correlates with disease severity. Upon activation, memory T cells express an increased spectrum of costimulatory molecules compared to naive T cells. Typically, naïve T cell

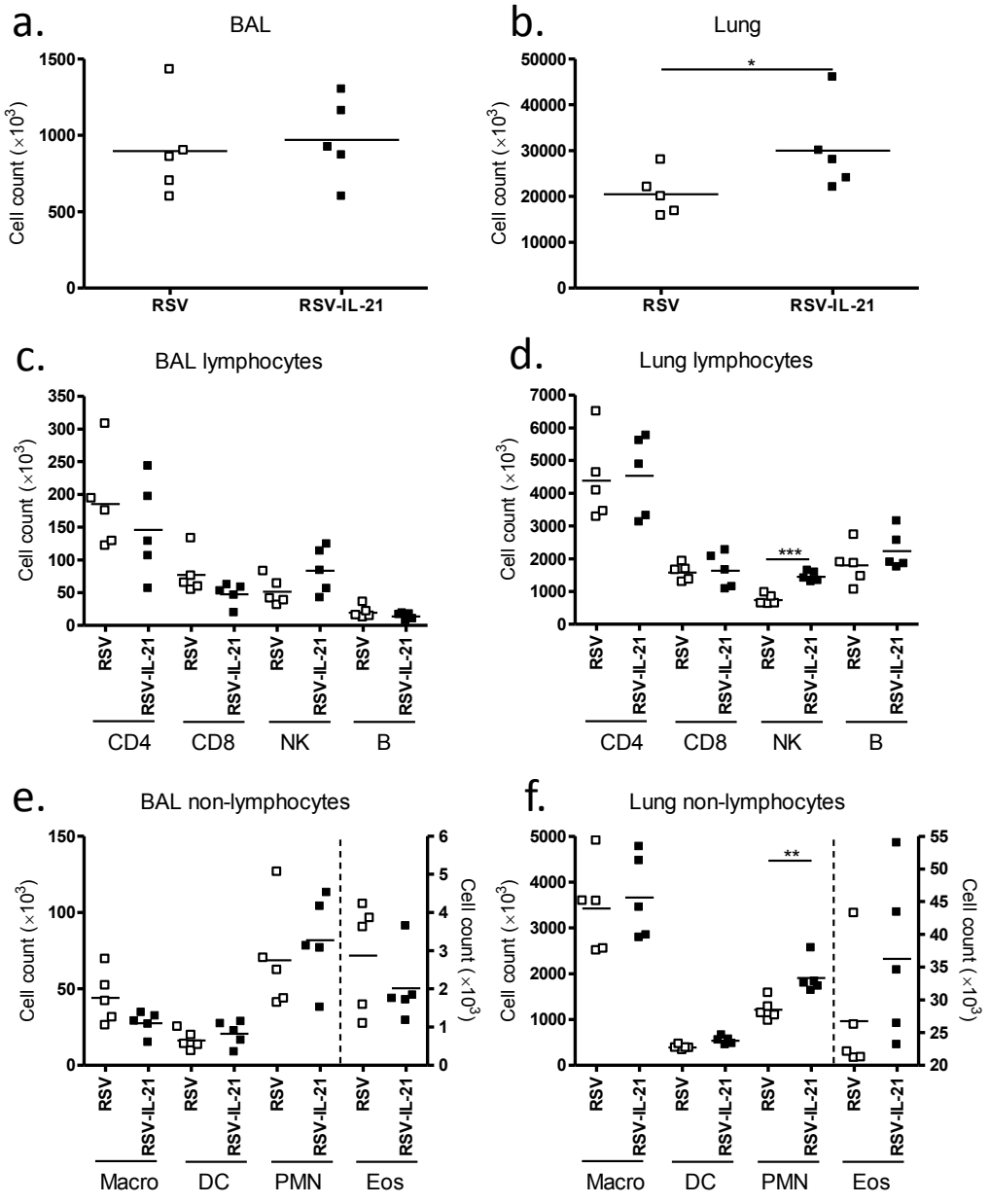

Figure 3.20. IL-21 expression during primary RSV challenge increases cell recruitment to lung tissue upon secondary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^6 pfu/mouse; i.n) on d0. Mice were left for 28 days and then rechallenged with RSV (1×10^6 pfu/mouse; i.n). BALF cells and lung tissue were harvested four days post challenge. Samples were processed and total cell counts in the BALF (a) and lung tissue (b) determined. CD4 T cells, CD8 T cells, NK cells, and B cells in BALF (c) and lung tissue (d) were phenotyped by flow cytometry and cell counts determined. Macrophages, DCs, neutrophils (PMN), and eosinophils in BALF (e) and lung tissue (f) were determined using the same method. At least 50×10^3 cells/sample were collected. Data are expressed as mean values. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

activation can be detected by measuring CD28 (768), CD69 (769), CD25 (770), and ICOS (771) expression, and it is thought that naïve T cell activation relies primarily on the CD28:CD80/86 costimulatory pathway (771). However, memory T cells have less dependence on the CD28:CD80/86 pathway because they express additional costimulatory molecules such as CD27 and OX40 (772), and can utilise additional costimulatory pathways upon interaction with antigen-presenting APCs. PD1 can also be used as a marker of antigen-experienced cells (i.e. they have been activated) (773). At d4 post-secondary challenge, the majority of infiltrating BALF CD4 T cells expressed both OX40 and ICOS and a minority expressed PD1 (Fig.3.21a). The expression of these molecules was significantly increased in mice previously challenged with RSV-IL-21. A similar phenotype was exhibited by BALF CD8 T cells, though there was no change in ICOS expression (Fig.3.21b).

21. IL-21 expression during primary RSV challenge increases BAL IFN-γ and IL-10 production upon secondary RSV challenge

An increase in disease severity correlates with increases in T cell activity and increased pro-inflammatory cytokine production. As T cell activity was increased in mice previously challenged with RSV-IL-21, I determined if cytokine production was also enhanced. BALF IFN-γ (Fig.3.22a), IL-4 (Fig.3.22b), IL-10 (Fig.3.22c), IL-17 (Fig.3.22d), granzyme B (Fig.3.22e), and IL-21 (Fig.3.22f) levels were determined by sandwich ELISA. I detected significant levels of IFN-γ and granzyme B in the BALF of mice previously challenged with RSV. There was also weak but detectable production of IL-10, but little IL-21, and no IL-4 or IL-17 production. However, in mice previously challenged with RSV-IL-21, I detected significantly enhanced production of BALF IFN-γ (Fig.3.22a), IL-10 (Fig.3.22c), and IL-21 (Fig.3.22f). BALF Levels of IL-

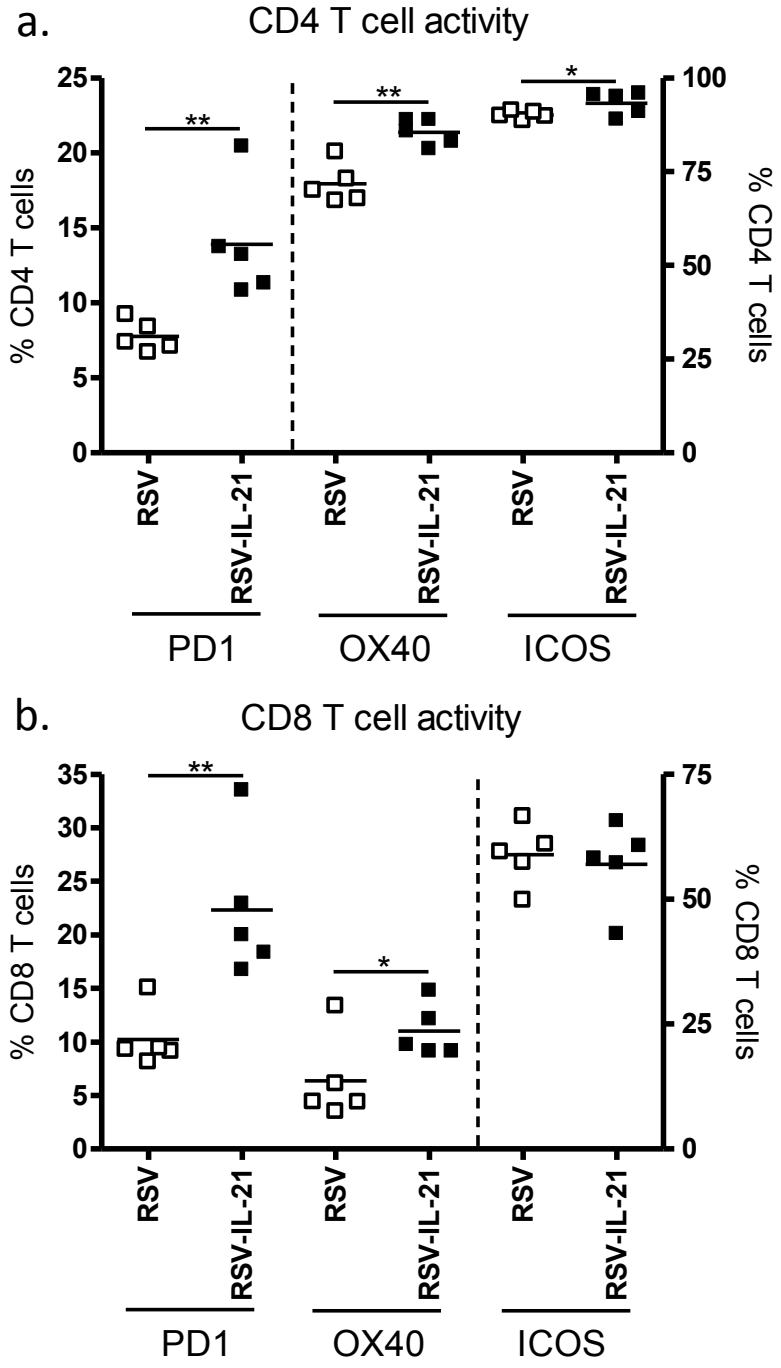

Figure 3.21. IL-21 expression during primary RSV challenge increases airway T cell activity upon secondary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^6 pfu/mouse; i.n) on d0. Mice were left for 28 days and then rechallenged with RSV (1×10^6 pfu/mouse; i.n). BALF cells were harvested four days post challenge. Samples were processed and PD1, OX40, and ICOS expression on CD4 and CD8 T cells was phenotyped by flow cytometry (a). At least 50×10^3 cells/sample were collected. Data are expressed as mean values. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: $p<0.05$, **: $p<0.01$.

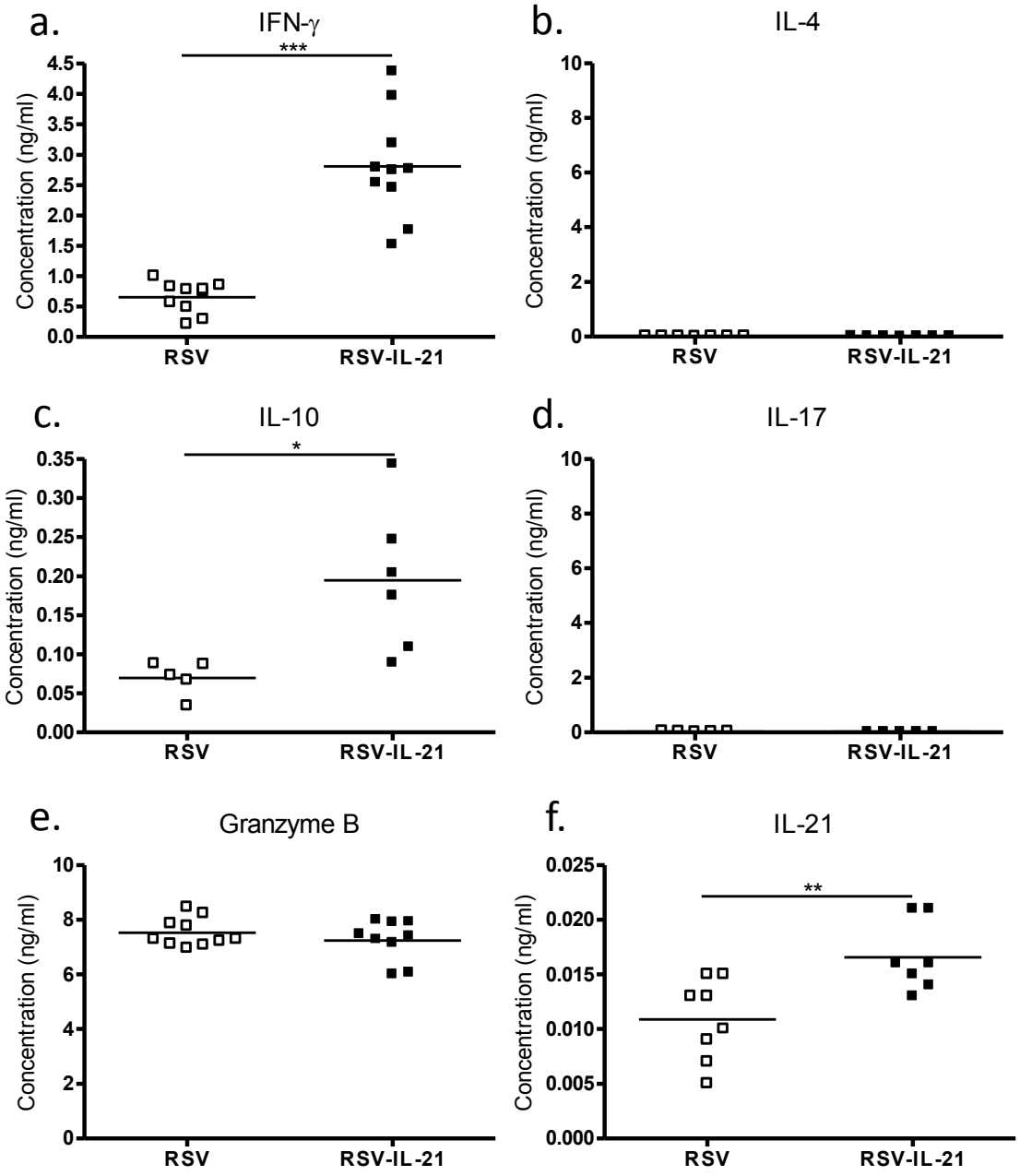

Figure 3.22. IL-21 expression during primary RSV challenge increases BAL IFN-γ and IL-10 production upon secondary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^6 pfu/mouse; i.n) on d0. Mice were left for 28 days and then rechallenged with RSV (1×10^6 pfu/mouse; i.n). BALF was harvested four days post challenge and IFN-γ (a), IL-4 (b), IL-10 (c), IL-17 (d), Granzyme B (e), and IL-21 (f) were determined by sandwich ELISA. Data are expressed as mean values. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

4, (Fig.3.22b), IL-17 (Fig.3.22d), and granzyme B (Fig.3.22e) were unchanged. Cytokine production by polyclonally-stimulated lung cells was similar between groups, though a reduction in IL-17 production by lung cells from mice previously-challenged with RSV-IL-21 was observed (Appendix 3.4).

22. Discussion

Previous data in this study had supported the hypothesis that IL-21 acted as an important regulator of CD4 T cell activation in primary, but particularly memory, responses to RSV challenge. Regulatory effects on dendritic cell (DC) activity may be responsible for these observations. On this basis, I hypothesised that IL-21 over-expression would further reduce DC activity, and therefore CD4 T cell responses to RSV challenge, consequently reducing disease severity. By using a novel IL-21-expressing RSV construct (RSV-IL-21), and comparing the immune responses of this virus to that of WT RSV I have confirmed that IL-21 has potent anti-inflammatory effects in the pulmonary compartment. Expression of supra-physiological IL-21 levels during primary infection ablated the T cell response and disease severity (including piloerection, hunched posture, and group isolation) after RSV challenge without affecting viral replication. However, there were detrimental effects on humoral immunity, memory development, and protection from rechallenge. Therefore, IL-21 is a powerful inhibitor of primary T cell responses in the pulmonary compartment but also reduces long-term protection which needs to be addressed in further studies.

Mice can be treated with cytokines using various methods, each with advantages and disadvantages as discussed in the introduction. My use of a 'viral vector' allows continuous cytokine production without further animal manipulation and the rate of production can be controlled by moving the gene upstream or downstream on the genome, utilising transcriptional polarity of the RSV genome (634). This contrasts favourably with daily exogenous administration, which is necessary as cytokines are rapidly taken up by receptor-expressing cells and filtered from the circulation by the kidneys prior to removal in urine (765). As such the serum half-life of many cytokines can be measured in hours. However,

once a viral vector has been administered there is no control over the amount of cytokine produced *in vivo* which may affect data interpretation. Therefore, it is important to measure cytokine production over a time course. Significant IL-21 production was confirmed *in vitro* and *in vivo* by ELISA. *In vivo*, BALF IL-21 was first detectable at d4 p.c when viral replication peaked and peaked at d7 p.c as virus was cleared. As epithelium is the primary target of RSV it is likely that most IL-21 is derived from these cells with initial detection of IL-21 at d4 p.c. This seems surprisingly slow as the first viral proteins are detectable only 6-8hrs post viral infection and L gene transcription within 48hrs (639). As RSV replicates using polysomes and transcriptional polarity, and the murine IL-21 gene was positioned between RSV F and G genes, then transcription should have occurred within 48hrs. Therefore, initial IL-21 protein production should have occurred within this time, but was undetectable in the BALF. The viral inoculum contains ~100-fold more IL-21 than that observed at the peak in the BALF, yet by d2 p.c this is undetectable. Most may have been lost by pinocytosis by macrophages and type I and II epithelial cells. A small proportion, however, is likely to have bound IL-21R on various cells including epithelial cells, macrophages and DCs and elicited a biological effect. There are other possible explanations for this lack of detection. First, the ELISA may not be sensitive enough to detect very low levels of IL-21 production, and the use of more sensitive techniques such as luminex, Meso Scale Discovery (MSD), or Europium detection may be more informative. Measure of murine IL-21 mRNA in lung tissue by qPCR would determine when the gene was transcribed. However, it is also possible that IL-21 was not released by infected cells until d4 p.c, possibly as a result of cell death. This would be difficult to test *in vivo*, but *in vitro* studies of epithelial cell death after RSV-IL-21 infection may help answer this. The IL-21 peak at d7 p.c occurs after viral replication has ceased, though measurements at other time points would more accurately determine this. Assuming peak production does

occur at d7 p.c, immune cells may be responsible for production after viral replication has ceased. This idea is supported by the fact that CD4 T cells are a major IL-21 source, and their recruitment and activity peaks at d7 p.c in this model. Although there were very few CD4 T cells present in the BALF of RSV-IL-21-challenged mice at this time they were present in the lung tissue, albeit at reduced levels. Moreover, there was significant IL-21 production upon stimulation of lung cells from RSV-IL-21-challenged mice with a polyclonal T cell stimulus. As IL-21 is known to enhance its own production in an autocrine and paracrine manner, it is likely that innate CD3-expressing cells had been induced to initiate IL-21 production. Such cells could include NKT cells, MAIT (Mucosal-Associated Invariant T) cells, and/or γδ T cells. Moreover, other cells may have been induced to produce IL-21 in a cytokine-dependent manner (rather than TCR-stimulation), including ILCs, NK cells, DCs, macrophages, and/or fibroblasts. Specific studies of IL-21 production by these cell types (e.g. by intracellular staining for IL-21) is necessary to determine the source(s).

Viral replication was unaffected by the addition of the IL-21 gene or inhibition of the immune response. This suggests that innate immune mechanisms that carry out viral clearance were unaffected by increased IL-21 production. IL-21 is known to increase NK cell cytotoxic functions in the presence of IL-15, and NKT cells by itself, but it is not known if this occurred *in vivo*. Moreover, γδ T cells may be affected but it is unclear how important they are in primary anti-viral immune responses (123). Macrophage and ILC activity may also be altered but these require experimental confirmation. Viral replication data suggests that the reduced transcription of the F, M2, and L genes due to the IL-21 gene insertion does not significantly affect 'viral fitness'. IL-21-expressing RSV could be cultured to the same number of plaque-forming units as RSV WT, indicating no reduction of viral fitness *in vitro*.

Moreover, the viral vector is antigenically-identical to RSV WT, therefore, antigen presentation, and antigen-specific immune responses should be identical.

Previous data in this study and from this chapter strongly supports a critical role for IL-21 in limiting DC maturation and antigen presentation to naïve T cells. Previous studies by Brandt *et al* have clearly demonstrated that IL-21 reduces MHCII expression by DCs that prevents them from activating CD4 T cells in the draining lymph nodes (622). As well as a lack of T cell influx into the BALF and lung tissue, there was ablation of DC recruitment. There are three possible explanations for this: IL-21 causes DCs to down-regulate MHCII, CD11b, and CD11c proteins used as markers in this study, DCs are failing to migrate back to the pulmonary compartment from the draining lymph nodes, or IL-21 over-expression induces DC apoptosis. Investigating the first possibility is difficult as another means of detecting these cells would be necessary. Extending the markers measured (e.g. Siglec H) or using alternative detection techniques (e.g. haematoxylin and eosin staining) may help. DC migration studies could answer the second possibility. Whether IL-21 over-expression affects specific DC subsets is another important question that was not addressed in this study, and the use of more markers (OX40L, CD40, and PD-L2 as described in the previous chapter) would help answer this question. DC migration from the BALF to the mediastinal lymph nodes was also not measured in this study, but *in* vivo CFSE labelling of DCs prior to viral challenge would enable DC tracking to the draining lymph nodes where costimulatory molecule expression on DCs, and activation markers (e.g. CD69) on T cells, could be measured to determine whether DC migration and activation potential are altered by IL-21 over expression. DCs returning from the mediastinal lymph nodes could be determined by measuring CFSE, assuming that DC proliferation has not reduced the signal to undetectable

limits. DC apoptosis could be addressed using TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) staining.

The lack of T cell influx in RSV-IL-21-challenged mice could reflect a direct effect of IL-21 on T cells, however there is contradictory evidence of this in previously published IL-21 studies. IL-21 has been shown to boost T cell activation in different models (626,774-777), but more recent studies have demonstrated that it has potent anti-inflammatory effects (704,729,731,778). In my studies, IL-21 significantly reduced ICOS expression on both BAL and lung CD4 and CD8 T cells, suggesting it reduced their activation (directly or indirectly). ICOSL expression on macrophages and DCs increased simultaneously, indicating that ICOS:ICOSL interactions reduce ICOSL molecule expression on these cells. ICOSL may be removed by endocytosis or trogocytosis. Although there was a clear reduction in cytokine production into the BALF there was significant cytokine production by stimulated lung cells. Indeed, IFN-γ, IL-17, and IL-21 levels were higher in samples from RSV-IL-21-challenged mice. This suggests that IL-21 may block migration of activated T cells from the lung tissue into the BALF, possibly by altering integrin and adhesion molecule expression on T cells as well as structural cells. The reduction in BALF chemokine production in RSV-IL-21-challenged mice also supports this idea. Analysis of these molecules on the surface of infiltrating cells and lung sections would help clarify this issue.

The data generated here support the concept that IL-21 does not alter T cell differentiation. No significant bias in cytokine production in the BALF or by stimulated lung cells was observed in RSV-IL-21-challenged mice. Moreover, no significant alterations in transcription factor expression were observed by CD4 T cells in either the pulmonary compartment or mediastinal lymph nodes. Only significant reductions in BALF IFN-γ and granzyme B were

noted, as well as a loss of T-bet expression in responding T cells, suggestive of a loss of pro-inflammatory T cell influx rather than diversion to an alternative T cell profile. Importantly, there is no data that supports regulatory T cell differentiation by IL-21 as no increase in FoxP3 expression was observed. Therefore, IL-21 is inhibiting the anti-viral response via other mechanisms. These data support the idea that IL-21 is acting as an anti-inflammatory factor rather than a T-cell-subset-biasing cytokine.

The lack of cell recruitment and migration observed in this study could also be explained by a reduction in chemokine expression, as was observed in the BALF. RSV infection of mucociliary epithelium induces production of several chemokines including RANTES, MCP-1, and MIP-1α (779,780). Similar results have been observed in human studies (781). Certain chemokines such as RANTES (663), CCL20 (782), and MIP-1α (661,783) have been associated with RSV disease. Other studies have suggested that RSV may inhibit early chemokine release, presumably to reduce the immune response and prolong the opportunity for viral replication (784). Moreover, the development of Th1 and Th2 responses against RSV is chemokine-driven (662,785). The loss of MIP-1α, MIP-3α, and IP-10 suggest that IL-21 may act to reduce chemotactic factors that prevent cellular recruitment. Indeed, changes in chemokine production could explain many of the observations in this study and is indicative of a major effect of IL-21. Previous studies in rheumatoid arthritis (786), and an air-pouch model of inflammation (632), have demonstrated that macrophages express IL-21R and are activated by IL-21. Other studies have demonstrated that IL-21 induces alternative activation of macrophages to boost Th2 responses (775), and can enhance antigen processing and presentation to CD4 T cells (715). They have also been shown to secrete MIP-1α (268) and CXCL10 (787) upon activation. Therefore, IL-21 may ablate RSV disease by

controlling T cell recruitment via chemokine production by macrophages. There are few studies that have focussed on changes in chemokine expression caused by changes in IL-21 expression, and those that have demonstrated that IL-21 increased chemokine expression, particularly CCL20 (713). Therefore, my finding that IL-21 inhibits chemokine production in the pulmonary compartment is a novel finding and warrants further study as it has therapeutic implications. As opposed to other sites, IL-21 may have a unique role in controlling cellular recruitment to the BALF via changes in chemokine production. An extensive analysis of BALF chemokine production [including TNF, another pro-inflammatory factor not assayed in this study known to increase RSV disease severity (788)] over a time course of RSV challenge should confirm the effect, compared to IL-21 depletion (where RANTES production was increased, supporting the general concept) and untreated animals. The mechanism as to how IL-21 controls chemokine production should then be investigated. It is likely that most chemokines are produced by infected epithelium or macrophages, as well as other innate cells. Therefore, future studies should focus here. If the mechanism can be determined then it may be utilised therapeutically to treat multiple lung diseases, not just viral infection.

Previous data in this study had predicted that IL-21 overexpression would boost B cell activity and increase virus-specific antibody production, thereby improving long-term protection from rechallenge with an antigenically-syngeneic virus. However, a significant loss in virus-specific antibody production was observed in both BALF and serum, and there was no indication that B cell activation had increased. This is surprising given previous studies that have identified crucial roles in follicular helper T cell development (599,600), B cell activation (738), isotype class switching (789), plasma cell generation (609,790), and

antibody production (625). Nevertheless, there is no data to suggest the IL-21 over-expression boosts B cell responses during RSV challenge. As B cell activation requires CD4 T cell help, it is likely that the loss of CD4 T cells in the primary response has decreased help given to B cells in the secondary lymphoid organs. This, in turn, has resulted in reduced antibody production, and memory B cell generation. Further specific studies measuring different B cell populations in challenged mice, and adoptively transferring CD4 T cells from RSV-challenged mice will address these issues.

IL-21 over-expression significantly reduced RSV disease by inhibiting T cell recruitment, cytokine production, and effector function. It is unsurprising therefore that it significantly reduced the development of T cell memory in the pulmonary compartment, specifically effector T cell memory. However, it only affected lymphocytic memory in the pulmonary compartment; the splenic compartment remained functional as measured by antigen-specific cytokine production (proliferation was not measured). This indicates that IL-21 over-expression only affects the local site where it is produced, and not systemically. There is conflicting data regarding the effect of IL-21 on the development of T cell memory, focussed mostly on CD8 T cell memory. Allard *et al* demonstrated that in the absence of infection, transgenic mice that over-express IL-21 have an enhanced central memory CD8 T cell compartment in the spleen, and both central and effector memory T cell compartment in the lymph nodes (791). In contrast, the B cell compartment was unchanged, and CD4 T compartments were reduced in number. This study suggests that IL-21 supports CD8 T cell memory but not CD4 T cell memory. Likewise, Kaka *et al* demonstrated that IL-21 over-expressing CD8 T cells augmented development of central memory CD8 T cells upon activation (792). In contrast, Sondergaard *et al* demonstrated that IL-21 restricted primary

CD8 T cell expansion in a model of tumour immunity, and had no effect on the memory responses to tumours (729). Further, in a model of LCMV infection, Yi *et al* found that IL-21 had no quantitative effect on the primary CD8 T cell response though it did enhance recall responses upon viral rechallenge (793). A study by Barker *et al* also observed important enhancing effects of CD4-T-cell-derived IL-21 on both primary and memory CD8 T cell responses (794). My studies suggest that pulmonary IL-21 over-expression inhibits B cell as well as CD4 and CD8 T cell memory in the pulmonary compartment, a contrasting conclusion. The reasons for this difference are unknown at present, and warrant further investigation. Why is lymphocytic memory not supported by IL-21 over-expression in the lung? A study of memory T cell differentiation in a mouse model of malaria suggested a linear pathway of development with $CD27^{+}CD62L^{+}$ central memory T cells differentiating into $CD27^{+}CD62L^{-}$ 'early' effector memory, and finally to $CD27^{-}CD62L^{-}$ 'late' effector memory T cells (795). If this occurs in T cell responses to RSV then my data suggest that IL-21 blocks the central to 'early' effector memory transition in the pulmonary compartment, as the proportion of $CD44^{hi}CD62L^{+}$ central memory CD4 T cells was unaltered but there was a significantly reduced $CD44^{hi}CD62L^{-}$ effector memory CD4 T cell compartment. This would also explain the effects of IL-21 depletion on memory CD4 T cell responses after rVV-G-priming: IL-21 depletion allows maximal transition from the central memory stage to the effector memory, resulting in an increased pro-inflammatory T cell response, and increased disease. Further studies of T cell memory development in IL-21KO & IL-21Tg mice would address this possibility.

Another possibility is that IL-21 affects the fate decision of T cells to die by apoptosis or survive to become memory. Previous studies have shown that IL-21 can induce CD8 T cell

apoptosis (778), and B cell apoptosis (586,610). Therefore, it is possible that IL-21 also induces T cell apoptosis during respiratory viral infection. Apoptosis measurements (e.g. TUNEL staining) of infiltrating cells during RSV vs RSV-IL-21 challenge would help answer this question. IL-21 could be directly inhibitory on T and B cells but the data from my studies do not fully support this. Certainly IL-21 depletion enhanced T cell responses, particularly CD4 T cells, but equally effects on DCs could account for the observations. Further, IL-21 depletion reduced B cell influx and virus-specific antibody secretion in the prime and challenge model, suggesting it is necessary despite the availability of increased CD4 T cell help.

Reduced chemokine production may also account for the specific memory loss in the lung, and may explain why there is intact memory in one compartment but not another. Chemokine administration (e.g. CXCL10, CCL20) into the BALF in RSV-IL-21-challenged mice would help demonstrate whether chemokine loss was responsible for the lack of T cell or DC influx.

Another promising mechanism is that IL-21 inhibits DC numbers in the BALF by inhibiting DC influx or survival in this compartment. If DC maturation and/or migration is negatively affected by IL-21, then naïve T cell activation in the mediastinal lymph nodes would be reduced and little migration to the BALF would occur. Consequently, there would be fewer T cells to form memory, not just in the BALF but also in the draining lymph nodes (an organ not tested in my studies). As there is less T cell help for B cells, antibody production was reduced and memory B cells did not form. Effects of other innate cells (e.g. macrophages) cannot be ruled out at this stage, and selective depletion studies of different cell types (particularly professional APCs) prior to RSV challenge would help ascertain which cells are

important for memory formation in this model. There may also be knock-on effects on B cell memory also. The specific effects of IL-21 on highlighted cell types can then be focused on.

The failure to generate an effector T cell memory population in the lung had detrimental effects on protection against viral rechallenge. Mice previously challenged with RSV-IL-21 exhibited increased T cell activation and more severe disease. There are two likely mechanisms for this phenotype: reduced virus-specific antibody levels failed to protect against rechallenge, and/or a pathogenic role for central memory T cells. Both of these possibilities are supported by previous studies. Early studies by Graham et al investigated the immunological determinants that correlated with RSV clearance upon rechallenge (796). Clearance correlated with the presence of lymphocytic aggregates around the bronchovascular bundles, and RSV-specific antibody levels. Depletion studies by the same group confirmed their importance (332). Depletion of either T cell subset during primary RSV challenge delayed viral clearance and reduced disease severity upon secondary RSV challenge. The necessity of antibody in protection against RSV was confirmed by other studies by Graham et al when they demonstrated that antibody depletion during primary challenge did not significantly affect viral clearance during primary challenge but delayed viral clearance, and increased disease severity, during secondary RSV challenge (797).

A recent study by Teijaro *et al* demonstrated similar mechanisms in influenza infection. Adoptive transfer of lung memory T cells, from mice challenged with influenza, into naïve recipients protected them upon challenge with the same strain, and enhanced viral clearance. However, transfer of splenic memory T cells failed to protect and exacerbated disease (746). Central memory T cells vigorously expanded in lung and spleen, increased weight loss and mortality in challenged mice, and compromised viral clearance. Those

differences observed by Teijaro *et al* are very similar to those observed in my studies after RSV-IL-21 challenge and indicate a similar mechanism (though I have no evidence that the infiltrating cells upon RSV rechallenge originated from the spleen). In my study IL-21 was administered locally, therefore systemic IL-21 administration may reduce memory development systemically rather than just the pulmonary compartment. This may allow enhanced memory retention in the lung and increased protection both from T cells and antibody production by B cells. These studies do not rule out effects by other cell types on the balance of pulmonary T cell memory after RSV-IL-21 challenge. It would be of interest to deplete specific cell types (e.g. macrophage depletion using chlodronate liposomes) in the pulmonary compartment after convalescence and determine if the balance of memory T and B cell populations is restored.

The results of this chapter extend the findings of the previous chapters. IL-21 has a crucial role in limiting T cell activation during respiratory viral infection. This may be a direct effect or mediated via inhibition of DC maturation, migration, and antigen presentation in the draining lymph nodes. This results in a lack of effector T cell memory in the lung tissue, as well as reduced B cell help and a failure to generate optimal virus-specific antibodies. Consequently, the host has reduced protection against rechallenge with an antigenically-syngeneic virus. Further work is required to fully elucidate the immunological mechanisms responsible for the observed effects. The data presented here do not address all possibilities, and definitively confirm how IL-21 acts during respiratory viral challenge. However, this study has highlighted the prospect of utilising IL-21 to control primary T cell responses in the pulmonary compartment, and alleviating disease, possibly in conjunction with other soluble factors.

K. Conclusions

My original hypothesis was that IL-21 potentiated CD8 T cell activity, as previous studies had clearly demonstrated this. In cancer studies, IL-21 enhanced the cytotoxic activity of NK cells, NKT cells, and CD8 T cells to increase regression of cancerous tissue. Thus, IL-21 was clearly beneficial in anti-cancer responses and remains a very exciting therapeutic target. Similar effects have been observed in models of chronic viral infection such as LCMV and HIV. In respiratory disease however, CD8 T cells are the main cause of loss of lung function after severe respiratory viral infection. As this is the major cause of clinical burden in respiratory disease, its alleviation by inhibiting CD8 T cell activation and function, without significantly affecting viral clearance, was a major therapeutic goal. Given its described effects, IL-21 it was an attractive candidate to achieve this.

The principal finding of my studies however, and perhaps most surprising, is that IL-21 plays a crucial role in limiting CD4 T cell responses to RSV infection, not CD8 T cells. In particular, it limits effector memory responses. How does it do this? My data point to multiple mechanisms including inhibiting chemokine secretion, and reducing T cell activation by DCs. This is described pictorially in Figure 4.1. Therefore, in the context of respiratory viral disease IL-21 has considerable therapeutic potential to alleviate disease, though my data suggest that IL-21 over-expression alone is insufficient and may need to form part of a combination therapy. However, my data do not conclusively prove any of the above and clearly further work is required to fully elucidate the mechanisms and therapeutic potential of IL-21.

Why does IL-21 function in such a distinct way during RSV infection compared to cancer studies? This is likely due to the nature of the diseases. The response to RSV is acute,

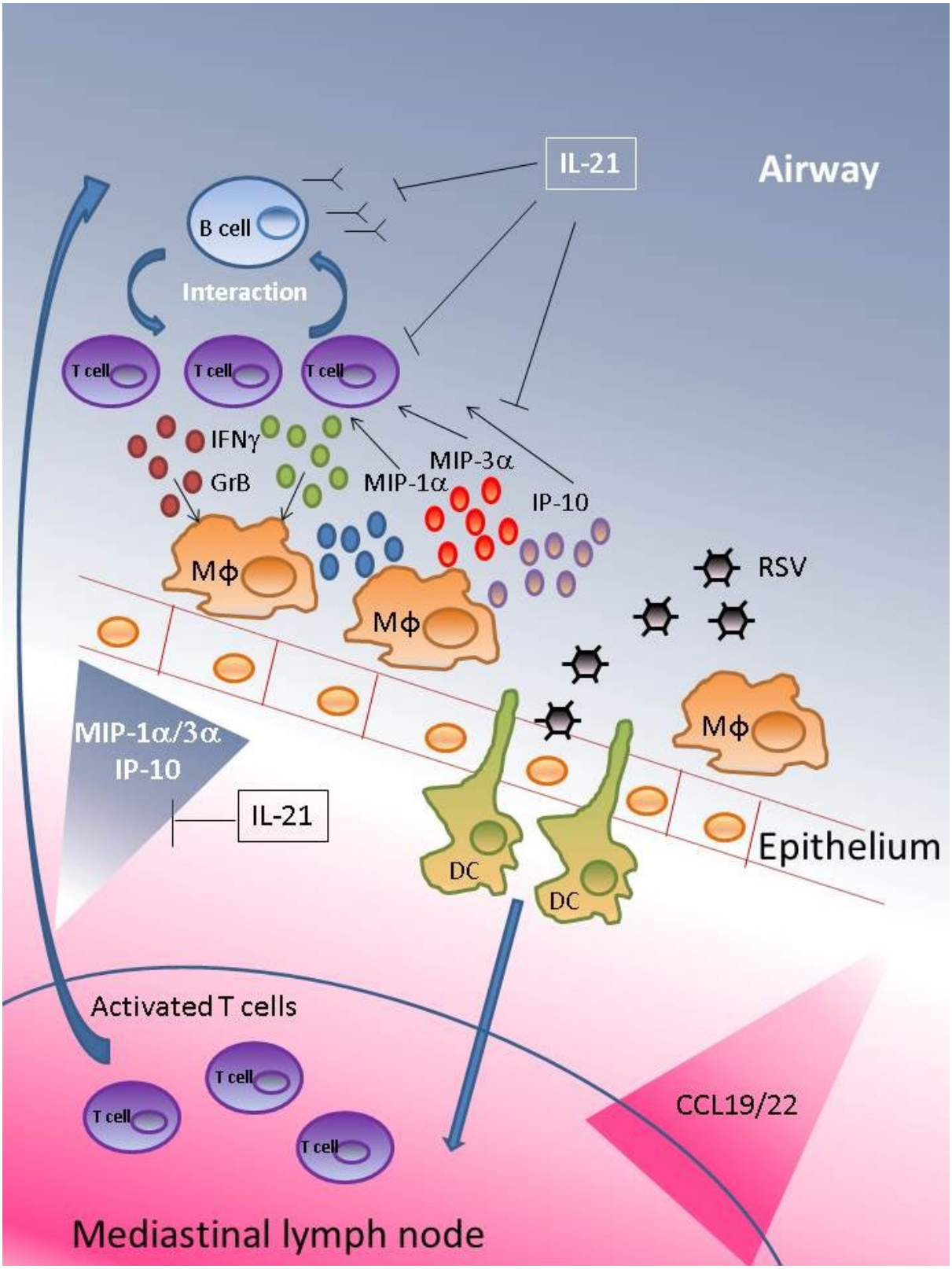

Figure 4.1. The effects of IL-21 on T cell responses to Respiratory Syncytial Virus (RSV). IL-21 inhibits macrophage-derived chemokines necessary to recruit activated T cells to the site of infection. Consequently, there is a lack of T-cell-derived pro-inflammatory mediators (e.g. IFN-γ and granzyme B) that elicit disease. The lack of T cells results in a lack of T cell help for B cells reduces antibody production, and memory T cell development. This negatively impacts on protection against rechallenge.

complete within two weeks of viral challenge, whereas responses to cancer may be months or years in length. Therefore, the requirement for continuous T cell support is paramount in anti-cancer responses but not so for anti-RSV responses. Consequently, other functions of IL-21 may be more critical. The lung may also be a site of unique immunology, given its continuous exposure to the environment for example. As such, the anti-inflammatory functions of IL-21 may be more important at this site than at other sites that are not directly exposed. Of the γc chain cytokine members, IL-21 is the only one that signals mainly via STAT3 (IL-2, -7, and -15 signal via STAT5, whereas IL-4 and -9 via STAT6) as do the anti-inflammatory cytokines IL-10 and IL-22, which may be indicative of its more anti-inflammatory nature.

What are the major anti-inflammatory functions of IL-21 in the context of RSV disease? The data from this study suggest three primary mechanisms: reduction in chemokine secretion, blockade of DC migration, and enhancement of regulatory T cell recruitment and/or function. However, these may not operate simultaneously, nor may they operate equally throughout a genetically heterogeneous human population. Moreover, the use of the rVV-G prime and challenge model may not accurately reflect memory responses derived from natural viral challenge. Therefore, further work using RSV challenge alone is recommended to confirm the findings of this study. It would also be of interest to establish if the IL-21 functions described here are present in other respiratory viral disease (e.g. influenza). The existence of common pathways would enhance the possibility of targeting IL-21 therapeutically in the lung.

What are the future directions of IL-21 research in the context of respiratory infection? A consensus on the effects of IL-21 in respiratory disease are required, in particular the

conflicting data between IL-21 function in PVM vs RSV disease requires attention. Direct comparisons of the responses to these two viruses are necessary to determine the critical parameters that are responsible for the differing effects of IL-21. For example, IL-21 has been shown to induce DC apoptosis, which should reduce T cell activation. However, the same study found that GM-CSF can rescue these DCs and increase inflammation. Therefore, differences between seemingly similar respiratory viruses may be due to the balance of IL-21 and GM-CSF produced during their respective responses. These possibilities need to be addressed.

Another important obstacle to the use of IL-21 therapeutically is the lack of immunoprotection when IL-21 is over-expressed. Given the central role of T cells in loss of lung function, it seems logical to boost antibody-mediated protection and enhance long term protection. There are many immunological factors that boost B cell activation and antibody production, and it would be a significant undertaking to identify a factor that works in combination with IL-21 to alleviate RSV disease while boosting protection. Moreover, as RSV is mostly a disease of infants these factors must be effective in an immature immune system, a critical factor that has not been addressed in this study. Nevertheless, there is some support for IL-21 being beneficial in neonates. A recent study found that IL-21 was instrumental in protecting adults from hepatitis B disease. However, IL-21 is largely absent in neonates and may well be the reason why this age range suffer significantly more disease. Whether this protective role of IL-21 extends to RSV disease remains to be determined.

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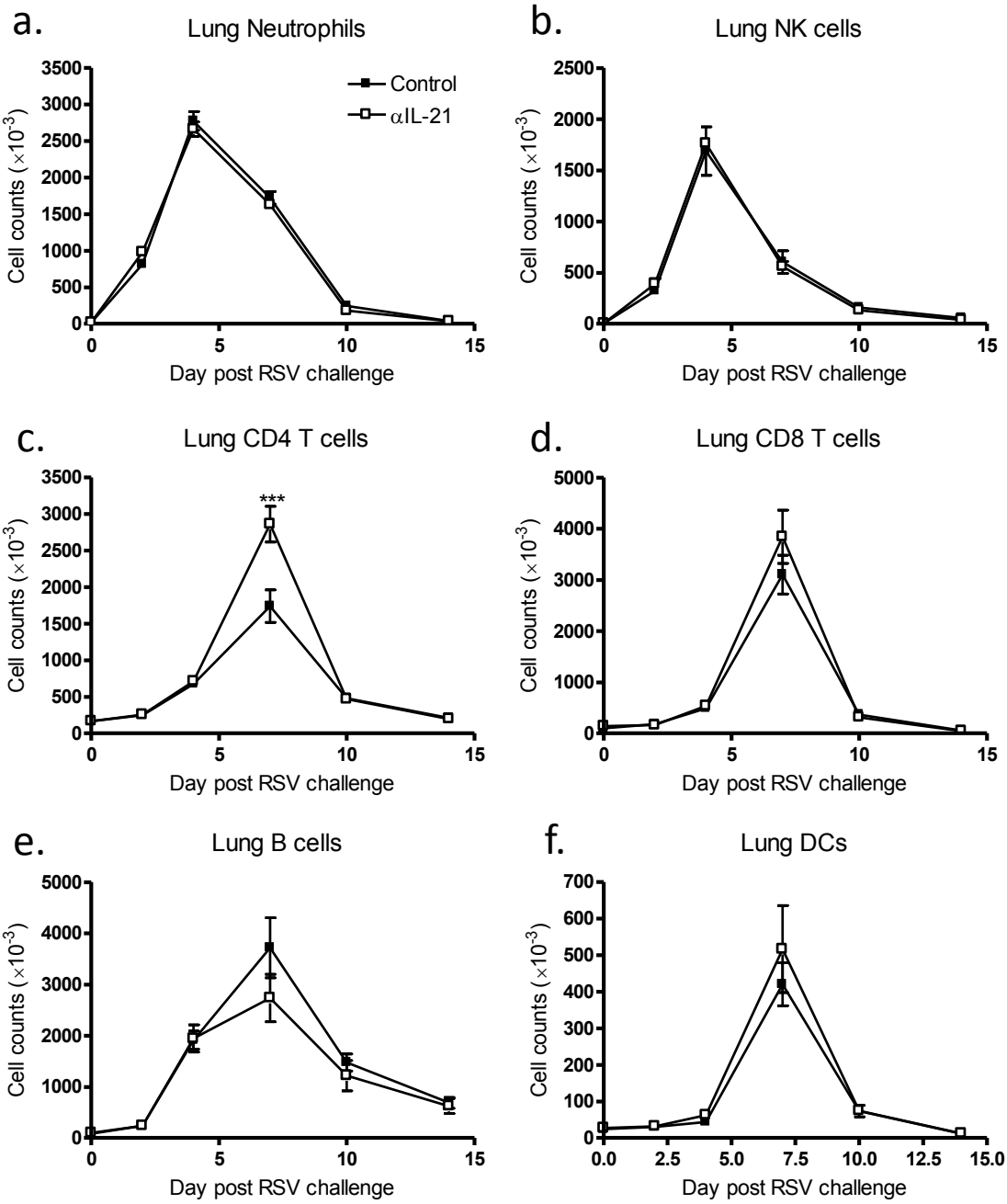
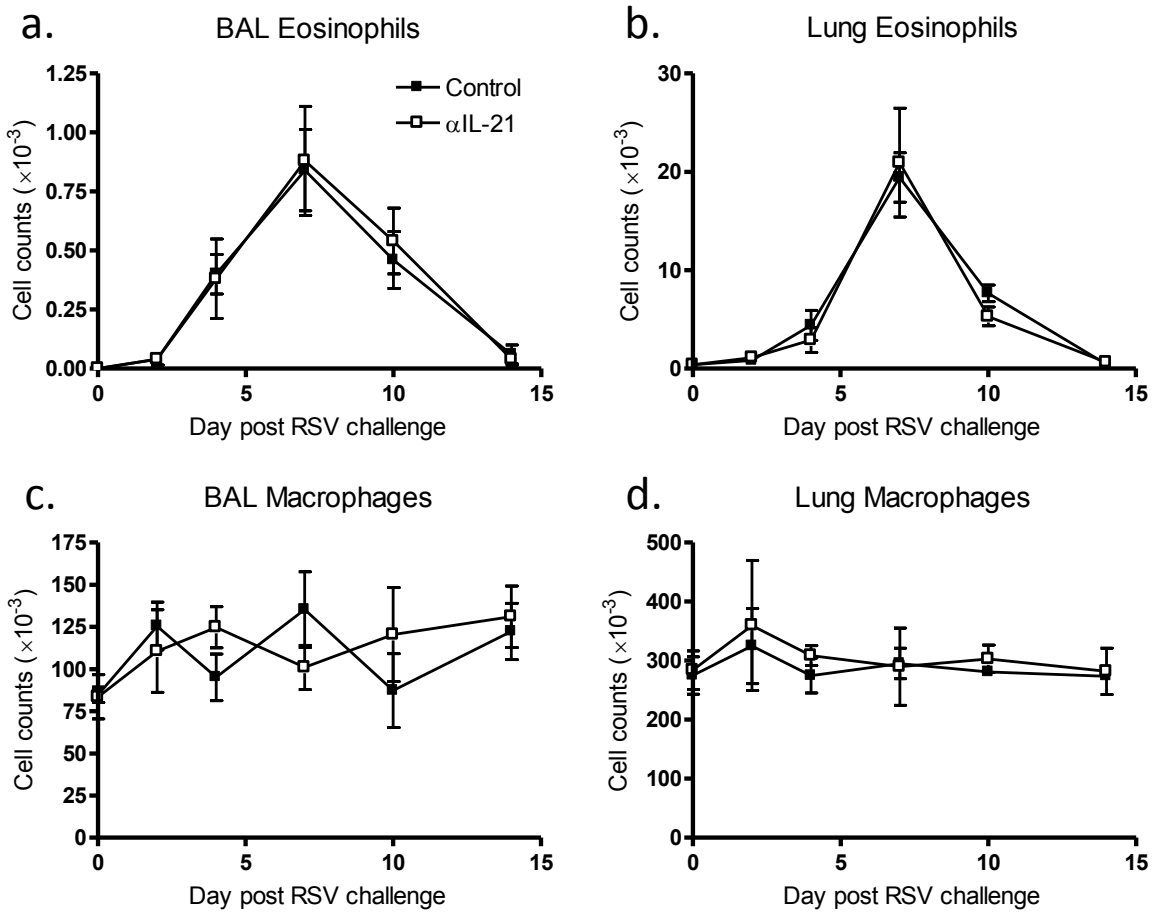
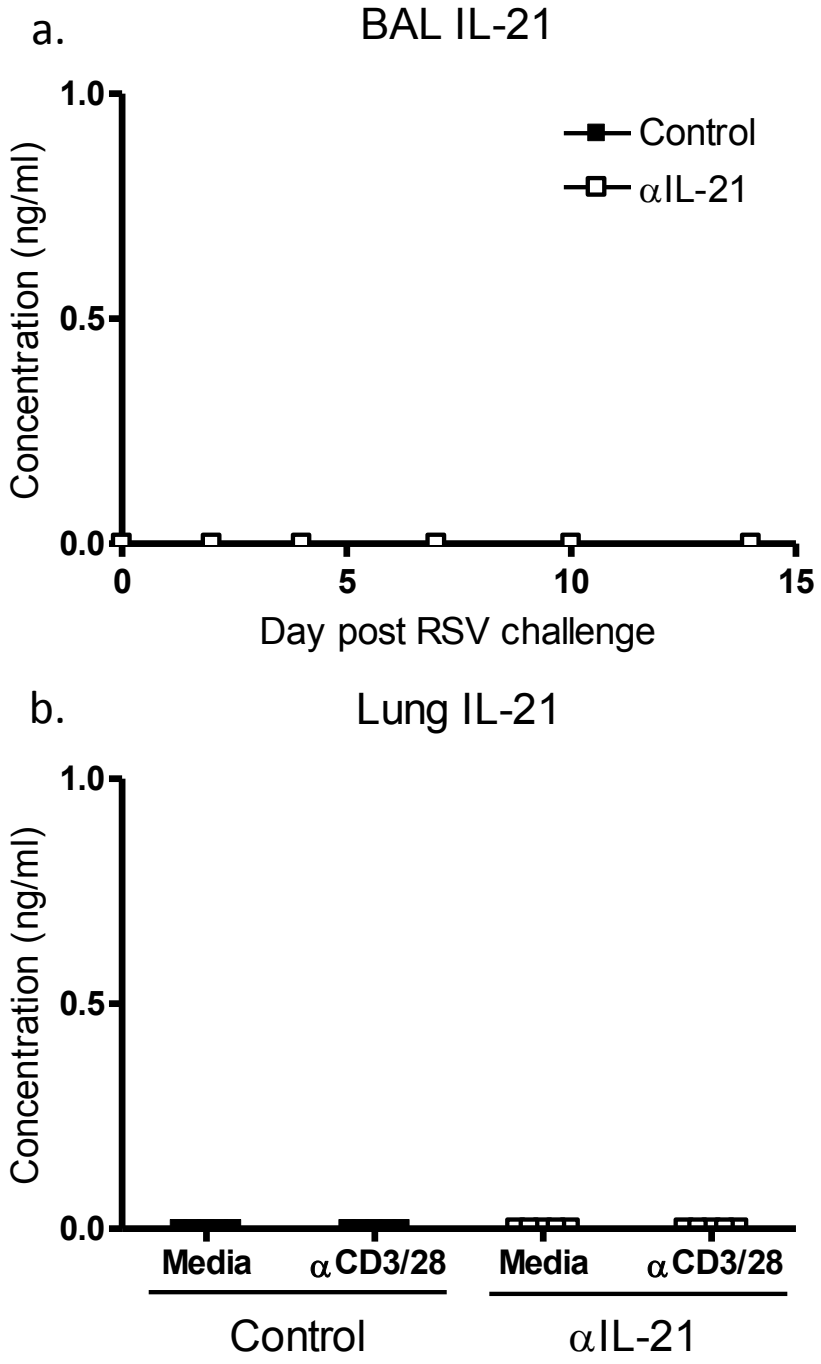
M. Appendix



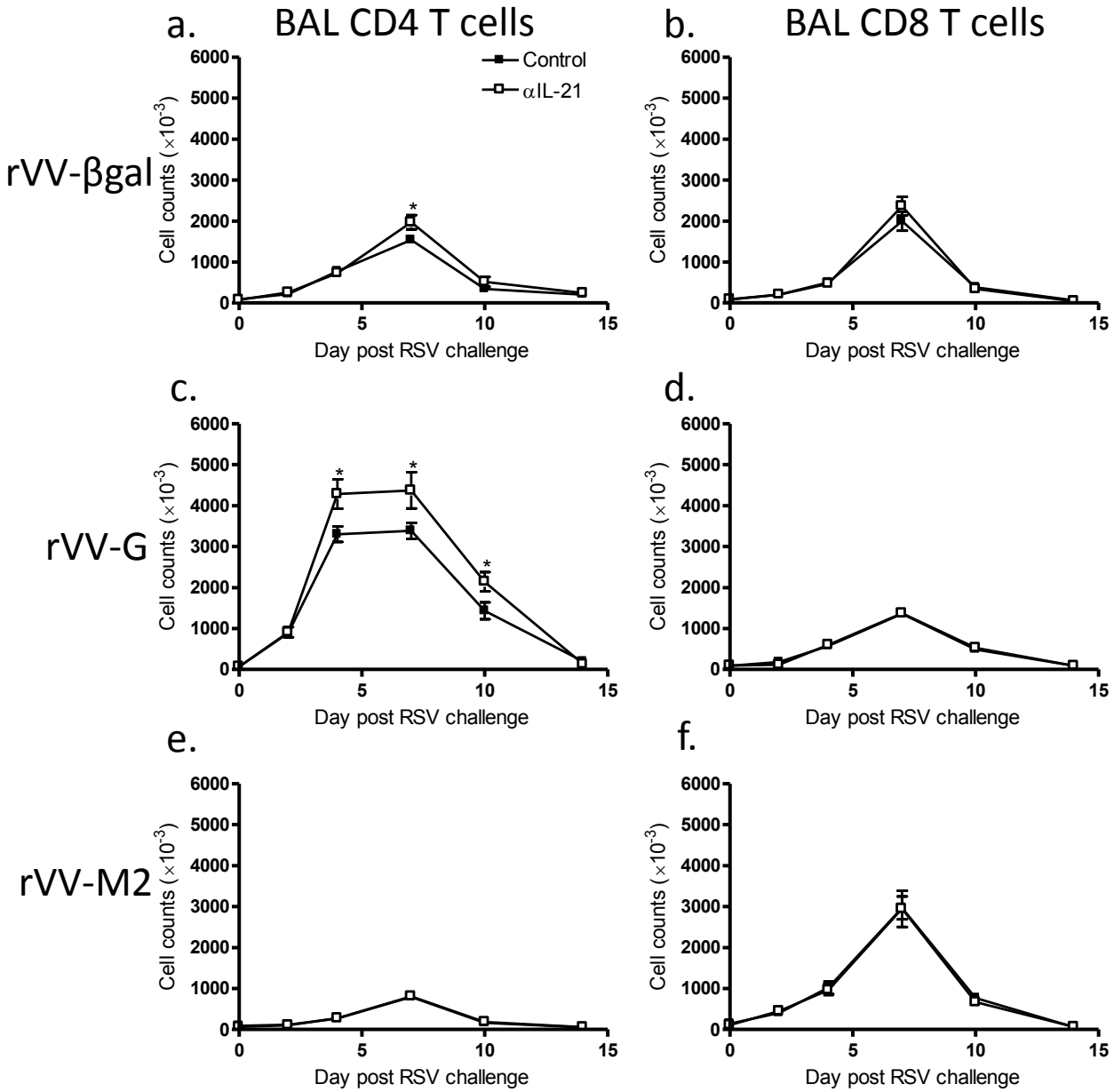
Appendix 1.1. IL-21 depletion increases CD4 T cell recruitment after primary RSV challenge. Mice were challenged with RSV on d0. Lung tissue was harvested on d0, 2, 4, 7, 10, and 14 post challenge. Samples were processed and lung cells were phenotyped by flow cytometry. Neutrophil (a), NK cell (b), CD4 T cell (c), CD8 T cell (d), B cell (e), and DC (f) cell counts determined. At least 50×10^3 cells/sample were collected. Data is expressed as mean±SEM. The graphs are representative of two independent experiments of five mice per group.



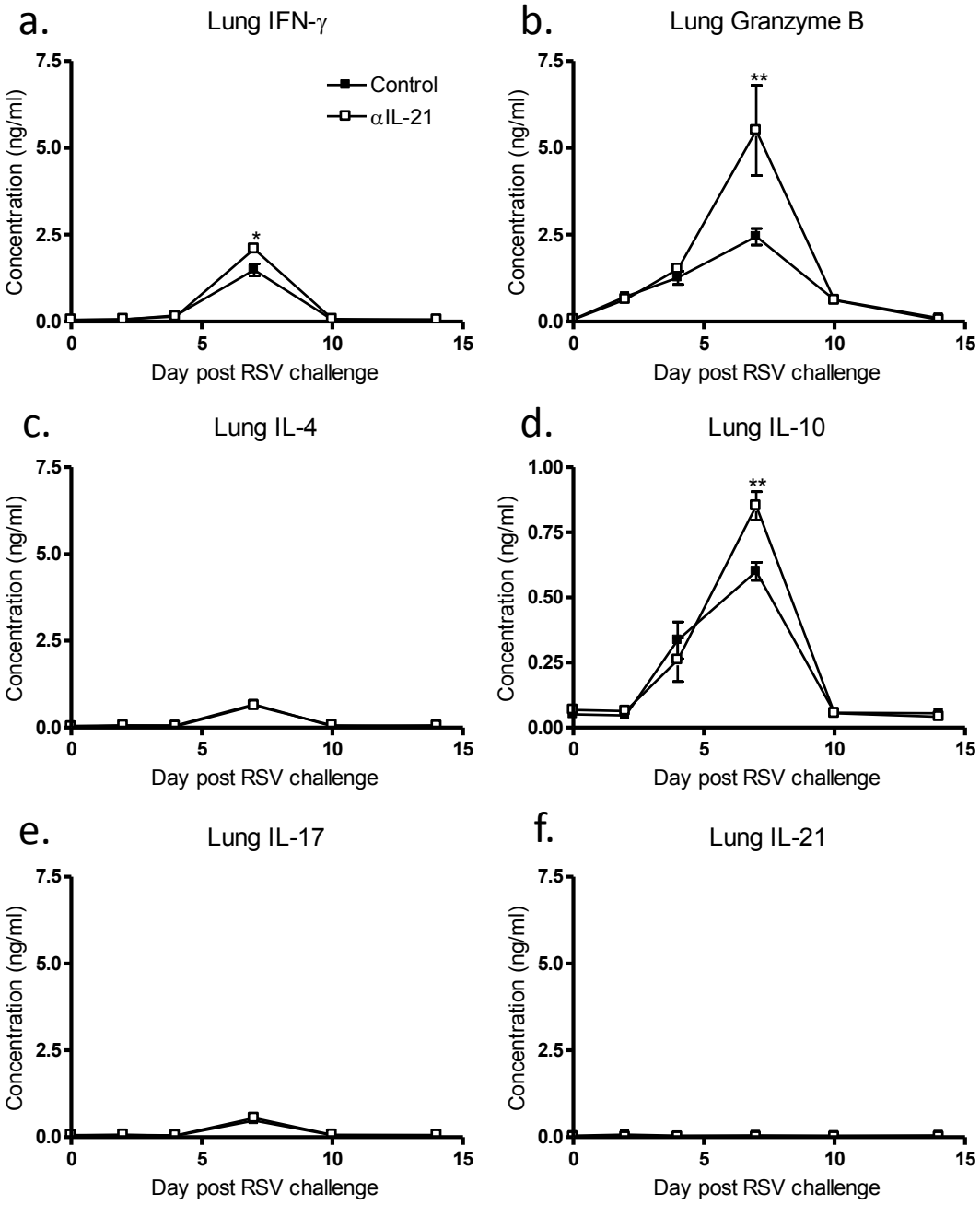
Appendix 1.2. IL-21 depletion has no effect on eosinophil and macrophage recruitment after primary RSV challenge. Mice were challenged with RSV on d0. BALF and lung tissue was harvested on d0, 2, 4, 7, 10, and 14 post challenge. Samples were processed and BALF and lung cells were phenotyped by flow cytometry. BAL (a), and Lung (b) eosinophil and BAL (c) and Lung (d) macrophage cell counts were determined. At least 50×10^3 cells/sample were collected. Data is expressed as mean±SEM. The graphs are representative of two independent experiments of five mice per group.



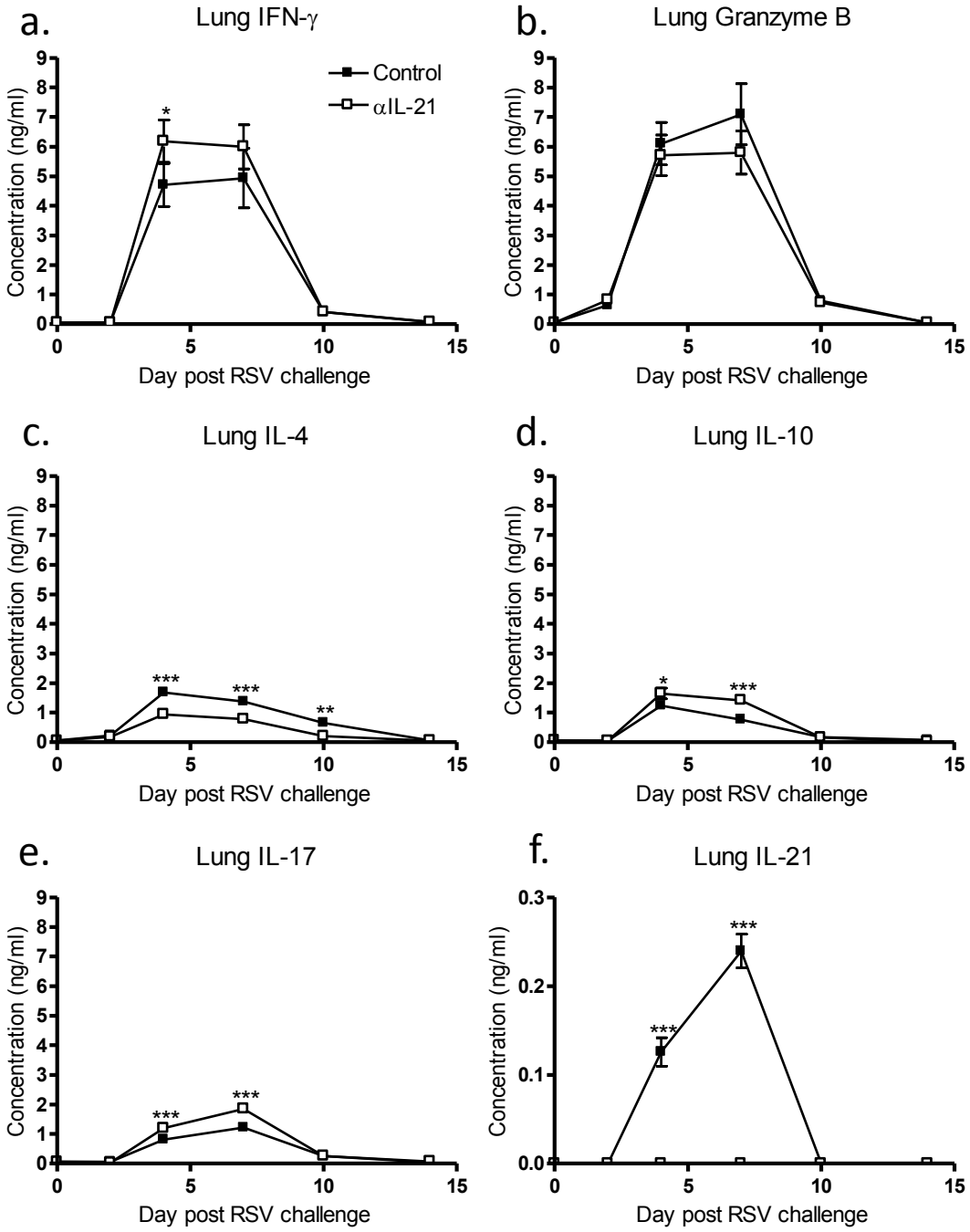
Appendix 1.3. IL-21 production is not detectable in the BALF or by stimulated lung cells after primary RSV challenge. Mice were challenged with RSV on d0. BALF was harvested on d0,2, 4, 7, 10, and 14 post challenge and IL-21 production (a) was determined by sandwich ELISA. Lung cells were harvested seven days post challenge, RBCs lysed, and single cell suspensions counted by trypan blue exclusion assay. Lung cells (2×10^6 cells/well) were stimulated with either media alone or αCD3/28-expressing beads (50µl/well) for 24hrs. Supernatants were harvested and IL-21 levels (b) determined by sandwich ELISA. Data is expressed as mean±SEM. The graphs are representative of three independent experiments of five mice per group.



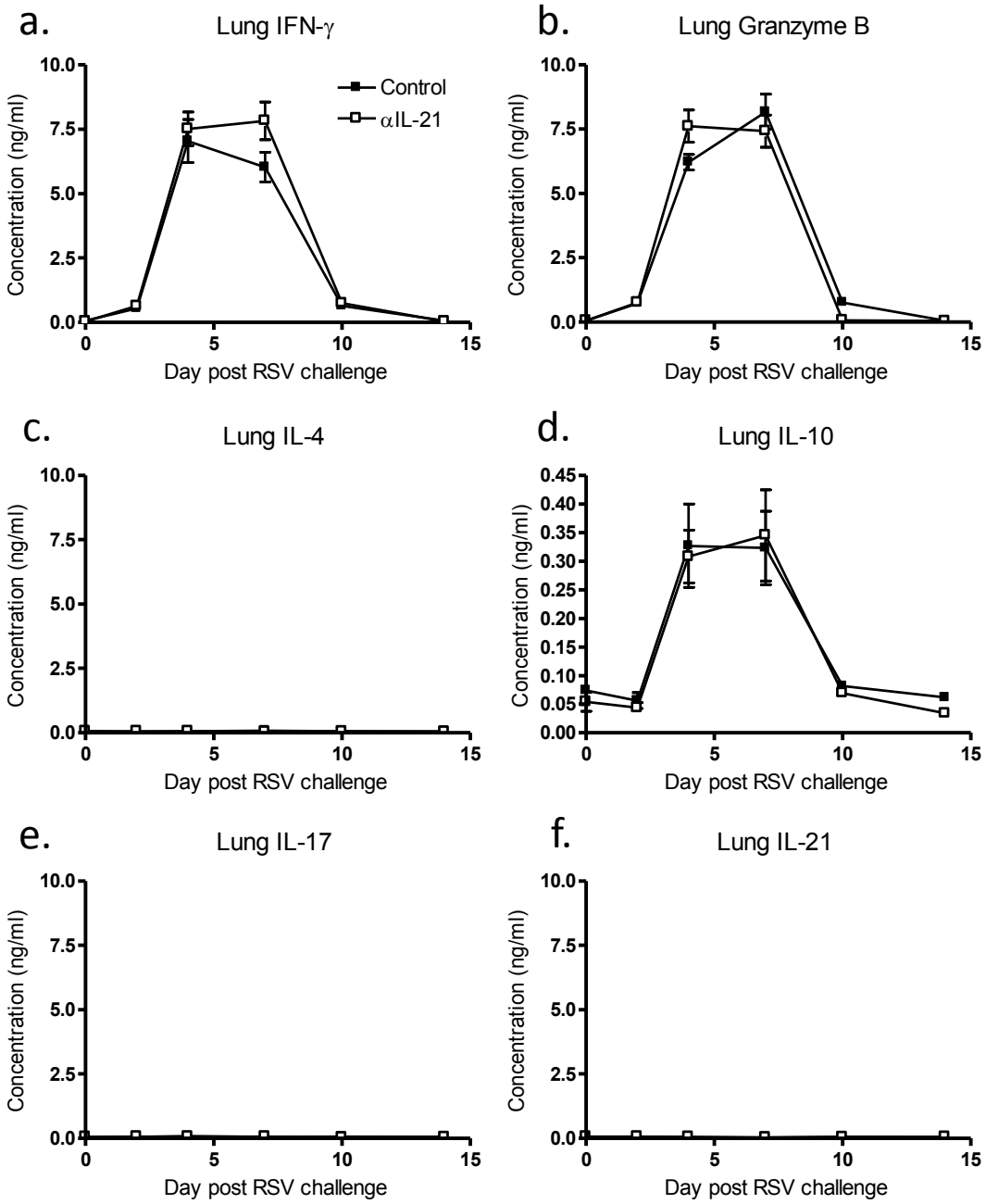
Appendix 2.1. IL-21 depletion significantly increases T cell recruitment in G-, but not M2-, primed mice after RSV challenge. Mice were immunised and challenged as described in Fig.2.8. Lung cells were harvested at several time points post challenge. Samples were processed, RBCs lysed, and live cells counted by trypan blue exclusion assay. Lung CD4 (a, c, e) and CD8 (b, d, f) T cells in rVV-βgal (a-b), rVV-G (c-d), and rVV-M2 (e-f) were phenotyped by flow cytometry and cell counts determined. At least 50×10^3 cells/sample were collected. Error bars represent SEM. The graphs are representative of three independent experiments of five mice per group. Student *t* test result *: $p<0.05$.



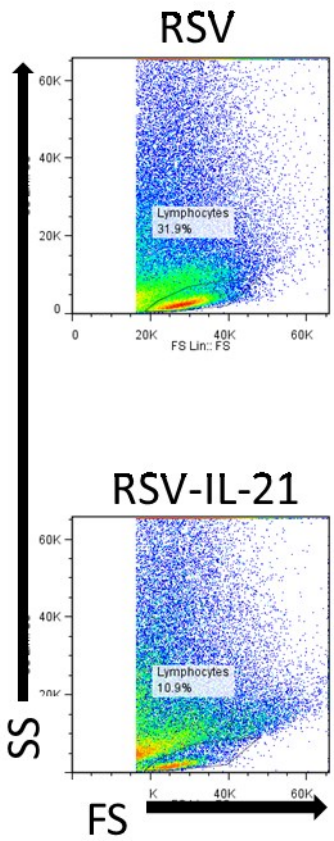
Appendix 2.2. IL-21 depletion during rVV-βgal priming increases IFN-γ, granzyme B, and IL-10 by lung cells after RSV challenge. Mice were immunised with rVV-βgal and challenged as described in Fig.2.8. Lung cells were harvested at several time points post challenge. Samples were processed, RBCs lysed, and live cells counted by trypan blue exclusion assay. Lung cells (2×10^6 cells/well) were stimulated with either media alone or αCD3/28-expressing beads (50µl/well) for 24hrs. Supernatants were harvested and IFN-γ (a), Granzyme B (b), IL-4 (c), IL-10 (d), IL-17 (e), and IL-21 (f) were determined by sandwich ELISA. Error bars represent SEM. The graphs are representative of three independent experiments of five mice per group. Student *t* test result *: $p<0.05$, **: $p<0.01$.



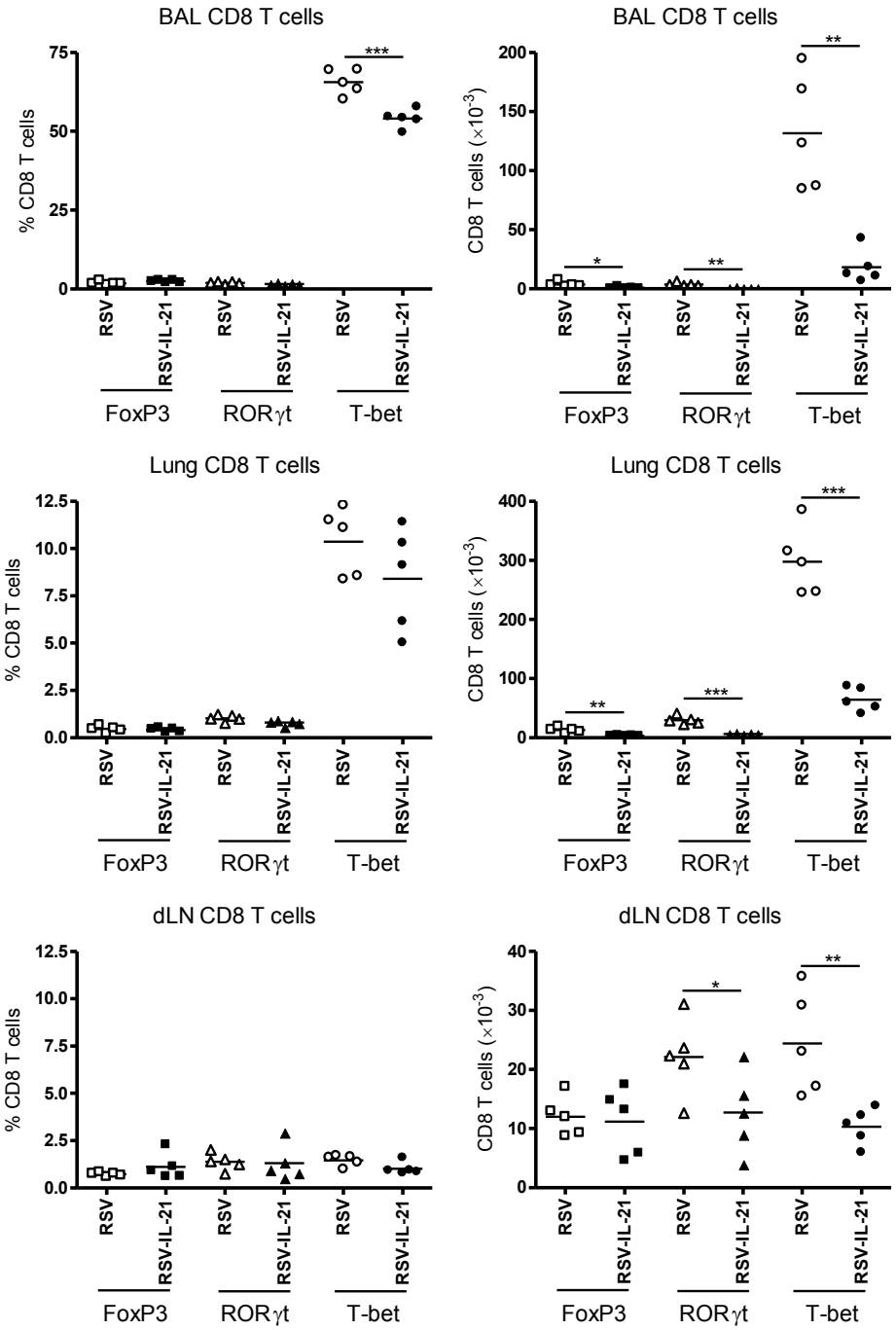
Appendix 2.3. IL-21 depletion during rVV-G priming increases IFN-γ, IL-10, and IL-17, and reduces IL-4 production by lung cells after RSV challenge. Mice were immunised with rVV-G and challenged as described in Fig.2.8. Lung cells were harvested at several time points post challenge. Samples were processed, RBCs lysed, and live cells counted by trypan blue exclusion assay. Lung cells (2×10^6 cells/well) were stimulated with either media alone or αCD3/28-expressing beads (50µl/well) for 24hrs. Supernatants were harvested and IFN-γ (a), Granzyme B (b), IL-4 (c), IL-10 (d), IL-17 (e), and IL-21 (f) were determined by sandwich ELISA. Error bars represent SEM. The graphs are representative of three independent experiments of five mice per group. Student *t* test result *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.



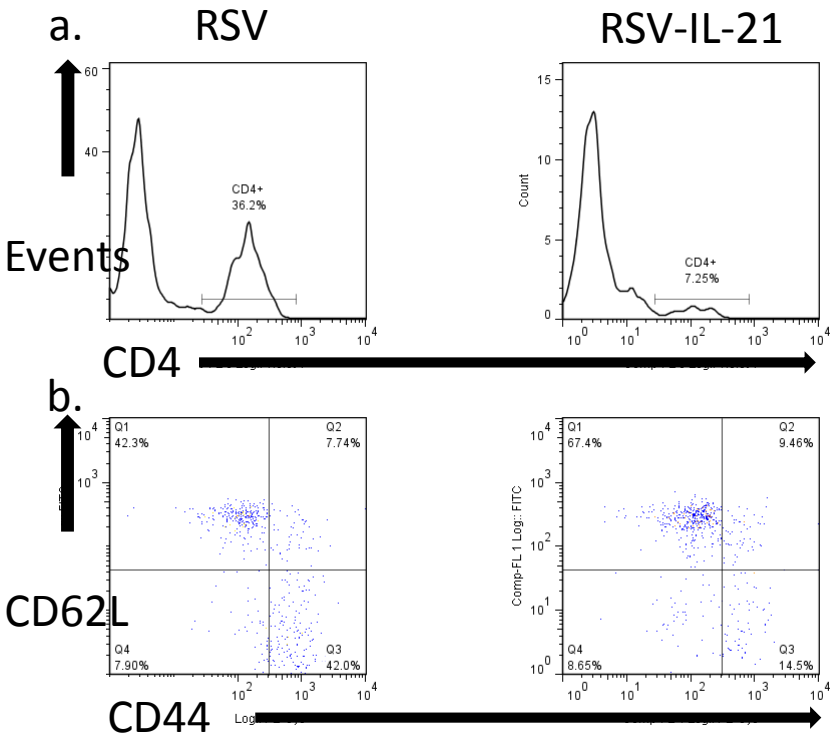
Appendix 2.4. IL-21 depletion during rVV-M2 priming has no effect on cytokine production by lung cells after RSV challenge. Mice were immunised with rVV-M2 and challenged as described in Fig.2.8. Lung cells were harvested at several time points post challenge. Samples were processed, RBCs lysed, and live cells counted by trypan blue exclusion assay. Lung cells (2×10^6 cells/well) were stimulated with either media alone or αCD3/28-expressing beads (50µl/well) for 24hrs. Supernatants were harvested and IFN-γ (a), Granzyme B (b), IL-4 (c), IL-10 (d), IL-17 (e), and IL-21 (f) were determined by sandwich ELISA. Error bars represent SEM. The graphs are representative of three independent experiments of five mice per group.



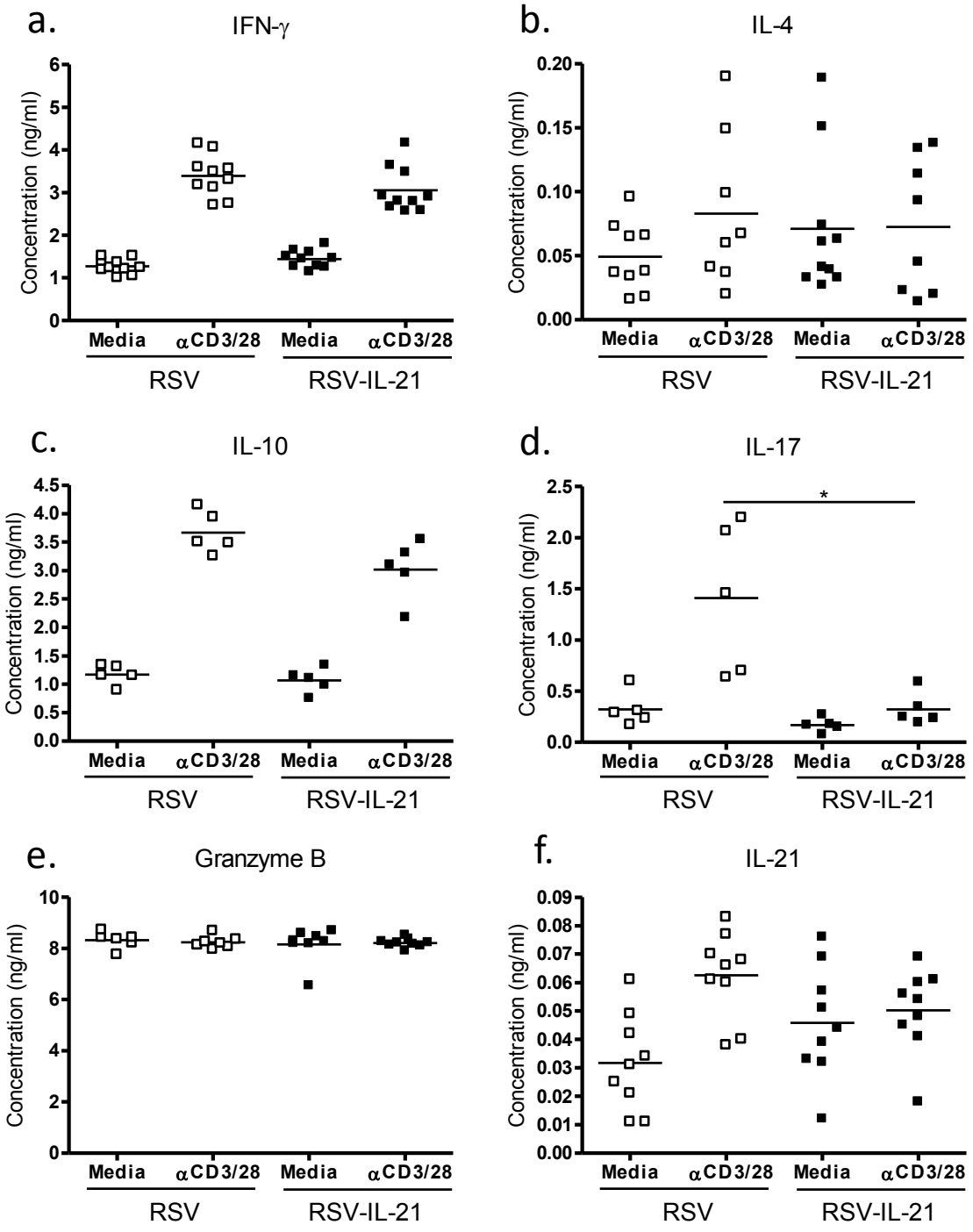
Appendix 3.1. IL-21 expression significantly inhibits cell recruitment after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^{6} pfu/mouse; i.n) on d0. BALF cells were harvested seven days post challenge. Samples were processed and total cell counts in the BALF determined. Forward (FS) and side scatter plots (SS) were phenotyped by flow cytometry. At least 50×10^{3} cells/sample were collected. The plots are representative of two independent experiments of five mice per group.



Appendix 3.2. IL-21 expression significantly reduces T-bet expression by CD8 T cells after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^6 pfu/mouse; i.n) on d0. BALF (a-b), lungs (c-d), and mediastinal lymph nodes (e-f) were harvested seven days post challenge and processed. CD8 T cells were stained for FoxP3, RORγt, or T-bet according to the manufacturer's instructions. The percentage of CD8 T cells expressing each transcription factor was determined by flow cytometry. Grouped data for percentage (a, c, e) and total number (b, d, f) is also shown. The graph is representative of two independent experiments of five mice per group. Student t-test result *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.



Appendix 3.3. IL-21 expression inhibits effector, but not central, memory T cell development in the lung tissue after primary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^{6} pfu/mouse; i.n) on d0. Lungs were harvested 28 days post challenge and samples processed. CD44 and CD62L expression (b) on $CD4^{+}$ cells (a) were phenotyped in the lung by flow cytometry. At least 50×10^{3} cells/sample were collected. The plots are representative of two independent experiments of five mice per group.



Appendix 3.4. IL-21 expression during primary RSV challenge increases cell recruitment to lung tissue upon secondary RSV challenge. Mice were challenged with RSV or RSV-IL-21 (1×10^6 pfu/mouse; i.n) on d0. Mice were left for 28 days and then rechallenged with RSV (1×10^6 pfu/mouse; i.n). Lung cells were harvested four days post challenge and samples processed. Lung cells (2×10^6 cells/well) were stimulated with either media alone or αCD3/28-expressing beads (50µl/well) for 24hrs. Supernatants were harvested and IFN-γ (a), IL-4 (b), IL-10 (c), IL-17 (d), Granzyme B (e), and IL-21 (f) were determined by sandwich ELISA. Data is expressed as mean±SEM. The graphs are representative of two independent experiments of five mice per group. Student *t* test result *: $p<0.05$.