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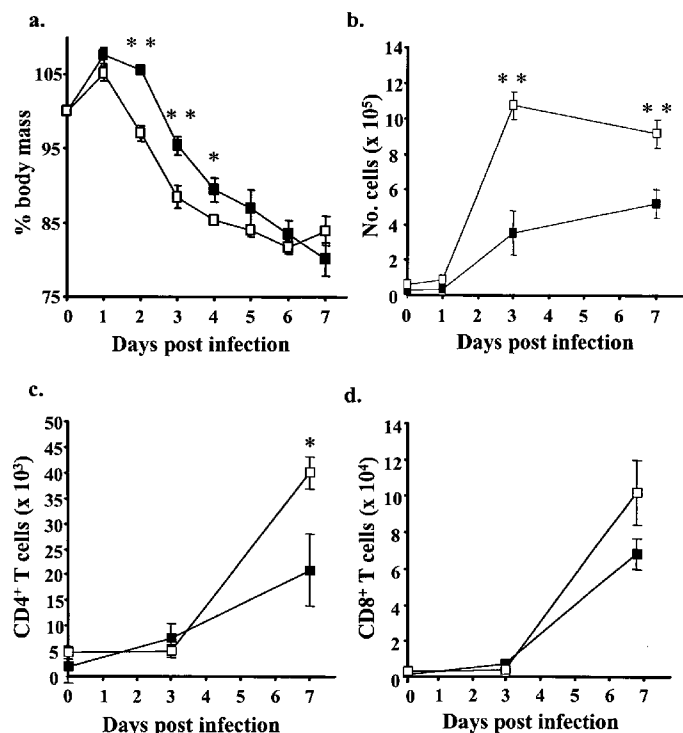
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(54) Title: COMPOSITIONS AND METHODS RELATING TO MANIPULATION OF THE MYELOID IMMUNE COMPARTMENT DURING RESPIRATORY INFECTION

Figure 3.8



(57) Abstract: The invention relates to therapeutic compositions for use in treating, ameliorating or preventing an infection such as a microbial infection in a subject, comprising a compound modulative of reactive oxygen species level, or CD200 level, or CD200R level, or a combination of two or more of said levels in said subject. The invention also relates to methods of treatment using such compositions. In addition, the invention relates to therapeutic compositions for use in treating, ameliorating or preventing an inflammatory pulmonary infection in a mammalian host having a CD200-CD200R axis comprising a compound modulative of said axis, and to methods of treatment using such compositions. Furthermore, the invention relates to therapeutic compositions for use in treating, ameliorating or preventing a microbial infection and/or pulmonary inflammation in a subject comprising a compound mimetic of superoxide dismutase, and to methods of treatment using such compositions.



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**Compositions and methods relating to manipulation of the myeloid
immune compartment during respiratory infection.**

The present invention relates generally to therapeutic compositions and methods for use in treating, ameliorating or preventing an infection, for example a pulmonary infection, in a subject. The therapeutic compositions comprise compounds that are capable of modulating the level of components of the myeloid immune response.

Introduction

Acute respiratory infections are a significant cause of global morbidity and mortality. Traditional strategies to combat such infections are vaccination and administration of anti-microbial agents, but these are frequently lacking in their efficacy and safety. In this thesis we examine the alternative strategy of manipulating the excessive immune response that occurs upon lung infection. Influenza is a viral respiratory pathogen that evokes a potent Th1 driven response and significant immunopathology. Conversely, *Cryptococcus neoformans* is an encapsulated yeast, which induces a non-protective Th2 immune response that fails to eradicate the pathogen and ultimately culminates in dissemination to the brain and meningoencephalitis. Here we describe the therapeutic benefit of manipulating the myeloid immune compartment.

The phagocyte NADPH oxidase assists in combating bacterial and fungal infections through the generation of superoxide radical. We demonstrate a role for NADPH oxidase (using knockout mice, Cybb tm1 mice) in macrophage homeostasis and the subsequent Th1 driven immunity in the airways during influenza infection resulting in improved viral clearance. Similarly, the non-protective Th2 response to *C. neoformans* is heavily skewed to a protective Th1 granulomatous response that promotes immunity to the fungus and limits dissemination to the brain. Similar results are obtained using a novel manganic porphyrin that scavenges reactive oxygen species.

CD200, through it's interaction with the myeloid specific CD200 receptor, delivers an inhibitory signal to the myeloid compartment. Our understanding of the basic biology

regarding the expression and function of this inhibitory interaction is still in its infancy. Here, we investigate these parameters both *in vitro* and *in vivo* in the context of respiratory infections. Subsequently, we elucidate how manipulation of such an interaction can modulate inflammation during respiratory infection with influenza and
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Abbreviations

A. fumigatus *Aspergillus fumigatus*

Ab Antibody

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	ADCC	Antibody dependent cellular cytotoxicity
	Ag	Antigen
	AICD	Activation induced cell death
	AIDS	Acquired immunodeficiency syndrome
5	AMB	Amphotericin B
	AP-1	Activating protein-1
	APC	Allophycocyanin
	APC	Antigen presenting cell
	ATP	Adenosine Triphosphate
10	BAL	Bronchoalveolar Lavage
	BALT	Bronchial associated lymphoid tissue
	BCG	Bacille Calmette-Guerin
	BCR	B Cell Receptor
	BH	Benjamini Hochberg
15	BM	Bone marrow
	Bp	Base pairs
	BSA	Bovine Serum Albumin
	<i>C. neoformans</i>	<i>Cryptococcus neoformans</i>
	CBA	Cytometric bead array
20	CD	Cluster of differentiation
	CD200:Fc	Mouse CD200:mouse IgG1 fusion protein
	CD200R	CD200 receptor
	CFSE	5-carboxyfluorescein diacetatesuccinimidyl ester
	CFU	Colony Forming Unit
25	CGD	Chronic granulomatous disease
	CIA	Collagen induced arthritis
	CNS	Central nervous system
	CpG	Cytosine Guanine
	CR	Complement receptor
30	CTL	Cytotoxic T cell
	CTLA-4	Cytotoxic T lymphocyte antigen-4
	Cy	Cychrome

	Cybb	Cytochrome b beta polypeptide
	DALY	Disability adjusted life year
	DC	Dendritic cell
	DMEM	Dulbecco modified eagle's medium
5	DNA	Deoxyribonucleic Acid
	ds	Double Stranded
	EAE	Experimental autoimmune encephalitis
	EAU	Experimental autoimmune uveoretinitis
	ELISA	Enzyme Linked Immunosorbent Assay
10	FAD	Flavin adenide dinucleotide
	FasL	Fas Ligand
	Fc	Fragment crystallisable
	FCS	Foetal calf serum
	FITC	Fluorescein Isothiocyanate
15	fMLP	N-Formyl-L-Methionyl-L-Leucyl-L-Phenylalanine
	FSC	Forward Scatter
	GM-CSF	Granulocyte macrophage-colony stimulating factor
	GXM	Glucuronoxylomannan
	HA	Haemagglutinin
20	H and E	Haematoxylin and Eosin
	HEPES	N-(2-Hydroxyethyl) Piperazine-N'-(2-Ethanesulphonic acid)
	HIV	Human immunodeficiency virus
	HLA	Human Leukocyte Antigen
	HPLC	High Performance Liquid Chromatography
25	HRP	Horse radish peroxidase
	HSP	Heat Shock Protein
	ICOS	Inducible co-stimulatory molecule
	ICOSL	ICOS co-stimulatory molecule ligand
	IDO	Indoleamine-2,3-dioxygenase
30	IFN	Interferon
	Ig	Immunoglobulin
	IgSF	Immunoglobulin superfamily

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	Ion	Ionomycin
	IL	Interleukin
	IRF	Interferon regulatory factor
	ITAM	Immunoreceptor tyrosine based activation motif
5	ITIM	Immunoreceptor tyrosine based inhibitory motif
	kb	Kilobase
	kD	Kilodalton
	KLH	Keyhole limpet haemocyanin
	L	Ligand
10	LAP	Latency associated protein
	LDH	Lactose dehydrogenase
	LN	Lymph node
	LPS	Lipopolysaccharide
	LTA	Lipoteichoic acid
15	LTK63	Modified heat labile toxin of <i>Escherichia coli</i>
	M	Mol
	M cell	Microfold cell
	MAC	Membrane Attack Complex
	MALT	Mucosal associated lymphoid tissue
20	MBL	Mannose-Binding Lectin
	MCP-1	Monocyte Chemotactic Protein-1
	M-CSF	Macrophage-colony stimulating factor
	MDCK	Madine Darby canine kidney
	MEM	Minimal essential medium
25	MHC	Major Histocompatibility Complex
	MIR	Macrophage immunoglobulin-like receptor
	MLN	Mediastinal lymph node
	MMP	Matrix Metalloprotease
	MnTE-2-PyP	Manganese (III) tetrakis (N-ethyl pyridinium-2-yl) porphyrin
30	mRNA	Messenger Ribonucleic Acid
	MW	Molecular weight
	μ g	Microgram

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	NA	Neuraminidase
	NADPH	Nicotinamide adenine dinucleotide phosphate
	NALT	Nasal associated lymphoid tissue
	NaOH	Sodium Hydroxide
5	NK- $\kappa\beta$	Nuclear factor- $\kappa\beta$
	Ng	Nanogram
	NK cell	Natural killer cell
	NO	Nitric oxide
	OD	Optical Density
10	OX40L	OX40 ligand
	OX40L:Ig	mouse OX40 ligand:mouse IgG1 fusion protein
	PAMP	Pathogen-Associated Molecular Patterns
	PBMC	Peripheral Blood Mononuclear Cells
	PBS	Phosphate Buffered Saline
15	PCA	Passive cutaneous anaphylaxis
	PCR	Polymerase chain reaction
	PE	Phycoerythrin
	PercP	Peridinin Chlorophyll Protein
	PFU	Plaque forming unit
20	Pg	Picogram
	Phox	Phagocyte NADPH oxidase
	pIg	Polymeric immunoglobulin
	pIgR	Polymeric immunoglobulin receptor
	PK	Protein Kinase
25	PLC	Phospholipase C
	PMA	Phorbol-12-Myristate-13-Acetate
	PRR	Pattern Recognition Receptors
	R	Receptor
30	RANTES	Regulated upon Activation in Normal T cells, Expressed and Secreted
	RBC	Red blood cell
	RNA	Ribonucleic acid

	RNS	Reactive nitrogen species
	ROS	Reactive Oxygen Species
	RPMI	Roswell Park Memorial Institute
	RSV	Respiratory syncytial virus
5	<i>S. aureus</i>	<i>Staphylococcus aureus</i>
	SD	Standard deviation
	SEM	Standard Error of the Mean
	SH	Src homology
	SHIP	SH2-containing inositol phosphatase
10	SIgA	Secretory immunoglobulin A
	SIRP	Signal regulatory protein
	SOD	Superoxide dismutase
	STAT	Signal transducers and activators of transcription
	TCR	T Cell Receptor
15	TGF- β	Transforming Growth Factor- β
	Th1	T helper 1
	Th2	T helper 2
	TLR	Toll-like Receptor
	TNF	Tumour Necrosis Factor
20	Tr1	Type 1 regulatory T cell
	Treg	Regulatory T cell
	TREM	Triggering receptor expressed on myeloid cells
	WHO	World health organisation
	XAO	Xanthine oxidase
25	XDH	Xanthine dehydrogenase
	XOR	Xanthine oxidoreductase

Chapter 1 – Introduction

1.1 Epidemiology of respiratory infections

30 The respiratory tract is a portal to the external environment, and as such is exposed to a vast array of potentially harmful antigens, with an estimated 10 000 micro-

organisms being inhaled every day. The respiratory tract can be viewed as a continuum that extends from the nasal cavity down to the alveoli with every site potentially being targeted by an array of bacterial, viral, fungal and other pathogenic organisms. The upper respiratory tract of most individuals is colonised by a diverse collection of commensal microflora which exert no detrimental effect on the host under normal physiological conditions. Conversely, the lower respiratory tract from the larynx down is essentially sterile so as to optimise the potential of the lung to partake in efficient gaseous exchange. Infection of the lungs would be highly deleterious to the host, and the development of an intricate and potent mucosal immune system generally restricts pathogen invasion and infection. However, pathogens have developed virulence factors to target and subvert immune effectors and facilitate infection. Respiratory infections can afflict anyone, but display an increased propensity when the immune status of the host is compromised or the invading pathogen is highly virulent.

The Global Burden of Disease study of 2002 highlights acute respiratory infections as the third leading cause of global mortality, resulting in an estimated 3.9 million deaths each year . The World Health Organisation (WHO) illustrate that such acute respiratory infections are the most extensive burden of disease, responsible for an estimated number of disability adjusted life years (DALYs) approaching 100 million in 2001, exceeding that of HIV/AIDS, tuberculosis and other eminent infections (Fig. 1.1a). Respiratory infections have remained a constant health burden for enumerable years, and are the most common and perhaps severe illness experienced by the populace, regardless of age or gender. In addition to the significant mortality associated with such infections, is the substantial morbidity and ensuing economic disruption. Furthermore, it must be appreciated that such infections are not simply an affliction of the Western world but are indeed a more sizeable dilemma in developing countries where effective methods of prevention and cure are more underdeveloped (Fig. 1.1b).

1.2 Innate Immunity to Respiratory Infections

It is credit to the efficient, robust and multifaceted immune system that so few infective organisms compromise the integrity of the respiratory epithelium, and that most respiratory diseases are cleared rapidly after an acute episode of infection. The immune system can be viewed as a co-ordinated interplay between innate/non-specific immunity and acquired/specific immunity. Innate immunity fulfils a highly significant role early in infection against a diverse array of pathogens and is critical to the development of the more pathogen specific adaptive immunity.

1.2.1 Physical barriers

The primary obstacle to micro-organisms entering the respiratory tract is an array of mechanical barriers that act to limit pathogen invasion beyond the respiratory epithelia. The nasal passage acts to filter any antigens greater than 10 μ m in diameter from progressing past the larynx, and further size exclusion is imparted by tight junctions between epithelial cells of respiratory mucosal surfaces, with membrane and paracellular spaces being impervious to large molecules. The respiratory epithelia are coated in a thick layer of mucus which acts to trap invading micro-organisms and prevent them gaining access to the underlying respiratory epithelia. Consequent ciliary movement and cough and gag reflexes act to displace foreign particles from the lumen of the respiratory tract.

1.2.2 Cells of the innate immune response

There are many effector cells that comprise the innate immune system, which exert potent anti-microbial activity, and are fundamental in the establishment of a rapid response to pathogen challenge. Some cells of the innate immune system are resident in the healthy lung of an uninfected individual, but there is further recruitment to inflammatory signals generated during infection. Such recruitment at appropriate times is dictated by the temporal expression of a diverse array of chemoattractants and adhesion molecules that mobilise inflammatory cells to the site of infection and facilitate their infiltration (Fig. 1.2). Recognition of conserved microbial structures by cells of the innate immune system augments their activation leading to more potent anti-microbial capacity and greater release of an array of chemokines and cytokines

that promote inflammation. Activation of innate mechanisms can be mediated by recognition of pathogen associated molecular patterns (PAMPs). PAMPs are expressed by a variety of pathogens but are not present in mammalian cells, allowing the distinction between self and non-self, and are recognised directly by the pattern recognition receptors (PRR) .

PAMPs have not evolved to interact with the host immune system, they perform essential functions within the infectious agent and are highly conserved across species, allowing a limited number of PRRs to detect infection . PAMPs include lipopolysaccharide (LPS), the cytosine guanine (CpG) dinucleotide DNA motif, lipoproteins, peptidoglycan, flagellin and lipoteichoic acid (LTA). Toll-like receptors (TLRs) constitute an important family of receptors that recognize such PAMPs, and are considered to represent a significant mechanism for inducing production of anti-microbial products and in the establishment of a rapid and potent immune response to infection. There are now acknowledged to be 10 distinct groups of TLRs, each recognizing distinct factions of microbial products. TLR2 recognizes lipoproteins, TLR4 LPS, TLR5 flagellin and TLR9 is specific for CpG motifs .

One mechanism of protection to respiratory pathogen infection is the production of anti-microbial peptides by resident leukocytes . Production of these soluble mediators may be constitutive or induced via recognition of pathogen motifs and can impart both direct and indirect immunity towards the invading micro-organism. Table 1.1 depicts some of the most significant microbicidal mediators that constitute the host's protective immune response .

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Anti-microbial agent	Source	Target	Method of protection
Lactoferrin	Neutrophils, macrophages, epithelial cells	Bacteria	Sequesters iron essential for microbes; Directly bactericidal ; Stimulates O_2^- production by macrophages
Lysozyme	Neutrophils, macrophages, epithelial cells	Gram-positive bacteria	Degradation of cell wall
Defensins	Phagocytes, lymphocytes, epithelial cells	Gram-positive.-negative bacteria, mycobacteria, yeast, fungi, some enveloped viruses	Permeabilises microbial membranes; Triggers alternative complement pathway
Surfactant protein-A/D	Alveolar type II cells	Carbohydrate PAMPs	Opsonisation, agglutination and neutralisation
Type 1 IFNs	Macrophages, epithelial cells	Viruses	Limit viral replication; Activate macrophages and NK cells; Induce activation and differentiation of DCs; Adjuvants for T and B cells

Table 1.1 Microbicidal mediators of the host's innate immune response to infection.

Macrophages are essential cells of the innate immune system and fulfil a pivotal role in immunosurveillance and are consequently fundamental to the establishment of a protective inflammatory response to infection. Alveolar macrophages are resident in the respiratory tract, comprising 95 % of all cells in the airways of uninfected individuals. They fulfil an important role in immune surveillance, recognizing invading pathogens and setting in motion microbicidal and inflammatory events necessary for the clearance of the invading pathogen . Macrophages also play an important role in the regulation of potentially harmful inflammation; maintaining immunological tolerance and respiratory homeostasis Alveolar macrophages express an assortment of receptors that facilitate recognition and phagocytosis of microbial pathogens, such as Fc, complement, mannose, Toll-like and an array of scavenger receptors. Ingested pathogens are subsequently exposed to toxic mediators, such as reactive oxygen species (ROS) and proteases that can kill the engulfed micro-organism . Macrophages also secrete an array of other molecules that are anti-microbial including lysozyme, lactoferrin, and defensins which together confer resistance to an array of potential infective agents .

Macrophages must communicate with other cells of the immune system not only to initiate inflammation but also resolve it. This is largely achieved through the secretion of cytokines. Cytokines derived from macrophages and proven to be important in pulmonary defence to infection include TNF, IL-12, IFN- γ , IL-10, IL-1 and a selection of chemokines that govern recruitment of cells . These cytokines increase the production of chemokines and promote the up-regulation of adhesion molecules on the endothelia and epithelia, facilitating the recruitment and diapedesis of neutrophils and lymphocytes into the lung. Macrophages are also an abundant source of IL-12, a cytokine fundamental to the establishment of a Th1 immune response, and NK cell activation. Through the production of such cytokines, as well as their potential to act as antigen presenting cells (APCs), macrophages clearly also fulfil a fundamental role in orchestrating the development of an acquired immune response, critical to specific cell mediated and humoral responses.

Dendritic cells (DCs) are another important APC that form a network in the submucosa of the nasopharynx, trachea and bronchial tree . These exhibit an enhanced capacity to present antigen as a result of their constitutively augmented expression of several co-stimulatory molecules important in the activation of lymphocytes. At the site of infection, naïve dendritic cells engulf and process pathogen for presentation, and are capable of migrating to draining lymph nodes to present the epitopes of the pathogen to effector cells of the adaptive arm of the immune response

Neutrophils represent another cellular component of the host's innate immune response, which like macrophages exhibit direct anti-microbial activity and release multiple cytokines. Neutrophils are an essential component of the host's armoury against many bacterial pathogens but also fulfil a significant role in protection against other microbial infections of the lung. Neutrophils are recruited rapidly from the periphery to the site of infection in response to various stimuli such as chemotactic proteins IL-8 and MIP-2 produced by lung macrophages and airway epithelial cells . Once at the infected site, neutrophils are activated by local pro-inflammatory cytokines. Like macrophages, neutrophils are strongly phagocytic and exhibit a propensity for ingesting and killing invading pathogens . They also produce a number of cytokines such as TNF, IL-1 β , IL-6 and MIP-2, which act to augment and refine the pro-inflammatory response . Eosinophils and basophils are other granulocytes found in the lung. Unlike neutrophils, these cells are poorly phagocytic, but secrete cytotoxic mediators such as major basic protein into the extracellular milieu in a process known as "frustrated phagocytosis".

Finally, Natural Killer (NK) cells respond rapidly to viral infection and express a variety of surface receptors that survey the levels of MHC class I. If the NK cell detects reduced expression levels of MHC I, as may be evoked by viral infection, then it will proceed to kill it. NK cell cytotoxicity is mediated by perforin and granzyme induced lysis, and there is also evidence that this cell type can mediate Fas dependent apoptosis. NK cells are activated by IL-12 derived from pulmonary macrophages and in turn generate IFN- γ , which further activates the macrophage population.

1.3 Reactive oxygen species and immunity to infection

1.3.1 The NADPH oxidase and respiratory burst

As described previously, one facet of innate immunity is the phagocytic barrier consisting of a variety of cells such as neutrophils and macrophages, which engulf the invading pathogen and subsequently destroy it via production of a number of antimicrobial and cytotoxic substances. One mechanism utilized by phagocytes to kill invading micro-organisms is the generation of reactive oxygen species (ROS) that are toxic to the pathogen. These ROS are generated via a membrane associated NADPH oxidase called the PHagocytic OXidase (PHOX) . NADPH oxidase is a multimeric membrane associated enzyme complex that utilizes molecular oxygen and the reducing power of NADPH to generate superoxide radical ($O_2^{\cdot-}$) :



The generation of superoxide is coincident with the so-named “respiratory burst” of the phagocyte, which evokes a sudden and dramatic increase in the rate of oxygen consumption. A host of external stimuli induce the phagocyte to undergo the respiratory burst and the concomitant activation of the NADPH oxidase, and ultimately the generation of superoxide. Cited examples of such external stimuli include phagocytic prey, galectin-3, agonists of complement receptor 3 (CR3), PMA, ionomycin and 7-transmembrane receptor agonists (e.g. IL-8) .

The phagocytic NADPH oxidase is located on the membranes of all professional phagocytes, but is also present on some poorly phagocytic cells such as B cells. It was originally anticipated that this enzyme was expressed primarily on the plasma membrane of such cells, proximally located to external environment. However, it is now known to also be located on the membranes of vesicles and granules, particularly in neutrophils

The phagocyte NADPH oxidase is a multi-component enzyme complex that is composed of membrane associated and cytosolic components (Fig. 1.3a). The core of the enzyme is comprised of the membrane bound flavocytochrome b558. This flavocytochrome is the site of the oxygen reduction that generates superoxide and comprises of two subunits, a 22 kDa protein (p22phox) and a 91 kDa glycoprotein (gp91phox). Gp91phox contains one flavin and two heme prosthetic groups, and it is these that mediate the transfer of electrons from the NADPH donor to the molecular oxygen substrate. When the phagocyte is in a resting state the cytosolic factors (p40phox, p47phox and p67phox) persist as a complex in the cytosol. Phagocytosis or appropriate stimuli can induce the union of all these components to form an active complex that catalyses the formation of superoxide. Activation elicits the phosphorylation of the cytosolic p47phox, which is subsequently translocated to the plasma membrane along with other cytosolic factors, where they interact via p67phox with flavocytochrome b558 to form an active complex that is capable of superoxide generation.

1.3.2 Xanthine oxidase as a source of superoxide

Xanthine oxidase (XAO) and xanthine dehydrogenase (XDH) are inter-convertible forms of the same gene product, namely xanthine oxidoreductase (XOR). The enzymes are complex molybdopterin containing flavoproteins that consist of two identical sub units of approximately 145 kDa. XOR is synthesised as the dehydrogenase form, which predominates in mammalian cells, but can be readily converted to the active oxidase form by oxidation of sulphydryl residues or by proteolysis. Expression of XAO/XDH is up-regulated in response to a variety of stimuli, including hypoxia, anoxia, LPS and cytokines such as IFN- γ and IL-1. XDH has subsequently been demonstrated to be converted to XO by several signals including C5a, fMLP and TNF.

One of XORs primary physiological roles is in the conversion of hypoxanthine to xanthine and xanthine to uric acid. Hypoxanthine or xanthine bind to the active oxidized form of the enzyme (XAO) and donates two electrons to the molybdenum

cofactor reducing it from MoVI to MoIV. In the process the substrate is hydroxylated by H₂O at the molybdenum site, as their electrons are passed via two iron-sulphide residues to flavin adenine dinucleotide (FAD) within the enzymes active site. The reduced FAD can subsequently be re-oxidised to produce superoxide or hydrogen peroxide. In this manner XAO is capable of generating ROS.

XOR is widely distributed through various tissues, present in the liver. Intestines, lung, kidney, myocardium, brain and plasma. XAO serum levels have been reported to be significantly elevated in various pathological states such as hepatitis, inflammation, ischemia-reperfusion and carcinogenesis and the ROS generated by this enzyme are anticipated to cause oxidative damage. XAO is reported to have a role in the pathogenesis of acute lung injury. Indeed, hypoxia up-regulates XAO/XDH mRNA expression in pulmonary arterial endothelial cells, and XDH/XAO activity and gene expression have been shown to be increased in response to hypoxia, endotoxin and IL-1 β in animal models of lung injury. Furthermore, inhibition of XAO/XDH was shown to prevent the development of pulmonary edema following the forementioned treatments, clearly implicating these enzymes in acute lung injury.

1.3.3 Superoxide and downstream radicals exhibit anti-pathogenic potential

The superoxide generated by the phagocytic NADPH oxidase exhibits potent anti-pathogenic action, mediating the destruction of a host of invading microorganisms. Furthermore, the superoxide can subsequently be converted to a vast array of ROS that are detrimental to the invading organism (Fig. 1.3b). The superoxide radical and other ROS are by their very nature reactive and can cause extensive and irreversible damage to cellular components with excessive superoxide being linked to lipid peroxidation, mitochondrial dysfunction and oxidation of other biomolecules. However, the pathogenic potential of reactive oxygen species has been suggested to be attributable to the role of these mediators in activating proteases that reside within the phagocytic vacuole. These proteases exhibit potent anti-microbial potential, and are activated through the generation of a hypertonic K⁺ rich alkaline environment within the phagocytic vacuole. The K⁺ crosses the membrane into these vacuoles

through large conductance Ca^{2+} activated K^{+} channels, which are opened due to an oxidase induced membrane depolarization and elevated Ca^{2+} concentration .

The critical role fulfilled by ROS in the control of pathogen burden is made clearly apparent by the genetic condition chronic granulomatous disease, whereby the afflicted individual is inherently deficient of a functional phagocytic oxidase, and consequently exhibits a greater propensity to infection with bacterial and fungal agents .

1.3.4 Reactive oxygen species as signalling moieties and regulators of the immune response

The anti-pathogenic action of superoxide is not the exclusive role of this molecule, and there have recently been a plethora of studies dissecting the role of ROS in intracellular signalling and in the regulation of immune cell function . Superoxide acts as a second messenger and has been implicated in the induction of pro-inflammatory and apoptotic events. A great number of transcription factors are regulated by their redox state, and consequently reactive oxygen species have a significant influence on transcription of a vast array of genes. One transcription factor whose action is dictated by redox state, and which is of particular prevalence as a consequence of its indicative role in the transcription of pro-inflammatory cytokines and adhesion molecules, is NF- κB . Furthermore, ROS are implicated in the induction of a programmed cell death pathway, an example being the cited potential of hydrogen peroxide to induce apoptosis in macrophages .

1.4 Control of myeloid cells

1.4.1 Receptors that modulate activity of the myeloid population

The immune system must evoke a rapid and potent immune response to pathogen challenge in order to eradicate the invading organism, but must also exhibit a degree of subtlety to minimize inappropriate or over-exuberant inflammation and immunopathology . As previously discussed, myeloid cells represent a population of immune cells with pleiotropic functions that form a formidable barrier to combat the

infective agent. However, their potency and permissiveness would implicate them in ensuing pathology if their activity is not regulated. This is achieved by soluble factors or cell contact dependent interactions . Cell contact dependent modulation of myeloid function offers more specificity and selectivity than the broad signals conferred by soluble mediators, capable of eliciting a signal at specific sites within a tissue and at specific stages of an immune response. Such cell contact dependent regulation of the myeloid compartment can be mediated through receptors that are members of the Immunoglobulin superfamily (IgSF). The IgSF domain is the most abundant domain type in leukocyte surface proteins and receptors bearing these domains are frequently involved in control of the immune system. Receptors with IgSF domains that regulate myeloid activity include CD200R, CD172a (SIRP- α), TREM and macrophage Ig-like receptor (MIR) (Fig. 1.4).

1.4.2 CD200 – CD200R interaction and the regulation of myeloid cells

The signal delivered to the myeloid cell through CD200 receptor is induced by ligation with CD200. CD200 (OX-2) was discovered approximately 20 years ago as an antigen present in rat brain and thymus . The rat CD200 protein was subsequently cloned and demonstrated to be a 41-47kDa surface glycoprotein . CD200 has also now been cloned and characterised in mice and humans . CD200 is a transmembrane protein and a member of the Immunoglobulin-superfamily with 2 extracellular Ig-SF domains . In mice, it is a protein of 269 amino acids constituting a hydrophobic signal peptide, a membrane distal Ig-V-like domain, a membrane proximal Ig-CH2-like domain, a transmembrane segment and a short cytoplasmic tail . It possesses 6 N-linked glycosylation sites and shares structural homology to CD80/CD86. The cytoplasmic tail is only 19 amino acids long and shares no homology to signalling kinases, which, combined with the lack of ITIM/ITAM sequences or SH2/SH3 domains would imply that it is incapable of inducing an intracellular signal.

CD200 exhibits an unusual distribution on specific cell types, which has been demonstrated to be virtually identical in mice, rats and humans . Thus far, CD200 expression has been reported on thymocytes, B cells, activated T cells, neurons, follicular dendritic cells, endothelium and cells in reproductive organs (Table 1.2).

The CD200 receptor was cloned by Barclay *et al* and shown to be closely related to CD200 and has most probably arisen through gene duplication . The receptor has subsequently been independently characterised in humans and mice . Like its ligand, CD200R is also a member of the immunoglobulin superfamily, with two extracellular IgSF domains . It possesses an unusually high number of N-linked glycosylation sites, with 8 identified in rats and 10 in mice . The cytoplasmic tail of CD200R is longer than that of the ligand being 67 amino acids in length. It contains three tyrosines residues as potential phosphorylation sites and is capable of signalling, implying that the action of CD200 is solely delivered through the receptor and is not bi-directional . One of these tyrosine residues is located within a NPXY motif, and a recent study in mast cells has demonstrated this residue to be phosphorylated upon ligation of the receptor . Furthermore, this phosphorylation leads to the recruitment of adaptor proteins Dok-1 and Dok-2, which are in turn phosphorylated and associate with RasGAP and SH2-containing inositol phosphatase (SHIP). Ultimately, this was demonstrated to inhibit the phosphorylation of ERKs, p38 and JNK . There have, however, been limited studies thus far citing the downstream signalling events from CD200R in other myeloid cells.

The expression of the receptor is far more restricted than that of the ligand being largely confined to myeloid cells (Table 1.2). The expression profile of the receptor again appears to be conserved between mice, rats and humans and has thus far been described upon macrophages, dendritic cells, neutrophils, basophils and mast cells . Though expression appears largely confined to myeloid cells, one study describes expression of CD200R on CD4⁺ T cells from peripheral blood in mice and humans, and further identified high levels of CD200R mRNA expression in Th2 polarised T cells . Two groups have subsequently described an extended family of CD200R molecules in addition to that previously discussed . It would thus appear that there exist four isoforms of CD200R in mice (R1-4) and two in humans (R1-2), yet the exact role of these other receptors as well as their natural ligands remains ambiguous.

1.4.3 CD200 knock out mice: a lesson in CD200R function

The generation of knock out mice lacking CD200 provided the first and best clues as to the role of the CD200-CD200R interaction . Naïve CD200 knock out mice exhibit an intrinsic defect, not in the cell types expressing CD200, but in those populations
5 expressing the receptor. CD200 knock out mice exhibit increased numbers of CD11b⁺ myeloid cells in the spleen (especially in young mice) with elevated numbers of splenic red pulp macrophages and a thicker marginal zone macrophage layer . Furthermore, elevated levels of the immunotyrosine activating motif (ITAM) containing intracellular DAP-12 were detected in CD200 knock out mice, within the
10 marginal zone and on dendritic cells in T cell areas of the red pulp. This implies an elevated level of myeloid cell activation. Lymph nodes are slightly enlarged and exhibit an unusual tubular structure with no demarcation between nodes. Macrophage populations are again expanded and exhibit heightened activation. Microglia (macrophage-like cells of the CNS) also exhibit heightened activity, forming clumps
15 reminiscent of that seen with inflammation or neural degeneration and express heightened levels of activation markers CD11b and CD45.

These mice also exhibited a heightened propensity for developing autoimmune conditions, showing increased susceptibility to collagen induced arthritis (CIA) ;
20 experimental autoimmune uveoretinitis (EAU) , a model for retinal inflammation; and experimental autoimmune encephalitis (EAE), a model of multiple sclerosis and a model for alopecia . Symptoms of these diseases are more rapid in CD200 knock out mice and lesions contain greater macrophage numbers in a heightened activation state. These findings imply that CD200 fulfils a significant role in regulating macrophage
25 activation and ensuing inflammation, and that the CD200-CD200R interaction is important in down-regulating the activity of myeloid cells expressing CD200R .

1.4.4 Manipulation of the CD200 induced signalling pathway

It is apparent that CD200 has the capacity to modulate myeloid cell activity in an inhibitory manner. Manipulation of the CD200-CD200R interaction has clearly
30 defined an important role for these molecules in dictating the phenotype of the myeloid response in conditions characterised by excessive immunity. Furthermore, it

has become apparent that delivery of this inhibitory signal can be utilised immunotherapeutically to alleviate symptoms attributable to such excessive immunity. Several studies have cited the potential of manipulating the CD200-CD200R axis during autoimmune conditions. CD200 knock out mice showed increased susceptibility to EAE, EAU and CIA and subsequently antibodies that block the inhibitory signal have also been shown to exacerbate EAE and CIA . Conversely, the administration of an agonistic antibody to CD200R or CD200:Fc fusion protein halts CIA disease progression and is also capable of reducing established arthritic disease .

Similarly there is a clear role for CD200 in governing alloimmunity. In a murine model of spontaneous abortion, whereby an unusually high level of fetal loss is observed by breeding CBA and DBA/2 mice, administration of CD200:Fc significantly reduces abortion rates . Furthermore, whereas blockade of CD200 signalling reduces graft survival , administration of CD200:Fc prolongs graft survival in both allotransplants and xenotransplants . Finally, with the observed expression of CD200R on mast cells , it would appear that there is a potential regulatory role for CD200 in governing allergy. Indeed, in a murine model of passive cutaneous anaphylaxis (PCA), the systemic administration of an agonistic antibody to CD200R inhibits FcεR1-dependent responses .

1.4.5 Basis of CD200 mediated inhibitory activity

Though beneficial effects of signalling through CD200R are undeniable, it is not precisely known how the myeloid cell is modulated to evoke these immunosuppressive events. Recent studies demonstrate that CD200 signalling inhibits degranulation in mast cells and basophils and production of IL-13 and TNF by mast cells . Furthermore, an agonistic antibody to CD200R was demonstrated to reduce IFN-γ and IL-17 induced production of IL-6 in murine resident peritoneal macrophages, and reduce cytokine and chemokine production of CD200R transfected U937 cells . Therefore, it would appear that signalling through CD200R diminishes the pro-inflammatory potential of the myeloid compartment. Finally, ligation of CD200R on plasmacytoid dendritic cells causes an up-regulation of indolamine-2,3-

dioxygenase (IDO) expression and activity . IDO mediates the catabolism of tryptophan leading to its immunosuppressive products being generated, and high levels of IDO expression are affiliated with inhibition of alloreactivity and enhanced Treg function .

5 ***1.5 Adaptive Immune response to respiratory Infection***

Fundamental to the resolution of infection and development of memory is the establishment of an acquired specific immune response, comprising both cell mediated T cell, and humoral B cell, responses.

1.5.1 T Cell Classification

10 T cells are a subset of lymphocytes that originate from the bone marrow and mature in the thymus. They are identified by the expression of CD3 and can be further categorised into two main subsets: CD4⁺ T helper (Th) lymphocytes and CD8⁺ cytotoxic (Tc) lymphocytes. CD4⁺ helper T cells exert their effector function primarily through the release of cytokines that activate and stimulate other cells. They
15 also fulfil a significant role in antibody production by providing a critical signal for B cell activation through the CD40-CD40L interaction. Cytotoxic T cells are accomplished killers of infected and tumour cells, lysing infected cells via a perforin/granzyme dependent mechanism, which punches holes in the membrane of the cells or a Fas-dependent mechanism. CD8⁺ T cells are critical mediators in the
20 resolution of viral infections, as a consequence of the intracellular nature of these pathogens.

Traditional CD4⁺ and CD8⁺ T cells recognize antigen MHC complexes via a T cell receptor that possesses an α and a β chain. However, there are distinct T cell subsets
25 that do not express the $\alpha\beta$ TCR, but rather a receptor that is $\gamma\delta$. These are often associated with mucosal sites and anticipated to infer protection of epithelial surfaces. Furthermore, there is a population of unique intraepithelial T cells (CD103⁺) in the respiratory tract, which may possess an $\alpha\beta$ or $\gamma\delta$ TCR. These T cells recognize generic immunological distress signals rather than pathogen specific motifs, and fulfil a
30 significant role in the recognition and eradication of infected epithelial cells .

1.5.2 MHC Restriction and T Cell Activation

Activation of both CD4⁺ and CD8⁺ T cell subsets requires interaction of the T-Cell Receptor (TCR) / CD3 complex with the antigenic peptide displayed on the Major Histocompatibility Complex (MHC) of the Antigen Presenting Cell (APC). Antigenic products can be processed by distinct pathways and presented in conjunction with MHC class I or class II molecules. MHC I expression is universally observed on all nucleated cells and such presentation is important in the activation of CD8⁺ T_C cells. Conversely, MHC II expression is restricted to APCs (although it can be up-regulated on other cell types), and such presentation of antigenic peptide is responsible for the activation of CD4⁺ T_H cells.

However, this TCR / MHC interaction is generally insufficient to effectively activate naïve T cells or the full repertoire of T cell responses. A second “co-stimulatory signal” is provided by surface molecules on the APC or by soluble factors (Fig. 1.5). The successful integration of two such signals results in the proliferation and differentiation of antigen specific T cells. The most well known co-stimulatory signal is the CD28/B7 pathway , which governs activation of naïve T cells. CD28 is constitutively expressed on the surface of both resting and activated T cells and interacts with B7-1 (CD80) or B7-2 (CD86) on the surface of an APC. Ligation of CD28 culminates in high-level interleukin 2 (IL-2) production, by increasing the transcription and the mRNA stability of this crucial T cell growth factor in both CD4⁺ and CD8⁺ T cell subsets . Such a co-stimulatory signal also up-regulates IL-4, IL-5, IL-8, IL-13, IFN- γ and TNF , and prevents anergy {Boussiotis, 1993 9952 /id; Gimmi, 1993 12443 /id;} or cell death by inducing the expression of the anti-apoptotic agent Bcl-X_L both *in vitro* and *in vivo* . CD28 fulfils a significant and fundamentally crucial role in the sustained proliferation of naïve T cells and hence in facilitating their development into effector cells.

1.5.3 The Ever-Growing Array of T Cell Costimulatory Molecules

An array of other potential co-stimulatory interactions has recently been reported. Though these surface molecules undoubtedly have an impact on T cell activation when stimulated in conjunction with the TCR, their function appears distinct from the

Name	Cell Expression	Naïve T cell expression?	Inducible cell surface expression	Ligand	Reference
CD28	T cells	✓	↑ 24hrs	B7.1/B7.2	116
ICOS	T cells, NK cells		↑ 12 hrs	ICOSL	117
OX40	T, B cells, DCs, eosinophils		↑ 24-48 hrs	OX40L	118,119
4-1BB	T, B cells, DCs, macrophages, NK cells, eosinophils		↑ 24-48 hrs	4-1BBL	118,119
CD27	T,B Cells	✓	↑ 24-48 hrs	CD70	120
CD30	T, B cells		↑ 24-48hrs	CD30L	121
CD40L	T, B, NK cells, DCs basophils, mast cells, eosinophils,		↑ 24-48hrs	CD40	122
CTLA-4	T cells		↑ 24-48hrs	B7.1/B7.2	123

Table 1.3 Summary of T cell co-stimulatory molecules.

classical CD28 pathway, in that they are predominantly incapable of inducing IL-2 production or preventing T cell AICD , rather they play distinct roles at different stages of T cell differentiation, on different T cell subsets and are crucial to the development of divergent effector functions . A summary of some key co-stimulatory molecules is shown in Table 1.3 and Fig 1.5.

1.5.4 T cell subsets

The T helper subset can be subdivided into two broad groups, namely Th1 and Th2, which are defined by the distinct cytokines that each subset produces (Table 1.4) . The cytokine expression profiles of each signify the discrete functions implemented by each of these populations in an immune response. Th1 derived cytokines are responsible for predominantly cell-mediated immune responses to intracellular pathogens , whereas the Th2 are important for antibody-mediated humoral response that combats extracellular pathogens . The distinction between Th1 and Th2 subsets is not, however, as unambiguous as first seems and there have been discrepancies regarding exactly how each subset are defined. Some view the Th1/Th2 dichotomy not as discrete subsets, but rather as a continuum of different combinations of cytokines . Nonetheless, in an *in vivo* model, T cell clones show a dramatic bias towards one of the cytokine patterns denoted above and hence this clearly defines an important functional division of the immune system.

Table 1.4 Cytokines produced by Th subsets

Cytokines produced exclusively by Th1 cells	IL-2, IFN- γ and LT- α /TNF- β (lymphotoxin)
Cytokines produced exclusively by Th2 cells	IL-4, IL-5 and IL-6
Cytokines produced predominantly by Th1 cells	TNF- α and GM-CSF
Cytokines produced predominantly by Th2 cells	IL-10, IL-13 and IL-21
Cytokines produced equally by both Th1 and Th2 cells	IL-3 and TGF- β

Both subsets are derived from a common naïve precursor that upon Ag stimulation produces predominantly IL-2 before passing, via an intermediate (Th0), into one of the distinct polarized subsets . The generation of a Th1 or Th2 directed response is dictated by the cytokine milieu at the time of T cell activation (Fig. 1.6), although the antigen dose and level of co-stimulation may also be important. The fundamental cytokines in the development of a Th1 polarised immune response are IFN- γ and IL-12. The source of IL-12 at the time of T cell activation is predominantly macrophages and dendritic cells that have been infected with intracellular pathogen. IL-12 can also act on NK cells to provoke IFN- γ production, which in turn drives macrophages to a heightened activational state augmenting their endogenous production of IL-12, which acts to further reinforce the Th1 polarisation .

The predominant cytokine in the development of a Th2 polarized response is IL-4, which is derived from a variety of sources . It is postulated that APC-derived IL-6 is capable of provoking IL-4 production by naïve T cells. Furthermore, NK T cells, mast cells, eosinophils and basophils are all potential sources of IL-4 .

Another feature of the Th1/Th2 paradigm is the strong antagonism observed between populations, with the cytokine products being mutually inhibitory towards the differentiation and effector functions of the reciprocal phenotype . Such antagonism reinforces development of distinct polarised T helper subsets and explains the strong bias observed in many infection models.

In recent years it has become apparent that another population of CD4⁺ T cells, called regulatory T cells, exist that is quite distinct from T helper cells in their phenotype and function . This regulatory T cell family are specific T cell populations with suppressive properties important in the control of auto-aggressive immune responses. Interest in this population has escalated in recent years owing to their ability to prevent autoimmune disease, allograft rejection and intestinal inflammation . CD4⁺ regulatory T cells (Tregs) can be divided into two distinct subsets that exert differential suppressive mechanisms: the naturally occurring CD4⁺CD25⁺ Tregs, whose suppressive effects are mediated via cell contact and the inducible Th3 and

Type 1 T regulatory cells (Tr1), whose suppressive effects are contact independent and mediated through cytokines IL-10 and TGF- β .

Naturally occurring CD4⁺CD25⁺ Tregs were originally described in the 1970s in mice
5 , and subsequent studies have shown that this subset of peripheral CD4⁺ T cells,
expressing CD25 on their surface, are critical in the control of autoreactive T cells *in vivo*, with their depletion resulting in the development of various autoimmune diseases . Comparable populations with identical phenotype and function have subsequently been identified in rats and humans . These naturally occurring Tregs
10 appear to originate in the thymus , and constitute 5-10% of peripheral CD4⁺ T cells. Studies *in vitro* have demonstrated this population to be anergic and suppressive, being capable of suppressing the proliferation of CD4⁺CD25⁻ T cells in co-culture . This suppression is dependent on the CD4⁺CD25⁺ Tregs being activated through their TCR and requires cell contact with the CD4⁺CD25⁻ T cells and evokes inhibition of
15 IL-2 transcription in the responder population . The naturally occurring CD4⁺CD25⁺ Tregs have been further characterised by the expression of several activation markers such as glucocorticoid induced TNFR (GITR) family related protein, OX40, CTLA-4 and CD62L . Whilst these markers have proved useful in the identification of this regulatory population in a naïve immune system, there persists controversy regarding
20 their role in mediating the suppression by these cells. It has been postulated that membrane bound TGF- β may also be responsible for mediating the observed suppression, although this too remains controversial . More recently, the transcription factor Foxp3 has been demonstrated to be critical in the development and function of these Tregs in mice .

25 The induced group of regulatory T cells are derived in the periphery from conventional CD4⁺CD25⁻ T cells and can be further subdivided into two types: Tr1 cells that produce large quantities of IL-10 and little TGF- β and Th3 cells that preferentially produce TGF- β . Tr1 cells have been generated from naïve CD4⁺ T cells
30 *in vitro* through culturing with IL-10 , IL-10 and IFN- α , or immunosuppressive drugs vitamin D3 and dexamethasone . The Tr1 cells generated proliferate poorly; generate

large amounts of IL-10, some IL-5 and IFN- γ and marginal or no IL-2 . Their immunosuppressive properties are largely mediated through IL-10 and they are capable of preventing the development of T cell mediated autoimmune responses . Th3 regulatory cells are induced *in vivo* through oral administration of antigen , and whilst their induction is antigen specific, their suppression is not and is mediated through TGF- β . They are suppressive towards both Th1 and Th2 cells and facilitate IgA production . Murine Th3 cells can be induced *in vitro* by administration of TGF- β to cultures and their expansion is further enhanced by the presence of IL-4 and IL-10 .

1.5.5 B cells and antibody production

The other arm of the acquired immune response is antibody-mediated immunity imparted by B cells. B cells are bone marrow matured leukocytes that are specialized in recognition of antigen and subsequent expression and secretion of antigen specific antibodies. Antibodies (immunoglobulins) are produced exclusively by B cells and are important in binding pathogen and neutralising its virulence and helping to eliminate infection. B cell deficiency or defective B cell function significantly reduces the host's capacity to control infection . Activation and maturation of the B cell is required for the production of antibodies specific to the antigen encountered. The activation of naïve B cells occurs in the T cells zones of secondary lymphoid organs, where the migrating B cell encounters antigen presented by follicular dendritic cells. The B cell is activated when antigen epitopes are specifically recognized by a surface bound immunoglobulin (IgM or IgD isotype). Further activation and maturation of the B cells into memory B cells and antibody producing plasma cells requires help from activated T cells through CD40:CD154 ligation, and is further aided and directed by the presence of T cell and follicular dendritic cell derived cytokines such as IL-2, IL-4, IL-5, IL-10 and IL-12 . This secondary stimulus facilitates the expansion of the antigen specific B cell, isotype switching and antibody secretion . This isotype switching involves a change in the Fc region of the B cell receptor, whilst its antigenic specificity is maintained. The isotype response is dictated by the localised T cell response and cause a switch to an IgA, IgG or IgE phenotype.

There are five major antibody isotype subclasses: IgM; IgD; IgG; IgA and IgE. The effector function of each subtype is defined by the interaction of its heavy chain constant region (Fc) with other serum proteins or leukocyte Fc receptors. In this manner, the antibody produced may actively participate in the host defence by a variety of effector mechanisms. Some antibody isotypes (specifically IgG) can opsonise a particular pathogen, whereby surface antigens are recognised and bound by the antibody in a manner that exposes the Fc region of the antibody to effector cells. Effector cells are subsequently brought into close proximity and the rate and efficiency of phagocytosis is increased, facilitating clearance of the pathogen. IgG isotypes can also bind pathogen and cause activation of the complement system, whereby a cascade of events ultimately leads to the formation of a membrane attack complex (MAC) where the antibody is bound leading to rupturing of the pathogens membrane . Antibodies may also recognize surface expression of pathogen epitopes on infected host cells and alert effector cells to eliminate the infected cell by secreting lytic enzymes and granules, in a process known as antibody dependent cellular cytotoxicity (ADCC) . Finally, certain antibody isotypes are important in the agglutination of foreign antigen. In this scenario secreted antibodies extensively cross-link antigens to form large complexes, which may hinder the ability of the pathogen to infect the host cell through steric hindrance or lead to clearance by complement fixation, ADCC or reticulo-endothelial filtration. IgM and IgA isotypes are excellent agglutinins and are subsequently found extensively at mucosal surfaces .

1.5.6 T and B cells at mucosal sites

The mucosal immune system can be structurally and functionally divided into two sites. Primarily, organised secondary lymphoid tissues confer the site for the sensitisation or induction phase of immunity, whereby antigen is taken up, processed and presented to naïve T cells and B cells . Secondly, induced lymphocytes traffick to effector sites to engage other immune cells and exert a protective response. The mucosal associated lymphoid tissues (MALT) that are the key inductive sites for mucosal immunity have predominantly been studied in the respiratory tract and gut and are similar in structural organisation and cellular composition (Fig 1.7). The secondary lymphoid tissue of the respiratory tract is the nasal associated lymphoid

tissue (NALT) and in some animals bronchial associated lymphoid tissue (BALT). NALT has been well characterised in rodents, but has not been seen in humans although the lymphoid tissue of the Waldeyers ring is arguably analogous. Likewise, BALT is only variably present and its functional significance is not conclusive, although it displays all the attributes of secondary lymphoid tissue.

MALT display conserved structural features with sub-epithelial B cell and T cell rich areas that are clearly defined and segregated, and proximal APCs, such as dendritic cells and macrophages . Both NALT and BALT are characterised by several follicles where B cells are preferentially found, which subsequently develop secondary germinal centres after antigenic stimulation. These follicles are surrounded by more diffuse lymphoid tissue with the T cell zone, and the luminal side is covered by follicle associated epithelium. Antigen is directly sampled from the luminal compartment into the MALT by specialised epithelial cells known as microfold cells (M cells). M cells, whilst present in the gut, appear to be absent from the normal respiratory tract and are anticipated to be induced when airway and lung tissues are exposed to antigen . Antigen is subsequently taken up and processed by underlying APCs and presented to resident naïve T cells and B cells. Having been sensitized to antigen, the activated lymphocytes mature and proliferate in draining lymph nodes. The effector cells exhibit a change in the expression of surface molecules L-selectin and $\alpha_4\beta_7$ whilst maturing, which consequently facilitates their exit from the inductive sites and into systemic circulation. Subsequently, the primed T and B cells migrate to mucosal effector sites where they mature further and exert effector functions that protect the mucosal surfaces . The predominant effector site of the mucosal immune response, where mature T cells and B cells migrate after induction, is the lamina propria. The lamina propria is a layer of connective tissue between the epithelia and the muscularis mucosa, and (at least in the gut) is the residence of a heterogenous group of lymphoid and myeloid cells. The large infiltrate of cells at this site confer protection should any pathogenic antigen compromise the integrity of the epithelial surface and besiege the mucosal site. The efferent inductive and effector phases of the mounted mucosal immune response culminates in a protective immune response that

is mediated by effector CD4⁺ and cytotoxic CD8⁺ T cells and blast cells capable of producing significant quantities of IgA.

1.5.7 Mucosal IgA.

IgA is the predominant antibody in secretions at mucosal surfaces and is critical in protection against an array of pathogens (Fig. 1.8). IgA is always present in the respiratory tract of healthy, uninfected individuals. At mucosal surfaces, a secretory immunoglobulin system operates, which allows the transport of polymeric immunoglobulins (pIg) across epithelia and into the lumen through interaction with poly immunoglobulin receptor (pIgR). The pIgR has a basolateral targeting sequence that directs the receptor from the trans Golgi network to the epithelial basolateral membrane where it can interact with J chain containing pIgs. The pIgR is subsequently endocytosed and delivered to basal early endosomes and may eventually be transported across the epithelia to the apical membrane in a process referred to as transcytosis. The pIgR/IgA complex is released after cleavage from the apical membrane into the mucosal lumen as secretory immunoglobulin-a (SIgA).

IgA binds and neutralises pathogens in the lumen of the respiratory tract and consequently renders them incapable of invading the mucosal epithelia in a mechanism known as “immune exclusion”. Relatively high levels of polyreactive natural SIgA are probably designed to offer a first line of defence and confer immediate protection before an adaptive response is elicited. Secondly, dimeric IgA may bind endocytosed pathogens during transcytosis across epithelial cells and transfer them back into the lumen (Fig. 1.8b). In this manner IgA mediates intraepithelial neutralisation of numerous viruses (e.g. influenza). Furthermore, it has been demonstrated that IgA can bind pathogens that have already compromised the epithelial barrier into the lamina propria, forming immune complexes, and expel them selectively back into the airways (Fig. 1.8c). Finally, IgA may also activate FcαR expressing leukocytes to facilitate clearance of pathogen, through phagocytosis of IgA immune complexes, opsonin induced killing, ADCC and production of ROS, cytokines and other inflammatory mediators.

1.6 Influenza

1.6.1 Epidemiology of influenza infection

Influenza infection is an acute respiratory disease that is one of the most persistent and important infectious diseases to have afflicted mankind throughout the ages. This extremely variable virus is highly contagious and is an omnipresent threat to the population, regardless of age or gender. Influenza is a global dilemma inducing seasonal epidemics that can affect 10 to 20% of the population with ensuing morbidity and mortality. Seasonal circulation occurs every winter and lasts between 8 and 12 weeks in the UK . It is estimated that influenza A viral infections are responsible for between 10,000 to 20,000 deaths in the United Kingdom each year . Furthermore, the World Health Organisation has recently decreed, “another influenza pandemic is inevitable and possibly imminent” (<http://www3.who.int/whosis>), an alarming prospect with reference to the devastation and fatalities experienced in the previous pandemics. The worst ever pandemic was the 1918 Spanish influenza which killed between 20 and 40 million people and infected approximately half of the world’s population. Unusually, this pandemic was particularly fatal in young adults. The source of the Spanish flu is thought to be an avian reservoir, and similar pathogenicity was observed during the recent “bird flu” outbreak caused by an avian influenza A (H5N1) strain. To date the cumulative number of confirmed human cases of avian influenza A/ (H5N1) reported to the WHO is 246, with 144 deaths (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2006_09_14/en/index.html).

Influenza infection is usually self-limiting, culminating in a local and systemic reaction, whereby the afflicted individual may experience fever, chills, headaches, coughing, myalgia and diarrhoea. However, there remain a significant proportion of patients who are considered to be at high risk of developing severe illness and complications, such as the elderly, the very young and the immuno-compromised . Individuals possessing underlying medical conditions, such as cardiovascular disease, chronic bronchitis and asthma, also possess a heightened susceptibility to complications, with a greater percentage developing secondary bacterial infections

that are frequently life threatening. Ultimately, the outcome of an infection is a competition between a virus of considerable genetic and antigenic diversity and a multifaceted immune system.

5 **1.6.2 Classification and structure**

Influenza virus is a member of the family *Orthomyxoviridae*, and can be classified into three genera, namely types A, B and C . Influenza A was first discovered in 1933 and acknowledged to be the most prominent and severe, being responsible for the seasonal epidemics and the devastating pandemics that sporadically afflict mankind .

10 Influenza B and C were identified in 1940 and 1950 respectively, and are not recognised to present the same threat as type A .

Influenza is an enveloped negative stranded RNA virus that is approximately 80-120nm in size (Fig. 1.9). The outer envelope comprises of a lipid bilayer from which
15 hemagglutinin (HA) and neuraminidase (NA) glycoproteins project. These outer membrane proteins are functionally significant and are essential to the viability and replicative potential of the influenza virion, governing host cell entry and ultimately exit. The HA protein is fundamental for binding and subsequent fusion to the host cell, whereas the NA protein fulfils a critical role in preventing viral aggregation and
20 aiding release of viral particles from the host cell post-replication. NA has also been implicated in aiding the transport of the virion through the respiratory tract .

Positioned within the viral envelope exists the matrix protein M1, which interacts with both the cell genome and the nuclear export factor and facilitates viral assembly .

25 Also located within the envelope is viral protein M2, which forms an ion channel between the interior of the viral particle and its immediate environment, dictating the localised pH . Maintenance of a low pH is mandatory for several viral activities, such as HA synthesis and virion uncoating. The RNA genome of influenza consists of 8-segmented genes, containing the genetic information for expression of 10 viral
30 proteins, each encased by nucleoprotein (NP) . In addition to the viral proteins already discussed, a further 3 proteins (PB2-, PB1- and PA-encoded proteins) form a

transcriptase complex and there are 2 non-structural proteins, NS1 and NEP, that govern post-transcription RNA control and viral assembly, respectively .

1.6.3 Antigenic diversity of influenza: Antigenic drift and shift

Influenza A is responsible for extensive illness in humans owing to the considerable antigenic variability that it displays enabling it to successfully evade the host's neutralizing antibody repertoire. As discussed, influenza A expresses HA and NA on its surface. These glycoproteins are subject to extensive antigenic variation that is important for immune evasion and it is changes in these surface glycoproteins that are largely responsible for the observed epidemics and pandemics. There are to date at least 15 haemagglutinin subtypes and 9 neuraminidase subtypes. Widely circulating influenza strains within the human populace are restricted to 3 HA subtypes (H1-3) and 2 NA (N1-2), with the remaining subtypes being seen in viruses that persist within avian reservoirs.

The antigenic variability that occurs within the influenza genome takes two forms: antigenic drift and antigenic shift. Antigenic drift occurs when genes encoding the surface glycoproteins undergo stepwise mutation, owing to the relatively low fidelity of the influenza RNA-specific RNA polymerase (replicase) which lacks proof reading 5'-3' exonuclease activity. These mutations may lead to attenuated or non viable influenza, but some may provide a survival advantage, which will be selected for and expand. If the surface glycoproteins are sufficiently different that the host's antibodies are incapable of neutralizing it then the new strain is capable of causing disease, and frequently the observed seasonal epidemics.

Antigenic shift is a far less frequent form of antigenic change but can lead to the devastating pandemics that occasionally plague mankind. Antigenic shift occurs when two different viruses from distinct host's (possibly different species) co-infect a single host and exchange genetic information. The ensuing reassortment of viral genome segments results in a new virus that possesses elements from each of the two original strains. Again the resulting virus may be attenuated or non-viable, but it may also be highly virulent and potentially pathogenic due to expression of novel surface proteins

derived from the animal host virus. In this scenario, the human host may possess little or no immunity to the novel surface proteins and the new variant can cause widespread disease through evasion of the host's immune response.

5 **1.6.4 Replication cycle**

Transmission of influenza between individuals is via virus laden liquid droplets that are expelled when the infected patient coughs or sneezes . The primary sites of infection of the infective virions are the epithelial cells of the upper respiratory tract, but influenza also possesses a propensity for invading macrophages and other
10 leukocytes within this localised area . The virus has the potential to invade any cell that possesses a suitable receptor, notably those expressing a sialic acid containing cell surface glycoprotein . As previously discussed, attachment and fusion to the host cell is mediated through HA. HA exists as a native precursor termed HA0, which attaches the virion to cell surface receptors containing sialic acid, and is subsequently
15 cleaved through the action of extracellular host proteases to yield HA1 and HA2 . The virion is subsequently endocytosed to form an endosome with HA2 mediating the union between virus membrane and host cell . The low pH of the endosome induces a conformational change in the HA2 protein, culminating in enhanced proximity of viral and host cell membranes, and ultimately the fusion event . Fusion subsequently
20 leads to the liberation and nuclear import of viral nucleocapsids, which contains the crucial genetic code that dictates the amplification and generation of substantial viral progeny (Fig. 1.10).

The viral RNA genome, once localised within the host nucleus, is transcribed by viral
25 RNA polymerases resulting in the synthesis of viral mRNAs. The mRNAs are subsequently transported to the cytoplasm of the host cell where they are translated to yield viral nucleoproteins and polymerases . The newly generated viral proteins are then re-directed towards the host nucleus where they assist in viral replication and further mRNA synthesis . This positive feedback loop greatly amplifies the quantity
30 of viral RNA and proteins, which are subsequently packaged to form viral nucleocapsids that are exported from the nucleus before budding from the host plasma

membrane to form active virions . Hence the virus envelope is derived from host cell plasma membrane and the budding event is mediated via influenza neuraminidase protein.

1.6.5 Immunity to influenza

5 Succeeding influenza infection, the multifaceted immune system of the host mounts an immediate response to restrict viral replication and limit the associated cytopathology and destruction. Infection of cells with influenza virus culminates in the activation of a host of transcription factors that co-ordinate the release of chemokines and cytokines. Subsequently, there is an induction of an anti-viral
10 defence program and the enhanced recruitment of inflammatory cells, which together form a formidable barrier to combat viral burden and resolve infection.

As previously discussed influenza primarily infects epithelial cells of the upper respiratory tract as well as proximally located leukocytes, and it is these cells that
15 initially evoke a targeted response. Influenza infection triggers the activation of a variety of transcription factors, and although the mechanism of induction is somewhat of an enigma, it is postulated that it may be mediated via Toll like receptors and protein kinase R . Among the transcription factors actively induced are NF-kB (nuclear factor-kB), IRFs (IFN regulatory proteins), STATs (Signal Transducers and
20 Activators of Transcription), AP-1 (Activating Protein-1), and NF-IL6 (nuclear factor of interleukin-6) . The combined effect of these transcription factors is to induce transcription of a host of cytokines and chemokines that are aptly suited to combat the viral burden .

25 Both epithelial cells and monocytes/macrophages are capable of eliciting chemokine release upon infection with influenza, although the exact nature of the evoked signal is divergent for the distinct cell types (Fig. 1.11). Epithelial cells primarily release RANTES, MCP-1 and IL-8, whereas monocytes/macrophages secrete MIP-1 α , MIP-1 β , RANTES, MCP-1, MCP-3, MIP-3 α , and IP-10 . The array of chemokines
30 released ultimately favours the recruitment of blood mononuclear cells to the site of infection . Similarly, the cytokine profile of the discrete host cell is again distinct,

with the monocytes/macrophages being the primary and most potent source of inflammatory and anti-viral cytokine release. IFN- α/β is a critical cytokine in the containment and resolution of an influenza infection due to direct anti-viral effects, and potential to mediate the further recruitment of inflammatory cells .

5 Monocytes/macrophages are the predominant source of type 1 interferons as well as other pro-inflammatory cytokines TNF, IL-6 and IL-1, whereas infected epithelial cells are considerably inferior at eliciting such a cytokine response .

10 IFN- α/β is a critical component of the hosts armoury against infection, as its intrinsic anti-viral activity fulfils a critical role early in infection in limiting viral replication and consequently providing sufficient time for a humoral and cell mediated response to be mounted. The function of IFN- α/β as an anti-viral mediator is enhanced through its ability to up-regulate production of PKR, oligoadenylate synthetase and Mx, all of which confer natural viral resistance . Furthermore, IFN- α/β has a broader role, as it
15 enhances the production of MCP-1, MCP-3 and IP-10, which augment the recruitment of monocytes/macrophages and Th1 cells to the site of infection . In addition, IFN- α/β enhances antigen presentation by dendritic cells and macrophages via the up-regulation of MHC . It is also postulated to act as a cofactor in the development of a Th1 response, being implicated in T cell survival, up-regulation of the IL-12 receptor
20 and in the expression of IFN- γ by NK cells and T cells .

Other pro-inflammatory cytokines play a significant role in the co-ordinated response critical to influenza immunity. IL-1 β and TNF function to enhance the inflammatory response through augmentation of MCP-1 and MCP-3 . These cytokines provoke the
25 maturation of tissue macrophages and dendritic cells, hence invoking a primed antigen presentation system for the recruited T cells . IL-18, derived from influenza infected macrophages, also confers protective anti-viral immunity and aids the generation of a Th1 response by acting in conjunction with IFN- α/β to induce IFN- γ production from NK cells and T cells .

Ultimately, to resolve viral infection effectively, the induction of an adaptive immune response is a pre-requisite and a combination of both cell-mediated and humoral components fulfil important roles. Antibodies specific to hemagglutinin and neuraminidase are fundamental to resistance to infection and subsequent recovery, as they act to neutralize viral infectivity and hinder viral release from infected cells. Cytotoxic T cells also confer a central role in resolution of viral infection via their cytopathic elimination of infected cells. These CD8⁺ T cells arrive at the site of infection at approximately day 3-4-post infection whereby they detect virus infected cells, through recognition of conserved HA, NP and PB2 epitopes, and subsequently lyse them. CD4⁺ Th cells also confer a significant role in resolution of influenza infection, primarily via the delivery of signals to the main effectors. In addition to this role in the facilitation of humoral and cell mediated responses, they may also have a lesser role as cytolytic effectors.

The majority of studies that define the phenotype of the immune response to influenza infection have been conducted in mice. It is important to question to what extent the mouse model of influenza imitates the infection seen in man. Standard research with influenza in a murine model utilises mouse adapted strains of influenza A and B. However, even viral strains that are not mouse-adapted induce a toxic pneumonitis that is reduced by early treatment with antivirals such as amantidine and ribavirin. In the murine model, influenza virus is readily recovered from the lungs and follows a similar kinetics of infection to that in man. Virus titres in the lungs peak 4-6 days after infection and then declines, so that virus cannot be recovered by plaque assay at day 14. Symptoms of disease, like that in man, are dependent on the influenza strain and the dose of virus administered. As discussed above, during the first 3 days of infection in mice, infected epithelial cells and macrophages produce type-I IFN and TNF α , respectively. NK cells, in addition to their cytotoxic function, also contribute to the cytokine milieu releasing IFN γ , TNF and GM-CSF, all of which activate APCs. This innate phase of the immune response contributes to the magnitude of subsequent T and B cell accumulation. Neutrophils are also prominent, and again, their numbers reflect the virulence and dose of virus. The cytokines produced, the composition of

cells infiltrating the lung, and the disease parameters all replicate those observed during infection of man.

1.7 *Cryptococcus neoformans*

5 1.6.1 Structure and epidemiology

Cryptococcus is a genus of encapsulated budding yeast (Fig. 1.12), of which there are at least 19 species existing as free-living saprophytes . The only species within the *cryptococcus* genus that is known to exert pathogenic potential is *Cryptococcus neoformans*, the causative agent of the potentially life threatening meningoencephalitis called cryptococcosis . Sanfelice and Busse first identified *Cryptococcus neoformans* independently in 1885 and there are now two recognized varieties, namely *Cryptococcus neoformans* var. *neoformans* and *Cryptococcus neoformans* var. *gattii* . These two varieties fall into 4 major serotypes, delineated A to D, which are defined by certain epitopes of the polysaccharide capsule. *Cryptococcus neoformans* var. *neoformans* possesses serotypes A or D, whereas serotypes B and C are restricted to *Cryptococcus neoformans* var. *gattii* .

Cryptococcus neoformans var. *gattii* is confined to tropical and subtropical regions, infecting human beings only under exceptional circumstances, and postulated to be derived from flowering eucalyptus trees . Conversely, *Cryptococcus neoformans* var. *neoformans* exhibits a global distribution, with soil and avian reservoirs the primary source of transmission to man . It is this latter variety that constitutes the most potent threat to humans and will be the focus of this report. *Cryptococcus neoformans* var. *neoformans* is capable of eliciting disease in immunocompetent individuals, but immunologically compromised are at greatest risk . The lung represents the primary site of infection for this basidiomyceteous fungus but failure to control pathogen burden results in dissemination to extra-pulmonary tissues, with heightened propensity to the brain (Fig. 1.13). In the last 10 years clinical cases of cryptococcosis have escalated, the increase in numbers believed to be related to an increase in patients with underlying T cell deficiencies, such as individuals infected with AIDS and those treated with chemotherapeutic or immunosuppressive drugs. It is within the

AIDS infected population that *Cryptococcus neoformans* is most devastating, with between 6 and 8 percent all AIDS patients developing cryptococcus-associated meningitis.

1.6.2 Replication

5 *C. neoformans* is an encapsulated spherical yeast, which in its vegetative state varies in size from 5-10um in diameter . This budding yeast has the potential to engage in both asexual and sexual reproduction, although there is discrepancy regarding the extent of sexual reproduction that occurs in nature . During this sexual reproduction, a and α mating haploid yeasts enter into a diploid state and subsequently undergo
10 meiosis to yield haploid basidiospores, which ultimately develop into yeast cells again . It has been postulated that infection is initiated by inhalation of either desiccated, poorly encapsulated yeast cells or basidiospores . It has been claimed that penetration through the lung parenchyma is governed by strict size limitations imposed by alveolar spaces, and therefore the smaller size of the basidiospores (1.8-2um), and
15 their resistance to desiccation, makes them a far more attractive candidate for the infectious particle . Furthermore, there has been recent evidence citing the dimorphism of *cryptococcus*, whereby the yeast form hyphae and haploid basidiospores in the absence of mating; an appealing prospect with regard to the confusion surrounding the extent of sexual reproduction that is seen *in vivo* .

20 Having gained entry into the host, the establishment of infection and the degree of pathogenicity is highly variable depending on the immunological status of the host and the virulence of the infecting strain of *C. neoformans* . The *cryptococcal* inoculum is invariably ineffective at establishing an infection in an immunocompetent
25 host and is efficiently controlled and eradicated by the immune response. However, where the individual is immunocompromised, the host of virulence factors exhibited by the pathogen are suitably effective at evading the mounted immunological response, and facilitate dissemination of the fungus from the primary site of infection.

1.6.3 Virulence factors

Upon entry into the lung, the infective particle becomes rehydrated and develops the polysaccharide capsule that is essential for survival . Prior to capsule formation the fungus is particularly susceptible to phagocytosis by alveolar macrophages. To aid survival, *C. neoformans* has sialic acid terminal units on a number of glycoproteins that display anti-phagocytic potential early in infection . The mechanism by which the sialic acid residues mediate protection is ambiguous, but could be a consequence of their negative charge, mediating a repulsion towards the phagocyte, or the masking of underlying galactose residues in the fungal glycoproteins that can be recognised macrophage receptors .

The capsule is composed of a high molecular weight polysaccharide referred to as glucuronoxylomannan (GXM). The polysaccharide has a primary backbone of α -1,3-D mannopyranose, with single residues of β -D-xylopyranosyl and β -D-glucuronopyranosyl attached , and at least 4 genes have been determined to be essential to its formation (CAP59, CAP60, CAP64 and CAP10). Acapsular mutants that have been generated are avirulent and have an enhanced disposition to being phagocytosed by neutrophils, monocytes and macrophages . The reduction in phagocytosis imparted by the capsule also has repercussions regarding alteration of phagocyte phenotype, function and role in the mounting of an immune response. Primarily, the inability of the macrophage to phagocytose the organism reduces antigen presentation to recruited T cells . Furthermore, highly encapsulated organisms evoke a less substantial release of pro-inflammatory cytokines from monocytes and macrophages . It has been postulated that the inability of these organisms to be phagocytosed limits the ensuing production of TNF, IL-1 β and IL-6, whose levels are normally augmented by the phagocytic process. These cytokines are believed to be central to the control of *cryptococcal* infection, with TNF of particular importance to the development of protective immunity .

GXM also activates the alternative complement pathway, eliciting the deposition of C3b and C3bi on the surface of *C. neoformans*. Such deposition would generally facilitate binding to the leukocyte specific C3 receptor with ensuing phagocytosis.

However, the encapsulated *Cryptococcus* masks the bound complement fragments at sites beneath the capsule surface hence protecting the organism and depleting the hosts complement repertoire . Similarly, GXM binds IgG, an interaction that would ordinarily facilitate recognition by the Fc receptor of host phagocytes. Once again,
5 however, the epitopes that interact with host antibody are primarily located beneath the capsular surface, thus hindering efficient targeting by phagocytes .

The *C. neoformans* capsule also inhibits leukocyte migration from the bloodstream to the inflammatory site . Intravascular GXM inhibits L-selectin being discarded from
10 the surface of neutrophils, hence preventing their infiltration into the tissue from the bloodstream . Furthermore, GXM and GalXM both bind CD18 (β chain of LFA-1) on the surface of leukocytes, preventing the ensuing interaction with ICAM-1 and consequently hindering extravasation into the inflammatory site .

Yet another mechanism of immune evasion, which is mediated by the capsule of
15 *Cryptococcus*, is induction of a regulatory T cell population induced by GXM that in turn reduces humoral and cell mediated responses . This inhibition is potentially mediated via IL-10 that is evoked following infection with the encapsulated organism. This diminishes the release of pro-inflammatory cytokines cytokines and is thought to
20 drive a Th2 rather than a protective Th1 response.

Another putative virulence factor utilised by *Cryptococcus* is the generation of melanin via the copper dependent enzyme laccase . Mutant *Cryptococcus*, deficient in melanin synthesis, possess no, or reduced, capacity to kill mice. The mechanism by
25 which melanin is believed to elicit protection is undefined yet evidence from *in vitro* studies suggest that it is laid down on the inner aspect of the yeast cell wall and acts as a free radical scavenger . Similarly, the *Cryptococcal* product mannitol is thought to enhance survival and pathogenicity, potentially through its ability to scavenge hydroxyl radicals . Limited evidence suggests the presence of a *cryptococcal* derived
30 superoxide dismutase (SOD) with free radical scavenging potential, a phospholipase that correlates with augmented virulence, and intrinsic proteolytic activities, which have been postulated to be implicated in pathogenicity .

1.6.4 Immunity to *Cryptococcus neoformans*.

In mice, protection from *C. neoformans* infection depends on the genetic background of the inbred strain used. Resistant mice (CBA, C.B-17, BALB/c) generally produce higher concentrations of type 1 cytokines in response to *C. neoformans* infection. In contrast, susceptible strains (C57BL/6, C3H and B10.D2) develop a Th2 driven pulmonary eosinophilia and at the peak of pathogen burden up to 40% of airway cells are eosinophils. This response is non-protective and results in tissue damage resulting from degranulation and crystal deposition by eosinophils. The role of eosinophils in anti-fungicidal activity is controversial. Though *in vitro* studies show them capable of phagocytosing *C. neoformans*, this event *in vivo* has been more difficult to elucidate. Eosinophils have been observed juxtaposed to *C. neoformans* and are thought to be involved in recruiting macrophages and T cells by the production of pro-inflammatory cytokines and chemokines.

The phagocytosis of the yeast cell by the alveolar macrophage is central to the ensuing immune response through the early production of TNF, MCP-1, MIP-1 α , which are fundamental for subsequent cellular recruitment to the lung. The protective infiltrate comprises of T cells, macrophages, NK cells and granulocytes, with neutrophils found early in the pulmonary infiltrate and subsequently replaced by monocytes. The inflammation is granulomatous, with the primarily intracellular fungi being encased by an accumulation of inflammatory cells in the alveoli. It is thought that granuloma formation is primarily dependent upon CD4⁺ T cells and macrophage accumulation, encasing the yeast and preventing the organism from replicating and disseminating into the bloodstream. Furthermore, the macrophages fuse around the yeast, forming giant multinucleated cells and releasing hydrolytic enzymes into the interstitium. Formation of the granuloma is considered to correlate with resistance to *Cryptococcus neoformans* infection, with its absence associated with dissemination and potentially death.

Specific T cell mediated immunity is critical to the protective immune response although no immunodominant antigen has been identified to date. CD4⁺ and CD8⁺ T cells are required for maximal recruitment, pulmonary clearance of pathogen and

protection from dissemination . IL-2 activated T cells, as well as localised NK cells, have the potential to directly bind and inhibit growth of *Cryptococcus neoformans* . Furthermore, there is evidence claiming that a protective T cell response is of a Th1 phenotype, with mice deficient in Th1 associated cytokines TNF, IFN- γ , IL-12 and IL-18, all succumbing to infection . TNF in particular is fundamental for cell recruitment, delayed type hypersensitivity and in direct *cryptococcal* killing. Furthermore, IFN- γ and IL-12 are implicated in the assembly of a granuloma and suppression of dissemination, respectively. The IFN- γ generated by the T cell infiltrate also augments the activation state of the resident macrophages, hence enabling them to eradicate infecting yeast, primarily through the generation of nitrite products .

1.8 Respiratory Infection Driven Pathology

1.8.1 Pathology: Pathogen induced or Immunopathology

Pathology and disease induced by respiratory infections is often attributed to the replication and cytotoxicity of the pathogen alone. However, it is now understood that many of the manifestations of respiratory disease are evoked by the host's immune response to infection. The relative contributions of pathogen induced pathology and this immunopathology are variable and defined by the infective agent. Immunopathology may occur as a consequence of over-exuberance, or the induction of an inappropriate counter that is damaging rather than protective. If the pathogen is to be successfully eradicated, then some immunopathology may be unavoidable, but it is desirable to get the optimal balance between fighting infection and restricting collateral damage to surrounding tissue architecture and ensuing impairment of respiratory function. In the case of a viral infection such as influenza, the clinical signs of infection are closely linked to the over-exuberant immune responses to the pathogen. In contrast, the clinical manifestations of disease caused by *Cryptococcus neoformans* are primarily caused by the pathogen itself. These two quite distinct pathogens therefore pose quite different problems and strategies to combat the pathogen and alleviate illness and disease must take this in to consideration.

1.8.2 Influenza driven pathology

Influenza is highly cytopathic and induces extensive tissue damage at the site of infection, with significant submucosal oedema and shedding of epithelial cells. Influenza directly elicits significant aberration of tissue architecture following infection, with extensive cell death by cytolytic and apoptotic means. Influenza is capable of subverting host cell function by suppressing host gene transcription and protein synthesis, whilst promoting viral replication and production of virion particles. Subsequently, infected host cells are subjected to a cytolytic death at approximately 20-40 hours post infection. Conversely, influenza can also elicit apoptosis of the invaded host cells, with virion proteins such as NA and NS1 implicated in the regulation of the apoptotic program.

There are many viruses that display minimal cytopathic potential, implying that a significant proportion of the extensive tissue damage and illness is attributable to the over-exuberance of the host's immune response. Whilst some pathology that accompanies influenza infection of the lung is directly attributable to the virus itself, a significant extent results from the over-exuberance of the host's immune response to the virus.

Influenza infection elicits a potent pro-inflammatory response, with an abundance of cytokines and chemokines being released from epithelial cells and leukocytes. The release of these soluble mediators is fundamental to the control and resolution of influenza infection, but is also central to the development of many of the observable clinical and pathological manifestations. Mediators that are provoked by influenza infection and that have subsequently been demonstrated to correlate with increased illness and disease are TNF, IL-6, IL-8, IL-1 α , IL-1 β , IFN- α , and various chemokines such as MIP-1 α . Investigations, using knock out mice and anti-cytokine strategies have implicated these cytokines and chemokines in lung inflammation, anorexia, excessive sleepiness and fever progression. The use of a neutralising antibody to TNF in a murine model of influenza induced pneumonia culminated in a significant reduction in lung pathology and prolonged survival. Similar investigations with anti-serum injection have defined IFN- α and IL-1 α as important mediators of the influenza

induced fever . Influenza infected IL-1 β and IL-6 knock out mice fail to display the same elevation in body temperature and MIP-1 α knock out mice possess reduced mononuclear infiltrate and pulmonary edema .

5 Both macrophages and neutrophils are recruited during influenza infection and as part of their anti-microbial arsenal they possess an NADPH oxidase that generates the highly reactive superoxide species. The superoxide generated by the phagocytic NADPH oxidase may be the causative agents of what may be referred to as “collateral damage”, and the underlying determinant of immunopathology and disease. This is
10 particularly evident in the lung, whereby oxidant-induced injury can cause extensive damage to lung epithelial cells and may in extremes lead to respiratory failure and death. ROS fail to discriminate between exogenous pathogens and the endogenous host tissue, and consequently are key mediators in pathology in a host of infections. Superoxide can react with nitric oxide, derived from inducible nitric oxide synthase
15 (iNOS) of macrophages, to form the peroxynitrite, which causes extensive tissue injury and mutagenesis through oxidation and nitration of various biomolecules . Indeed, excessive superoxide has been linked to lipid peroxidation, mitochondrial dysfunction, and inflammatory injuries. Treatment with superoxide dismutase (SOD), which breaks down superoxide, improves lung pathology and reduces lethality in
20 influenza induced pneumonia .

Though an efficient T cell response is crucial to the resolution of many viral infections, over-exuberance leads to immunopathology, airway occlusion and significant injury to alveoli epithelia. Many infection induced lung diseases are
25 believed to be largely T cell mediated, with substantial accumulation of both CD4⁺ and CD8⁺ T cells in the alveolar spaces and / or the interstitium, which subsequently impedes efficient oxygen transfer and compromises lung function. CD8⁺ T cells are of particular prevalence as both key effectors of viral clearance due to their potent cytotoxicity, and as significant mediators of pathology and illness. Influenza infection
30 of T cell deficient mice compromises viral clearance, but attenuates inflammation with reduced histological evidence of lung injury. Furthermore, adoptive transfer of haemagglutinin specific CD8⁺ CTLs into transgenic mice that constitutively express HA

antigen in lung cells, leads to profound and lethal lung injury, with extensive alveolitis and abated gas exchange . Furthermore, lung inflammation and impairment of lung function following influenza infection is impingent upon the production of the Th1 cytokine IFN- γ by CD8⁺ T cells .

5 **1.8.2 *Cryptococcus neoformans* Driven Pathology**

Pathology attributable to *C. neoformans* infection is a combination of pathogen induced and immunopathology, but is primarily attributable to the persistence of the pathogen itself. The persistence of the pathogen coupled with its considerable size and vast numbers represents a serious problem in an organ such as the lung whereby open
10 airways must be maintained to support efficient gas exchange. However, the persistence of the pathogen will ultimately lead to chronic inflammation which will subsequently evoke considerable immunopathology. In mice, resolution of *C. neoformans* infection and prevention of dissemination to other sites is dependent on development of a Th1 immune response (as seen in resistant BALB/c mice) .
15 Conversely, the generation of a Th2 response (as seen in susceptible C57BL/6 mice) leads to chronic pulmonary eosinophilia, unchecked fungal growth and dissemination to other organs . Similarly, in humans there is evidence that eosinophilia correlates with a non-protective response and an inability to clear *C. neoformans* infection. Eosinophils have been identified in the BAL fluid of patients infected with this fungal
20 pathogen , and hypereosinophilia has been reported with disseminated cryptococcal disease . Similarly, *C. neoformans* causes summer type hypersensitivity pneumonitis in Japan and pulmonary eosinophilia is observed even in *C. neoformans* infected immunocompetent individuals . The chronic pulmonary eosinophilia that occurs with failure to clear *C. neoformans* is strongly affiliated with the subsequent pathology.
25 Persisting tissue eosinophilia results in tissue destruction due to the release of eosinophil derived mediators such as ROS, major basic protein and eosinophil cationic protein and the deposition of Charcot Leyden crystals .

1.9 Treatment of Respiratory Infections

Classically, vaccination and the administration of anti-microbial agents have been the primary strategies utilised to directly target the invading pathogen and confer protection. However, with ubiquitous problems of efficacy and safety, and the significance of immunopathology to disease status there is now intense research into new therapeutics that manipulate the host's mounted immune response.

1.9.1 Vaccination.

Many vaccination strategies have been applied worldwide with variable success . Whole killed influenza vaccines are successful and well tolerated, if under utilised, and afford 60-90% protective efficacy in children and adults . However, there is some evidence that these vaccines induce adverse reactions in juveniles and confer limited protection in the elderly . There is immense antigenic variability in influenza as a result of the antigenic drifts and shifts in the surface proteins HA and NA. Consequently, the WHO is continually monitoring this variability in order to predict the influenza strains that will circulate the following year. These strains are then incorporated into a tri-valent vaccine currently with H1N1, H3N2 influenza A virus and one influenza B virus. In addition, split influenza vaccines containing ether or detergent treated viral products, are currently in widespread use. However, there are concerns regarding the level of protection they confer, with limited immunogenicity in naïve individuals . Furthermore, infrequent hypersensitivity reactions such as oculorespiratory syndrome and Guillain-Barre syndrome have been reported.

Cold-attenuated live vaccines have been used extensively for many years in the former Soviet Union with considerable success and are now starting to be utilised in the US. It is anticipated that these vaccines may afford greater protection as they more intimately imitate natural influenza infections and should therefore elicit a broader immunological response and confer more long-lasting protection. Preliminary studies have shown live attenuated vaccines confer high protection , but concern persists regarding their safety, as it is feared that genetic exchange, as seen in pandemics, may occur between the attenuated vaccine and co-infecting virulent strains. DNA vaccines have been constructed encoding one or multiple influenza antigenic determinants,

such as HA, NA, MP 1 and NSP-1. In mice, such vaccines evoke significant protection, eliciting a full range of humoral and cellular immunities .

Due to the antigenic variability continually displayed by influenza virus, a conserved M2 protein vaccine appears to be extremely promising, since its efficacy will be unperturbed by the extensive antigenic shift and drift in other proteins . The protection of many traditional vaccines is now being augmented by the co-administration of appropriate adjuvants. Subunit influenza vaccines are now delivered with the adjuvant MF59 in some European countries, and afford increased neutralising antibody responses to both influenza A and B. Mucosal delivery of vaccines may be advantageous, but such delivery systems have displayed limited success. However, the incorporation of adjuvant derived from bacteria improves immunogenicity.

There are at present no licensed vaccines currently available for the prevention of fungal infections. The insurgence of fungal opportunistic infections has highlighted the urgent requirement to develop effective treatments to combat such diseases. New methods that aim to enhance immunity to the fungal pathogen are therefore a prerequisite for an applicable and effective therapy, and much focus is now being directed towards the generation of a protective vaccine.

Cryptococcus neoformans exemplifies the problems that persist with existing treatments and the efforts that are being made to develop plausible vaccine candidates. There is at present a paucity of chemotherapeutic agents that offer relief from *cryptococcal* driven disease, and active vaccination has been heralded as a real possibility of preventing cryptococcosis in high risk patients. The cryptococcal polysaccharide capsule would represent a logical vaccine antigen , being capable of evoking a protective antibody response. Indeed, there exists unequivocal evidence that antibodies directed against the cryptococcal polysaccharide capsule enhance host protective immunity , and a murine monoclonal antibody to GXM is currently in Phase 1 clinical trials in the USA .

Vaccine candidates are also being investigated that will elicit a protective cell mediated response to *C. neoformans*, rather than merely a humoral response. Cell mediated immunity is critical to the resolution of this fungal pathogen and hence strategies to heighten this immune compartment would be desirable. Levitz and colleagues have demonstrated the potential of *cryptococcal* mannoprotein to stimulate T cell responses , and Mandel *et al* have shown the DHA 1 gene product to elicit delayed type hypersensitivity in mice .

1.9.2 Anti-microbial drugs

The WHO has warned that a new influenza pandemic is possibly imminent, and there are concerns that current vaccines will be insufficiently immunogenic. In the outbreak of a pandemic there will be limited time between the detection of the causative strain and the requirement for a protective vaccine. The development of anti-viral drugs could be crucial at conferring protection in such a scenario, and are now receiving extensive research. There are currently two distinct classes of anti-influenza drugs available, those that inhibit the M2 ion channel and those that are neuraminidase inhibitors. Existing M2 ion channel inhibitors are amantidine and rimantidine, which while exhibiting definitive anti-viral potential have significant disadvantages. Amantadine displays poor oral bioactivity with ensuing difficulties in successful administration, and there are serious deleterious side effects in the CNS, liver and kidneys. The drugs ineffectiveness against influenza B strains, and the rapid onset of post-treatment drug resistance also raise considerable concerns over its applicability as a plausible drug candidate . Rimantadine displays reduced neurotoxicity relative to amantadine but its availability is limited as are extensive investigations profiling its activity. Neuraminidase inhibitors, zanamivir and oseltamivir, function to prevent release of new infective virions from the host cell and subsequent dissemination. Clinical studies demonstrate the potential of these anti-viral agents to alleviate some early symptoms of influenza . Though generally regarded as being safe there persist some concerns, with zanamivir being implicated in occurrence of bronchospasms. Worryingly, there have been reports of the emergence of influenza strains that are now also resistant to neuraminidase inhibitors Therefore, though the development of anti-viral drugs directed against influenza are highly desirable there still persist

concerns over safety and increasing resistance. Furthermore, many of the symptoms of influenza may only become apparent when the influenza burden has started to diminish, questioning the applicability of such drugs in the practical prevention of illness and viral spread.

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No vaccine to elicit protection from *C.neoformans* is currently available and although still somewhat lacking in efficacy, infection is currently treated with a number of antifungal agents. Amphotericin B (AMB) is successfully used in all forms of cryptococcosis from pneumonia to meningitis and in the case of the latter, has a 60-70% success rate . It is used on its own or in combination with the other widely used agent, flucytosine. Flucytosine has a superior penetration into the CSF than AMB but fungal resistance to this drug is common and treatment in meningitis results in a number of side effects, especially in AIDS patients . Due to the requirement for intact immune responses to control infection, *C. neoformans* is an ideal infectious model to explore the potential benefit of immuno-stimulatory drug strategies in the treatment of an ongoing infection.

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1.9.3 Immunotherapeutics

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Due to the limited success of vaccination and anti-microbial agents, modulation of the host's immune response is now an intense area of research in the development of therapeutics for respiratory infections . Various strategies have been utilised to manipulate the over-exuberance of the host's response such as modulation of levels of inflammatory cytokines, T cell responses and education of the lung environment. The two respiratory pathogens influenza and *C. neoformans* pose quite different problems when elucidating potential immunotherapeutic strategies that will alleviate symptoms of disease. In the case of a viral infection such as influenza, the clinical signs of infection are associated with over-exuberant immune responses to the pathogen. In such situations, a *reduction* of this inflammation presents a novel strategy to treat infected individuals. In contrast, the clinical manifestations in diseases such as cryptococcosis are primarily caused by the pathogen. Therefore in this situation it would be beneficial to *enhance* immunity to the infectious agent.

Cytokine therapy is a popular method utilised to combat pathology and illness induced by an inappropriate immune response. Cytokines play a paradoxical role in establishment of host immunity and frequently the ensuing pathology. Studies in antibody depletion have yielded promising results within the murine influenza model.

5 TNF depletion alleviates weight loss, illness and pathology that are evoked by influenza infection . In addition, depletion and knock out studies of IFN- α , IL-1 α , IL-1 β , IL-6, IL-8 and various MIPs and MCPs implicate these cytokines and chemokines in influenza associated fever, lethargy and anorexia .

10 In some instances, insufficient immunity results in inadequate host defence and pathogen clearance. In such a scenario, depletion of immunosuppressive cytokines is beneficial. TGF- β is an immunosuppressive cytokine that deactivates macrophages and suppresses T cell function. TGF- β is secreted as an inactive homodimer bound to latency associated protein (LAP), and is activated extracellularly by dissociation from
15 LAP. LAP can therefore be utilised as a novel method of cytokine depletion through reduction of TGF- β activity. Administration of LAP to BCG infected mice inhibits growth of BCG in the lung and enhances the antigen specific proliferation of T cells and IFN- γ secretion . It is therefore apparent that the immunosuppressive cytokine TGF- β partially mediates the susceptibility of the lung to primary BCG infection, and
20 depletion of TGF- β represents a plausible mechanism to confer heightened immunity in this and potentially other infections such as *C. neoformans*. Indeed, administration of DNA encoding TGF- β 1 to *C.neoformans* mice reduced inflammation but compromised pathogen clearance Similarly, chemokines and other molecules that mediate the trafficking and accumulation of cells at an infected site provide attractive
25 targets for such neutralisation therapy .

ROS and RNS are potent pathogenic mediators elicited by the host's immune response. Anti-oxidants therefore confer a feasible therapeutic agent since they will not only limit oxidative stress but will also limit the exuberance of the elicited
30 response by suppressing ROS evocation of pro-inflammatory sequelae. Peroxynitrite is anticipated to be a causative agent of significant pathology and illness during influenza infections and various studies demonstrate the potential to limit lung

damage and inflammation in murine models by reducing superoxide or nitric oxide levels. For example, over-expression of extracellular superoxide dismutase (SOD) reduces inflammation and lung injury during influenza infection . Suppression of ROS/RNS levels is complicated practically due to the paradoxical role of these mediators in protection and pathology, and the multifaceted functions they fulfil in host physiology and immunomodulation, which are only now being appreciated.

T cells are another component of the host's immune response that are frequently the cause of extensive pathology. Several strategies have been employed to modulate host T cell responses to respiratory infection, such as depletion of T cells, reduced antigen presentation and inhibition of co-stimulation by B7. However, all of these mechanisms will not only target T cells activated in response to antigen challenge, but also the peripheral T cell repertoires and may render the host immunologically compromised. A novel mechanism to dictate host T cell responses is via modulation of the co-stimulatory signal imparted by OX40 . OX40 is absent on naïve T cells and up-regulated 1-2 days after antigen encounter. Therefore, investigation into this co-stimulatory signal has received heightened interest since its successful blockade could result in the selective targeting of only those T cell subsets that have recently encountered antigen. We have demonstrated the potential to interrupt OX40/OX40L signalling, through the administration of OX40:Ig, during influenza infection of the lung. Administration of OX40:Ig ameliorated T cell mediated immunopathology and illness . Furthermore, murine and human OX40 display significant homology, and human OX40 also reduces influenza-induced immunopathology in mice. Therefore, it is plausible that such a treatment may be applicable in humans.

It is sometimes necessary to promote a protective T cell response if an infection is to be successfully resolved. During *C. neoformans* infection in susceptible strains (C57BL/6, C3H and B10.D2) of mice, Th2 driven pulmonary eosinophilia develops. This response is non-protective and the mice fail to clear the pathogen leading to dissemination to the brain. It would therefore be advantageous to promote a Th1 response if we are to successfully eliminate *C. neoformans* infection in this model. We have again manipulated the OX40 signalling pathway through the addition of an

OX40L:Ig which binds OX40 on the surface of T cells and promotes a heightened response . Addition of OX40L:Ig during *C. neoformans* infection selectively enhances IFN- γ secreting CD4⁺ T cells, reducing pulmonary eosinophilia and controlling *C. neoformans* burden. Therefore, one can envisage a plausible therapeutic role for manipulation of this late co-stimulatory molecule in promoting T cell immunity and selectively protecting from Th2 driven immunopathology.

Selective T cell depletion offers another strategy to manipulate the host's immune response and limit pathology. In several diseases, discrimination must be made between a Th1 and a Th2 response, with one conferring protection and the other eliciting damage, as is the case with *C. neoformans*. Selective depletion of a Th1 or Th2 population of CD4⁺ cells may be beneficial, but it is first mandatory to determine stable, reliable markers for each subset against which therapy can be targeted. Markers specific to the Th1 subset include chemokine receptors 1, 3 and 5 and interleukin-18 , whereas those specific to Th2 CD4⁺ T cells are chemokine receptors 3 and 4, transcription factors c-maf and GATA-3 and orphan receptor T1/ST2 . Selective depletion of T1/ST2 expressing T cells alleviates eosinophilic lung disease to RSV

In conclusion the effective management and treatment of respiratory infections persists as a ubiquitous dilemma, with current therapeutics deficient in efficacy and practicality (Table 1.5). The development of protective vaccines and potent anti-viral agents, whose primary objective is eradication of the invading pathogen, would be optimal for conferring widespread protection and avoidance of all infection-induced pathological sequelae and illness. However, there remain significant deficiencies in these treatments with current vaccines incapable of conferring protection against the considerable heterogeneity elicited by the infective agent, and doubts persevering concerning the safety and immunogenicity of new vaccine candidates. Furthermore, current anti-viral agents are in many cases largely ineffective and there are increasing incidents of microbial resistance. More significantly, much of the illness and symptoms of respiratory infections only become apparent when viral burden has begun to subside, leaving the practicality of administering these drugs a considerable

dilemma. The significant immunopathological component of respiratory illness makes modulation of immune responses an attractive therapeutic candidate. These treatments would be highly applicable in alleviation of symptoms and recuperation from illness, following failure of traditional vaccines and anti-viral agents to rapidly resolve infection. However, immune manipulation is not without its downside and treatments must consider the specific pathogen infection, and the genetics, age and immunological status of the host. Infection history and the presence of co-infections must also dictate the applicability and direction of such treatments as manipulation of a particular facet of the immune response may alleviate illness associated with one infection, yet enhance the severity of another. Furthermore, many of the plausible targets of immune modulation possess natural physiological roles and their manipulation may alter the homeostasis or viability of the host.

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Treatment Strategy Advantages	Vaccination	Anti-microbial Agents	Immunotherapy
	-Directly targets pathogen -Long term protection -Relatively inexpensive -Relatively easy and quick production -Excellent safety record -Public acceptance	- Directly targets pathogen -Generally well tolerated -Relatively inexpensive -Relatively easy and quick production	-Induce appropriate immune response to pathogen -Effective in clinically ill patients -Single treatment may be effective against many infections -Unaffected by antigen diversity
Disadvantages	-Antigen diversity -Efficacy -Reversion to pathogenic strain -Incomplete deactivation of pathogen -Safety occasionally an issue -Ineffective against patients already infected	-Antimicrobial resistance -Ineffective against patients already infected -Safety occasionally an issue -Efficacy	-Largely untested -Effect on co-infections -Infection history -Genetic status of patient -Health of patient -Relatively expensive

Table 1.5. Treatment strategies to alleviate respiratory pathogen driven illness and pathology: advantages and disadvantages.

1.10 Objectives.

Illness and pathology induced by respiratory infections may be attributable to the cytotoxicity and persistence of pathogen and/or the over-exuberance or inappropriate nature of the host's response to infection. We have previously manipulated T cell responses as an immunotherapeutic strategy during respiratory infection: blockade of the late co-stimulatory signal OX40 reduced influenza induced inflammation and illness, whereas promotion of OX40 signalling boosted immunity to *C. neoformans*. Myeloid cells fulfil a critical role in the establishment and maintenance of an inflammatory response to respiratory infection, and as such offer plausible therapeutic targets. We hypothesise that myeloid cells can be manipulated to enhance anti-pathogen immunity and/or reduce immune pathology in the lung. In the following thesis I will seek to address the following aims:

1. To elucidate the role of ROS during influenza and *C. neoformans* infection, and ascertain the therapeutic potential of limiting the levels of these species.
2. To investigate the basic expression and biology of CD200 and its CD200R, which deliver an inhibitory signal to the myeloid compartment.
3. To determine effect of manipulating the CD200/receptor interaction during influenza and *C. neoformans* infection.

Chapter 2 - Materials and Methods

All chemicals were purchased from Sigma, Poole, Dorset, UK unless stated otherwise.

2.1 Mice

8-12 week old female BALB/c and C57BL/6 mice were purchased from Harlan Olac Ltd (Bicester, UK). Cybb tm1 mice (back-crossed to C57BL/6 background at least 10 times) were originally purchased from Jackson laboratories (Bar Harbour, ME, USA) but bred in house thereafter. OX-2 (CD200) knock out mice were a kind gift from J. Liversidge (University of Aberdeen, Aberdeen, Scotland). DO11.10 transgenic mice

were a kind gift from C. Lloyd (Imperial College, London, England). SKG mice were a kind gift from S. Sakaguchi (Kyoto University, Kyoto, Japan). All mice were kept in pathogen-free conditions according to Home Office guidelines.

5 **2.2 *Cybb tm1* Mutation Detection by PCR**

DNA was extracted from 10mg spleen of tissue from naïve C57BL/6 and *Cybb tm1* mice using a DNeasy kit (Qiagen, Hilden, Germany). Flanking probes specific for either exon 3 of wild type gp91phox or for the-neomycin gene cassette that replaces exon 3 in *cybb* mice were used, producing 240bp and 195bp bands representing wild type and mutated gp91 genes, respectively. The PCR conditions were as follows for a 10µl reaction: 94°C for 3 minutes> 35 x 1.5min cycles (30sec at 94°C > 30sec at 56°C > 30sec at 72°C) > 72°C for 2 min. Using wild type splenic DNA, the volumes of 25mM magnesium chloride and DNA was optimized for the reaction. The PCR products were then detected in the presence of ethidium bromide on a 1% agarose 15 (Invitrogen, California, USA) gel in Tris-Borate EDTA (TBE).

2.3 *Pathogen Stocks*

2.3.1 *Influenza*

Recombinant influenza A strain X31 (hemagglutinin [HA] titre 1024) was a kind gift from Dr Alan Douglas (National Institute for Medical Research, London, UK). The virus was titrated by haemagglutination assay. Human group 'O' red blood cells (RBC) were collected in Alsever's solution to prevent clotting (ratio 1:1). Cells were washed three times in Alsever's solution, each time centrifuging for 5 min at 1200 rpm, and re-suspended as a 10% stock solution in PBS. Influenza stock solution was doubly diluted in PBS in a round-bottom plate. An equal volume (50µl) of 0.5% 25 RBC/PBS was added and incubated at room temperature for 1 hour, or until RBCs had settled at the bottom of the plate. The HA titre was defined as the highest dilution of virus capable of causing agglutination, i.e. inhibit RBC precipitation into a defined button at the bottom of the well. The titre was expressed as the reciprocal of the highest dilution of virus showing agglutination, and represents 1 HAU/50 µl of virus.

2.3.2 *Cryptococcus neoformans*

C. neoformans strain 52, was obtained from the American Type Culture Collection (Rockville, USA) and for murine infection, grown to stationary phase (48-72 hours) at room temperature on a shaker in Sabouraud dextrose broth (1% neopeptone and 2% dextrose; Difco, Detroit, USA). The cultures were washed in saline, counted on a haemocytometer and diluted in sterile PBS to the required infective dose. Stock concentrations and viability were confirmed by plating on Sabouraud agar plates (Difco) and incubated for 48 hours at room temperature prior to colony counts.

2.4 *Reagents for in vivo studies*

2.4.1 *CD200 reagents*

OX90 (rat blocking anti murine CD200 IgG2a) and OX110 (rat agonistic anti murine CD200R IgG1) hybridomas were a kind gift from Neil Barclay (Sir William Dunn School of Pathology, Oxford, UK). Hybridomas were cultured in RPMI 1640 with 20% FCS, L-Glutamine, non-essential amino acids (Invitrogen, Paisley, UK) and 50µM 2-β-Mercaptoethanol acids (Invitrogen, Paisley, UK) in Integra CL1000 bioreactor flasks. Culture media for the OX110 hybridoma was supplemented with 10% IL-6 tissue culture soup from Sp.2 mIL-6 cell line (ATCC, Teddington, UK). CD200 fusion protein (CD200:Fc) was obtained from Trillium Therapeutics Inc. (Toronto, Canada). Murine CD200:mIgG1 (CD200:Fc) fusion protein was constructed by using a chimeric cDNA that contained the extracellular domain of CD200 fused to the constant region of murine IgG1. This construct was used to transfect clonal chinese hamster ovary cells and fusion proteins was purified from the culture supernatant using protein G sepharose .

2.4.2 *Manganese (III) tetrakis (N-ethyl pyridinium-2-yl) porphyrin (MnTE-2-PyP)*

MnTE-2-PyP was purchased from Merck Biosciences AG (Weidenmattweg, Switzerland).

2.4.3 CpG ODN

Highly purified CpG ODN 1826 (sequence 5' TCCATGACGTTTCCTGACGTT) was provided by Coley Pharmaceutical Group (Wellesley, MA) and had a fully phosphorothioate-modified backbone for nuclease resistance and no detectable endotoxin. CpG 1826 was also methylated between nucleotide positions 8-9 and 17-18 (5'-TCCATGACoGTTTCCTGACoGTT-3') in order to nullify the unmethylated immunostimulatory capacity. The methylated 1826 CpG is referred to as control ODN

2.4.4 Modified heat labile toxin of *Escherichia coli* (LTK63)

Escherichia coli non-toxic heat-labile toxin, LTK63, was provided by Chiron S.p.A. (Siena, Italy). LTK63 was free of physiologically significant levels of endotoxin as measured by the LAL system; 6.4 endotoxin U/mg

2.4.5 OX40 reagents

Pegylated antibody to OX40 (human gamma 1 A9 Fab'-PEG) was provided by UCB Celltech (Slough, UK). A9 is a human IgG1 Fab fragment linked to polyethylene glycol, and is 40KDa. A9 was free of physiologically significant levels of endotoxin as measured by the LAL system; 0.08 endotoxin U/mg. The murine OX40L: mIgG1 fusion protein, OX40L: Ig, was obtained from Xenova Research Ltd (Cambridge, UK) and was constructed using a chimeric cDNA that contained the extracellular domain of OX40L fused to the constant region of murine IgG1. This construct was used to transfect clonal Chinese hamster ovary cells and fusion proteins was purified from the culture supernatant using protein G sepharose.

2.5 Mouse Infections and Treatment

On day 0, BALB/c or C57BL/6 mice were anaesthetised with isoflourane and intranasally (i.n) infected 50 HA units of influenza virus (in 50 μ l PBS). In some experiments, virus-infected mice were re-infected with an identical titre of homologous virus 4-5 weeks after the initial infection. *C. neoformans* infections were performed in C57BL/6 mice. Anaesthetised mice were infected i.n with 1×10^4 cfu *C. neoformans* in 50 μ l sterile PBS. In some experiments, wild type C57BL/6 mice

were treated with the indicated dose of MnTE-2-PyP (Merck Biosciences AG, Weidenmattweg, Switzerland) or PBS i.n on day 0 and then at varying days after influenza or *C. neoformans* infection, as indicated in the text. In some experiments, mice were injected i.p. with 10 μ g CD200:Fc, 100 μ g OX90 or 100 μ g rat IgG (Caltag, South San Francisco, CA) on various days as indicated in the figure legend. In some experiments mice were treated i.p. with 100 μ g anti-IL-12 (rat mAb C15.6.7) 1 day before and 1, 4, 8 and 11 days following *C. neoformans* infection. Mice were sacrificed on days indicated in the text by injection of 3 mg pentobarbitone and exsanguinated via the femoral vessels.

2.6. Quantification of Weight Loss and Illness Severity

Weight loss was monitored daily and percent reduction calculated from their original weight on day 0. Illness severity of influenza- infected mice was scored daily using the following criteria: 0 = healthy, 1=barely ruffled fur, 2 =ruffled fur but active, 3 = ruffled fur and inactive, 4 = ruffled fur, inactive and hunched, 5 = dead (never allowed due to home office restrictions). In some experiments lung function was measured in unrestrained animals by whole body plethysmography (Buxco Technologies, Petersfield, UK) as described previously .

2.7 Serum, Nasal Wash and Cell Recovery

BAL, lung tissue and serum were harvested by methods described previously . Blood was first harvested by exsanguination via the femoral vessel and centrifuged for 8 min at 8000 rpm. The serum was isolated and frozen at -70°C for analysis of antibody and cytokines. The lungs of each mouse were then inflated 6 times with 1.5ml 1mM EDTA in MEM and placed in sterile tubes on ice. 100 μ l BAL fluid from each mouse was cytocentrifuged onto glass slides and stained with Haematoxylin and Eosin (H and E). The remainder was centrifuged and the supernatant removed and stored at -70°C for analysis of cytokines by ELISA. Cell viability was assessed using trypan blue exclusion and the pellet resuspended in R10F at a final concentration of 10⁶ cells per ml. Lung tissue, spleen, thymus, Peyer's patch and lymph node were disrupted to a single cell suspension by passage through a 100 μ M sieve (BD labware, New Jersey, USA). The cell suspension was then spun for 5 min at 1200rpm and red blood cells lysed

by resuspending pellets in ACK buffer (0.15M ammonium chloride, 1M potassium hydrogen carbonate and 0.01mM EDTA, pH 7.2) for 3 minutes at room temperature before spinning (1200rpm 5 min) and washing with R10F. Cell viability was assessed by trypan blue exclusion and cells resuspended in R10F at 10^6 cells/ml. The nasal cavity was also sometimes washed with 200 μ l PBS, collected and frozen at -70°C for antibody ELISA analysis.

2.8 Differential Counts of Cellular Composition of BAL Fluid

The proportion of eosinophils induced by *C. neoformans* infection was first enumerated as granulocytes by flow cytometry, using forward and side scatter. Percentages were confirmed by counting eosinophils in H and E stained cytocentrifuge preparations of BAL fluid. The proportion of macrophages and lymphocytes in both *C. neoformans* and influenza infections was also enumerated by morphological analysis of H and E stained BAL fluid preparations.

2.9 Isolation of lung of Type II epithelial cells

Lung type II epithelial cells were isolated as described previously . Briefly, lungs were perfused by the infusion of 10 ml PBS into the right ventricle of the heart. Lungs were subsequently inflated with 2.5 ml of 5 mg/ml dispase II solution (Roche, Basel, Switzerland) and then allowed to collapse naturally. 0.5ml of 1% low melting point agarose was then slowly injected into the lungs and immediately allowed to solidify by packing the lungs in ice. Lungs were then removed and incubated for 40 minutes in dispase solution before being transferred to DMEM containing 50 $\mu\text{g/ml}$ of DNase I solution (Roche, Basel, Switzerland) and the digested tissue teased away from the upper airways. Digested lung tissue was disrupted to a single cell suspension by passage through a 100 μM sieve (BD labware, New Jersey, USA). Epithelial cells were enriched by negative selection, with removal of hematopoietic and endothelial cells by MACS separation (section 2.15). Briefly, lung homogenate was incubated with rat anti-mouse CD45 or CD90 antibodies (BD Pharmingen, Heidelberg, Germany), followed by anti-rat Dynabeads (Invitrogen) and applied to an MS column (Miltenyi Biotec, Gladbach,

Germany) in the presence of a magnetic field. Unlabelled epithelial cells were washed through with buffer (PBS containing 0.5% BSA and 2mM EDTA).

2.10 Generation of bone marrow (BM) derived macrophages and DCs

5 Femurs were removed from BALB/c or C57BL/6 mice and bone marrow extracted by flushing extensively with RPMI-1640 media. RBC depleted bone marrow cells were washed and counted by trypan blue exclusion. To generate BM macrophages, 3×10^5 cells/ml were plated out with 20ng/ml M-CSF in 10 mls RPMI with 20% FCS, 0.1U/ml penicillin / 0.1µg/ml streptomycin and 10% HEPES, and incubated at 37°C
10 for 72 hours. At this time, a further 5mls of medium containing M-CSF (20ng/ml; PeproTech Inc., Rocky Hill, NJ) was added on top of the existing 10mls and incubated at 37°C for another 72 hours. Media was subsequently removed and cells incubated in versene for 20 minutes at room temperature. Cells were then scraped from the plates, washed and resuspended in R10F and counted. To generate BM
15 dendritic cells, 2×10^5 cells/ml were plated out in 10 mls RPMI with 10% FCS, 0.1U/ml penicillin / 0.1µg/ml streptomycin and 10% J558 supernatant (containing GM-CSF), and incubated at 37°C for 72 hours. At this time, 5 mls of media were carefully removed and replaced with 5mls of fresh media with 10% J558 supernatant and incubated at 37°C for another 72 hours. Floating and weakly adherent cells were
20 collected by gentle pipetting, washed and resuspended in R10F and counted.

2.11 Stimulation of BM macrophages/DCs

BM derived macrophages and DCs were cultured at 2×10^6 cells/ml in 200µl R10F in 96 well flat bottomed plates. Cells were stimulated with IFN-γ (10ng/ml or
25 100ng/ml), LPS (10ng/ml or 100ng/ml) or influenza (8 HA or 16 HA). Some cells were also cultured with OX110 to a final concentration of 10µg/ml. Cells were incubated at 37°C and at varying times after stimulation (as indicated in the text), supernatants were removed for cytokine/chemokine analysis and cells stained for surface markers by flow cytometry as described below.

2.12 Flow Cytometric Analysis

2.12.1 Cell Surface Antigens

Cells were stained for surface markers and analysed by flow cytometry as described previously. All antibodies were purchased from BD Pharmingen (Heidelberg, Germany) except anti-CD200-FITC/PE and anti-CD200R-PE (Serotec UK, Oxford, UK). Briefly, 1×10^6 BAL, lung, spleen, thymus, Peyer's patch or lymph node derived cells were stained using APC-conjugated anti-CD4 and anti-CD8-PerCP. Cells were also stained with combinations of FITC-labelled antibodies specific for CD44, CD45RB, CD200, $\alpha\beta$ TCR, $\gamma\delta$ TCR, CD3 or B220, and PE-labelled anti-CD45RB, anti-CD25, anti-CD200, anti-CD200R, anti-CD62L, anti-CD103, or anti-DX-5 and biotinylated anti-OX40 and anti-ICOS. Some cells were also stained with anti-Ly6G-FITC, anti-CD11b-PerCP, anti-CD11c-APC and one of anti-MHC class II-PE, anti-CD200/R-PE, anti-CD80/86-PE, anti-CD40-PE and anti-OX40L-biotin. All antibodies were diluted in PBS containing 1% BSA/0.05% sodium azide (PBA). Cells were stained for 30 min on ice, washed with PBA and spun for 5 min at 1200rpm. Where appropriate, secondary stains with streptavidin constructs were performed. After washing, cells were then fixed for 20 min at room temperature with 2% formaldehyde/PBS. Cells were then washed in PBA and data was acquired on a FACS Calibur and 30,000 lymphocyte events analysed with CellQuest Pro software (BD Biosciences, Belgium).

2.12.2 Intracellular Cytokine Expression

To detect intracellular cytokines, 10^6 cells/ml were incubated with 50 ng/ml PMA, 500 ng/ml ionomycin (Calbiochem, Nottingham, UK), and 10mg/ml brefeldin A for 3 hours at 37°C. Cells were then stained with anti-CD4-APC and anti-CD8-PerCP and fixed as described above. After permeabilization with PBS containing 1% saponin/1% BSA/0.05% azide (saponin buffer) for 10 min, cells were stained with anti-IFN- γ -FITC/PE, anti-TNF-FITC/PE, or PE-conjugated anti-IL5 or anti-IL-4 diluted 1:50 in saponin buffer. 30 minutes later cells were washed once in saponin buffer and once in PBA. Data was then acquired as described above.

2.12.3 *FoxP3*

Binding of PE-labelled intracellular FoxP3 was detected according to manufacturers' instructions (BD Pharmingen, Heidelberg, Germany). Briefly, 1×10^6 cells were stained for surface CD4-PercP as described above and washed extensively with PBA. Cells were subsequently incubated for 16 hours in 200 μ l Fix/Perm working solution at 4°C. Cells were permeabilized with permeabilization buffer for 15 min at 4°C (containing Fc block), and then stained with anti-Foxp3 (FJK-16s)-PE antibody diluted 1:50 in permeabilization buffer for 30 min at 4°C.

2.12.4 *Apoptosis Analysis*

For apoptosis analysis, binding of PE-labelled Annexin V was detected according to manufacturers' instructions (BD Pharmingen, Heidelberg, Germany). Briefly, cells were washed in PBS and resuspended in Annexin-binding buffer containing Annexin V-PE, anti-CD4-FITC and anti-CD8-PercP. After washing in binding buffer, 7-AAD was added to determine dead cells. Apoptotic cells were identified as 7-AAD⁻/Annexin V⁺. Data was then immediately acquired, collecting 30,000 lymphocyte events.

2.13 *Hematoxylin and Eosin staining of lung tissue*

In some studies, lungs were inflation fixed with 2 % formalin in PBS, and spleen, thymus and Peyer's patches excised and placed in 2% Formalin. The organs were then embedded in paraffin wax and 4 μ M sections stained were with H and E (Lorraine Lawrence, Imperial College, UK).

2.14 *Purification of cells by MACS separation*

CD4⁺ T cells were purified from single cell suspensions from C57BL/6 or DO11.10 spleens. Cells were resuspended at 10^8 cells/ml in PBS containing 0.5% BSA and 2mM EDTA (buffer), with 10% CD4 microbeads (Miltenyi Biotec, Gladbach, Germany). Cells were incubated for 15 minutes at 4°C. Cells were washed and

resuspended in buffer up to 2×10^8 cells/ml and applied to an MS column (Miltenyi Biotec, Gladbach, Germany) in the presence of a magnetic field. Unlabelled cells were washed through with buffer. The column was removed from the magnetic field and the fraction containing the magnetically labelled cells was flushed out with a
5 plunger. Cells were counted and purity assessed by flow cytometry.

2.15 T cell activation

2.15.1 OVA peptide stimulation of DO11.10 TCR-Tg CD4⁺ T cells

RBC depleted splenocytes (2×10^5 / well) of naïve DO11.10 TCR-Tg mice were cultured in 96 well flat-bottomed plates in a final volume of 200 μ l RPMI
10 supplemented with 10% FCS. Cells were stimulated with OVA peptide (SIINFEKL) at a final concentration of 0.1, 1, 10 and 100 μ g/ml. At 0, 24, 48, 72 and 96 hours after stimulation cells were stained for the surface expression of CD4, CD200, ICOS and OX40 as described above and analysed by flow cytometry. In some experiments, T cells were stimulated with 10 μ g/ml OVA peptide in the presence of neutralizing
15 antibodies to IL-12 (10 μ g/ml), IL-4 (10 μ g/ml) or IL-10 (10 μ g/ml). In some experiments, cyclosporin A (1 μ g/ml) was added to the splenocyte cultures at 24 hours after addition of OVA peptide.

2.15.2 Generation of Th1 and Th2 biased CD4⁺ T cells

For short-term polarized T cell lines, CD4⁺ cells were purified from the spleen of
20 naïve DO11.10 TCR-Tg mice as described above. Cells were cultured with OVA peptide (SIINFEKL, 100 μ g/ml) and irradiated BALB/c spleen cells in the presence of murine recombinant IL-12 (10 ng/ml) plus neutralizing anti-IL-4 antibody (10 μ g/ml) for Th1 phenotype, or recombinant IL-4 (10 ng/ml) plus anti-IL-12 (10 μ g/ml) and anti-IFN- γ (10 μ g/ml) antibodies for Th2 phenotype, for 5 d at 37°C.

2.15.3 PMA/Ion induced activation of T cells

25 CD4⁺ T cells (2×10^5 / well) were isolated from the spleens of naïve C57BL/6 mice as described above and were cultured in 96 well flat-bottomed plates in a final volume of 200 μ l RPMI supplemented with 10% FCS. Cells were stimulated with 20nM PMA

and 500nM Ionomycin. At 0, 24, 48, 72 and 96 hours after stimulation cells were stained for the surface expression of CD4 and CD200 as described above and analysed by flow cytometry. In some experiments, PMA/Ion were washed out of the cultures after 24 hours and the cells re-cultured in RPMI supplemented with 10% FCS alone for a further 48 hours before being analysed by flow cytometry.

2.15.4 Activation of T cells through CD3/CD28

CD4⁺ T cells (2×10^5 / well) isolated from the spleens of naïve C57BL/6 mice, or total RBC depleted splenocytes (2×10^5 / well) of naïve BALB/c or SKG mice were cultured in 96 well flat-bottomed plates in a final volume of 200µl RPMI supplemented with 10% FCS. Cells were cultured with different combinations, as defined in the text, of plate bound anti-CD3 antibody (5µg/ml; BD Pharmingen, Heidelberg, Germany), soluble anti-CD28 antibody (1µg/ml; BD Pharmingen, Heidelberg, Germany) and / or OX40L:Ig (10µg/ml). At 0, 24, 48 and 72 hours after stimulation cells were stained for CD4, CD200 and OX40 as described above and analysed by flow cytometry.

2.15.5 Antigen presentation assay

Following purification, DO11.10 OVA-specific CD4⁺ T cells were labelled with the intracellular fluorescent dye 5-carboxyfluorescein diacetate succinimidyl ester (CFSE) to analyse cell division. Cells were resuspended in PBS at 5×10^7 /ml and CFSE added quickly to a final concentration of 10µM. Cells were incubated for ten minutes at room temperature and washed twice in R10F to block the reaction. Cells were then resuspended in R10F for plating. BM DCs (2×10^5 /ml) were loaded with ovalbumin (1µg/ml) and stimulated with LPS (100ng/ml) for 3 hours at 37°C. CFSE labelled OVA-specific CD4⁺ T cells were subsequently added to the DCs along with control IgG or OX110 (to a final concentration of 10µg/ml). Cells were incubated for 96 hours at 37°C and CFSE incorporation assessed by flow cytometry.

2.16 Immunoprecipitation and immunoblotting

BM macrophages (1×10^7 cells/ml) were stimulated at 37°C with 100ng/ml IFN- γ for 16 hours. The cells were subsequently treated with control IgG or OX110 (10 μ g/ml) for indicated periods of time at 37°C. Cells were then rinsed once with ice-cold PBS containing 1 mM Na_3VO_4 and lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 5 mM EGTA, 50 mM NaF, 1 mM Na_3VO_4 , plus protease inhibitor mixtures) for 20 min on ice. Lysates were clarified at 14,000 rpm for 10 min. The protein concentration of the supernatant was determined by Bradford assay. For immunoprecipitations, polyclonal anti-Dok2 Ab (Upstate Biotechnology, Lake Placid, NY) was incubated with 0.5–1 mg of cell lysate for 2 at 4°C. The immune complexes were recovered by incubation with protein G Plus-agarose beads (Santa Cruz Biotechnology) for 1 h at 4°C. After washing three times in lysis buffer and once in PBS containing 1 mM Na_3VO_4 , the immune complexes were dissociated in SDS sample buffer. Equal amounts of protein were analyzed by Nu-PAGE (Invitrogen Life Technologies) and Western blotting. For Western blotting, primary Abs (anti-phosphotyrosine mAb (4G10) and polyclonal anti-Dok2 Ab, Upstate Biotachnology) were detected with HRP-conjugated secondary Abs and chemiluminescence (Pierce, Rockford, IL).

2.17 Immunofluorescence microscopy

2.17.1 Immunofluorescent staining for CD200/CD200R

Lungs from 8- to 12-wk-old C57BL/6 mice, at days 0, 3 and 7 after influenza infection, were inflated with 1.5ml OCT (Tissue-Tek) and snap frozen in OCT freezing medium by liquid nitrogen flotation. All frozen tissues were stored at -80°C. Cryostat sections (5–8 μ m) were fixed in cold 80% acetone and 20% methanol, air dried, then blocked with 15% normal goat serum for 30 min at room temperature. Sections were then incubated in primary Abs to murine CD200 (OX90) or CD200R (OX110) (3 μ g/ml) for 2 h at room temperature, extensively washed in PBS, and then incubated for 1 h in Alexa-488 conjugated goat anti-rat IgG (Molecular Probes). Sections were then washed three times in PBS and wet mounted for fluorescent

microscopy. Sections were examined under a Nikon ECLIPSE TE2000-U fluorescence microscope.

2.17.2 Immunofluorescent staining for pro-surfactant C

Isolated epithelial cells, as described in section 2.9, were cytocentrifuged onto
5 poly-lysine coated slides and fixed in 4% paraformaldehyde for 20 mins at room
temperature. Cells were subsequently washed in PBS and permeabilized with 0.2%
Triton-X-100 in PBS for 30 mins. Cells were again washed with PBS and blocked
with 10% normal goat serum for 30 min at room temperature. Rabbit anti-human pro-
surfactant protein C antibody (Chemicon Europe Ltd., Hampshire, UK) diluted 1/500
10 in 10% goat serum was added to cells and incubated for 16 hours at room
temperature. Cells were washed extensively with PBS and cells incubated for 1 hour
in Alexa 488 conjugated goat anti-rabbit IgG (Molecular Probes) in PBS with
propidium iodide (1/1000). Cells were washed again in PBS and mounted in PBS with
10% glycerol and 0.02% azide and examined under a Nikon ECLIPSE TE2000-U
15 fluorescence microscope.

2.18 Measurement of Pathogen Load in Lung Homogenates

2.18.1 Influenza-Specific Plaque Assay

Lung homogenates were freeze thawed three times, centrifuged at 4000 g and
supernatants titrated in doubling dilutions on Madine Darby canine kidney (MDCK)
20 cell monolayers in flat bottomed 96 well plates. After incubation at room temperature
for 3 hours, samples were over-layed with 1% methycellulose and incubated for 72
hours at 37°C. Cell monolayers were washed and incubated with anti-influenza
antibody (Serotec) followed by anti-mouse-HRP (Dako) and infected cells were
detected using 3-amino-9-ethylcarbazole substrate. Infectious units were then
25 enumerated by light microscopy and total plaque forming units per lung quantified
(number of plaques × dilution factor × total volume of lung homogenate).

2.18.2 Enumeration of *C.neoformans* Colony Forming Units (cfus)

Lungs were homogenized by passage through 100 μ m cell strainers. 100 μ l of cell suspension was diluted in sterile PBS and incubated at room temperature for 48 hours on sabouraud dextrose agar plates (Sigma). The total colony forming units per lung were then determined (number of colonies x dilution factor x original cell suspension volume).

2.19 Cytokine detection

2.19.1 ELISA

IL-4, IL-5, IFN- γ , IL-10, IL-12 and TNF in BAL fluid, and in some cases serum, were quantified using OptEIA kits (Pharmingen). Briefly, microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 μ l of capture antibody diluted in the recommended buffer overnight at 4°C. After 5 washes with PBS containing 0.5 % Tween-20, plates were blocked with 200 μ l PBS containing 10 % FCS and left for 1 hour at room temperature. Samples and standards (diluted in PBS + 10 % FCS) were then incubated for a further 2 hours at room temperature. After 5 washes, bound cytokine was detected using biotinylated antibodies pre-mixed with avidin-HRP followed by tetramethylbenzidine and hydrogen peroxidase. Optical densities were read at 450 nm. The mean optical density of wells containing no cytokine was subtracted from the results obtained for samples and standards. The concentration of cytokine in each sample was calculated from a standard curve.

2.19.2 Cytometric Bead Array(CBA)

Levels of interleukin (IL)-6, IL-10, IL-12p70, TNF, monocyte chemoattractant proteins (MCP)-1, and interferon (IFN)- γ in murine serum, BAL fluid and supernatants from macrophage cultures were assayed by mouse inflammation CBA kit (BD Pharmingen, Heidelberg, Germany) using a flow cytometer (FASCalibur, Becton Dickinson). IL-2, IL-4, IL-5, TNF and IFN- γ in lung homogenate were measured by mouse Th1/Th2 CBA. In CBA, five/six bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for five/six

different cytokines. These bead populations could be resolved in the fluorescence channels of the flow cytometer. After the beads had been incubated with 50 µl of supernatant, different cytokines or chemokines in the sample were captured by their corresponding beads. The cytokine/chemokine captured beads were then mixed with phycoerythrin-conjugated detection antibodies to form sandwich complexes. Following incubation, washing and acquisition of fluorescence data, the results were generated in graphical format using the BD CBA software.

2.19.3 *Luminex*

The mouse cytokine Twenty-Plex kit (BioSource International, Inc., California, US.) was utilized to determine the levels of cytokines in BAL fluid, according to the manufacturer's instructions. Briefly, 25µl of Twenty-Plex beads were applied to each well of a pre-wet 96 well filter plate and washed with 200 µl of wash buffer by aspiration with a vacuum manifold. 50µl of incubation buffer and 50µl of standard/sample were subsequently added to the beads in each well and incubated for 2 hours at room temperature. After 2 hours, wells were washed and 100µl of 1x Biotinylated Detector Antibody added to each well and incubated for 1 hour at room temperature. Wells were subsequently washed again and beads incubated with 100µl of 1x Streptavidin-RPE for a further 30 minutes at room temperature. Beads were then extensively washed and resuspended in 100µl of 1x working wash solution. Data was collected with a minimum of 100 beads per analyte using StarStation 1.0 (Applied Cytometry, Sacramento, CA). Standard curves for each cytokine were generated by using the reference cytokine concentrations supplied by the manufacturers. The analyzed data were graphed and statistically analyzed using PrismGraph 4.0 (GraphPad Software, Inc., San Diego, CA).

2.20 *Antibody ELISAs*

2.20.1 *Influenza-Specific Antibody ELISAs*

Microtiter plates were coated with 100HA/well UV inactivated influenza overnight at 4°C. Plates were washed with PBS/tween and blocked with 10%BSA/PBS and diluted nasal wash or serum from naïve and influenza-infected mice was incubated for 3hrs at

room temperature. Plates were washed and incubated with either biotinylated anti-mouse IgG1 or IgG2a (both serotec), anti-mouse immunoglobulin-HRP (Dako) or, in the case of nasal washes/BAL, anti-mouse IgA-HRP (Serotec) for 90 min at room temperature (all diluted in 5%BSA/PBS). In the case of IgG1 and IgG2a ELISAs, plates were washed and incubated with streptavidin-HRP (Dako) for 60 min. After washing, bound antibody was detected on all plates by incubating with *O*-phenylenediamine (OPD, Sigma) substrate in the dark for 20 minutes. The reaction was then stopped with 50 μ l 2M sulphuric acid and plates were read at 490nm. Absorbance values of naïve mice were subtracted from all values.

2.20.2 *C.neoformans*-specific ELISAs

2 x 10⁵ cfu/ml heat killed *C.neoformans* in PBS was used to coat 96 well microtiter plates overnight at room temperature on a shaker. After blocking with 3% BSA/PBS for 2 hours at room temperature, dilutions of sera from naive and *C.neoformans*-infected mice were added for a further hour at room temperature. Bound antibody was detected as described in section 2.20.1. Optical densities were read at 490 nm and mean BSA/PBS blank values were subtracted from the optical density values of test samples.

2.20.3 Total IgE Antibody ELISA

Nasal washes/BAL from *C.neoformans*-infected mice were used to coat microtiter plates overnight at 4°C. Plates were washed with PBS tween, blocked for 2 hours with 3%BSA/PBS and incubated for 2 hrs at room temperature with anti-IgE-HRP in BSA/PBS (Serotec). Plates were washed again and bound antibody detected with OPD substrate. The reaction was stopped with 2M sulphuric acid and absorbance read at 490nm.

2.21. Protein detection

BAL fluid protein concentration was determined using the Pierce BSA protein assay kit. Working reagent was added to samples and standards at a 4:1 ratio and incubated at 37 °C for 30 minutes. Absorbance was measured at 490 nm, and protein concentration calculated by comparison with an albumin standard.

2.22. Lactate Dehydrogenase (LDH) detection

The amount of LDH in the BAL was measured using Sigmas *in vitro* toxicology LDH based assay kit. Samples (tested in triplicate) were added to an equal volume of LDH assay mixture (assay dye, substrate and enzyme) and incubated at RT for 30 minutes. Absorbance was measured at 490nm.

2.23. Nitric oxide detection

The concentration of nitrite in BAL and macrophage culture supernatants was used as a measure of NO production and was quantified using the Greiss reagent kit. Briefly, samples and standards (50µl) were added to a microtiter plate and treated with 1% sulphanilamide for 10 minutes at room temperature. Subsequent addition of 0.1% naphthylethylenediamine (NED) in 2.5% H₃PO₄ yielded a magenta colour in the presence of NO. Optical densities were read at 550 nm. The mean optical density of wells containing media alone was subtracted from the results obtained for samples and standards. The concentration of NO in each sample was calculated from a standard curve.

2.24 Microarrays

2.24.1 RNA extraction

Total RNA was extracted from C57BL/6 and Cybb tm1 adult female mouse lung using RNA STAT-60 (AMS Biotechnology, Oxon, UK) according to the manufacturer's instructions. Briefly, snap frozen lung tissue was homogenised under liquid nitrogen and incubated for 5 minutes at room temperature in RNA STAT-60 to allow nucleoprotein complexes to dissociate. RNA was subsequently separated from protein and DNA using chloroform and precipitated with isopropanol. RNA was washed with 75% ethanol and resuspended in DEPC-treated RNase-free water. RNA integrity was quantified by measuring its absorbance at 260nm and its integrity confirmed by running a small aliquot on a 1% agarose gel.

2.24.2 Labelling of RNA

RNA was labeled using a standard reverse transcriptase labelling method whereby 100 μ g of total RNA was heated for 5 min at 95°C in the presence of 4 μ g oligo(dT₁₅) (total volume 12 μ l). Samples were cooled to 42°C and 18 μ l labelling reaction mixture added [6 μ l 5x RT buffer (BRL); 3 μ l 0.1 M DTT (BRL); 5 mM dATP, dGTP and dTTP, 2 mM dCTP (Abgene); 2 mM Cy3 or Cy5-dCTP (Amersham); 400 U M-Superscript II(Invitrogen)]. Samples were subsequently incubated for 10 minutes in the dark at room temperature followed by 90 minutes in the dark at 42°C. Following first-strand cDNA synthesis, 20 μ g RNase A was added to each sample and incubated at 37°C for 20 min. Reactions were stopped by the addition of 5 μ l 0.5 M EDTA.

2.24.3 Hybridisations

Following termination of the labelling reactions individual samples were mixed with the correctly labelled corresponding sample and passed through a nucleotide removal kit (Qiagen). Mouse Cot-1 DNA (10 μ g) was then added to the mixed samples and sample volume was made up to 30 μ l with hybridisation solution to give a final concentration of 40% formamide, 5x SSC, 0.1% SDS. Samples were heated to 100°C for 5 min then allowed to cool for 10 minutes. Samples were briefly subjected to centrifugation and pipetted beneath coverslips onto HGMP total mouse genome v2 microarray slides (MRC). Microarrays were placed into hybridisation chambers (Corning), the end wells were filled with 16 μ l hybridisation buffer and the chambers sealed. Microarrays were placed in a waterbath at 50°C overnight.

2.24.4 Microarray washing

Microarrays were removed from the hybridisation chambers and washed twice in 2x SSC for 5 min; 2x 0.1x SSC, 0.1% SDS for 5 min; 2x 0.1x SSC for 5 min. Slides were dried by centrifugation for 5 min at 60 g and entered for scanning.

2.24.5 Data capture and analysis

Microarrays were scanned using a GenePix 3000 scanner. BlueFuse image analysis software was used to generate Cy3 and Cy5 expression values for the genes on each of the arrays. During image processing, an automatic flagging process was used to

remove spots whose background-subtracted mean signal intensity was less than four times the standard deviation of the background pixel intensity. Manual flagging was also used to highlight spot irregularities such as dust, scratches and misaligned features. The BlueFuse output data was then imported into GeneSpring GXv7.3. The data was normalised using the Lowess algorithm and the normalised data was checked for any sample anomalies. The data was filtered on confidence, using the p-value ($p < 0.05$) of a t-test as the confidence value, with a Benjamini and Hochberg (BH) correction applied to reduce the false discovery rate. This was first applied to the wild type data to determine which genes were up-regulated and down-regulated with respect to the reference sample. This was repeated for the cybb arrays

2.25. Statistics

All experiments were performed at least twice, analysing 5 individual mice per time point. Statistical significance was evaluated using the student t test, 2 tailed assuming unequal variance within the Minitab software program. The Bonferroni correction was applied where appropriate.

Chapter 3 – The Role of Reactive Oxygen Species in Pulmonary Influenza Infection

3.1 Introduction

3.1.1 The NADPH Oxidase and the generation of Reactive Oxygen Species

The generation of reactive oxygen species (ROS) represents an important component of the host's arsenal to combat invading microorganisms. In addition to their potent anti-microbial activity, ROS also possess a significant cell-signaling role in biological systems, capable of regulating the phenotype and function of immune cells. However, the extreme toxicity and lack of specificity, means they are also capable of eliciting significant immunopathology to surrounding tissue.

The most abundant sources of ROS following pathogenic challenge are the NADPH oxidase of professional phagocytes and xanthine oxidase. Both are potent producers

of superoxide, which can subsequently be converted into a host of other ROS. The NADPH oxidase is a multimeric enzyme complex, composed of membrane associated and cytosolic components, that utilize molecular oxygen and NADPH to generate the superoxide radical ($O_2^{\cdot-}$). The core of the enzyme contains the membrane bound flavocytochrome b558. This flavocytochrome is the site of the oxygen reduction and is comprised of 2 sub units, a 22kDa protein (p22phox) and a 91kDa glycoprotein (gp91phox). After appropriate stimulation, cytosolic factors (p40phox, p47phox and p67phox) migrate to the membrane-associated components to form an active complex that catalyses the formation of $O_2^{\cdot-}$.

3.1.2 The Role of ROS in Respiratory Infections

A combination of transgenic and knock out mice and scavengers of reactive oxygen species have been utilised to elucidate the role of reactive oxygen species in protection and pathology to respiratory infections. Cybb tm1 mice offer a murine model of the autosomal genetic condition of chronic granulomatous disease (CGD). These mice lack the gp91phox sub-unit of the phagocyte NADPH oxidase rendering them incapable of eliciting a respiratory burst with ensuing production of superoxide through this route. These mice demonstrate enhanced susceptibility to certain fungal and bacterial pathogens with increased susceptibility to *Aspergillus fumigatus* and *Staphylococcus aureus*, and compromised clearance of *Escherichia coli*. Mice lacking the p47phox sub unit of the NADPH oxidase display impaired NF- κ B activation and host defence to *Pseudomonas pneumonia*, and a transient loss of resistance to pulmonary tuberculosis with a significant increase in bacterial growth over the early period of infection.

Whilst the production of ROS in pulmonary bacterial and fungal infections constitutes significantly to the host's protective immune response, their role in viral infections is less clear. In 1979, Peterhans *et al* first demonstrated the potential of viral infection to induce production of ROS by phagocytes with *in vitro* studies utilising Sendai virus. Ten years later there was documented evidence citing for the first time the potential of influenza to elicit superoxide production by PMNs and monocytes *in vitro*. Influenza infection induces the production of superoxide from phagocyte NADPH

oxidase and xanthine oxidase pathways that may have partial virucidal activity or limit viral propagation by inducing apoptosis of the infected cell . Conversely, low levels of ROS may facilitate viral replication because of their mitogenic effects on cells. Most viruses grow better in proliferating cells, and indeed, many viruses induce in the host cell changes similar to those seen early after treatment with mitogenic lectins. Furthermore, ROS may inactivate the anti-proteases that are localised on the surface of the alveoli . These anti-proteases are protective to the host as they inhibit proteases that exist in the pulmonary surfactant, which proteolytically cleave the naïve viral coat protein HA0 into HA1 and HA2. Influenza virions expressing HA1 and HA2 possess enhanced infectivity and virulence, and hence the inhibition by ROS on anti-proteases will culminate in more cleavage of this viral coat protein.

ROS, however, fail to discriminate between exogenous pathogens and endogenous host tissue, and consequently are key contenders in the induction of pathology in a host of infections. Superoxide is a precursor for an array of more potent oxidants such as the hydroxyl radical, and furthermore can react with nitric oxide to form peroxynitrite which causes extensive tissue injury and mutagenesis through oxidation and nitration of various biomolecules . Indeed, excessive superoxide is linked to lipid peroxidation, mitochondrial dysfunction, and inflammatory injuries . Furthermore, a decrease in levels of peroxynitrite reduces mortality rates with influenza induced pneumonia , and transgenic mice over-expressing extracellular superoxide dismutase (SOD), exhibit reduced inflammation and lung injury similar to influenza pneumonia.

3.2.3 Scavengers of ROS as potential therapeutics

The potential of reactive oxygen species to elicit oxidative stress and pathology has resulted in the generation of compounds that confer protection through scavenging these reactive and toxic species. Previous studies that have attempted to limit oxidative stress via manipulation of endogenous SODs for therapeutic purposes have had limited success, due to the large molecular weight and short half life of such compounds. A new generation of SOD mimetics offer a more plausible therapeutic approach owing to their increased half life and lower molecular weight, which facilitate entry to intracellular sites. Furthermore, such a mimetic would be ideal as a

therapeutic as it can be administered via a variety of routes. Manganese (III) tetrakis (N-ethyl pyridinium-2-yl) porphyrin (MnTE-2-PyP) is a low molecular weight, cell permeable, catalytic metalloporphyrin antioxidant with potent superoxide dismutase (SOD) mimetic activity . This anti-oxidant has a broader range than SODs, also
5 possessing catalase activity and the ability to scavenge lipid peroxides and peroxynitrite. The potential of this molecule to scavenge superoxide and other ROS provides an alternative strategy to elucidate the role of these mediators in immunity and pathology to respiratory infection. Though MnTE-2-PyP offers protection in a variety of oxidative stress injuries, such as liver ischemia, diabetes, lung radiation
10 injury and stroke , this is the first study to describe its use in the treatment of respiratory infection.

3.2.4 Hypothesis.

The failure of ROS to discriminate between exogenous pathogens and endogenous
15 host tissue implicates them in pathology following influenza infection . Furthermore, ROS act as second messengers evoking the activation of redox sensitive transcription factors implicated in the establishment of an inflammatory response suggesting they may add to the over-exuberance of the elicited immune response . This chapter tests the hypothesis that reducing the levels of ROS during influenza infection will reduce
20 immunopathology and offer a potential immunotherapeutic strategy. Whilst there have been some studies investigating the role of ROS in influenza infection, they have failed to define the role these species have on the phenotype of the host's immune response and in dictating the balance between protection and pathology.

3.2 Results

3.2.1 Influenza induced pathology and illness is linked to the exuberant immune response elicited by the host

Influenza infection of C57BL/6 and BALB/c mice elicits an acute weight loss that peaks at day 6-7 after infection. The inflammatory infiltrate into the airways is also maximal at this time point implying a strong correlation between illness severity and
30 the exuberance of the host's immune response (Fig. 3.1a). By day 10 post infection

pulmonary inflammation has largely subsided and mice have returned to their original weight. A significant extent of the observed illness and pathology evoked by influenza infection of the lung is attributable to the over-exuberance of the host's immune response. T cells are critical to viral clearance but are also a significant component of the observed pathology, causing occlusion of airways and producing inflammatory mediators that cause the observed cachexia and fever. The dependence of illness on T cell accumulation can easily be demonstrated by inhibiting the late co-stimulatory signal delivered through OX40 (Fig. 3.1b). Administration of a pegylated anti-OX40 blocking antibody significantly reduced weight loss following influenza infection of BALB/c mice (Fig. 3.1c). Those mice treated with the pegylated anti-OX40 antibody also exhibited significantly reduced inflammatory infiltrate in their airways at day 7 post infection (Fig. 3.1d). Flow cytometric analysis confirmed that blockade of the OX40 signal to the T cell reduces the number of CD4⁺ and CD8⁺ T cells in the airways (Fig. 3.1e), and their production of intracellular IFN- γ and TNF cytokines (data not shown), 7 days after infection.

3.2.2 Influenza infection elicits infiltration of myeloid populations into the lung and airways

The dominance of T cells in influenza induced pathology is likely to be directly influenced by the activity of the myeloid compartment. Myeloid cells are central to the establishment and maintenance of the inflammatory response to infection by secreting cytokines and chemokines and presenting antigen to recruited T cells. Manipulation of different facets of the myeloid response to influenza may be beneficial in quelling the immune response and reducing pathology. The numbers of myeloid cells in the lung and airways of C57BL/6 mice infected with influenza was assessed by flow cytometry, with distinction primarily based upon differential expression of CD11b and CD11c as described by Gonzalez-Juarrero *et al* (Fig. 3.2a and b). Cells were also sorted on the basis of differential CD11b and CD11c expression and cytocentrifuged onto glass and stained with H and E to confirm their morphology (data not shown). Differential CD11b and CD11c staining of a naïve mouse lung, excluding lymphocytes, revealed four distinct populations:

CD11b^{int}CD11c^{hi} (R1), CD11b^{hi}CD11c^{hi} (R2), CD11b^{int}CD11c^{lo} (R3),
CD11b^{hi}CD11c^{lo} (R4) (Fig. 3.2a). Cells in the region R1 exhibited the same
CD11b/CD11c profile as the majority of cells isolated in the BAL (Fig. 3.2b) of a
naïve mouse and appeared morphologically like alveolar macrophages (data not
shown). Cells in region R2 were also determined to express low Gr-1 and high MHC
II and appeared morphologically like dendritic cells, with cytoplasmic processes (data
not shown). Cells in region R3 expressed an intermediate level of Gr-1 and appeared
to be highly autofluorescent. Morphologically they were fairly small cells with horse-
shoe shaped nuclei and we believe them to be monocytic in nature. Finally, cells in
region R4 also stained very strongly for Gr-1 and were negative for MHC II and
appeared morphologically to be neutrophils, possessing multilobed nuclei (data not
shown). This differential expression profile was subsequently utilised to distinguish
between distinct myeloid populations during influenza infection. However, during the
course of influenza infection macrophages within the lung up-regulate CD11b upon
activation. Subsequently, activated macrophages were distinguished from neutrophils
by differential Gr-1 staining.

The number of each myeloid population was determined in the lung (Fig. 3.2c) and
airways (Fig. 3.2d) at days 0, 2, 7 and 14 after influenza infection. The number of
neutrophils in both the lung and airways appeared to peak at day 2 after influenza
infection and had subsided significantly by day 7. The number of DCs was low
throughout the course of infection and remained largely unaltered, although a small
increase was seen at day 7 in the lung and airways. Alveolar macrophages exhibited a
drop in numbers in the airways at day 2, possibly reflecting virus induced death, and
were then shown to peak in the lung and BAL at day 14. The numbers of monocytes /
small macrophages peaked at day 7 after infection in the lung and BAL. However, the
most striking increase in myeloid infiltrate was the significant increase in the number
of activated macrophages in the lungs and airways at day 7 after influenza infection.

3.2.3 Phenotypic characterisation of mice lacking gp91phox (Cybb tm1)

After defining the recruitment of myeloid cells to the lung during influenza infection, we next examined the influence of depleting the phagocyte NADPH oxidase. Cybb tm1 mice lack the gp91phox sub-unit of the NADPH oxidase and are subsequently incapable of generating superoxide through the respiratory burst. Much research utilising knock out mice fails to acknowledge the phenotype of the naïve animal and hence the ensuing repercussions on immunity to the infection of interest. We show that in the absence of infection, naïve Cybb tm1 mice exhibit a generalised inflammation with a Th1 bias. There was a significant increase in cellular infiltrate in the lungs, Peyer's patch and spleen of naïve Cybb tm1 mice (Fig. 3.3; Table 3.1). The increment in cell numbers in the lungs of Cybb tm1 mice was not as extensive as seen in the Peyer's patch and spleen, but became considerably more pronounced with increased age where airways were also affected (data not shown).

Enhanced cellularity encompassed both the CD4⁺ and CD8⁺ T cells (Table 3.1), which were more activated in Cybb tm1 mice (defined as CD45RB^{lo}, Fig. 3.4a and b), and produced significantly higher levels of intracellular IFN- γ (Fig. 3.4c and d) and TNF (data not shown). The number of CD11b⁺ myeloid cells (predominantly macrophages) in the lungs, Peyer's patches and spleens of the Cybb tm1 mice was also increased (Table 3.1 and Fig. 3.5a and b), an effect more significant with age, with the appearance of multinucleated giant cells (Fig. 3.5c). Hexagonal colourless crystals were observed in lung tissue of 4 month old mice either extra-cellular or intracellular imparting significant tissue disruption (Fig. 3.5d and e).

It was therefore apparent that in the absence of any antigenic stimuli Cybb tm1 mice exhibited significant alterations in hematopoietic populations resident in the naïve lung. Microarray analysis was subsequently utilised to investigate differential gene expression in the lungs of Cybb tm1 mice relative to wild type controls. RNA was extracted from the naïve lung of wild type or Cybb tm1 mice and directly labelled with Cy3 dCTP. The labelled RNA from the wild type or Cybb tm1 lung was subsequently mixed with Cy5 dCTP labelled universal mouse reference RNA and hybridised to a HGMP total mouse genome v2 array, which was subsequently

scanned with Genepix 3000. Three biological replicates and two technical replicates were performed for each of wild type and *Cybb* tm1 RNA. BlueFuse image analysis software (<http://www.cambridgebluegnome.com/bluefuse.htm>) was used to generate Cy3 and Cy5 expression values for the genes on each of the arrays. The BlueFuse output data was then imported into GeneSpring GXv7.3. The data was normalised using the Lowess algorithm and the normalised data was checked for any sample anomalies. All the arrays exhibited a good even normal distribution centred around 1 on the log ratio scale (an assumption which should be satisfied for t-test to perform correctly), with no evidence of skewing and a similar spread of data. The Pearson correlation was used to develop a sample cluster tree, which is a useful indicator of how replicates are behaving. As would be expected the technical replicates had the highest level of correlation, but a good correlation (> 0.75) was also observed between biological replicate samples. The data was filtered on confidence, using the p-value ($p < 0.05$) of a t-test as the confidence value, with a Benjamini and Hochberg (BH) correction applied to reduce the false discovery rate. This was first applied to the wild type data to determine which genes were up-regulated and down-regulated with respect to the reference sample. This was repeated for the *cybb* arrays. It was apparent that a significant number of genes were specifically up-regulated (1416) (Fig. 3.6a) or down-regulated (1216) (Fig 3.6b) in the lungs of the *Cybb* tm1 mice. Therefore, the absence of a single gene, encoding the gp91 subunit of the phagocyte NADPH oxidase, alters the expression of a striking number of unrelated genes. Analysis into the differential expression in naïve wild type and *Cybb* tm1 mouse lung is on-going.

3.2.4 Influenza infected *Cybb* tm1 mice exhibit heightened inflammation in their airways

Having determined the phenotype of naïve *Cybb* tm1 mice, the profile of the immune response to intranasal influenza infection was characterised in the knock outs and wild type controls (for experimental protocol see Fig. 3.7). *Cybb* tm1 mice displayed enhanced weight loss compared to wild-type C57BL/6 mice in the early stages of infection (days 2-4), but were ultimately comparable to wild-type mice 6 days after

infection (Fig. 3.8a). As discussed previously, weight loss during influenza infection is attributed largely to an over-exuberant immune response and release of Th1 cytokines such as $\text{TNF-}\alpha$. Accordingly, the Cybb tm1 mice possessed greater numbers of cells in their airways from day 3 onwards (Fig. 3.8b).

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Flow cytometric analysis was utilised to determine the extent of T cell infiltrate into the airways of wild type and Cybb tm1 mice throughout the course of the influenza infection. The numbers of CD4^+ and CD8^+ T cells were low and comparable in the BAL of both groups of mice at day 3 after infection (Fig. 3.8c and d). Whilst the numbers of both T cells subsets increased dramatically by day 7 after influenza challenge in wild type and Cybb tm1 mice, the knock out mice possessed a significantly greater number of CD4^+ T cells and a marginal increase in the number of CD8^+ T cells at this point relative to the wild type controls (Fig. 3.8c and d). Both the proportion (Fig. 3.9a and c) and total number (Fig. 3.9b and d) of activated CD4^+ (Fig. 3.9a and b), as defined as $\text{CD45RB}^{\text{lo}}$, and CD8^+ (Fig. 3.9c and d) T cells were increased 7 days after infection in Cybb tm1 mice compared to wild type controls. Furthermore, an increased percentage of CD4^+ and CD8^+ T cells were producing Th1 cytokines $\text{IFN-}\gamma$ (Fig. 3.9e) and TNF (data not shown), in the airways of Cybb tm1 mice from day 3 of influenza infection. This in turn led to a significant increase in the total numbers of Th1 cytokine producing T cells in Cybb tm1 mice by day 7 after influenza infection (Fig. 3.9f).

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The elevation of total cell numbers 3 days after influenza infection of Cybb tm1 mice was not explained by expanded T cell numbers unlike day 7. Instead, at day 3 numbers of macrophages (Fig. 3.10a) and neutrophils (Fig. 3.10b) were increased, which correlated with higher levels of TNF , IL-6 , and MCP-1 in the BAL (Fig. 3.11a-c) and serum (Fig. 3.11e-g) and most likely explains the enhanced weight loss observed in Cybb tm1 mice relative to their wild type counterparts early in infection. Furthermore, elevated levels of IL-12 were present in the BAL of Cybb tm1 mice relative to wild type controls (Fig. 3.11d), which may lead to the observed Th1 bias seen in the knock out mice. The heightened macrophage population may be responsible for the elevated IL-12 in Cybb tm1 mice, although dendritic cells are

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generally the predominant source of this cytokine during influenza infection and it is plausible that the activity of this cell type is also modified.

3.2.5 Influenza infected Cybb tm1 mice have reduced cellularity in the lung parenchyma

Despite the heightened Th1 inflammatory infiltrate observed in the airways of Cybb tm1 mice compared to wild type controls, the number of cells in the lung tissue itself was significantly reduced in the knock out mice (Fig. 3.12a). Hematoxylin and eosin stained lung sections revealed a striking reduction in perivascular and peribronchiolar infiltrate in Cybb tm1 mice at days 3 and 7 after influenza infection relative to wild type controls (Fig. 3.12b). Flow cytometric analysis revealed a significant reduction in both CD4⁺ and CD8⁺ T cells in the lung tissue of Cybb tm1 mice relative to wild type controls at day 7 after influenza infection (Fig. 3.13a and b, respectively). A greater percentage of those T cells present in the lung tissue, like in the BAL, of Cybb tm1 mice were in a heightened activation state (defined as CD45RB^{lo}) relative to those in the lungs of wild type mice (Fig. 3.13c and e), but the total number of activated T cells was still significantly lower in Cybb tm1 mice (Fig. 3.13d and f). Similarly, the percentage of both CD4⁺ and CD8⁺ T cells producing Th1 cytokines was higher in the lungs of Cybb tm1 mice than wild type controls, but again total numbers were still reduced (data not shown). This would imply that Cybb tm1 mice focus the immune response to influenza in the airways and not the parenchyma. Despite the general reduction in inflammatory infiltrate into the lung tissue of Cybb tm1 mice relative to wild type controls, the numbers of macrophages and neutrophils were comparable at this site (data not shown).

3.2.6 Cybb tm1 exhibit improved influenza clearance and reduced lung damage

Whilst a Th1 immune response is the cause of extensive immunopathology during influenza infection, it is also a pre-requisite for viral clearance. It was intriguing to assess how the distinct immune profile observed in Cybb tm1 mice would affect resolution of viral infection. The peak viral titres in the lung of Cybb tm1 mice were

significantly lower on day 3 post infection compared to wild type controls, and were undetectable at day 7 post infection in the knock out mice (Fig. 3.14a). The reduced viral titres within the lung tissue of Cybb tm1 mice may be a consequence of the heightened macrophage driven Th1 response in the airways restricting viral replication and promoting clearance or the elevation of mucosal IgA we detected in the BAL (Fig. 3.14b) and nasal wash (Fig. 3.14c) of Cybb tm1 mice. IgG sub-types in the BAL and serum, however, were similar in knock out and wild type mice (data not shown).

Total protein (Fig. 3.15a) and lactate dehydrogenase (Fig. 3.15b) levels in BAL fluid were reduced in Cybb tm1 mice relative to wild type controls, reflecting a reduction in tissue damage. This could be a function of the reduced levels of ROS, the lower lung viral titres or the reduced inflammatory infiltrate resident to the lung tissue itself. Furthermore, whole body plethysmography was utilised to assess the lung function of both groups of mice at day 7 post influenza infection, and we observed an improvement in both the tidal volume (Fig. 3.15c) and peak expiratory flow (Fig. 3.15d) in Cybb tm1 mice compared to wild type controls.

3.2.7 Reduced apoptosis in influenza infected Cybb tm1 mice

It was of interest to determine what caused the elevated myeloid response and T cell activation observed in Cybb tm1 mice in response to influenza infection. Since the generation of superoxide by phagocytes has been affiliated with the induction of apoptosis, we enumerated the number of Annexin V⁺ CD11b⁺ myeloid cells and CD4⁺ and CD8⁺ T cells in lungs and airways of Cybb tm1 mice and found them reduced compared to wild-type controls (Fig. 3.16a). Furthermore, lung cells derived from Cybb tm1 mice exhibited a greater proliferative response to influenza antigen compared to those of wild type C57BL/6 mice, as determined by ³H-thymidine incorporation (Fig. 3.16b). Enumeration of CD200 expression provided another explanation for the heightened macrophage compartment in Cybb tm1 mice. CD200 is widely expressed by a number of cell types. Binding of CD200 to CD200R on myeloid cells delivers an inhibitory signal to myeloid compartment. Interestingly,

Cybb tm1 influenza infected mice displayed a significant reduction in the levels of CD200 expression on the surface of B lymphocytes and CD4⁺ and CD8⁺ T cells (Fig. 3.16c).

5 **3.2.8 Recall responses to influenza are not compromised in Cybb tm1 mice**

We next examined whether recall responses to a second influenza challenge was affected in Cybb tm1 mice. Wild type and Cybb tm1 mice were re-infected with influenza 35 days after a primary influenza infection. Three days after re-challenge influenza virus was undetectable in lung homogenates in wild type and Cybb tm1
10 mice (data not shown). Furthermore, no signs of illness or weight loss were observed (Fig. 3.17a). Cybb tm1 mice did, however, exhibit reduced lung inflammation, with decreased numbers of CD4⁺ and CD8⁺ T cells (Fig. 3.17b). Of those T cells present, a greater percentage were in a heightened activation state (CD45RB^{lo}) (data not shown) and were producing Th1 cytokines (Fig. 3.17c and d). The levels of influenza specific
15 IgG1 and IgG2a in the serum were assessed by ELISA and found to be comparable between wild type and Cybb tm1 mice (Fig. 3.17e and f).

3.2.9 The cell permeable mimetic MnTE-2-PyP prevents pulmonary inflammation during influenza infection

Since multiple immune compartments are disrupted in Cybb tm1 mice we next tested
20 whether comparable results were observed with a scavenger of ROS. MnTE-2-PyP is a cell permeable manganic-porphyrin that acts as a potent anti-oxidant and superoxide mimic, and has previously been reported to confer protection in a variety of oxidative stress injuries such as liver ischemia, diabetes and stroke. Mice administered with 25 μ g or 50 μ g of mimetic on days 0 and 3 of influenza infection (Fig. 3.18) showed a
25 comparable immune response to Cybb tm1 mice, with heightened cellularity in the airways and reduced inflammation of the lung tissue at day 7 after infection relative to control treated mice (Fig. 3.19a and c). Mice treated with MnTE-2-PyP exhibited significantly reduced perivascular and peribronchiolar infiltrate compared to PBS treated mice as depicted by H and E stained lung sections (Fig. 3.19b). Viral titres
30 were slightly reduced by administering 25 μ g mimetic although not significant, but

actually heightened with a 50 μ g dose (Fig. 3.19c). The increase in viral titres at a higher dose of mimetic may reflect the scavenging of ROS from other sources (such as xanthine oxidase) than just the phagocyte NADPH oxidase disrupted in Cybb tm1 mice.

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Similar to Cybb tm1 mice, MnTE-2-PyP caused a reduction in CD4⁺ and CD8⁺ T cell numbers in the lung parenchyma (Fig. 3.20a), but numbers of both T cell subsets were comparable in the airways (Fig. 3.20b). Of those T cells that remained in Cybb tm1 mice a greater percentage were producing intracellular Th1 cytokines relative to PBS treated controls (data not shown) although this was not as striking as seen in Cybb tm1 mice and failed to be significant. However, the macrophage response induced by influenza infection was greater in mice treated with MnTE-2-PyP, with a greater percentage of the cellular infiltrate present in the lung and airways being macrophages (Fig. 3.20c and e). Ultimately, the total number of macrophages present in the lung tissue was comparable in treated and untreated mice (Fig. 3.20d) but significantly greater in the airways of MnTE-2-PyP treated mice (Fig. 3.20f). It was also demonstrated that J774 macrophages exhibited reduced apoptosis in response to an array of stimuli when incubated in the presence of MnTE-2-PyP, as adjudged by staining with Annexin-V (Fig. 3.20g). This supports our findings from Cybb tm1 mice that ROS have a critical role in controlling macrophage apoptosis.

3.3 Discussion

3.3.1 Modulation of myeloid cells in influenza infection

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This chapter has shown that exaggerated immune responses contribute significantly to lung pathology. Blockade of the co-stimulatory signal delivered through OX40 to the T cell with a pegylated blocking antibody ameliorates weight loss and significantly reduces the T cell infiltrate into the airways. Since the early myeloid response will ultimately dictate the induction and potency of the acquired immune response targeting such cells could prove a powerful strategy of immune manipulation. One facet of the myeloid response to pathogenic infection is the generation of ROS through the phagocyte NADPH oxidase. The potential of ROS to elicit direct

immunopathology as well their pleiotropic functions in immune modulation and inflammation makes them ideal targets for immunotherapies that seek to alleviate influenza driven illness and pathology.

3.3.2 Characterisation of naïve *Cybb tm1* mice

5 The preceding results show that *Cybb tm1* mice, a murine model for X-linked chronic granulomatous disease, display a pronounced augmentation in inflammatory infiltrate in mucosal sites such as the lung and peyers patch, as well as in the spleen in the absence of infection. The expanded macrophage population in naïve animals is associated with an increase in activated T cells producing IFN- γ and TNF. The
10 influence on macrophages becomes more pronounced with increasing age with the appearance of multinucleated giant cells, which form by the fusion of monocytes or macrophages, but little is known about the fusion process itself . It is hypothesised that multinucleated giant cells have an advantage in microbicidal activity over macrophages; in part due to enhanced oxidative capacity . The abundance of these
15 cells in *Cybb tm1* mice is most likely attributable to a condition known as acidophilic macrophage pneumonia which has been reported to be common in immunocompromised mice by 6 months of age , and has actually been cited to occur in very old *Cybb tm1* mice . Another feature of older naïve *Cybb tm1* mice is the abundance of crystal like structures within their respiratory system. Unstained the
20 crystals appeared colourless and were of varying sizes and hexagonal in shape, but with H and E staining the crystals were pink denoting their positive charge and ensuing attraction of eosin. The crystals were found both extra-cellularly and located intra-cellularly within the macrophages and multinucleated giant cells, frequently in stacks and distorting the natural morphology of the cell. It has previously been
25 suggested that these crystals represent a protein known as Ym1 which possesses homology with chitin . Acidophilic macrophage pneumonia is again characterised by accumulation of macrophages that are laden with such crystals, and hence are again an artefact of macrophage dysfunction .

30 The general systemic state of heightened inflammation in *Cybb tm1* mice may imply a pre-disposition to infection by some other pathogen, and susceptibility to *A.*

fumigatis, *Paecilomyces sp.*, *Pseudomonas aeruginosa* and *Enterococcus* has been noted . However, Cybb tm1 mice utilised in all experiments were confined in strict pathogen free conditions and regularly screened for infection by the most common murine pathogens. Heightened reactivity to commensal organisms however cannot be ruled out. Indeed, multinucleated giant cells display enhanced microbicidal activity against natural fauna such as *Candida albicans*, and hence as the Cybb tm1 mice age there may be an increase in macrophages and multinucleated giant cells in an attempt to control lung fauna.

It is also plausible that ROS fulfil a role in the macrophage homeostasis even in the absence of infection. ROS are linked with apoptosis through the non-specific bystander damage they evoke onto lipids, proteins and nucleic acids, the release of cytochrome c from mitochondria and the activation of caspase-3 . ROS may therefore assist self-enforced homeostasis of cell numbers by apoptosis in the absence of infection explaining the enhanced cellularity we observed in Cybb tm1 mice. A lack of immune regulation could also explain the enhanced susceptibility of patients suffering from CGD to autoimmune disorders such as lupus and polyarthritis . It is generally acknowledged that ROS have a detrimental role in the progression of rheumatoid arthritis, with SOD administration proving beneficial in arthritis induced by streptococcal cell wall components or immunization with collagen type II and the observed reduction in joint inflammation in mice given apocynin (an NADPH oxidase inhibitor) . Experimental arthritis induced by intra articular zymosan or poly-L-lysine coupled lysozyme is significantly enhanced in mice lacking a functional phagocyte NADPH oxidase, with more severe joint inflammation, granulomatous synovitis and multinucleated giant cell accumulation . These discrepancies could be attributable to the defect that develops in knock-out mice owing to a lack of homeostatic control in the absence of NADPH oxidase derived superoxide.

We show considerable dysregulation in gene expression in the lungs of Cybb tm1 mice by microarray analysis. Whilst investigation into this differential gene expression is in its infancy it is clear that the absence of the cybb gene, encoding the gp91phox sub-unit of the phagocyte NADPH oxidase, results in the up-regulation and

down-regulation of a significant number of genes in these knock out mice. This likely reflects in part the significant role fulfilled by ROS in immune regulation. In addition to their potent anti-microbial activity, ROS also possess a significant cell-signaling role in biological systems, capable of regulating the phenotype and function of immune cells . Even in a naïve animal it would appear that ROS may fulfil a role in regulating gene expression. These analyses may facilitate our understanding of why people with CGD exhibit aberrant inflammatory responses and an increased susceptibility to autoimmune conditions. Furthermore, it proves an important point that the depletion of a single gene in knock out animals can have serious repercussions in dictating expression of other genes even in a naïve setting. This altered resting state phenotype should always be considered when utilizing knock out or transgenic animals to elucidate the role of a specific gene.

Infection of *Cybb* *tm1* mice with influenza provided the opportunity to assess the role of phagocyte derived oxidants in the resolution of this strongly Th1 skewed viral infection, for which there have been discrepancies concerning the protection and pathology afforded by ROS. Furthermore, we can gain insight into whether the dysfunctional immune regulation is persistent and modulated following such an infection and how the immune response may be modulated in patients suffering from CGD.

3.3.3 Heightened cellularity in the airways of *Cybb tm1* and MnTE-2-PyP treated mice in response to influenza

Cybb tm1 mice exhibited heightened cellularity in the airways in response to influenza infection from day 3 onwards with a pronounced early increase in macrophage and neutrophil numbers and an increased T cell infiltrate by day 7. It is important to stress that the augmented infiltrate seen in *Cybb tm1* mice compared to wild type controls is significantly greater in infected mice than that seen in the naïve animals. Similarly, MnTE-2-PyP treated mice elicited a greater macrophage response in their airways in response to influenza infection. This increase in numbers of macrophages and neutrophils may arise due to a reduction in apoptosis. We report reduced apoptotic macrophages in influenza infected *Cybb tm1* mice, and reduced

apoptosis of J774 macrophages to an array of stimuli when incubated in the presence of the ROS scavenger MnTE-2-PyP. In support of our observations neutrophils from CGD patients demonstrate reduced spontaneous and Fas-induced apoptosis *in vitro* compared to those derived from healthy individuals . Furthermore, oxidants fulfil an important function in phosphatidyl serine exposure on neutrophils and their subsequent clearance by macrophages .

Alternatively, enhanced macrophage activity could also be explained by reduced inhibitory signals from other immune cells. For example, we observed reduced expression of CD200 on B cells, CD4⁺ and CD8⁺ T cells in the lung of Cybb tm1 mice infected with influenza. CD200 delivers an inhibitory signal on binding CD200 receptor on the surface of myeloid cells and CD200 knock out mice display a similar phenotype to Cybb tm1 mice with heightened macrophage numbers and a Th1 bias . CD200 is up-regulated on apoptosing cells to restrict the induction of inappropriate inflammatory sequelae by the phagocytosing macrophage . Therefore, a reduction in apoptosis and CD200 may in turn hinder the ability to switch off myeloid cells.

The enhanced cellularity we describe to viral infection in NADPH oxidase knockout mice is also evident during bacterial and fungal infection. Furthermore, NADPH oxidase knockout mice exhibit enhanced inflammatory gastritis, and sterile heat killed fungal products cause excessive inflammation in the lungs. The phagocyte oxidase may therefore fulfil some anti-inflammatory role and of significance it has been implicated in oxidatively inactivating chemotactic factors , inducing anti-inflammatory cytokine production by neutrophils and macrophages and in modulation of Th1 cytokine production . However, it is pertinent to question the extent to which the heightened inflammatory infiltrate present in the naïve knock out mice dictates the potency of the subsequent response to infection. Do the augmented macrophage numbers and Th1 skewed immunity facilitate a more exuberant response to influenza challenge? The similarities in immune phenotype between Cybb tm1 mice and those treated with MnTE-2-PyP would imply that this is not solely the case, although could explain why the heightened cellularity is more potent in the knock outs than in those treated with mimetic.

3.3.4 Reduced cellularity in the lungs of Cybb tm1 and MnTE-2-PyP treated mice in response to influenza

Despite the significantly enhanced cellularity in the airways of Cybb tm1 mice, the lungs of these mice exhibited a marked reduction in inflammatory infiltrate compared to those of wild-type controls. However, it should be noted that regardless of the general reduction in inflammation in the lung tissue the number of macrophages remained comparable and there persisted to be an augmentation in the percentage of activated T cells producing Th1 cytokines. The reduction of inflammation in the lung despite heightened cellularity in the airways is interesting and may be explained by: 1) better containment of influenza in the airways such that cells do not accumulate in the lung tissue and/or 2) faster movement of cells from the blood, through the lung parenchyma and into airways, especially since the absence of ROS would release chemokines from oxidative inactivation .

Influenza induces apoptosis in macrophages and neutrophils, partially through the generation of ROS in these cells. Since apoptosis of myeloid cells was reduced leading to elevated numbers of macrophages and neutrophils in the airways early infection, it is plausible that influenza was contained within the airways without requirement for further cellular recruitment. The heightened Th1 environment seen in the absence of ROS will act to further promote and activate resident macrophages. Indeed, elevated levels of TNF, IL-6, MCP-1 and IL-12 in the airways at day 3 of influenza infection in Cybb tm1 mice were observed.

3.3.5 Reduced influenza titres in the absence of a functional NADPH oxidase

We show that the absence of a functional phagocyte NADPH oxidase results in reduced lung viral titres. Though we are the first to document the accelerated clearance of a viral infection in the absence of NADPH oxidase, a similar effect during *Helicobacter pylori* infection has previously been demonstrated, with knock out mice exhibiting an enhanced inflammatory gastritis and reduced bacterial load . This effect, however, is not observed for all pathogens. Despite amplified macrophage reactivity in CGD patients increased susceptibility to *Aspergillus fumigatus* and *Staphylococcus aureus* arise . Furthermore, mice lacking a functional phagocyte

oxidase exhibit compromised clearance of *Escherichia coli* , impaired host defence to *Pseudomonas pneumonia* , and a transient loss of resistance to pulmonary tuberculosis . The divergent effects upon protection to different infections may depend on whether the pathogen has developed sophisticated strategies for avoiding intracellular killing machinery.

Improved viral clearance in Cybb tm1 mice may be explained by heightened immune activation in the airways facilitating clearance. Macrophages are critical to the establishment and maintenance of the host's protective response to influenza. Activated macrophages are capable of phagocytosing infective virions and producing Type I IFNs that are critical in conferring viral protection. Furthermore, a Th1 response is imperative to the resolution of an influenza infection, and Cybb tm1 mice display enhanced numbers of activated T cells producing Th1 cytokines in their airways. Finally, the role of IgA in conferring protection to influenza is significant, and elevated levels of this antibody were measured in the BAL and nasal washes of Cybb tm1 mice during influenza infection. The elevated levels of IgA in these mice may also reflect the heightened macrophage response, with potentially augmented levels of CD40 and IL-6, which is known to induce differentiation of B cells into antibody producing plasma cells.

Alternatively, the absence of ROS could lead to the reduced titres of influenza due to the ability of these species to modulate the infectivity and replication of the virus itself. Low levels of ROS may facilitate viral replication because of their mitogenic effects on cells, with most viruses growing better in proliferating cells. Alternatively, another plausible explanation could be the potential role of superoxide in promoting viral infectivity by inactivating the anti-proteases that prevent cleavage of the influenza attachment HA0 protein to HA1/HA2 . Only viruses expressing HA1/HA2 are infective and the higher the proportion of infective virions, the faster the virus will spread throughout the lungs and airways

Mice treated with MnTE-2-PyP did not show the improved viral clearance seen in Cybb tm1 mice. At a lower dose the levels of virus were marginally lower in treated

relative to untreated mice. At higher doses of MnTE-2-PyP, however, viral clearance was compromised. This would imply that ROS do in fact have some virucidal role against influenza, and the compromised clearance observed with mimetic may reflect its capacity to scavenge ROS from other sources than the NADPH oxidase, such as xanthine oxidase. It is plausible that NADPH oxidase and xanthine oxidase systems are mutually redundant and that pathogen clearance is only compromised if both are absent, similar to that reported for *Burkholderia cepacia*. In this scenario, ROS may fulfil some virucidal role but the levels generated through xanthine oxidase are sufficient to deal with the virus, or the heightened macrophage response and Th1 bias can compensate for the reduction in ROS.

3.3.6 The role of ROS in T cell responses and the Th1 / Th2 paradigm

Another salient feature of the naive Cybb tm1 lung was the heightened activation of the resident T cell population, which was more pronounced after influenza infection. A slight increase in T cell activation was observed in MnTE-2-pyP treated mice although it failed reach significance. Signals delivered by the larger population of macrophages in the lungs and airways of Cybb tm1 mice could enhance the recruitment of pulmonary T cells populations or elevated T cells may reflect the observed reduction in apoptosis and enhanced proliferation. ROS have been linked to T cell hypo-responsiveness with peroxynitrite (formed by the reaction of superoxide with nitric oxide) inhibiting T cell activation and proliferation by nitrating tyrosine residues in downstream TCR signalling molecules. Similarly manganic porphyrins with anti-oxidant ability (such as the mimetic used in our study) protect T cells from superoxide generation and apoptosis. Thus it can be envisaged how a reduction in the levels of ROS would facilitate the observed increase in T cell numbers and activation.

The enhanced Th1 phenotype observed in Cybb tm1 and mimetic treated wild type mice is likely to reflect the heightened levels of IL-12 production observed. The mimetic used in this study reduces eosinophilia in a mouse model of asthma, implying a potential for ROS in defining the Th1/Th2 paradigm. A role for superoxide in maintenance of homeostasis and in immune regulation is reminiscent of the potential of nitric oxide to exert immunosuppressive capabilities. NO has been shown to inhibit

T cell proliferation during the G1/S transition. Furthermore, NO may also govern the balance of the Th1/Th2 paradigm, with mice lacking iNOS developing an enhanced Th1 response and peritoneal macrophages from these mice generating elevated levels of IL-12. ROS/RNS are highly potent and toxic mediators with the potential to be detrimental to bystander tissue. Consequently, low levels of such mediators may play a role in minimising or blunting the immune response to prevent heightened, potentially damaging, production of themselves.

3.3.7 Conclusion

In summary, mucosal sites must efficiently respond to harmful organisms but not the myriad of other harmless antigens. The data presented suggests that ROS may fulfil a fundamental role in making this distinction: low basal levels acting in an autocrine manner to induce a homeostatic or immunosuppressive role, whereas up-regulation after a more serious threat directs the establishment of an inflammatory response. The absence of ROS results in improved illness during influenza infection, with reduced inflammation of the lung parenchyma, reduced viral titres and oxidative stress, ultimately leading to reduced lung damage, as denoted by the reduced levels of total protein and LDH in the BAL wash, and improved lung function. This may also be advantageous in improving resistance to bacterial co-infections, which are believed to gain a foothold as a consequence of the extensive cytopathology caused by the virus, and the immune response to it. In particular, the respiratory burst induced in neutrophils by influenza is thought to induce apoptosis and hence limit the bactericidal capacity of these important cells. The *Cybb* *tm1* mice also seem to respond equally well to an influenza re-challenge, with negligible weight loss and rapid resolution of viral burden, with a reduced T cell recruitment into the lung. MnTE-2-PyP could therefore offer therapeutic potential by evoking a “poised” macrophage and subsequent development of Th1 mediated immunity that is better equipped to limit influenza virus replication resulting in reduced lung consolidation and bystander tissue damage.

Chapter 4 – The Role of Reactive Oxygen Species in Pulmonary *Cryptococcus neoformans* infection

4.1 Introduction

4.1.1 The role of ROS in pulmonary fungal infection

5 The protection afforded by reactive oxygen species (ROS) to respiratory fungal pathogens has been highlighted by the enhanced susceptibility of CGD patients to infection with *Aspergillus fumigatus*. *A. fumigatus* is capable of eliciting disease in mice lacking the NADPH oxidase at significantly lower doses than in wild type controls, and higher doses of this fungal pathogen prove fatal to Cybb tm1 knock out mice. Similarly, phagocytes unable to generate superoxide exhibit reduced ability to 10 kill *C. albicans* and *Sporothrix schenckii* infection of CGD mice leads to systemic infection and death.

Despite the recurring fungal infections in CGD patients, there are no clinical studies 15 showing enhanced susceptibility to *C. neoformans*. There are discrepancies regarding the efficacy of superoxide and its derivatives in the eradication of *C. neoformans*, yet there is cited evidence defining the potential of PMNs and macrophages to kill the organism by oxidative mechanisms. Furthermore, this fungus displays an array of protective mechanisms (Superoxide Dismutase (SOD), mannitol, melanin) to confer 20 resistance to ROS, suggesting that in some instances they may be toxic and adverse to its survival. *C. neoformans* deficient in melanin exhibit reduced virulence *in vivo*, and CuSOD lacking mutants are more susceptible to oxygen radicals in macrophages.

4.1.2 Murine models of *C. neoformans*: protection versus patholog.

25 The genetic background of the inbred mouse strain determines the outcome of the immune response to *C. neoformans*. Some murine strains (CBA, C.B-17 and BALB/c) are inherently resistant to infection, producing significant levels of Th1 cytokines that confer protection. Conversely, other strains (C57BL/6, C3H and B10-D2) are susceptible to *C. neoformans*, producing significantly lower levels of Th1 cytokines and developing a Th2 driven pulmonary eosinophilia. These mice fail to

clear infection and the fungus disseminates to extra-pulmonary tissues. Furthermore, the chronic eosinophilia leads to tissue damage as a result of degranulation and crystal deposition. These murine models emphasize the clear dichotomy that exists between a protective Th1 response and a non-protective Th2 response

5 **4.1.3 The role of ROS in defining the Th1 / Th2 paradigm**

Several studies have demonstrated that NADPH knock out mice do not show a shift in T cell cytokine responses. Although p47phox^{-/-} mice show reduced clinical signs of EAE, this was not attributed to any change in bias of Th subsets . Similarly, p47phox^{-/-} mice display a normal cytokine response to *Mycobacterium avium* (potent stimulus of Th1 responses) and *Schistosoma mansoni* eggs (elicits Th2 mediated granuloma) . However, this lack of an effect is not true for all scenarios. King *et al* have demonstrate a role for oxidative stress in defining human T cell differentiation to Th1/Th2 subsets, with DMNQ (induces oxidative stress) shown to cause the up-regulation of Th2 cytokines IL-4, IL-5 and IL-13 but not Th1 cytokines . Similarly, exposure of peripheral blood T cells to anti-oxidant Vitamin E reduces IL-4 production, intake of Vitamin E reduces IgE serum levels and allergic asthma is linked to a low intake of anti-oxidant vitamins . Furthermore, in a model of experimental arthritis, NADPH oxidase knock out mice display increased TNF and IL-1 and we have previously seen an enhancement of Th1 responses in Cybb tm1 mice to influenza.

20 **4.1.4 Hypothesis**

The prevalence of fungal infections amongst patients suffering from chronic granulomatous disease implies that ROS are critical to the control and eradication of fungal pathogens. Whilst this appears to be the case with certain fungi, such as *A. fumigatus*, there is little conclusive data defining the role of ROS in protection versus pathology in an *in vivo* model of *C. neoformans* infection. We have demonstrated that ROS would appear to fulfil a role in dictating the Th1 / Th2 paradigm, with deprivation of these mediators leading to enhanced macrophage numbers and Th1 bias. A Th1 driven immune response to *C. neoformans* is protective, with a Th2 response giving rise to unchecked fungal growth and dissemination to the brain. It is

anticipated that the absence of ROS, either through the utilisation of Cybb tm1 mice or administration of MnTE-2-PyP, may therefore paradoxically prove beneficial in the control of this respiratory fungal pathogen.

4.2. Results

5 4.2.1. *C. neoformans* infection of C57BL/6 mice induces pulmonary infiltrate consisting of T cells and eosinophilia

Intranasal infection of C57BL/6 mice with *C. neoformans* elicits a non-protective Th2-dominated immune response in the lung characterized by extensive pulmonary eosinophilia . We first characterized the kinetics of this infection over a 48-day time-course experiment. *C. neoformans* burden peaked at 14 days after infection following
10 exponential growth from day 3 (Fig. 4.1a). As reported previously, C57BL/6 mice do not clear the pathogen completely and *C. neoformans* was still present in the lungs 48 days post-infection. Pulmonary eosinophilia is associated with *C. neoformans* infection in this mouse strain. The accumulation of eosinophils in the airways (Fig.
15 4.1a) and lung tissue (not shown) closely mirrored that of fungal burden. It is interesting to note that percent eosinophilia preceded that of peak pathogen burden. This probably reflects the fact that only low antigen doses are required to induce their recruitment. Eosinophilia was evident in H and E stained lung sections 11 days post-infection with extensive eosinophilic accumulation around the peribronchiolar and, to
20 a lesser extent, the perivasular regions of the lung (Fig. 4.1b). *C. neoformans* also elicited a substantial expansion of both CD4⁺ and CD8⁺ T cells. The increase in T cell numbers in the lung also peaked at the time of maximum pathogen burden and pulmonary eosinophilia (Fig. 4.1c). The number of CD4⁺ T cells at this time was approximately ten times higher than that of CD8⁺ T cells, underlining the CD4⁺ T cell
25 dominated response in this mouse strain; which probably reflects the extracellular life cycle of this pathogen.

4.2.2 Manipulation of the innate immune compartment can promote immunity to C. neoformans through induction of a Th1 dominated response

Protective immunity to *C. neoformans* critically depends on the development of a Th1 driven response, with depletion of Th1 cytokines being shown to severely compromise resolution of infection . Immune manipulation that promotes immunity to *C. neoformans* or encourages a more protective Th1 response offers a plausible immunotherapeutic strategy. Modulation of the T cell response to *C. neoformans* has been demonstrated to improve pathogen clearance , but would manipulation of earlier innate immune compartments offer a similar or greater effect?

We have shown that a modified bacterial labile toxin (LTK63) affords protection against an array of subsequent respiratory pathogens . Administration of intranasal LTK63 two weeks prior to infection with *C. neoformans* greatly reduces pathogen driven pulmonary inflammation as evident in of H and E stained lung sections (Fig. 4.2a). Furthermore, clearance of *C. neoformans* within the lung is actually improved at day 12 after infection (Fig. 4.2b). This reduced inflammation and improved pathogen clearance correlates with the development of a more pronounced Th1 response and a reduction in airway eosinophilia (Fig. 4.2c). The protective effect conferred by LTK63 lasted for up to 12 weeks after administration and is associated with the maturation of myeloid cells (data not shown). The protective effects conferred by LTK63 does not depend on T and B cells, since innate imprinting can be induced in mice lacking T and B cells (RAG knock out mice) (data not shown).

Similarly, administration of Cytosine-phosphate-guanosine-containing oligodeoxynucleotides (CpG ODN) confers protection against pulmonary challenge with *C. neoformans* . CpGs are motifs present within bacterial DNA that are recognized by TLR 9 on the surface of a variety of cells of the innate immune system. Immunization with CpG ODN 3 days prior to intranasal challenge with *C. neoformans* greatly reduces the pathogen burden within the lung (Fig. 4.3a) and BAL (data not shown) and reduces dissemination to the brain (data not shown) 12 days after infection. This promotion of anti-cryptococcal immunity was attributable to a shift in the immune phenotype from a Th2- toward a Th1-type response with a significant

reduction in eosinophilia in the lung (Fig. 4.3b) and BAL (data not shown). Intracellular cytokine staining revealed a significant shift in the ratio of IFN- γ to IL-5 production by CD4⁺ and CD8⁺ T cells, with significantly more IFN- γ production in those mice treated with CpG ODN (Fig. 4.3c and d). This improved pathogen clearance is T cell-independent and may reflect a direct effect of CpG on enhancement of macrophage antimicrobial activity. These strategies clearly demonstrate that manipulation of the innate immune compartment offers clear therapeutic potential against *C. neoformans*.

4.2.3 *Cybb tm1* mice develop pulmonary granulomas that encompass *C. neoformans* and promote pathogen clearance

Having elucidated the role of the phagocyte NADPH oxidase and ROS in dictating the phenotype and resolution of a strong Th1 driven intracellular viral infection such as influenza, we next assessed their role in immunity to a potent Th2 inducing infection, *C. neoformans*. Wild type C57BL/6 and *Cybb tm1* mice were infected with *C. neoformans*, and culled at various times after infection to elucidate differences in immune phenotype and pathogen burden (Fig. 4.4). Both *Cybb tm1* mice and C57BL/6 controls exhibited comparable lung cellular infiltrate at days 5, 8 and 12 after infection (Fig. 4.5a), but the distribution of the infiltrate within the lung tissue was distinct between groups. Wild type mice displayed a generalised cellularity with perivascular and peribronchiolar infiltrate with significant occlusion of alveoli. Conversely, the *Cybb tm1* mice exhibited small distinct pockets of infiltrate, granulomatous in nature, surrounding cryptococci (Fig. 4.5b). It is important to stress the numbers of cells in the lungs of naïve mice are at least an order of magnitude lower than that recruited during *C. neoformans* infection (at peak infection, wild type: $8.9 \times 10^6 \pm 2.9$ SEM; *Cybb tm1* $11.5 \times 10^6 \pm 2.5$ SEM).

Strikingly, the *Cybb tm1* mice exhibited minimal recruitment of cells into their airways compared to wild type controls (Fig. 4.6a). This was clearly visible from observation of H and E stained cytocentrifuged BAL samples, which clearly show a reduction in airway cellularity in *Cybb tm1* mice (Fig. 4.6b). Furthermore, it was clearly apparent that the *Cybb tm1* mice exhibited a marked reduction in the number

of cryptococci in their airways (Fig. 4.6b), which may imply that containment within the lung tissue hinders dissemination into the airways with consequently reduced number of cells recruited into the BAL.

5 Cybb tm1 mice have previously been demonstrated to develop a granulomatous response to other fungal pathogens, but have been compromised in their ability to resolve infection. In contrast, Cybb tm1 mice showed an enhanced ability to combat *C. neoformans* infection with a significantly reduced fungal load in the lung tissue (Fig. 4.7a), the BAL (Fig. 4.7b) and, importantly, the brain (Fig. 4.7c) at days 5, 8 and
10 12 after infection. The pathogen load within the spleen was low compared to that observed in the lung but comparable between wild type and Cybb tm1 mice (Fig. 4.7d).

4.2.4 Cybb tm1 mice exhibit reduced eosinophilia and a more pronounced Th1 response to C. neoformans

15 Macrophages are central to the development of a granulomatous response orchestrating cellular recruitment and containment of the pathogen. We have previously observed an aberrant macrophage response in naïve Cybb tm1 mice, which is augmented upon pathogen challenge. Analysis of H and E stained lung sections (Fig. 4.5b) demonstrates an accumulation of macrophages and multinucleated giant
20 cells at the centre of the granuloma formations in Cybb tm1 mice. Conversely, the cellularity in the wild type lung, whilst containing macrophages, was dominated by lymphocytes and eosinophils. This was confirmed by FACS analysis of lung tissue, which clearly showed a significantly greater percentage of macrophages in Cybb tm1 mice (Fig. 4.8a), which ultimately led to an enhanced number of these cells
25 throughout the course of infection (Fig. 4.8b).

Differential cell counts of H and E stained cytospin preparations (Fig. 4.6b) and FACS analysis (Fig. 4.8c and d) revealed a marked reduction in the percentage eosinophilia in the lung and airways in Cybb tm1 mice. This in turn led to a reduction
30 in the total numbers of eosinophils in the lung and airways of Cybb tm1 mice relative to wild type controls (data not shown). Eosinophilia is dependent on CD4⁺ T cells. In

Cybb tm1 mice, however, the percentage of CD4⁺ T cells was reduced at day 12 after infection whereas that of CD8⁺ T cells was increased (Fig. 4.9a). Cybb tm1 mice exhibited a significant increase in the total number of CD8⁺ T cells in their lungs at day 12 after infection (Fig. 4.9b). Both CD4⁺ and CD8⁺ T cells exhibited a heightened activation state (CD45RB^{lo}) in Cybb tm1 mice at days 8 (data not shown) and 12 (Fig. 4.9c and d) after infection.

To determine whether a shift in the cytokine profile accounted for reduced eosinophilia in Cybb tm1 mice we employed intracellular cytokine staining. Both CD4⁺ and CD8⁺ T cells produced increased levels of IFN- γ (Fig. 4.10a) and TNF (data not shown) in Cybb tm1 mice compared to wild type mice. Conversely, Cybb tm1 derived T cells produced lower levels of IL-5 (data not shown). The ratio of IFN- γ producing T cells to IL-5 producing T cells was greatly augmented in Cybb tm1 mice (Fig. 4.10b) showing a significant Th1 bias. Soluble cytokine levels in lung homogenate were also monitored by Cytometric Bead Array (CBA) throughout the course of infection and displayed a similar Th1 bias in Cybb tm1 mice. There were significantly elevated levels of TNF at days 8 and 12 after infection in Cybb tm1 mice and reduced levels of IL-5 at days 5 and 8 (Fig. 4.10c).

Since CD4⁺ T cells assist in anti-cryptococcal antibody production we next compared the effect in Cybb tm1 mice. Cybb tm1 mice exhibited significantly reduced B cells in the lung and airways (Table 4.1). Furthermore, reduced pathogen specific total antibody and IgG1 isotype in the serum at day 12 post infection, and IgE isotype in the BAL fluid was observed at day 5 post infection (Table 4.1).

The severe pulmonary eosinophilia that occurs during *C. neoformans* infection is largely attributable for the observed pathology owing to degranulation and crystal deposition. Furthermore, *C. neoformans* is directly responsible for much of the observed lung pathology observed during the course of infection owing to its considerable size and extensive numbers. To assess whether the reduced eosinophilia and pathogen load observed in Cybb tm1 mice reduced lung damage we assessed the levels of total protein (Fig. 4.11a) and lactate dehydrogenase (LDH) (Fig. 4.11b) in

the BAL fluid. Both markers of pathology were significantly reduced in Cybb tm1 mice.

4.2.5 The Th1 bias and enhanced protection observed in Cybb tm1 mice is IL-12 independent

IL-12 is a critical cytokine in the induction of a Th1 response . IL-12 is also critical in controlling dissemination of *C. neoformans* to the brain . The heightened macrophage response observed in Cybb tm1 mice could presumably affect levels of this cytokine and thus control dissemination with greater aptitude. Neutralising anti-IL-12 antibody was thus utilised to infer the role of IL-12 in the enhanced protection observed in Cybb tm1 mice.

The administration of a neutralising anti-IL-12 antibody to *C. neoformans* infected wild type mice had no effect on total cell numbers in the lung (Fig. 4.12a), but gave rise to a general increase in cellularity in the airways (Fig. 4.12b) of the treated mice. Furthermore, administration of this neutralising antibody to Cybb tm1 mice failed to reverse the reduced airway cellularity seen in these mice relative to wild type controls (Fig. 4.12b). The enhanced ability of Cybb tm1 mice to control cryptococcal infection was not compromised by anti-IL-12 antibody treatment. Wild type mice administered anti-IL-12 antibody exhibited a dramatic increase in *C. neoformans* dissemination to the brain, but this was not the case with Cybb tm1 mice (Fig. 4.13a). CFUs in the lung and BAL were not affected by anti-IL-12 treatment in either strain, and Cybb tm1 mice continued to exhibit a reduced pathogen burden at these sites (Fig. 4.13b and c).

The Th1 bias observed in Cybb tm1 mice relative to wild type controls persisted even with administration of anti-IL-12 antibody by intracellular cytokine staining of T cells. Anti-IL-12 treatment of wild type mice evoked heightened IL-5 production and reduced IFN- γ production, but the cytokine profile of Cybb tm1 mice receiving the treatment remained unaltered. This was most prominent in the percentage CD8⁺ T cells producing IFN- γ (Fig. 4.14a). Furthermore, macrophage numbers were augmented in the lungs of both treated and untreated Cybb tm1 mice relative to wild

type controls (data not shown), and eosinophil numbers markedly reduced in the lungs (data not shown) and BAL (Fig. 4.14b).

4.2.6 Cybb tm1 mice continue to show a heightened Th1 response and improved C. neoformans clearance in the airways at day 35 after infection.

5 C57BL/6 mice fail to clear *C. neoformans* infection, whereas BALB/c mice elicit a protective Th1 response and do so. To infer whether the enhanced Th1 driven protective immunity observed in Cybb tm1 mice at day 12 would eventually lead to complete resolution of *C. neoformans* infection, we extended the infection to day 35. At this time point, the Cybb tm1 mice actually possessed a greater cellular infiltrate
10 within their lungs compared to wild type controls (Fig. 4.15a), which continued to be highly granulomatous in nature (Fig. 4.15c). There was also reduced cellularity within the airways of the Cybb tm1 mice at this time point compared to wild type controls (Fig. 4.15b). Cybb tm1 mice continued to exhibit a strong Th1 bias, with a greater percentage of both CD4⁺ and CD8⁺ T cells producing IFN- γ (data not shown). As
15 seen at earlier time points, the Cybb tm1 mice also possessed a greater percentage (Fig. 4.15d) and number (data not shown) of macrophages relative to wild type mice and a reduction in the percentage of eosinophils (Fig. 4.15e).

At this late time point the number of *C. neoformans* cfus were comparable in the
20 lungs of wild type and Cybb tm1 mice (Fig. 4.16a), but persisted to be significantly lower in the airways of the knock out mice (Fig. 4.6b). The number of cfus in the lung at day 35 after infection were comparable in wild type mice to that seen at day 12 but had significantly increased in the knock outs at this later time point. The number of cfus in the airways was comparable in both groups of mice at days 12 and 35.
25 Pathogen burden in the brain of both groups of mice was low at day 35 after infection and whilst generally lower in Cybb tm1 mice this difference failed to be significant (data not shown).

4.2.7 Mice treated with MnTE-2-PyP exhibit reduced inflammation in response to C. neoformans infection and show reduced dissemination to the brain

We have previously noted marked differences in the phenotype of naïve wild type and Cybb tm1 mice, and appreciate many inherent problems of utilising knock out mice to elucidate the role of immune components in the outcome of infection. We therefore further assessed the role of ROS in *C. neoformans* infection utilising the metallic manganese porphyrin MnTE-2-PyP, which acts as an SOD mimic and possesses ROS scavenging potential. C57BL/6 mice were inoculated intranasally with *C. neoformans* and subsequently administered either PBS or 50µg of the manganic porphyrin MnTE-2-PyP at days 0, 4 and 8 after infection (Fig. 4.17). Mice administered MnTE-2-PyP exhibited a marked reduction in cellular infiltrate into the lung tissue (Fig. 4.18a) and airways (Fig. 4.18b) in response to *C. neoformans* infection, relative to controls. This is distinct to the scenario in Cybb tm1 mice which possessed a significant granulomatous lung infiltrate. Visualisation of H and E stained lung sections revealed a striking reduction in perivascular and peribronchiolar infiltrate in MnTE-2-PyP treated mice, with reduced occlusion of alveoli (Fig. 4.18c). The reduced cellularity evoked by MnTE-2-PyP treatment encompassed a significant reduction in total numbers of eosinophils (Fig. 4.19a and b) and lymphocytes (Fig. 4.19c and d) in the lungs and airways. The percentage of cells that were macrophages was slightly augmented in the mice treated with MnTE-2-PyP, but total numbers were generally reduced owing to the significant reduction in total cell numbers in these mice (data not shown). Similar to Cybb tm1 mice, those treated with MnTE-2-PyP showed a marked bias towards a CD8⁺ T cell response relative to CD4⁺ when compared to control mice (Fig. 4.20a-c). Furthermore, both CD4⁺ and CD8⁺ T cells from mimetic treated mice exhibited a Th1 cytokine bias with a greater percentage of cells expressing intracellular IFN-γ relative to PBS treated mice (Fig. 4.20d). The percentage of CD8⁺ T cells activated, as defined by being CD45RB^{lo}, was elevated in MnTE-2-PyP treated mice, as seen in Cybb tm1 knock outs (Fig. 4.20e). Conversely, the percentage CD4⁺ CD45RB^{lo} T cell was significantly reduced in mimetic treated mice (Fig. 4.20e). Finally, MnTE-2-PyP treated mice showed comparable fungal loads in their lungs and airways (Fig. 4.21a), but exhibited reduced dissemination to the brain relative to control treated mice (Fig. 4.21b).

The Th1 bias and improved protection seen in Cybb tm1 mice or those administered MnTE-2-PyP suggests a role for ROS in defining the Th1 / Th2 paradigm. This may be partly explained by the heightened macrophage numbers in *C. neoformans* infected Cybb tm1 mice. Since ROS have been implicated in apoptosis we have investigated macrophage apoptosis during *C. neoformans* infection with or without administration of MnTE-2-PyP. Whilst we found the total number of annexin V⁺ macrophages were slightly reduced, this was a reflection of the total reduction in cellular infiltrate rather than an alteration in the proportion of cells undergoing apoptosis (data not shown). It would appear that a reduction in macrophage apoptosis is therefore not the sole reason for the elevated Th1 response and improved pathogen clearance.

At day 45 after infection with *C. neoformans*, mice that had been administered MnTE-2-PyP continued to exhibit a reduction in cellular infiltrate into the lung tissue (Fig. 4.22a) and airways (4.22b). Despite a significantly reduced CD4⁺ and CD8⁺ T cell infiltrate into the lung tissue (Fig. 2.22c), those mice treated with the mimetic showed a striking increase in fungal clearance from this site (Fig. 2.22d). As seen at day 12, MnTE-2-PyP treated mice also exhibited a significant decrease in *C. neoformans* dissemination to the brain (Fig. 2.22e). The mimetic treated mice continued to exhibit a Th1 bias, and it was of interest that pulmonary macrophages in these mice were in a heightened activation state and expressing elevated MHC II on their surface (Fig. 2.22f and g).

4.3 Discussion

4.3.1 The role of ROS during pulmonary *C. neoformans* infection

In this study we have investigated the anti-microbial action of ROS against *C. neoformans*, utilising mice that lack a functional phagocyte NADPH oxidase (Cybb tm1 mice) or through administration of a manganic porphyrin with broad anti-oxidant activity. ROS are believed to fulfil a critical role in conferring protection against fungal infections; the most important evidence provided by patients with chronic granulomatous disease, who lack a functional NADPH oxidase, and display

heightened susceptibility to fungal pathogens such as *Aspergillus fumigatus* . We have previously demonstrated that mice lacking a functional phagocyte NADPH oxidase possessed a basal Th1 skewed immunity affiliated with an augmented macrophage population. Furthermore, this phenotype was promoted in response to a pulmonary influenza challenge. A non-protective response to *C. neoformans* shows Th2 bias with prominent airway eosinophilia, whereas a Th1 response is linked with pathogen clearance and prevention of dissemination to the CNS. It is plausible that the absence of a NADPH oxidase may therefore actually prove to be beneficial in the control and resolution of infection from this fungal pathogen.

4.3.2 Pulmonary granuloma formation and containment of *C. neoformans*

We show that Cybb tm1 mice elicit a lung granulomatous response to *C. neoformans* with enhanced macrophages and a distinct shift towards a protective Th1 driven response. In this environment, *C. neoformans* is contained in the lung and dissemination to the brain dramatically reduced. The appearance of a granulomatous response to *C. neoformans* in Cybb tm1 mice is not unexpected, since one of the clinical manifestations of CGD patients is aberrant inflammation and the formation of granulomatous lesions at disparate sites . *A. fumigatus* evokes enhanced inflammatory responses in the lungs of both CGD patients and in murine models of CGD . This correlates with heightened macrophage responses leading to granuloma formation. Similarly, mice lacking a functional phagocyte NADPH oxidase display enhanced inflammatory gastritis to *Helicobacter pylori* , and elevated levels of PMN sequestration in an *Escherichia coli* sepsis model . The aberrant inflammatory response is not restricted to pathogenic challenge, with excessive inflammation and granuloma formation observed in the lungs of Cybb tm1 mice following injection of sterile heat killed fungal products and enhanced peritoneal leukocytosis in response to thioglycolate . The occurrence of granulomatous responses in Cybb tm1 mice is poorly understood but appears to be tightly affiliated with an exuberant macrophage response.

Macrophages are central to granuloma formation and their numbers were significantly elevated in *C. neoformans* infected Cybb tm1 mice relative to wild type controls. As

discussed previously, naïve *Cybb* *tm1* mice possess an intrinsic increase in the number of macrophages in lymphoid tissues implying a possible role in the homeostasis of this cell population in resting conditions. This basal increase is further augmented following infection with *C. neoformans*. The elevated macrophages would appear at least in part to be attributable to a reduction in apoptosis of macrophages, with ROS having been implicated in cellular damage and induction of the apoptotic pathway. The absence of ROS ultimately leads to persistence of this population and greater clustering and activation in response to *C. neoformans* infection.

4.3.3 *Th1 skewed immunity in C. neoformans infected Cybb tm1 mice*

Perhaps the most significant alteration in the phenotype of the immune response elicited to *C. neoformans* in *Cybb* *tm1* mice is the distinct shift in the Th1/Th2 balance. It is notable that *Cybb* *tm1* mice exhibit a marked bias towards a Th1 response, relative to the wild type controls. This is conveyed by the significant increase in CD8⁺ T cells, a striking shift towards a Th1 cytokine expression profile, and a marked reduction in B cells and total antibody, specifically Th2 associated IgG1 and IgE. Most prominent, however, is the marked reduction in lung and airway eosinophilia, a perpetual trait of the non-protective Th2 response normally elicited in C57BL/6 mice. Therefore, it would appear that the absence of ROS leads to a more potent Th1 response.

Macrophages are an abundant source of IL-12, a potent Th1 skewing cytokine with the potential to inhibit the development of the Th2 driven chronic eosinophilia elicited by *C. neoformans* in C57BL/6 mice. IL-12 and IFN- γ have been reported to act at several levels to reduce eosinophilia, with cited potential to restrain eosinophil differentiation from bone marrow stem cells, and inhibit expression of eotaxin at the mRNA and protein level. Thus it is foreseeable how an enhanced macrophage number, possibly due to reduced apoptosis, and ensuing Th1 response would impede the non-protective Th2 phenotype. Furthermore, there is evidence claiming that superoxide is necessary for the activation of very late antigen-4 (VLA-4) on the surface of eosinophils, which is required for the targeted adhesion to VCAM-1 and recruitment to tissues. The absence of a functional NADPH oxidase and ensuing

superoxide production would therefore reduce eosinophil recruitment to the lung. It has also been reported that reactive oxygen intermediates can induce NK cell apoptosis and dysfunction. NK cells are a potent source of IFN- γ and as such could also facilitate the skew towards a Th1 phenotype, as well as being critical cytotoxic mediators in the resolution of *C. neoformans* infection.

The increased CD8⁺ T cell response in *C. neoformans* infected Cybb tm1 mice probably reflects the heightened macrophage numbers and augmented Th1 response. The activation of naïve CD8⁺ T cells and ensuing proliferation and effector CTL function requires both TCR engagement and co-stimulatory signal imparted by CD28 or IL-2. Priming of naïve CD8⁺ T cells will largely occur in secondary lymphoid organs and MHC class I antigen presentation will be predominantly mediated by mature dendritic cells that express co-stimulatory molecules. *C. neoformans* is an extracellular pathogen and as such APCs will internalize and process the antigen from other cells or the extracellular space. This processing of exogenous antigen for MHC I “cross-presentation” occurs by mechanisms that are termed alternate MHC class I processing and leads to the cross priming of CD8⁺ T cell responses. It is plausible that cytokines present during infection will alter MHC class I antigen presentation. Indeed, IFN- γ has been demonstrated to regulate the expression of multiple components of MHC class I antigen presentation, including MHC class I heavy chain, β_2 microglobulin, TAP-1, TAP-2, LMP2 and LMP7, generally leading to heightened expression of MHC class I on various cells. Furthermore, IFN- γ has been shown to promote assembly and surface expression of stable MHC class I complexes through more rigorous quality control during their progress through the endoplasmic reticulum. The heightened Th1 immunity seen in Cybb tm1 mice infected with *C. neoformans* would therefore be anticipated to give rise to elevated MHC class I expression and a greater CD8⁺ T cell response in these animals. Furthermore, type I IFNs play an important role in increasing MHC class I mRNA and protein levels and it would be of interest to determine whether the augmented macrophage numbers seen in Cybb tm1 mice would increase the levels of these cytokines. We also saw elevated TNF levels in the *C. neoformans* infected Cybb tm1 mice, and this cytokine has also been linked with stimulation of MHC class I expression.

Cytokines present can also have a direct effect on the amplitude of the CD8⁺ T cell response. It is argued that an effective CD8⁺ T cell response actually requires three distinct signals: TCR engagement, co-stimulation / IL-2 and a third signal which can be provided by IL-12. This third signal appears to be a requisite for complete activation of naïve CD8⁺ T cells both *in vitro* and *in vivo* . It is anticipated that IL-12 is required for optimal IL-2 dependent proliferation and clonal expansion of CD8⁺ T cells. IL-12 stimulates the expression of the IL-2R α chain (CD25) to much higher levels than TCR and co-stimulatory signals, and to maintain CD25 expression for longer. Subsequently, CD8⁺ T cells proliferate more effectively in response to low levels of IL-2 . Another study has also revealed that the primary expansion of effector CD8⁺ T cells is greater following IL-12 priming owing to a reduced susceptibility to apoptosis . It is possible that the greater macrophage response seen in *C. neoformans* infected Cybb tm1 mice will lead to elevated IL-12, which could be one factor that dictates the heightened CD8⁺ T cell response.

Whilst the CD8⁺ T cell response is augmented in Cybb tm1 mice, deprivation of ROS appears to give rise to a relative reduction in the percentage of CD4⁺ T cells. This is likely to be largely attributable to the increase in the number of CD8⁺ T cells in the knock out mice since total numbers of the CD4⁺ subset are comparable between the two groups. However, administration of MnTE-2-PyP appeared to cause a definite shift from a CD4⁺ T cell to a CD8⁺ T cell bias. It is plausible that the shift away from a CD4⁺ T cell predominated response is attributable to reduced MHC II expression. Superoxide reacts very rapidly with NO to produce peroxynitrite, but in the absence of superoxide NO will persist for longer. Therefore, it is plausible that there would be elevated levels of nitric oxide in the Cybb tm1 or MnTE-2-PyP treated mice. There is a surfeit of cited literature that argues a role for nitric oxide in the inhibition of IFN- γ induced increase in MHC II expression . This regulatory role of NO is mediated through its potential to inhibit the IFN- γ stimulated expression of CIITA. CIITA regulates a family of genes that are implicated in antigen processing and presentation with MHC II . Similarly, TNF has been demonstrated to have variable effects on MHC II expression, in a manner that is dependent upon the cell type and cellular

differentiation. In peritoneal macrophages TNF has the potential to suppress MHC II expression , whilst other literature states that TNF will only suppress IFN- γ induced increment in MHC II expression if NO is also present . The TNF mediated down-regulation is again ascribed to its potential to act at the transcriptional level to suppress the IFN- γ induced increment in CIITA production . We see elevated levels of TNF in the *C. neoformans* infected Cybb tm1 mice, and it would be of interest to infer if this leads to reduced CD4⁺ T cell priming.

4.3.4 Enhanced clearance of *C. neoformans* in *Cybb tm1* mice

Whilst an augmented inflammatory response to pathogen is not atypical in the absence of a functional phagocyte NADPH oxidase, reduced burden of the pathogen is uncharacteristic. *Cybb tm1* mice limit *C. neoformans* in the lung tissue and airways reducing dissemination to the brain. CGD is characterised by recurring life threatening bacterial and fungal infections such as *Staphylococcus aureus* and *A. fumigatus*, with neutrophils from CGD patients demonstrated to display defective killing against certain bacterial and fungal pathogens. Whilst compromised pathogen clearance is the norm in the absence of a phagocyte NADPH oxidase, improved immunity and reduced bacterial load has been reported for *Helicobacter pylori* , and we have reported an improved clearance of influenza virus from the lungs of *Cybb tm1* mice. The improved killing of *C. neoformans* in the absence of ROS has been described previously *in vitro*, whereby incubation of peritoneal macrophages with SOD and catalase augments anti-cryptococcal activity owing to a concomitant increase in nitric oxide production .

The differences in susceptibility of NADPH oxidase deficient mice to *C. neoformans* relative to other fungal pathogens is probably partially attributable to the fact that protection against *A. fumigatus* and *C. albicans*, at least in the early stages of infection, is mediated primarily through ROS of phagocytes, whereas protection against *C. neoformans* is dependent on Th1 cell-mediated immune response, which we have demonstrated to be augmented in *Cybb tm1* mice. It is plausible that other non-oxygen mediated cytotoxic mechanisms are more important to eradicate *C. neoformans*, and in an *in vivo* scenario these may compensate for the absence of a

functional NADPH oxidase. The array of protective mechanisms exhibited by *C. neoformans* may negate the potential of superoxide and other ROS to kill this fungus, and oxidative cytotoxicity will only become apparent if these oxidative defences are absent. *C. neoformans* is an encapsulated yeast, with the large high molecular weight polysaccharide capsule protecting against different facets of the host's immune response. Such a capsule is not present around *A. fumigatus* and could explain the heightened significance of ROS in conferring protection to this species. The capsule surrounding the *Cryptococcus* renders the pathogen less susceptible to phagocytosis and so may deprive ROS accessibility to the fungus. *C. neoformans* also has the potential to elicit deposition of opsonins, which may trigger the phagocyte respiratory burst, at sites below the capsule surface, where they will be incapable of binding complement or Fc receptors on the surface of myeloid cells.

The improved clearance of *C. neoformans* is probably largely attributable to the enhancement of macrophages and Th1 cytokines in *Cybb* *tm1* mice. In addition, we report elevated CD8⁺ T cells that have been shown to directly bind *C. neoformans* and inhibit growth. The Th1 phenotype of the recruited T cell population in *Cybb* *tm1* mice is significant as mice lacking Th1 cytokines succumb to cryptococcal infection. IFN- γ will contribute to granuloma formation and the activation of macrophages. Furthermore, the chronic eosinophilia normally observed during a *C. neoformans* infection elicits extensive tissue disruption and damage through degranulation and crystal formation and facilitates replication and spread of *Cryptococcus*. The significant reduction in eosinophilia in *Cybb* *tm1* mice correlates with reduced lung pathology and damage and could also explain the enhanced ability to control this pathogen.

4.3.5 Enhanced protection observed in *Cybb* *tm1* mice is IL-12 independent

Considering the pivotal role of IL-12 in the generation of Th1 responses it was surprising that anti-IL-12 treatment had a negligible effect on the immune profile observed in *Cybb* *tm1* mice; especially since similar treatment of wild type mice enhanced Th2 cytokines and impaired pathogen clearance. *Cybb* *tm1* mice still displayed a potent Th1 response and enhanced protection even when deprived of IL-

12. It would appear that the aberrant immune phenotype evoked by the absence of the NADPH oxidase is able to overcome the customary requirement for IL-12. TNF in Cybb tm1 mice may assist in the maintenance of a protective Th1 response. TNF knock out mice are more susceptible to *C. neoformans* infection, and Huffnagle and colleagues have demonstrated a significant early role for TNF in recruitment of leukocytes and the development of Th1 biased immune response. Indeed, it has previously reported that CpG promotes a heightened Th1 response to *C. neoformans* and prevents dissemination to the brain, an effect that was abolished by depleting TNF.

4.3.6 MnTE-2-PyP and control of pulmonary *C. neoformans* infection

The manganic porphyrin antioxidant, MnTE-2-PyP, also enhanced Th1 cytokines to *C. neoformans*, again with heightened CD8⁺ T cells, Th1 cytokines and significantly reduced eosinophilia. Furthermore, treated mice exhibited reduced dissemination of *C. neoformans* to the brain. However, compared to Cybb tm1 mice, some differences were apparent. Whereas Cybb tm1 mice displayed a granulomatous response that contained the pathogen within the lung, those treated with MnTE-2-PyP did not. The granulomatous response observed in Cybb tm1 mice appears to be a function of the cumulative absence of a functional NADPH oxidase since birth leading to a heightened basal macrophage population that subsequently expanded greatly in response to encountered stimuli. A permanent lack of superoxide may compromise homeostatic regulation of this myeloid immune compartment. MnTE-2-PyP, however, was only administered during infection and so would not be anticipated to elicit the same extent of macrophage deregulation. The fact that inflammation was so significantly reduced in mice administered the anti-oxidant most likely reflects the pro-inflammatory action of ROS in activating certain redox sensitive transcription factors. This will be of particular importance with administration of MnTE-2-PyP since it targets superoxide generated by the phagocyte oxidase, xanthine oxidase and that derived from the NOX family that are more classically affiliated with a role in signalling. The effects of MnTE-2-PyP reported here are analogous to its effects in a murine model of asthma, where it reduced inflammation and, significantly, eosinophilia. Significantly, mice administered the MnTE-2-PyP anti-oxidant for a

longer period were not compromised in their ability to combat *C. neoformans* infection with treated mice exhibiting reduced pulmonary inflammation as well as reduced lung and brain fungal titres at day 45 after infection.

4.3.7 Conclusions

5 In summary, it would appear that the absence of a functional phagocyte NADPH
oxidase paradoxically results in enhanced *C. neoformans* clearance and most
significantly, reduced dissemination to the brain. This is in contrast to the
compromised immunity that such mice show to other fungal pathogens, and is a
function of a heightened macrophage infiltrate and the ensuing Th1 driven immune
10 response. These findings emphasize a significant role for ROS in defining the Th1 /
Th2 paradigm and offer a novel mechanism to modulate myeloid immune responses.

Chapter 5 – The Basic Biology of CD200 and its Receptor

5.1 Introduction

5.1.1 Control of the myeloid immune compartment

15 The development of an efficient and robust immune response is critical to the
successful resolution of infection, but a degree of subtlety and tolerance is required to
distinguish self from non-self and prevent bystander tissue damage . Myeloid cells are
central to the induction, maintenance and subsidence of the inflammatory response to
20 infection, as well as defining regulatory mechanisms that are crucial in the prevention
of an inappropriate immune response. The phenotype and function of leukocytes is
controlled by both secreted factors and more intimate cell contact interactions . The
role of such signals in dictating immunity and tolerance at the level of the T cell are
well characterised , but we know comparatively little regarding the interactions that
25 govern cells of the myeloid lineage despite them having a critical role in immunity
and immunopathology.

Myeloid cells are an extremely heterogeneous population; their phenotype and function exquisitely regulated through the multitude of receptors on their surface . Among such receptors are pattern recognition receptors for the recognition of pathogen derived products, cytokine and chemokine receptors, complement and Fc receptors to facilitate pathogen clearance, accessory molecules that facilitate T cell activation, receptors that mediate phagocytosis, and those that dictate the migration of cells from the circulation and into tissues. There are a family of receptors that dictate myeloid cell function through the action of paired activating and inhibitory receptors. These receptors are members of the Immunoglobulin Superfamily (IgSF) and include paired immunoglobulin-like receptors (PIRs), signal regulatory proteins (SIRPs), triggering receptors expressed my myeloid cells (TREMs) and the CD200 receptor family . Little is known about many of these receptors and in a lot of cases the physiological ligand remains unknown. However, the CD200-CD200R interaction is beginning to be characterised, with the majority of data demonstrating it to invoke a potent inhibitory signal to the myeloid immune compartment . Interest in this myeloid inhibitory interaction has escalated owing to the clear immunotherapeutic potential in alleviation of autoimmunity, allergy and immunopathology, however, much of our knowledge thus far is contradictory, speculative and in many cases completely absent.

5.1.2 CD200 and receptor expression

CD200 (OX-2) was first isolated and characterised in rats in 1982 as a type 1 transmembrane glycoprotein with two IgSF domains . CD200 was demonstrated to be expressed on a variety of cells in distinct tissues: thymocytes, recirculating B cells, some T cells, follicular dendritic cells, neurons in the central nervous system, endothelium, the granulosa of the degenerating corpora lutea, trophoblasts and smooth muscle . This unusual and specific distribution was found to be highly conserved in mice and humans, with expression also seen in the kidney glomeruli in humans and reported in murine keratinocytes . This conserved expression of CD200 on specific cellular populations would suggest a significant biological role for this molecule.

The CD200 receptor (OX-2 receptor) was subsequently purified from rat splenocytes using a high affinity monoclonal antibody (OX102) to a macrophage antigen, which

was demonstrated to block CD200 binding . This novel protein was subsequently cloned and shown to have homology to CD200, being a type 1 transmembrane protein with two IgSF domains, but possessing a longer cytoplasmic tail with signalling capacity . The CD200 receptor is almost exclusively expressed by cells of the myeloid lineage . However, RT-PCR of cDNA libraries from a variety of cell types of mice and humans demonstrate that whilst primarily on myeloid cells there is also receptor expression on polarized Th2 T cells . Flow cytometric analysis confirm these findings, with high levels of CD200R expression on cells of the myeloid lineage, with weak labelling of T cells, some NK cells, NKT cells and B cells. No other studies, however, report CD200R expression on populations other than myeloid cells.

5.1.3 Biological function of the CD200-CD200R interaction

Much of our knowledge concerning the role of the CD200-CD200R axis in immunity comes from studies performed in CD200 knock out mice generated by Sedgwick at DNAX . The only phenotypic difference found in the leukocyte compartments is an enlarged CD11b⁺ population. The spleens of CD200 knock out mice contain twice the number of CD11b⁺ myeloid cells with elevated numbers of macrophages and granulocytes . The red pulp is significantly enlarged and the marginal zone expanded from a single cell layer to a multilayer. Higher levels of the ITAM containing DAP12 are present in the marginal zone macrophages and in dendritic cells within T cell areas of the white pulp, implying heightened cellular activation. Lymph nodes of CD200 knock out animals are also enlarged, especially mesenteric lymph nodes, and exhibit a marked alteration in organisation. Wild type lymph nodes are interconnected spherical structures, but the knock outs nodes exhibit a tubular formation with no demarcation between nodes. Once again macrophages are expanded and more activated in the nodes of the CD200 knock outs. Microglia, the macrophages of the CNS, also exhibit alterations in CD200 knock out mice forming aggregates that are generally only seen in inflammation or neurodegeneration. The microglia also shows many features of activation with shorter glial processes and elevated expression of CD11b and CD45. The fact that the phenotype is observed in those cells that express the CD200R rather than those that express the knocked out gene led Hoek *et al* to postulate that the

CD200 knock out phenotype was a consequence of myeloid cells lacking a restraining signal from CD200 .

Subsequently, CD200 knock out mice have been demonstrated to exhibit a more severe or accelerated disease phenotype in a variety of conditions where macrophage products are critical elements of the disease process, such as experimental autoimmune encephalitis (EAE) and collagen induced arthritis (CIA) . However, much of the findings concerning the role of CD200 in immune regulation are observational and there is little in the literature to divulge the mechanisms by which CD200 exerts a suppressive signal to the myeloid compartment. One recent study demonstrates the potential of signalling through CD200R to suppress cytokine production by CD200R transfected U937 cells, and the potency of this suppression to correlate with density of CD200R expression . Furthermore, the same study demonstrates the potential of agonists to CD200R to suppress IFN- γ and IL-17 induced secretion of IL-6 by peritoneal macrophages. Similarly, engagement of CD200R in murine bone marrow derived mast cells over-expressing the receptor reduces degranulation and secretion of IL-13 and TNF . Similar findings were subsequently confirmed in human mast cells expressing normal levels of CD200R. Finally, some evidence supports a role for CD200R ligation in up-regulating indole dioxygenase (IDO) in plasmacytoid dendritic cells resulting in heightened tryptophan catabolism and tolerance .

5.1.4 Hypothesis

The CD200-CD200R interaction appears to deliver a potent inhibitory signal to the myeloid immune compartment, and many studies have manipulated this interaction to alleviate illness and disease in conditions characterized by excessive inflammation. There is clear therapeutic potential for manipulation of this inhibitory myeloid signal in respiratory infections, whether by delivering the inhibitory signal to suppress immunity during influenza infection or blocking the signal to promote immunity to *C. neoformans*. However, at present studies describing this therapeutic potential are significantly outpacing our understanding of the basic biology that underpins the observed phenotype. CD200 expression is unusual yet specific and conserved.

However, its expression is yet to be defined within the lung and little is known regarding the parameters that modulate the levels of its expression. Similarly, our knowledge of what regulates the levels of expression of the receptor on myeloid cells is still in its infancy. These findings would be crucial if we are to understand the physiological role this interaction may fulfil during infection and how we may optimally manipulate the signal therapeutically. Furthermore, very little is known regarding the biological function of CD200R signalling, with research required into its effect on myeloid cell cytokine/chemokine production, proliferation, survival, phagocytosis, chemotaxis and generation of microbial products. Much of our understanding of the immunological role of this interaction comes from CD200 knock out mice, but we still do not know why these mice exhibit heightened numbers of myeloid cells and many organs and tissues have yet to be studied, including the lung. I seek to address some of these issues and in so doing gain a fuller understanding of the physiological significance of CD200 and its receptor before attempting to manipulate this potent signal during respiratory infection.

5.2 Results

5.2.1 CD200 expression on distinct cellular populations within the lung

The expression of CD200 within the lung has not been definitively investigated. We therefore examined the CD200 expression patterns on both stromal and hematopoietic cells within the lung compartment. Single cell suspensions were obtained from the lungs of C57BL/6 mice and hematopoietic cells were defined by expression of CD45 by flow cytometric analysis (Fig. 5.1a). Hematopoietic cells were shown to exhibit differential levels of CD200 expression, reflecting the distinct subsets of cells within the hematopoietic population (Fig. 5.1b). The lung is a highly vascular organ and it was of interest to determine whether CD200 is expressed on endothelial cells in the lung, as has been reported at other sites. Endothelial cells were identified as CD31⁺CD45⁻ by FACS analysis (Fig. 5.1a) and were demonstrated to express high levels of CD200 on their surface (Fig. 5.1c). Fibroblasts, identified by the expression of CD90, were also shown to express CD200 on their surface (data not shown).

Comparable expression profiles were observed in the lungs of BALB/c mice (data not shown).

Expression of CD200 has been reported on some subsets of epithelial cells in murine skin . Antibodies to specific epithelial cell markers stain poorly for flow cytometry, making it difficult to investigate CD200 expression on epithelial cells by this method. We have therefore utilised a method that selectively isolates epithelial cells from lower aspects of the lung, primarily the alveoli and terminating bronchioles. Lungs were digested with dispase and CD45⁺, CD31⁺ and CD90⁺ cells were selectively depleted by MACS separation (Fig. 5.1d). The remaining cells appeared morphologically like epithelial cells and exhibited an appropriate FSc / SSc profile as adjudged by flow cytometry (data not shown). Furthermore, immunofluorescent staining for the type II epithelial cell marker pro-surfactant C, before and after depletion, showed a significant enrichment in these cells. Approximately 15% of cells freshly isolated from a naïve lung stained positive for pro-surfactant C, compared to around 65% after depletion (Fig. 5.1e). FACS analysis of isolated cells revealed CD200 to be expressed, but at two distinct levels of intensity (Fig. 5.1f). The differing CD200 expression levels most likely reflect distinct epithelial cell types and will demand further attention.

Immunofluorescent CD200 staining of OCT inflated naïve lung corroborated our findings by flow cytometry with strong staining visible upon endothelia surrounding blood vessels (Fig. 5.1g). Furthermore, epithelia around large bronchioles exhibited strong staining for CD200, whereas alveoli epithelia showed minimal expression (Fig. 5.1g-i). These may reflect the differing levels of CD200 expression observed on epithelial cells by flow cytometric analysis, with lower expression corresponding to alveoli.

We next investigated CD200 expression on hematopoietic cells. Myeloid cells in the lung did not express CD200 (data not shown), but expression was seen on lymphocytes. Flow cytometric analysis revealed that over 90% of C57BL/6 splenic B220⁺ cells expressed CD200 (Fig. 5.2a and c). CD19⁺ cells exhibited comparable

CD200 expression (data not shown). This high expression was also observed on lung B cells (Fig. 5.2b) and conserved in numerous tissues including those B cells circulating in the blood (data not shown). However, a significantly lower percentage of B220⁺ cells in the bone marrow expressed CD200 (approximately 25%), implying that CD200 is up regulated during a developmental stage of B cells at this site (data not shown).

CD200 is also reportedly expressed on some T cells (approximately 20% of human peripheral blood CD3⁺ T cells, and a significant percentage of murine splenic T cells (especially the CD4⁺ subset)). We showed expression on approximately 40 % of CD4⁺ and about 5-10 % of CD8⁺ T cells from the spleen (Fig. 5.2a) and lungs (Fig. 5.2b) of C57BL/6 mice. BALB/c splenic and lung T cells also expressed comparable levels of CD200 expression (data not shown). No CD200 expression was detected on NK or NK T cell populations within the lung (data not shown).

Studies in humans, rats and mice have reported CD200 expression on thymocytes . Indeed, almost all human thymocytes are CD200 positive with higher levels on single positive (SP) cells than double positive (DP) and more intense staining on CD4 SP than CD8 SP T cells. We found that levels of CD200 expression on thymocytes were considerably lower in C57BL/6 mice, and almost absent on DP thymocytes. Furthermore, comparable percentages of CD4 and CD8 SP T cells expressed CD200 to that seen in the lung and spleen (30-35% on CD4⁺ and 10% on CD8⁺) (Fig. 5.2e and f). Whilst CD200 expression on mice thymocytes was lower than in humans the same pattern of relative levels of expression was conserved with CD4 SP > CD8 SP > DP T cells.

Since CD200 expression was not ubiquitous on all T cell populations in the lung and spleen it was of interest to further dissect those populations that were positive. Flow cytometric analysis showed that whilst the majority of splenic CD200⁺ T cells expressed the $\alpha\beta$ TCR, all CD4⁺ and CD8⁺ T cells possessing a $\gamma\delta$ TCR were CD200 positive (Fig. 5.3). CD200 was not expressed on CD103⁺ intraepithelial T cells (data not shown), and remarkably no expression was detected on CD25⁺ or Foxp3⁺ (data

not shown). Comparable CD200 expression patterns were observed in T cells derived from the lung and in both lymphoid organs of BALB/c mice (data not shown). We did, however, observe some bias in CD200 expression with V β usage. Whilst the majority of V β expressing T cells showed the same 35-40% expressing CD200, V β 6, 7, 9 and 11 showed an elevated percentage of CD200⁺ cells (Fig. 5.4).

5.2.2 CD200 is up-regulated on T cells during pulmonary influenza infection

We next examined expression of CD200 during influenza infection of C57BL/6 mice. CD200 expression remained unaltered on all stromal cells investigated (endothelia, epithelia, fibroblasts) throughout the course of the infection and no CD200 expression was observed on myeloid cells at any point (data not shown). A significant increase in CD200 expression was observed on T cell populations in the lungs of influenza infected mice; up to 80% of CD4⁺ and 40% of CD8⁺ T cells by day 7 after infection (Fig. 5.5a). The intensity of CD200 expression (determined by the geometric mean) also increased (data not shown). During an influenza infection, there is a significant T cell infiltrate into the lungs and subsequently the number of CD200⁺ T cells was greatly enhanced throughout the course of infection (Fig. 5.5b). The up-regulation of CD200 on T cells correlated with increased activation markers on these cells (data not shown). Similarly, there was a comparable increase in the percentage and number of CD200⁺ T cells in the draining mediastinal lymph node through out the course of infection (data not shown). In contrast, T cells sampled from the airways by BAL at the peak of inflammation (day 7) showed a marked drop in CD200 expression compared to the lung (Fig. 5.5c). Thus it would appear that in the most inflammatory of sites during an influenza infection CD200 expression is strikingly down regulated. By day 14 post influenza infection, CD200 expression upon T cells within the lung tissue and BAL had returned to normal levels (data not shown).

The percentage CD200⁺ B cells in the lung remained high throughout the course of influenza infection (Fig. 5.5a), and only increased in numbers with the general increase in cellularity (Fig. 5.5b). However, once again CD200 expression was significantly reduced on B cells sampled from the BAL at day 7 post influenza infection (Fig. 5.5c). We have conducted numerous studies to infer what stimuli may

cause down-regulation of CD200 on the surface of B cells *in vitro*, treating both purified B cells and those in splenic cultures with an array of TLR ligands, cytokines, CD40, and crosslinking antibodies to surface Ig. All stimuli failed to evoke any alteration in the levels of CD200 on B cells (data not shown).

During influenza infection there is a dramatic influx of lymphocytes to the infected lung, peaking at day 7 after infection. This cellular infiltrate has largely subsided by day 10, largely due to apoptosis by activation induced cell death. It would be rational for these apoptotic cells to up-regulate CD200 so as to deliver an inhibitory signal to macrophages that clear them from the inflamed site. Indeed, we observed a significant percentage of annexin V positive lymphocytes expressing CD200 in the draining lymph nodes, lung and BAL (MLN>lung>BAL) (Fig. 5.6). Interestingly, virtually all CD200⁺ cells in the airways (BAL) are apoptotic, which may offer one plausible reason why the % CD200 expressing cells drop markedly at this site (Fig. 5.6b).

5.2.3 CD200 expression on T cells is dependent on dose and exposure to antigen

It has been shown previously that approximately 60% of CD25⁺ human peripheral blood CD3⁺ T cells express CD200 after a 72 hour exposure to Concanavilin A, indicating that CD200 is up-regulated on activated T cells. To further characterise the induction of CD200 on activated T cells we used the DO11.10 transgenic system, where ~95% of CD4⁺ T cells possess a TCR that recognizes Ovalbumin. DO11.10 splenocytes were cultured with different doses of OVA peptide and CD200 expression assessed at different time points by flow cytometry. The percentage of CD200⁺ CD4⁺ T cells and the geometric mean of CD200 expression increased with the dose of OVA peptide and with exposure time (Fig. 5.7a and b). The percentage of CD4⁺ T cells expressing CD200 showed a significant increase as early as 6 hours at the higher doses (10 and 100µg/ml) of OVA peptide and peaked by 48 hours (Fig. 5.7a). The intensity of CD200 expression, as determined by the geometric mean, did not show an increment until 24 hours, before peaking at 48 hours and then dropping again by 72 hours (Fig. 5.7b).

We then examined the kinetics of T cell CD200 expression relative to other activation markers. ICOS and OX40 are T cell co-stimulatory molecules that are induced on the surface of T cells after antigen exposure, with ICOS expressed by 12 hours after CD28 ligation and OX40 at 24 to 48 hours. Very little ICOS or OX40 expression was observed on the surface of CD4⁺ T cells until 24 hours from which point both increased dramatically as a function of both time and dose of OVA peptide. ICOS was induced quicker than OX40 as may be anticipated but was seen to decline at higher doses of antigen (Fig. 5.8a and b). Interestingly, with increasing doses of OVA peptide (for 24 hours) there appeared to be a shift from ICOS single positive CD4⁺ T cells to those co-expressing ICOS and CD200 (Fig. 5.8c). Conversely, most OX40⁺ CD4⁺ T cells expressed CD200 (Fig. 5.8c). The same pattern of sequential expression was apparent if the levels of ICOS, CD200 and OX40 were assessed at sequential time points (data not shown). This would imply that upon TCR triggering, ICOS is up regulated first, followed by CD200 and then OX40.

We next examined CD200 expression on Th1 or Th2 skewed D011.10 T cells. Intriguingly, whilst both Th1 and Th2 cells expressed CD200, levels of expression were significantly higher on Th1 cells (Fig. 5.9a). To ascertain the role of Th1 and Th2 cytokines in defining the expression levels of CD200, D011.10 splenocytes were cultured with OVA peptide for 48 hours in the presence of neutralising antibodies to IL-12, IL-4 and IL-10 and CD200 expression investigated by flow cytometry. Neutralisation of IL-12, a strong Th1 defining cytokine, had no effect upon CD200 expression (Fig. 5.9b). Conversely, neutralisation of IL-4 and IL-10 led to an increase in the intensity of CD200 expression (Fig. 5.9c and d). Thus it would appear that the presence of Th2 cytokines limits the expression of CD200 on activated T cells. Lymphocyte expression of CD200 was subsequently investigated in the lungs of C57BL/6 mice at day 12 after *C. neoformans* infection, which induces a strong Th2 response. Again, only limited induction of CD200 on CD4⁺ and CD8⁺ T cells was observed in this Th2 environment (Fig. 5.9e), relative to the potent up-regulation seen in the strongly Th1 driven influenza infection (Fig. 5.5). CD200 expression on B cells was high and comparable throughout the course of *C. neoformans* infection (Fig. 5.9e).

5.2.4 CD200 expression on T cells is TCR dependent

To confirm TCR signalling is required to induce CD200 on T cells, CD4⁺ T cells were purified from the spleens of C57BL/6 mice by magnetic bead separation and incubated with antibodies to CD3 and CD28 for 48 hours. CD3 ligation alone was sufficient to increase the % and GM of CD200 expression (Fig. 5.10a, b and c). Co-incubation with antibodies to CD3 and CD28 caused a modest, but not significant increase in CD200 expression (Fig. 5.10a), and no alteration in the intensity of CD200 expression (Fig. 5.10b). Addition of OX40L:Fc fusion protein, which has been shown to impart a signal through OX40, to this culture induced a striking increase in the CD200 GM (Fig. 5.10b), implying that late co-stimulatory signals can augment expression of CD200. To confirm that induction of CD200 on T cells is TCR signalling dependent we utilised SKG mice. SKG mice possess a mutation in the gene encoding ζ -associated protein of 70 kDa (ZAP-70). These mice have a diminished T cell response to antigen due to the attenuated signal transduction through ZAP-70, and are hypo-responsive to polyclonal TCR stimulation *in vitro*. Splenocytes from wild type and SKG mice were incubated with combinations of anti-CD3 and anti-CD28 antibodies for 48 hours and CD200 expression levels assessed by flow cytometry. Whereas CD3 ligation caused an increase in CD200 expression on both CD4⁺ (Fig. 5.11a, b and e) and CD8⁺ (Fig. 5.11c, d and e) T cells from wild type mice, no such induction was observed on those from SKG mice (Fig. 5.11). Reassuringly, PMA/Ionomycin, which transduces a signal to TCR distal pathways, induced a comparable increase in CD200 expression on T cells derived from both wild type and SKG mice (data not shown).

5.2.5 Down-regulation of CD200 expression

Splenic T cells purified by MACS separation exhibited a potent induction of CD200 expression when incubated with PMA / Ionomycin. However, as seen with the DO11.10 system whilst the percentage T cells expressing CD200 plateaus with time, the intensity of expression declines (Fig. 5.12a). Comparable results were also observed following treatment with agonistic anti-CD3 antibody (data not shown). The reduced CD200 expression at later time points may reflect the natural kinetics of CD200 expression or exhaustion of antigen. In the presence of PMA/Ion T cells

showed reduced CD200 expression at 72 hours compared to 24 hours (Fig. 5.12b). T cells were subsequently stimulated with PMA/Ionomycin for 24 hours, washed to remove the activating stimuli and re-cultured for a further 48 hours. Removal of PMA/Ion after 24 hours, further reduced CD200 expression at 72 hours relative to when PMA/Ion was maintained in cultures (Fig. 5.12b). This would imply that the reduction in CD200 expression may be a result of reduced antigenic stimulus rather than just the natural kinetics of expression.

Cyclosporin was subsequently utilised to study the effect of terminating T cell signalling on CD200 expression. DO11.10 splenocytes were cultured with different doses of OVA peptide for 24 hours to induce CD200 expression, and then for a further 72 hours with or without cyclosporin. Upon addition of cyclosporin to the splenocyte cultures, CD200 expression was largely suppressed at later time points and was generally maintained at a comparable level to that seen at 24 hours after initial stimulation (Fig. 5.12c and d). This once again proves the necessity for TCR signalling in the induction of CD200 expression.

5.2.6 CD200R expression in the naïve lung

The CD200 receptor is primarily restricted to the myeloid lineage with studies in mice, rats and humans describing expression on monocytes, macrophages, dendritic cells, neutrophils, basophils and mast cells. However, some studies in mice and humans have reported weak CD200R expression on T cells, with RT-PCR analysis of cDNA libraries of a variety of purified cell types showing preferential expression on Th2 cells. As with CD200, the levels of the receptor are poorly described in the murine lung although RT-PCR analysis has demonstrated its existence at this site. We therefore characterised CD200R expression on a variety of cell types within the lung and at other distal sites by flow cytometry.

Distinction between efferent myeloid compartments within the lung was achieved based upon the variable expression of CD11b and CD11c as discussed previously, with further discrimination being based upon levels of Gr-1 and MHC-II expression. Neutrophils within the lung compartment of C57BL/6 mice were devoid of CD200R

expression (Fig. 5.13), in stark contrast to granulocytes derived from the spleen, blood and bone marrow that were all demonstrated to exhibit some levels of expression (data not shown). Monocytes/ small macrophages in the lung were demonstrated to express some CD200R on their surface (Fig. 5.13), at comparable levels to that seen in the spleen, blood and bone marrow (data not shown). Dendritic cells were present in very low numbers, but did appear to exhibit minimal levels of CD200R expression (Fig. 5.13). Most striking was the significant CD200R expression on the surface of alveolar macrophages (Fig. 5.13), which was present at a higher level to that observed on myeloid cells from any other site investigated. Immunofluorescent staining for CD200R on OCT inflated naïve lungs verified this high level of receptor expression on alveolar macrophages (Fig. 5.14d). CD200R expression was also analysed on T cells, B cells, NK cells and various stromal cells at different sites and contrary to some previous reports no CD200R expression was observable (data not shown).

5.2.7 Increase in CD00R expression during influenza infection

We next examined CD200R expression during influenza infection. Lungs were isolated from naïve and influenza infected C57BL/6 mice at days 2, 7 and 14 after infection. As seen in the naïve mice, CD200R was not expressed on lymphoid populations, being restricted to the myeloid compartment (data not shown). The majority of alveolar macrophages expressed CD200R in naïve mice, but the intensity of this expression was increased during influenza infection peaking at day 14, a time when inflammation has largely resolved (Fig. 5.14a-c). The percentage of monocytes / small macrophages expressing CD200R in the lung also increased during influenza infection (Fig. 5.14a), and upon activation / maturation (adjudged by elevated CD11b, but still intermediate Ly-6G), macrophages showed a dramatic increase in CD200R expression, which peaked at day 14 (Fig. 5.14a-c). Neutrophils did not express CD200R in the naïve lung, but the percentage and intensity of expression increased during the course of influenza infection (Fig. 5.14a-c). Likewise, dendritic cells expressed minimal CD200R in a naïve lung, but the percentage bearing the receptor is increased slightly upon influenza infection (Fig. 5.14a) and the intensity of expression again peaked at day 14 (Fig. 5.15b). Immunofluorescent staining for CD200R in OCT inflated lung from naïve and day 7 influenza infected C57BL/6 mice showed an

increase in the number of cells bearing CD200R as may be anticipated and staining appears to be more dense implying greater expression levels (Fig. 5.14d).

As with our CD200 studies we next investigated what may up-regulate CD200R. using bone marrow derived macrophages. The majority of bone marrow (BM) macrophages expressed a basal level of CD200R (Fig. 5.15), but stimulation of the macrophages with IFN- γ and LPS, and to a lesser extent influenza, caused an augmentation in the % positive cells (Fig. 5.15a and c) and more strikingly the level of expression (Fig. 5.15b and c). The induction of CD200R expression by the different stimuli appeared to be largely dose dependent with 10ng/ml IFN- γ causing only a modest increase in the CD200R expression, but 100ng/ml causing a striking up-regulation (Figure 5.15a, b and d). Increasing the dose of influenza also caused a modest increase in the extent of CD200R up-regulation on BM macrophages, but LPS seemed to be a potent inducer of expression even at low concentrations. Furthermore, the increase in CD200R expression induced by these stimuli was temporally dependent, with a greater level of expression observed at 48 hours than at 24 hours (Fig. 5.15a, b and e); although CD200R expression seemed to subsequently drop marginally by 72 hours with IFN- γ and LPS treatments.

5.2.8 CD200R signalling in bone marrow macrophages reduces the production of pro-inflammatory cytokines

Manipulation of CD200R signalling has been utilised therapeutically to deliver an inhibitory signal to the myeloid compartment in conditions characterised by excessive inflammation. However, the exact manner by which the myeloid cell phenotype and function is modulated is poorly defined. We therefore investigated the effect that signalling through CD200R had on BM macrophages stimulated with a variety of inflammatory products/mediators.

Cytokine production by BM macrophages was assessed by performing cytokine bead array (CBA) on cell supernatants at 24 and 48 hours following stimulation. Comparable cytokine production profiles were observed at 24 and 48 hours but only the latter time point are presented and discussed here. Unstimulated BM macrophages

produced negligible cytokines either in the presence of a control antibody or an agonistic antibody to CD200R (OX110) (Fig. 5.16). IFN- γ stimulated BM macrophages produced significantly less TNF, IL-6 and MCP-1 when co-incubated with the agonistic OX110 antibody. There were no differences in the levels of IL-12 and IL-10 with incubation with OX110 (data not shown). Similar, but more potent, reductions in TNF, IL-6 and MCP-1 were observed in influenza treated macrophages that were incubated with OX110 (Fig. 5.16). Again no differences in IL-12 or IL-10 production were apparent, although CD200R signalling did cause a modest reduction in IFN- γ production (data not shown). Treatment of BM macrophages with LPS caused potent cytokine production that remained largely unaltered by treatment with OX110, although there was a small but significant reduction in IL-6 (Fig. 5.16). Signalling through CD200R also caused a subtle but reproducible reduction in the levels of nitric oxide produced by untreated and IFN- γ and LPS treated bone marrow macrophages. Reduction in pro-inflammatory cytokine production following signalling through CD200R was not attributable to enhanced macrophage apoptosis as negligible differences were seen with TUNEL assay or with annexin-V staining (data not shown).

A previous study conducted in BM derived mast cells demonstrated that signalling through CD200R reduced production of TNF and IL-13. Furthermore, the signalling pathway induced by CD200R ligation was partially characterised, with the receptor being tyrosine phosphorylated and Dok-1 and Dok-2 adaptor proteins subsequently recruited. Dok-1 and -2 are then phosphorylated and bind to RasGAP and SHIP, leading to inhibition of Ras and ERK (Fig. 5.17a). We determined whether the same signalling pathway was utilised in BM macrophages stimulated with IFN- γ overnight to up-regulate CD200R expression and then incubated with a control or an agonistic antibody to CD200R for different periods of time. Dok-2 was immunoprecipitated from cell lysates and immunoblotted with an antibody to Dok-2 or to phosphotyrosine. Signalling through CD200R did not alter the levels of Dok-2, but there was an observable increase in the phosphorylation of this adaptor protein (Fig. 5.17b). Whilst this would corroborate the pathway seen with mast cells, further investigation is required to investigate other signalling partners in the postulated pathway.

Signalling through CD200R did not cause a striking universal reduction in any BM surface activation markers in the same manner it did pro-inflammatory cytokines. Indeed, with IFN- γ and LPS stimulation, addition of OX110 appeared to slightly
5 augment the surface expressed levels of CD86, CD11b and OX40L (Fig. 5.18). Representative histogram overlays of CD86, CD11b and OX40L are shown following IFN- γ stimulation in the presence of a control antibody or OX110 (Fig. 5.18c, f and i). However, there did appear to be a general reduction in CD86 and OX40L expression with the addition of OX110 to influenza stimulated bone marrow macrophages (Fig.
10 5.18a, b, g and h).

5.2.9 Effects of CD200R agonists on bone marrow dendritic cell phenotype and function

We next determined whether signalling through CD200R altered the phenotype or function of dendritic cells similar to macrophages. BM derived dendritic cells were
15 stimulated with different concentrations of LPS, flagellin, CpG, Poly:IC, influenza, IFN- γ , IL-12 and IL-10 in the presence of a control antibody or OX110. None of these stimuli altered the level of CD200R expression on the surface of bone marrow dendritic cells (data not shown). We also analyzed expression of the cell surface markers CD11b, CD11c, CD80, CD86, OX40L and MHC II, as well as secretion of
20 IL-1 α , IL-6, IL-10, IL-12p70, MCP-1, IFN- γ and TNF. We did not observe any effect of CD200R agonists on these parameters (data not shown). CD200R agonists also had no effect on DC-induced CD4⁺ T cell proliferation, with ovalbumin loaded, LPS stimulated dendritic cells providing comparable CFSE incorporation and activation of CD4⁺ T cells in the presence of control antibody or OX110 (Fig. 5.19a). Cytokine
25 production by these dendritic cell activated CD4⁺ T cells were also assessed by intracellular cytokine staining and a small but reproducible reduction in IFN- γ production was observed with CD200R agonist, but no differences were apparent in IL-4 production (Fig. 5.19b). In the context of these experiments it is important to remember that TLR4, the ligand for LPS, is also expressed by activated T cells.
30 However, it has been demonstrated that LPS fails to induce significant T cell proliferation or cytokine production upon binding to the T cell TLR4 . It is likely

dendritic cells will be modulated in other ways through CD200R, however, to date, we have not identified the relevant *in vitro* conditions needed to observe CD200R-mediated modulation of dendritic cell function.

5.3 Discussion

5.3.1 CD200R signalling reduces pro-inflammatory cytokine production by macrophages.

We show that addition of an agonistic antibody to CD200R is capable of reducing pro-inflammatory cytokine production by BM macrophages *in vitro* in response to IFN- γ and influenza but not LPS. We do not, however, see such a marked reduction in the activation associated surface markers on BM macrophages following ligation of CD200R. These findings are largely supported by a recent study that demonstrates the potential of CD200R agonists to suppress TNF and IL-6 production by mouse peritoneal macrophages stimulated with IFN- γ . Similarly, IL-17 induced IL-6 production by peritoneal macrophages is also suppressed. However, once again, LPS induced production of TNF is unaffected by signalling through CD200R. The authors postulate that the inability of CD200R agonist to suppress LPS induced cytokine production may signify that TLR ligand induced signalling pathways are not susceptible to this form of inhibition. However, it is becoming increasingly apparent that much of the influenza induced cytokine production may be mediated via TLR signalling, which would contradict this hypothesis. However, it is possible that influenza induced cytokine production is also largely evoked simply by the infection and replication of the pathogen within the macrophage. Signalling through CD200R also suppresses IFN- γ induced IL-8, MIG and IP-10 at the RNA and protein level by the human monocytic cell line U937 transfected with hCD200R. These chemokines are strongly induced during influenza infection and are important in the recruitment (often excessive) of neutrophils and Th1 cells to the inflammatory site, thus further stressing the therapeutic potential of CD200R agonist administration during an influenza infection.

IgE mediated degranulation and production of IL-13 and TNF can also be suppressed in mouse and human mast cells through addition of a CD200R agonist . Furthermore, through this *in vitro* mast cell based system the signalling behind CD200R mediated inhibition had begun to be unravelled . Within the tail of the CD200R are three tyrosines that are phosphorylated upon CD200R ligation. These tyrosine residues are not in conventional inhibitory ITIM motifs but one tyrosine, within an NPXY signature, seems to be of particular prevalence. Upon phosphorylation of this tyrosine residue, adaptor proteins Dok1 and Dok2 are recruited and phosphorylated. This ultimately leads to the inhibition of the RAS/MAPK pathway. It would seem probable that signalling through CD200R in macrophages is comparable since we demonstrate enhanced Dok-2 phosphorylation in BM derived macrophages following CD200R ligation.

It is surprising that the influence of CD200R ligation on macrophages is not also true of dendritic cells since it is known that they express this inhibitory receptor and that ligation induces the immunosuppressive pathway of tryptophan catabolism in plasmacytoid dendritic cells . Other studies also confirm the inability of CD200R ligation to modulate dendritic cell cytokine production or surface expression of activation markers . It is plausible that 1) other aspects of DC phenotype or function that we have not investigated are altered, 2) we have not identified the relevant *in vitro* conditions needed to exert suppression, and / or 3) additional factors are contributing in an *in vivo* context such as contact with other cells or extracellular matrix proteins. Nevertheless, the role of CD200R signalling in dendritic cells is something that needs to be further addressed.

5.3.2 A role for CD200 in governing myeloid cell responsiveness and homeostasis in the lung?

We are the first to report a role of CD200 and its receptor within the lung environment. The lung is a mucosal site where a tolerogenic response to innocuous antigens is fundamental to maintaining an environment that is optimal for supporting gas exchange. CD200 and its receptor may maintain tolerance and impose subtlety in immune responses at this site. Furthermore, the role of CD200/R during infection has

not previously been addressed and it is plausible that CD200 may fulfil a role in shaping the host's immune response to pathogen challenge and perhaps in the resolution of inflammation.

5 The strong CD200 expression on endothelial cells within the lung, and at other sites ,
is interesting and may fulfil a role in restraining CD200R expressing neutrophils in
the circulation ; preventing lethality due to their heightened activation as seen in the
systemic inflammatory response syndrome (SIRS). To support this regulatory role for
CD200 in the circulation, we see that CD200R expression is lost from neutrophils
10 once actually in the lung, which may subsequently facilitate their full activation at this
potential effector site. Subsequently, during the course of an influenza infection
CD200R is restored on neutrophils, conferring a negative feed back loop. As yet,
nothing is known regarding what dictates the expression of CD200R on the neutrophil
surface and the effect CD200R signalling has upon neutrophil function. Circulating
15 monocytes also express CD200R and it is plausible that their function may also be
modified by interaction with CD200 bearing endothelium. Monocytes are wired
differently to neutrophils in that they do not possess their full effector potential until
they migrate into a tissue and mature into macrophages. However, CD200 on
endothelium may fulfil a role in restraining maturation of monocytes whilst in the
20 circulation and this is again a concept that requires further investigation.

We also present evidence for CD200 expression on lung epithelial cells. Whilst the
specific cells expressing CD200 requires clarification, this observation could prove
significant in our understanding of how the lung maintains a homeostatic state and
25 does not respond inappropriately to the myriad of innocuous stimuli it encounters
everyday. CD200 expression upon epithelial cells is poorly understood, although one
study describes its expression on epithelial cells of the murine hair follicle ; suggesting
that it may play a role in maintaining immune tolerance to hair follicle associated
autoantigens. It is possible that CD200 on epithelial cells in the lung plays a role in
30 tolerance by suppressing resident macrophages until a pathogenic challenge overrides
such suppression. Indeed, alveolar macrophages express a very high level of CD200R
in a naïve lung in accordance with their important role in lung tolerance. It is also

interesting that the level of CD200R expressed by myeloid cells is greatly augmented by influenza infection, which may suppress their inflammatory phenotype and facilitate resolution of inflammation. Indeed, the potency of CD200R mediated suppression correlates with the density of receptor expression .

5.3.3 *CD200 expression on T cells*

We demonstrate CD200 expression on certain populations of T cells within the naïve mouse. In both the spleen and lung approximately 35-40% of CD4⁺ T cells expressed CD200 with 5-10% of CD8⁺ T cells possessing this inhibitory molecule. This level of expression appeared to be defined at the generation of single positive T cells within the thymus where similar percentages of CD4 and CD8 T cells were seen to express CD200 as in the periphery, whereas double positive T cells did not express CD200. It is intriguing that only a percentage of CD4 and CD8 T cells expressed CD200 in a naïve mouse but what defines these CD200 positive T cells remains elusive. The majority of CD200 positive T cells appear to express an $\alpha\beta$ TCR, but interestingly all T cells with a $\gamma\delta$ TCR are CD200 positive in the lung and spleen. Expression of CD200 on $\gamma\delta$ T cells would substantiate the accumulating evidence of a role in immune regulation and in the protection of host tissues against the damaging side effects of immune responses . $\gamma\delta$ T cells preferentially accumulate at mucosal sites where there is a critical requirement to limit excessive immunity that may cause bystander tissue damage. It would appear that $\gamma\delta$ T cells are important in reducing pulmonary tissue damage associated with inflammation. One study demonstrates an increase in pulmonary injury in the absence of $\gamma\delta$ T cells in two distinct models of epithelial cell damage . Furthermore, $\gamma\delta$ T cells are required for normal airway responsiveness to metacholine following OVA stimulation . Within the spleen, $\gamma\delta$ T cells localise within the red pulp , a site where old and damaged RBCs are cleared by macrophages. CD200 on $\gamma\delta$ T cells at this site may act to suppress macrophages from evoking an undesirable inflammatory response. Indeed, CD200 knock out mice possess an increased number of macrophages in the splenic red pulp, with those present being in an elevated activational state.

5.3.4 Induction of CD200 expression on activated T cells

We show conclusively that CD200 is induced on activated T cells, similar to human peripheral blood CD3⁺ T cells incubated with Concanavalin A . Induction of CD200 expression occurs on both CD4⁺ and CD8⁺ T cells and is activation dependent. Using the DO11.10 transgenic system we verify that CD200 expression on activated T cells is antigenic dose and time dependent and have further verified the TCR dependency of this expression with agonistic anti-CD3/anti-CD28 antibody induced activation, PMA/Ionomycin stimulation and using SKG mice. CD200 is up-regulated after the early co-stimulatory molecule ICOS , but before the late co-stimulatory molecule OX40 , a molecule important in the maintenance of activated T cells.

It is interesting that Th1 cells express higher CD200 levels than Th2 cells. To support this Th1 bias, influenza elicits a very strong Th1 driven immune response and is associated with high CD200 expression on T cells in the lung. To understand why Th1 cells may express more CD200 than Th2 cells one should consider the scenarios in which each T cell subset is evoked. A bacterial or viral infection is classically associated with Th1 immunity . Such pathogens generally elicit a rapid and potent Th1 response that represents an immediate and severe threat to the host through immunopathology and as such it would make evolutionary sense for them to express high levels of CD200 to switch off the inflammatory myeloid cells. Conversely, Th2 cells would have evolutionarily been induced to target larger pathogens such as helminths, which generally evoke a chronic and sometimes largely asymptomatic infection . Such infections would not require the same level of immediate restriction on myeloid cell activation and cytokine production. Indeed, *C. neoformans* elicits a Th2 skewed immune response in C57BL/6 mice and in this scenario there is little up-regulation of CD200 on T cell populations, an idea that may be relevant to allergy and atopy. Though yet to be linked in this way, CD200 is capable of suppressing cytokine release and degranulation in mast cells and histamine release in basophils . As such administration of CD200 signalling in an allergic experimental setting would be anticipated to improve clinical outcome as seen with passive cutaneous anaphylaxis (PCA) . What causes Th1 cells to express CD200 at a greater level than Th2 cells is unclear, but may reflect differences in the strength or dose of the initial antigenic

signal or a potential role for cytokines in dictating the levels of CD200 expression. Indeed, we see that neutralisation of IL-4 and IL-10 permits a greater induction of CD200 expression on activated CD4⁺ T cells.

5 It is interesting that CD200 is dramatically lost in the airways of influenza infected mice at day 7 of infection. What causes CD200 to become down regulated is still debateable, but is also observed *in vitro* following long term T cell activation. The reduction in CD200 expression may reflect its natural kinetics whereby after initial induction, it is subsequently lost again to restore homeostasis. T cells in the airways
10 may be at their terminal end point and as such have been activated for a greater period of time. However, this is unlikely to be the only factor. Other contributing factors include: 1) a lack of organised lymphoid tissue in the airways, 2) the presence of high TLR ligand loads, 3) reduced co-stimulatory signals at this site, 4) sustained inflammatory environment and / or 5) heightened apoptosis at this site.

15 Virtually all B cells are seen to express CD200 in a naïve mouse, with in excess of 90% expressing CD200 in the blood, lung and spleen. A significantly lower percentage of B220 positive cells in the bone marrow express CD200 implying that its expression is induced at some early developmental stage from a B cell progenitor.
20 Throughout the course of an influenza infection, CD200 levels on B cells remain largely unaltered within the lung and mediastinal node but do again show a striking drop in expression on the limited number of B cells in the BAL. We have thus far been unable to determine what modulates the levels of CD200 on B cells.

5.3.5 T cell mediated suppression of the myeloid inflammatory response

25 The up-regulation of CD200 expression on activated T cells may provide a mechanism whereby they can actively “switch off” the pro-inflammatory phenotype of the myeloid compartment. Indeed, in the course of influenza infection, CD200 expression on T cells and CD200R expression on myeloid cells are increased significantly. It is feasible that through CD200, the adaptive immune response can
30 suppress the innate response, promoting resolution of inflammation. It is plausible that such an interaction occurs at the immunological synapse during T cell activation .

The optimal spacing between the APC and the T cell to support efficient T cell activation is 4 IgSF domains . The combined extracellular domains of CD200 and its receptor is 4 IgSF and as such would spatially be compatible with a model whereby the T cell can direct a signal to the myeloid compartment through CD200R at the immunological synapse .

Figure 5.20 depicts the events we believe may occur during an inflammatory response whereby an activated T cell can suppress the myeloid compartment. IFN- γ and TLR ligands evoke pro-inflammatory cytokine production by macrophages, critical to the establishment of an inflammatory response. We see that both IFN- γ and TLR ligands up-regulate CD200R expression on the myeloid cell. In the course of the inflammatory response, T cells become activated and will subsequently sequentially up-regulate ICOS, followed by CD200, then OX40. The activated T cell can then interact with the myeloid cell through CD200/R and reduce pro-inflammatory cytokine production by the myeloid compartment. The temporal expression of CD200 post ICOS, a co-stimulatory signal important in the early response of activated T cells, enables a sizeable adaptive response to be mounted before inflammation is switched off. Furthermore, CD200R signalling does not reduce expression of myeloid activation markers involved in antigen presentation, and with some stimuli augmented CD86 and OX40L are observed. Therefore, CD200R signalling does not only limit the inflammatory phenotype of the myeloid cell but also supports its role in antigen presentation. To support this concept, OX40 ligation on the T cell acts to further increase CD200 expression. Furthermore, the reduction in TNF and IL-6 by CD200 signalling may also free regulatory T cells from the suppression that is exerted upon them by these mediators, which may further facilitate resolution of inflammation .

It is appealing to envisage such a scenario during influenza infection, whereby the sizeable T cell infiltrate expressing CD200 can dampen down inflammation by targeting CD200R on myeloid cells. However, in an *in vivo* scenario, it is important to consider the level of structural organisation that is required to support a meaningful interaction between a CD200 expressing T cell and a CD200R expressing myeloid cell, and whether such an interaction can occur in the node, the lung parenchyma or

the airways. Indeed, the extent of structural organisation in distinct immune compartments could contribute to the level of control mediated by CD200 and hence the potential to evoke an inflammatory response. There is an increase in structural organisation going from the airways to the lung and to the node, and a concomitant reciprocal reduction in the inflammatory environment elicited at these sites. It is appealing to consider whether the high density of CD200 bearing lymphocytes within a node can actively repress myeloid cells at this site through CD200R, dampening inflammation and supporting antigen presentation.

5.3.6 Conclusion

Our basic understanding of CD200 is still in its infancy and we appear to be generating as many questions as answers. Nonetheless, we have described for the first time the expression profiles of CD200 and its receptor in the naïve lung. Furthermore, we have demonstrated how these expression profiles are modified in the course of an influenza infection, and dissected the parameters that can cause this modulation. The role for CD200 in delivering an inhibitory signal to myeloid cells, specifically through suppression of pro-inflammatory cytokine production is now irrevocable. It will be of great interest to ascertain the role of CD200 in maintaining myeloid homeostasis in the lung, and its function in dictating the phenotype of the host's elicited immune response to infection.

Chapter 6 – The Role of CD200 in Respiratory Infections

6.1 Introduction

In the previous chapter, we describe the expression profiles of CD200 and its receptor in both the naïve and infected lung, and define the parameters that dictated changes in expression. Furthermore, we demonstrate the potential of signalling through CD200R to reduce the production of inflammatory cytokines by macrophages. We subsequently postulate a potential role for the CD200-CD200R interaction in governing the phenotype of the myeloid response to respiratory infection. Myeloid cells constitute a significant arm of the host's armoury against respiratory infections, governing the induction, maintenance and ultimately resolution of inflammation, in

addition to acting as important effector cells against the invading pathogen. In this chapter, we investigate the impact of manipulating the CD200-CD200R axis during the respiratory infections induced by influenza and *C. neoformans*.

6.1.1 Myeloid cells in protection and pathology of pulmonary influenza infection

5 Pulmonary macrophages represent a significant primary site of infection by influenza virus. Infection of monocytes or macrophages with influenza virus induces an array of chemotactic, pro-inflammatory and anti-viral cytokines including IFN- α/β , which possesses potent anti-viral activity and is critical in limiting early viral replication . Influenza infected monocytes/macrophages produce large quantities of chemotactic
10 cytokines including MIP-1 α , MIP-1 β , RANTES, MCP-1, MCP-3, MIP-3 α and IP-10 , which favour the recruitment of blood mononuclear cells, as well as pro-inflammatory cytokines including IL-1 β , IL-6, TNF and IL-18 .

IL-1 β and TNF further enhance inflammation through augmenting production of
15 MCP-1 and MCP-3 and up-regulating the expression of various cellular adhesion molecules that facilitate further extravasation of inflammatory cells into the infected site . They also promote maturation of tissue macrophages and dendritic cells which gives rise to further inflammation and enhanced antigen presentation . Furthermore, IL-18 re-enforces these inflammatory sequelae by promoting the production of IL-1 β ,
20 TNF and various chemokines . Influenza infected DCs do not produce the same level of pro-inflammatory and chemotactic cytokines as macrophages but are an abundant source of IL-12, a critical cytokine in Th1 development . Much of the tissue damage and illness elicited by influenza infection is attributable to the over exuberance of the host's immune response, and as such myeloid cells are closely affiliated with the
25 pathology of the disease. Indeed, depletion of many myeloid cell derived products has proved beneficial in alleviating inflammation and symptoms of influenza induced illness .

6.1.2 Myeloid cells in protection and pathology of pulmonary *C. neoformans* infection

Resolution of *C. neoformans* infection and prevention of dissemination to other sites is dependent on development of a Th1 immune response (as seen in resistant BALB/c mice) . Conversely, the generation of a Th2 response (as seen in susceptible C57BL/6 mice) leads to chronic pulmonary eosinophilia, unchecked fungal growth and dissemination to other organs . Macrophages are essential to the development of a protective immune response to *C. neoformans*. Upon entering the respiratory tract the fungus is phagocytosed by resident alveolar macrophages eliciting the production of pro-inflammatory cytokines and chemokines (TNF, MCP-1 and MIP-1 α) . These cytokines are critical for the subsequent recruitment of T cells, macrophages, NK cells and granulocytes that will confer protection against *C. neoformans*. MIP-1 α knock out mice develop a Th2 immune phenotype with pulmonary eosinophilia, high serum IgE, increased IL-4 and IL-13 and significantly elevated fungal burden . TNF, in particular, is of great importance in evoking cell recruitment to *C. neoformans* and also possesses direct cryptococcal killing capacity . The protective response to *C. neoformans* is granulomatous in nature, with granuloma formation dependent upon accumulation of CD4⁺ T cells and macrophages encasing the fungi . Subsequently, macrophages fuse around the cryptococci to form multinucleated giant cells and releasing hydrolytic enzymes. Macrophages are therefore central to the control and resolution of *C. neoformans* infection and promotion of a greater macrophage response would be optimal.

6.1.3 CD200 is an inhibitory regulator of myeloid cell function with therapeutic potential

CD200 is now acknowledged to be a potent inhibitory regulator of myeloid cells. Naïve CD200 knock out mice exhibit heightened myeloid numbers that are present in an augmented activation state . Furthermore, these mice develop more rapid and severe pathologies in various autoimmune models such as CIA, EAE, EAU and a facial nerve transfection model, with disease progression characterised by greater myeloid infiltrate and activation . The nature of this inhibitory signal is still poorly defined but we have described the potential of CD200 to suppress macrophage pro-

inflammatory cytokine production in response to IFN- γ and influenza but not LPS. These findings are corroborated by another group that has described a similar reduction in pro-inflammatory cytokines by peritoneal macrophages stimulated with IFN- γ or IL-17 .

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The inhibitory potential of CD200 has been manipulated immunotherapeutically to alleviate inflammation and illness in conditions characterised by excessive immunity. Administration of a CD200 fusion protein (CD200:Fc) or an agonistic antibody to CD200R reduces disease severity of CIA, halts disease progression and also reduces established disease, with concomitant reduction in levels of serum IFN- γ and TNF and anti-collagen antibodies . In a murine model of spontaneous abortion, foetal loss is driven by excessive Th1 cytokine production, and administration of CD200:Fc dramatically reduces abortion rates implying a role for CD200 in tolerance of the foetus . Gorczynski and colleagues demonstrate that administration of soluble CD200 protein prolongs the survival of allotransplants of skin and kidney between mice and kidney xenotransplants from rats to mice . A clear therapeutic potential for CD200 signalling in allergy has also been demonstrated, with administration of an anti-mouse CD200R antibody inhibiting Fc ϵ R1-dependent responses in a murine model of passive cutaneous anaphylaxis (PCA) . It would therefore appear that CD200 offers therapeutic potential in many conditions characterised by dysregulated immunity through suppression of the myeloid compartment. The potential of manipulating the CD200 signal in infectious disease has not yet been addressed. Illness and pathology elicited by many respiratory infections, such as influenza, is attributable to the excessiveness of the host's immune response and as such would be a plausible target for CD200 driven therapeutic intervention. Conversely, with infections where pathogen persistence is the predominant concern it may be plausible to block CD200 signalling so as to evoke a more potent immune response.

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6.1.4 Hypothesis

We anticipate that CD200 plays an important role in dictating the potency of the immune response to infection and the resolution of inflammation. We postulate that CD200 knock out mice will exhibit heightened inflammation and more severe illness

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in response to influenza, whilst administration of CD200:Fc to influenza infected mice will alleviate symptoms. Conversely, we hypothesise that *C. neoformans* infected CD200 knock out mice or mice administered a blocking antibody to CD200 will show heightened immunity with improved clearance of the pathogen.

6.2 Results

6.2.1 Naïve OX-2 (CD200) knock out mice exhibit heightened myeloid numbers in their spleens

OX-2 (CD200) knock out mice, developed by Sedgewick *et al* at DNAX (Fig. 6.1a), provide an excellent tool to determine the role of CD200 during respiratory infection.

We first investigated the phenotype of the naïve lung in CD200 knock outs relative to wild type mice. Previous studies have highlighted enhanced myeloid numbers in the spleens and mesenteric lymph nodes of these mice but the lung compartment has not previously been investigated. Confirmation of the CD200 knock out phenotype was obtained by flow cytometric analysis with a complete absence of CD200 expression on splenic CD4⁺ and CD8⁺ T cells in knock out mice (Fig. 6.1b).

In accordance with previous studies naïve OX-2 knock out mice showed a significant increase in the number of CD11b⁺ myeloid cells in their spleens (Table 6.1). Further dissection of the myeloid population revealed that this increment was largely attributable to increased numbers of macrophages and neutrophils (data not shown). Conversely, there were comparable numbers of myeloid cells in the lungs and airways of the OX-2 knock out mice relative to the wild type controls (Table 6.1), although there was a small but significant increase in MHC II expression on macrophages within the lungs (data not shown). There were no differences in the number of lymphoid cells in the spleens or lungs of wild type and knock out mice with comparable numbers of CD4⁺ and CD8⁺ T cells (Table 6.1). Similarly, no differences were apparent between the two groups of mice in the activation status of the resident T cell populations (data not shown). It has previously been reported that OX-2 knock out mice exhibit heightened numbers of myeloid cells in their mesenteric nodes. The mediastinal nodes that drain the lymphatic from the lungs are, unlike the messenterics,

inducible upon infection, and neither wild type nor OX-2 knock out mice possessed these lymph nodes. H and E staining of naïve OX-2 knock out mice lung tissue showed comparable morphology to that observed in wild type mice, with large open air spaces and limited cellular infiltrate (Fig. 6.2).

5 ***6.2.2 Influenza infected OX-2 knock out mice show greater weight loss early in infection and fail to recover as quickly as wild type controls***

Age matched C57BL/6 wild type and OX-2 knock out mice were infected intranasally with 50 HA influenza (Fig. 6.3) and weight loss recorded daily and expressed as a percentage of original weight. OX-2 knock out mice exhibited greater weight loss
10 relative to wild type controls, particularly in the first three days of influenza infection (Fig. 6.4a), but significant until day 7. However, the OX-2 knock out mice were visibly sicker at day 7, appearing very hunched and non-motile and had to be culled in accordance with home office guide lines. In order to investigate the three phases of influenza infection (induction, peak illness and resolution) in OX-2 knock out mice, it
15 was therefore necessary to use a lower influenza inoculum of 25 HA (Fig. 6.3). At this dose of influenza, OX-2 knock out mice continued to exhibit greater weight loss in the early stages of infection with a significant difference at days 1 and 2 after influenza infection (Fig. 6.4b). However, weight loss was comparable in wild type and knock out mice from day 3 through to 7. Wild type mice subsequently recovered
20 significantly quicker than the OX-2 knock outs. From day 7 onwards wild type mice showed a steady increase in weight, whereas OX-2 knock outs continued to lose weight until day 9 before gaining weight at a slower rate (Fig. 6.4b).

6.2.3 Influenza infected OX-2 knock out mice exhibit heightened cellularity in their mediastinal lymph nodes at day 2 after influenza infection

25 Influenza infected wild type and OX-2 knock out mice were culled at day 2 after infection to investigate any deviation in immune phenotype at this early stage of infection. Lung, BAL and the draining mediastinal lymph nodes (MLN) were sampled and whilst cell numbers in the lung and BAL were comparable, the OX-2 knock out mice possessed a significantly greater number of cells in the MLNs (Fig. 6.5a). H and
30 E staining of lung tissue at day 2 after infection corroborated the comparable infiltrate

in the lungs of wild type and knock out mice at this time point. In both wild type and knock out lung tissue there still appeared to be minimal cellularity at day 2 after infection, but some limited peribronchiolar and perivascular infiltrate was observable (Fig. 6.5b). CD200 delivers an inhibitory signal to the myeloid immune compartment, and therefore the number and phenotype of different myeloid cells was assessed in each immune compartment. The OX-2 knock out mice possessed a significantly greater number of macrophages and dendritic cells in their MLNs relative to wild type controls (Fig. 6.5c and d), but myeloid cell numbers in the lungs were comparable. However, an increase in the number of alveolar macrophages in the airways was observed in OX-2 knock out mice relative to wild type controls (Fig. 6.5e). The phenotype of the myeloid cells at each site was further investigated by flow cytometry for a variety of activation markers. There was a general increase in the levels of MHC II expression on different myeloid compartments with elevated levels observed on alveolar macrophages in the BAL (Fig. 6.5f and g) and on macrophages and dendritic cells in the lung and MLN (data not shown). No differences were observed in the expression levels of CD80, CD86, CD40 and OX40L (data not shown).

The T cell populations at each site were also analysed by flow cytometric analysis. Total numbers of CD4⁺ and CD8⁺ T cells were comparable in the lung and BAL of wild type and knock out mice at day 2 after influenza infection, but a significantly greater number of both subsets were observed in the MLNs of OX-2 knock out mice (Fig. 6.6a and b), which were more activated (CD45RB^{lo}) (Fig. 6.6c, e and f). Subsequently, there were a significantly greater number of CD45RB^{lo} CD4⁺ and CD8⁺ T cells in the nodes of the OX-2 knock out mice (Fig. 6.6d). This elevated T cell activation in the OX-2 knock out mice was also observed in those T cells present in the lung and airways (data not shown).

6.2.4 OX-2 knock out mice exhibit heightened cellularity in their airways at the peak of influenza infection

Inflammation and illness to pulmonary influenza infection classically peaks at day 6-7 after infection. We therefore examined the influence of CD200 at this phase of infection. The number of MLN cells continued to be higher in the OX-2 knock out

mice relative to the wild type controls at day 7 after influenza infection (Fig. 6.7a). The number of cells in the lungs and airways at day 7 was considerably higher in both groups of mice than that seen at day 2 after infection. Furthermore, the number of cells in the BAL at day 7 was significantly greater in the knock outs than in the wild type controls (Fig. 6.7a). H and E staining of lung sections showed extensive perivascular and peribronchiolar infiltrate and significant occlusion of airways in both groups of mice (Fig. 6.7b). However, the infiltrate did generally appear be more extensive and dense in the wild type mice. Consistent with the inhibitory role of CD200 on myeloid cells, there were an increased number of macrophages and DCs in the MLNs and airways of the OX-2 knock out mice relative to the wild type controls (Fig. 6.7c and d). Whilst there was a general increase in the number of myeloid subsets in the lung tissue, this difference was not significant (data not shown). Similarly, there was a general increase in the levels of MHC II on the surface of these myeloid populations in the knock out mice, but this increment was not as pronounced as seen at day 2 after infection and failed to be significant (data not shown).

The number of CD4⁺ (Fig. 6.8a) and CD8⁺ (Fig. 6.8b) T cells was also significantly greater in the MLNs and airways of the OX-2 knock outs relative to the wild type controls at day 7, but comparable in the lungs. As seen at day 2 after infection, the percentage of CD8⁺ T cells that were CD45RB^{lo} was significantly greater in OX-2 knock outs in the airways (Fig. 6.8c), lung and MLN (data not shown). The percentage of CD4⁺ T cells that were CD45RB^{lo} was, however, comparable between the two groups of mice, although an increase in the total number of both CD4⁺ and CD8⁺ T cells that were CD45RB^{lo} was seen in the BAL (Fig. 6.8d) and MLNs (data not shown). Furthermore, there was an increase in the total numbers of both T cell subsets expressing intracellular IFN- γ and TNF in the airways of the OX-2 knock outs relative to the wild type controls (Fig. 6.8e). Since TNF and IL-6 are thought to suppress regulatory T cells we ascertained whether levels of these regulatory cells were altered in the OX-2 knock out mice. FoxP3 expressing cells were comparable in the lung (Fig. 6.8f and g) and MLN (data not shown) of both groups of mice.

6.2.5 OX-2 knock out mice show a reduced capacity to resolve inflammation relative to wild type controls

We next determined whether resolution of inflammation was compromised in OX-2 knock out mice. Therefore, wild type and OX-2 knock out mice were culled 14 days after infection and resident immune cells phenotyped in the lung, BAL and MLN. The total number of cells in the lungs and airways, whilst reduced from day 7, was significantly greater in the OX-2 knock outs relative to the wild type controls (Fig. 6.9a). The number of cells in the MLNs of both groups of mice was comparable at day 14 after infection (data not shown). The enhanced cellularity of the lung was observed in H and E stained lung sections (Fig. 6.9b). Enhanced pulmonary cellularity in OX-2 knock outs was partially attributable to increased macrophages (Fig. 6.9c) and dendritic cells (Fig. 6.9d) at these sites as seen at previous time points. Furthermore, the level of MHC II expression was once again elevated on macrophages (Fig. 6.9e) and dendritic cells (Fig. 6.9f and g) in the lung and airways.

Greater numbers of lung and airway CD4⁺ (Fig. 6.10a) and CD8⁺ (Fig. 6.10b) T cells were also detected in the OX-2 knock out mice at day 14 after infection, which expressed more intracellular IFN- γ and TNF (Fig. 6.10c and d). The total number of activated (CD45RB^{lo}) CD4⁺ and CD8⁺ T cells were also elevated in the airways (Fig. 6.10e) and lung (data not shown) of OX-2 knock out mice relative to wild type controls. The phenotype and function of T cells can be inferred from differential expression of CD44 and CD62L. CD4⁺ and CD8⁺ T cells from the lung (Fig. 6.11a-d) and airways (data not shown) of OX-2 knock out mice exhibited a bias towards CD44⁺CD62L⁻ (R2; Fig. 6.11a-d) expression compared to wild type mice, with a concomitant reduction in T cell populations that were CD44⁻CD62L⁺ (R1; Fig. 6.11a-d) in the knock outs. A reduced percentage of T cells derived from the MLNs of OX-2 knock out mice were CD44⁻CD62L⁺, as seen in the lung, but at this site a greater percentage of knock out T cells expressed both CD44 and CD62L (data not shown).

6.2.6 OX-2 knock out mice produce elevated levels of inflammatory cytokines in response to influenza relative to wild type controls

We have previously demonstrated that a potent effect of CD200R signalling on macrophages is suppression of pro-inflammatory cytokine production. It was anticipated that the absence of the inhibitory signal imparted by CD200 in OX-2 knock out mice would result in greater myeloid derived cytokine production in response to influenza infection and may explain the heightened inflammation seen in these mice. The levels of an array of inflammatory cytokines and chemokines in the BAL were therefore determined by Luminex (Fig. 6.12). We detected a small increase in the levels of IL-6 (Fig. 6.12a), TNF (Fig. 6.12b), MIP-1 α (Fig. 6.12c) and IFN- γ (Fig. 6.12d) in the OX-2 knock out mice, probably reflecting the heightened myeloid and subsequent T cell response. However, it must be recognized that in an *in vivo* scenario there will be multiple cell types both producing and ligating cytokines, and thus results may not fully represent the potency of CD200 in suppressing pro-inflammatory cytokine production. It would be of interest to ascertain production of pro-inflammatory cytokines within the lung at the level of mRNA, and also determine the systemic levels of these cytokines.

6.2.7 OX-2 knock out mice show improved influenza viral clearance

We next determined viral titres in wild type and OX-2 knock out mice at days 2, 7 and 14 after influenza infection by plaque assay. OX-2 knock out mice had a significantly reduced viral burden in their lungs at day 2 after influenza infection relative to wild type controls. By day 7 after influenza infection viral titres were very low and comparable in both groups of mice and by day 14 no virus was detectable by plaque assay (Fig. 6.13a). It was of interest to ascertain whether the absence of CD200 induced signalling in OX-2 knock out mice would alter their ability to develop an influenza specific antibody response. The levels of influenza specific IgG in the serum (Fig. 6.13b) and IgA in the BAL (Fig. 6.13c) were comparable in wild type and OX-2 knock out mice.

6.2.8 Administration of CD200:Fc alleviates influenza driven weight loss and inflammation

We next performed the opposite experiment by administering CD200:Fc to wild type mice, which ligates CD200R and transmits a negative signal to APCs. Mice were infected intranasally with 50 HA influenza and 10 μ g IgG or CD200:Fc administered i.p. on days 0, 2, 4 and 6 (Fig. 6.14). Those mice administered CD200:Fc exhibited reduced weight loss (Fig. 6.15a) and a significant reduction in the number of cells in their lungs and airways (Fig. 6.15b). H and E staining of lung sections from control and CD200:Fc treated mice also clearly showed a striking reduction in lung cellular infiltrate with reduced perivascular and peribronchiolar infiltrate (Fig. 6.15c).

A general reduction in the total number of CD11b⁺ cells in the airways (Fig. 6.16a) of the CD200:Fc treated mice failed to be significant, as did the total number of CD11b⁺ cells expressing MHC II (Fig. 6.16b) and CD86 (Fig. 6.16c). However, there was a reduction in the levels of the pro-inflammatory cytokines IFN- γ (Fig. 6.16d) and IL-6 (Fig. 6.16e) in the BAL fluid of CD200:Fc treated mice, possibly reflecting the potential of CD200 to deliver an inhibitory signal to the myeloid compartment.

The most striking reduction in pulmonary infiltrate in the CD200:Fc treated mice was observed in the T cell compartment with a reduction in CD4⁺ and CD8⁺ T cell numbers in the lungs (Fig. 6.17a) and airways (Fig. 6.17b), their activation (Fig. 6.17c and d) and their expression of IFN- γ (Fig. 6.17e) and TNF (Fig. 6.17f). Importantly, influenza virus was cleared in both control and CD200:Fc treated mice with infective virus undetectable in the lungs of both groups by day 7. Furthermore, levels of influenza specific IgG in the serum and IgA in the BAL was equivalent in the wild type and knock out mice (data not shown).

6.2.9 Blockade of CD200 signalling boosts immunity to *Cryptococcus neoformans* and prevents dissemination to the brain

Since *Cryptococcus neoformans* is a persistent lung fungal pathogen and we have previously shown that a heightened myeloid response in Cybb tm1 mice improves pathogen clearance, we next administered a blocking antibody to CD200 (OX90) on

days 0, 4 and 8 after infection (which should enhance myeloid activity) and mice were culled on day 12 to assess the immune phenotype and pathogen clearance (Fig. 6.18). The mice administered OX90 had comparable cell numbers in their lungs (Figure 6.19a) relative to control treated mice, but elevated numbers of cells in their airways (Fig. 6.19b) and spleen (Fig. 6.19c). Whilst the heightened immune infiltrate observed with administration of OX90 did not reduce pathogen burden in the lungs (Fig. 6.19d) and airways (Fig. 6.19e), dissemination to the brain was completely blocked (Fig. 6.19f).

As may be anticipated the blockade of CD200 signalling with OX90 caused an increase in myeloid cell numbers in the lungs, airways and spleen. Despite comparable cellular infiltrate in the lung, there was a general increase in the number of macrophages at this site (Fig. 6.20a) and a significant increase in the number of dendritic cells (Fig. 6.20b) and eosinophils (Fig. 6.20c and d). This increase in myeloid cells was more striking in the airways and spleen reflecting the general heightened cellularity at these sites (data not shown). To ascertain how blocking CD200 increases the number of lung eosinophils we investigated CD200R expression on eosinophils by flow cytometric analysis. No CD200R expression was apparent on the surface of eosinophils implying that the increase in the numbers of this cell type is not a direct consequence of the removal of the inhibitory signal imparted by CD200 (Fig. 6.20e).

The T cell response to *C. neoformans* is critical for pathogen control with the development of a Th1 skewed response leading to eradication of this yeast and prevention of dissemination to extra-pulmonary sites. Those mice administered OX90 possessed comparable numbers of both CD4⁺ and CD8⁺ T cells in their lungs (Fig. 6.21a) but a significant increase in the numbers of both subsets in the airways (Fig. 6.21b). Furthermore, a greater percentage of the CD4⁺ T cells in the airways of the OX90 treated mice were more activated (Fig. 6.21c). T cells present in the airways of those mice treated with OX90 did not exhibit any shift in Th1/Th2 bias with a greater percentage producing both IFN- γ and IL-5 (Fig. 6.21d-g).

6.3 Discussion

6.3.1 Heightened myeloid response in naïve OX-2 knock out mice

Studies with naïve OX-2 knock out mice show an elevated number of myeloid cells in spleens relative to wild type controls , and may imply a role for CD200 controlling myeloid cell homeostasis at the resting state. We, and others have reported the expression of CD200 on endothelia, B cells and some T cells within the spleen and it is plausible that some or all of these cell types interact with myeloid cells and restrain their activation through CD200R. Interestingly, unlike the spleen, the number of myeloid cells in the lungs and airways of OX-2 knock out mice were comparable to wild type. These differences may reflect the abundance of cell types bearing CD200 and its receptor or the fact that the spleen is highly compartmentalised whereas the naïve lung is not. Consequently, within the splenic compartment, CD200 may fulfil an important homeostatic role in controlling myeloid cell turn over. This lack of homeostatic control in effector, compared to primary lymphoid sites, may underlie the amplified inflammation versus organised immunity that occurs at these distinct sites.

6.3.2 More rapid and potent induction of immunity to influenza infection in OX-2 knock out mice

The investigation into the role of CD200 during influenza infection is extremely novel and topical. In the absence of CD200, influenza infected OX-2 knock out mice exhibit a more rapid weight loss early during infection and then fail to recover in line with wild type mice; implying a role for CD200 in controlling induction and resolution of lung inflammation. This reduced myeloid control leads to enhanced production of inflammatory cytokines in OX-2 knock out mice infected with influenza, which may ultimately cause the greater weight loss and illness observed in these animals . This idea is supported by our *in vitro* studies showing that signalling through CD200R suppresses production of pro-inflammatory cytokines by IFN- γ or influenza activated BM macrophages, whilst others have shown a reduction in IL-6 and TNF production in peritoneal macrophages in response to IFN- γ stimulation .

Furthermore, there are elevated numbers of activated myeloid cells in various lung compartments during influenza infection in OX-2 knock out mice at day 2 after infection. The increase in alveolar macrophages is interesting. It is possible they are normally restrained during respiratory challenge by CD200 expressed on epithelia or other CD200 expressing cells in the lung and in its absence there is expansion of this myeloid population. CD200 mediated control of alveolar macrophages may be more potent during infection relative to in a naïve animal owing to up-regulation of CD200/R expression. Likewise, myeloid cells within the nodes of wild type mice are possibly restrained by the high density of lymphocytes that bear CD200. The heightened myeloid numbers in OX-2 knock out mice at day 2 of influenza infection, may reflect the heightened inflammatory environment or another, as yet unknown, role for CD200 such as controlling myeloid cell apoptosis, persistence in the BM, differentiation/maturation or deflection from the circulation into tissues.

It would be of interest to further analyse the potential of epithelia to modulate and inhibit macrophage function via CD200 and in future we will seek to address this *in vitro* by incubating epithelia isolated from wild type and knock out mice with influenza infected alveolar macrophages. Furthermore, it would be of interest to ascertain how myeloid cells resident in mediastinal nodes differ in their potential to generate cytokines in the absence of CD200. Whilst it is likely that they generally do not produce pro-inflammatory cytokines *in vivo* owing to their limited exposure to TLR ligands and activating stimuli, it is possible that they are also actively restrained by the high density of surrounding CD200 expression and should be investigated further.

The increased activated CD4⁺ and CD8⁺ T cells in the MLNs of OX-2 knock outs at 2 days after influenza infection is likely to be a consequence of the heightened myeloid response and induction of inflammatory cytokines. Though we did not directly assess adhesion molecules, these are also likely to be raised by enhanced inflammatory cytokines. Alternatively, increased T cell numbers in OX-2 knock out mice may reflect enhanced presentation by the elevated numbers of CD200R bearing cells in the MLNs, which may be further matured by elevated cytokines. Indeed, TNF

contributes to DC activation , maturation and migration to and accumulation in draining lymph nodes .

The increased myeloid response and more rapid onset of weight loss observed in OX-2 knock out mice following influenza infection is comparable to the earlier and more severe onset of various autoimmune diseases previously reported in these mice. In a facial nerve transfection model, the microglial response of the OX-2 knock outs is accelerated with disease detectable at day 2 and peaking at day 4, relative to wild type mice where disease is first observed at day 4 and most severe at day 7 . Similarly, the onset of EAE in OX-2 knock out mice is seen 3 days earlier than in wild type controls and is characterised by increased macrophage numbers and activation . Experimental autoimmune uveoretinitis (EAU) also shows accelerated onset in OX-2 knock out mice although overall disease severity is not increased . Therefore, it would appear that CD200 exerts an inhibitory potential over myeloid cells and in its absence the inflammatory response is more rapid and potent.

Enhanced MLN cellularity persists to day 7 of influenza infection, at which time there is also an increase in the total number of cells in the airways, accounted for by increased numbers of macrophages, DCs and T cells. This is likely to be a consequence of enhanced immunity observed at day 2 in the MLN coupled with the inability to “switch off” the pro-inflammatory phenotype of pulmonary macrophages. It is interesting to note that comparable or even lower cellularity was observed in the lungs of OX-2 knock out mice than in the wild type controls. This is comparable to the scenario observed in Cybb tm1 mice previously whereby a greater myeloid response early in infection seems to draw cells directly into the airways rather than lung tissue. Furthermore, the primary site of influenza infection is the upper respiratory tract and as such a more rapid and potent immune response, as seen in OX-2 knock out mice, is likely to be targeted to this compartment to combat infection.

Interestingly, at day 14 after influenza infection there was a significant shift in lung resident T cells of the OX-2 knock out mice from a naïve (CD44⁺CD62L⁺) to a CD44⁺CD62L⁻ phenotype relative to wild type controls. CD44⁺CD62L⁻ expression

has been identified as the profile of effector memory T cells, which may indeed be expanded in the absence of CD200. Alternatively, these cells may simply be recently activated. MLNs in OX-2 knock out mice contained a greater number of T cells expressing both CD44 and CD62L, the definition of central memory, 14 days after influenza infection. The generation of a greater memory response in the absence of CD200 could potentially mean that CD200 blockade could fulfil an adjuvant-like capacity with vaccination, and requires further investigation.

6.3.3 Failure to resolve influenza induced pulmonary inflammation in OX-2 knock out mice

Intriguingly, OX-2 knock out mice fail to recover and regain weight as rapidly as wild type controls and possessed elevated numbers of cells in their lungs and airways (myeloid cells and activated T cells). This would imply that CD200 plays an important role in resolving inflammation, which would be an entirely novel concept. Previous studies with OX-2 knock out mice have investigated autoimmune conditions, which are chronic in nature and as such resolution is not investigated. This failure to resolve inflammation may reflect a lack of inhibition by CD200 at this stage and/or prolonged presence of signals maintaining inflammation such as co-stimulatory molecules or inflammatory cytokines. Alternatively, it may simply take more time to clear an expanded inflammatory infiltrate.

We have previously seen how CD200R is up-regulated on the surface of myeloid cells during the course of influenza infection, peaking at day 14, and subsequently enhancing their susceptibility to CD200 mediated inhibition and resolution of inflammation. It is plausible that CD200 expressing stromal cells or recruited lymphocytes are important in switching off myeloid cells that have up-regulated CD200R. However, in the absence of CD200, myeloid cells are not as effectively “switched off” and inflammation is slower to resolve. Finally, the absence of CD200 leads to elevated levels of pro-inflammatory cytokines during influenza infection, some of which have been demonstrated to inhibit the suppressive effects of regulatory T cells. Whilst we failed to see any difference in the numbers of FoxP3 T cells in

OX-2 knock out mice during influenza infection, it is plausible that failure to resolve inflammation may in part be attributable to aberrant Treg mediated suppression.

5 The heightened cellularity that persists in the lungs and airways of the OX-2 knock out mice at day 14 of infection is not a consequence of an underlying failure to clear influenza virus. Indeed, viral titres are reduced in OX-2 knock out mice at day 2 after infection, which is again likely to reflect heightened myeloid activity. Macrophages are critical to the establishment and maintenance of the host's protective response to influenza with activated macrophages capable of phagocytosing influenza virions and producing Type 1 IFNs that are critical to control of viral infection . The latter needs further investigation. Indeed, various other viruses such as those of the Herpes family express a CD200 homologue that signals through CD200R on the host myeloid cell and is anticipated to offer some form of survival advantage against the virucidal activity of macrophages .

15 ***6.3.4 Induction of CD200 signalling as an immunotherapeutic strategy during influenza infection***

Administration of a CD200 fusion protein (CD200:Fc) to influenza infected mice offers a plausible immunotherapeutic strategy since the development and maintenance of inflammation is directly linked to the activation of the myeloid cells. We have shown *in vitro* that signalling through CD200R suppresses the production of pro-inflammatory cytokines by BM macrophages stimulated by influenza or IFN- γ . Furthermore, we and others have previously demonstrated that neutralisation of pro-inflammatory cytokines during influenza infection alleviates illness and inflammation and as such it is no surprise that manipulation of CD200 signalling is beneficial. Mice administered CD200:Fc showed a significant reduction in weight loss and pulmonary infiltrate. It is plausible that the CD200:Fc binds myeloid CD200R that would not normally be exposed to natural CD200 ligand and in so doing represses the myeloid response to influenza infection. It is likely that the reduced pulmonary T cell infiltrate seen in mice treated with CD200:Fc reflects a reduction in pro-inflammatory cytokine production by myeloid cells early in infection. However, 1) phagocytosis of the influenza virion, 2) migration of the myeloid cell to the draining lymph node, 3) the

maturation of the myeloid cell into a professional APC capable of successfully activating the adaptive arm of the immune response and / or 4) reduced migration competent T cells in the node (Fig. 6.22), may also account for reduced T cells.

5 There have been various reports citing the potential of manipulating CD200 signalling as an immunotherapeutic strategy to alleviate excessive inflammation with autoimmunity, alloimmunity and allergy and we can now add inflammatory lung disease to this list. However, it is essential that administration of CD200:Fc therapeutically be investigated as the delayed administration of the fusion protein may
10 be less effective since the myeloid cells would have already produced large quantities of pro-inflammatory cytokines and the immune response already set in motion. However, it is more likely that CD200:Fc will continue to offer some relief in suppressing inflammation, although the earlier the administration the better. Furthermore, it is always a concern that reducing the potency of immune responses
15 through immunotherapeutic strategies will compromise immunological memory and the development of an antibody response upon any subsequent exposure to the pathogen. This is again something that would require investigation with CD200:Fc, although it is reassuring that influenza specific antibody responses are not compromised during a primary infection.

20 **6.3.5 Blockade of CD200 signalling to boost immunity to *C. neoformans***

Finally, if we can administer CD200:Fc to transmit the inhibitory signal, we can also block it to enhance immunity. This we present for *C. neoformans* infection and show a rise in immunity with augmented macrophages, DCs and T cell responses (their activation and cytokine production). It is interesting that the heightened T cell
25 cytokine production did not show any bias towards a Th1 or Th2 phenotype since previous studies show that CD200 signalling skews immunity from a Th1 towards a Th2 response . An increase in eosinophils was observed in *C. neoformans* infected mice administered OX90, and probably reflects the increase in IL-5 production by T cells since IL-5 is an eosinophil differentiation and activation factor .

Despite the general increase in immunity seen by blockade of CD200 signalling, there were comparable *C. neoformans* cfus in the lung and BAL of both groups of mice. As discussed previously, a Th1 driven response against *C. neoformans* is protective and leads to clearance of the pathogen, whereas a Th2 response is non-protective. Whilst there appears to be heightened Th1 cells, there is also an increase in Th2 cells, which are likely to cancel each other out. Furthermore, the elevated number of eosinophils in OX90 treated mice will cause significant lung tissue damage and facilitate replication of *C. neoformans*. Persisting tissue eosinophilia results in tissue destruction due to release of eosinophil-derived mediators at the site of inflammation including reactive oxygen metabolites, major basic protein, eosinophil cationic protein and crystal deposition. However, blockade of CD200 led to the prevention of *C. neoformans* dissemination to the brain, which depends on the development of a Th1 immune response. IL-12 is a critical cytokine in the induction of a Th1 response and controlling dissemination of *C. neoformans* to the brain. The heightened myeloid response observed with OX90 administration could affect levels of this cytokine and thus control dissemination with greater aptitude. TNF may also assist in the prevention of dissemination. TNF knock out mice are more susceptible to *C. neoformans* infection, and Huffnagle and colleagues have demonstrated a significant early role for TNF in leukocyte recruitment of leukocytes and the development of Th1 biased immune response.

6.3.6 Conclusions

In conclusion, we have shown several important concepts relating to lung immunity/pathology:

1. The CD200-CD200R interaction is important in the induction and resolution of inflammation to respiratory pathogens.
2. Innate immunity requires active signals, potentially delivered by the acquired immune response, for inflammation to resolve.
3. The inhibitory signal delivered via CD200R can be harnessed immunotherapeutically to dampen excessive inflammation.
4. Myeloid inhibitory receptors can be blocked to facilitate clearance of persistent pathogens.

Chapter 7 – General Discussion

Manipulation of the myeloid immune response during respiratory infection offers plausible therapeutic potential. The form of this manipulation will differ depending on the nature of the pathogen: stimulation of the myeloid immune response promotes general immunity and pathogen clearance, whilst suppression can alleviate immunopathology. We have utilised two distinct strategies to target the myeloid compartment: 1) By modulating the levels of toxic, inflammatory mediators (ROS) produced by the cell and 2) By ligating or blocking the inhibitory CD200R on myeloid cells. There are many concepts arising from this work that could merit further consideration, but in this final discussion I will address the following:

- Active suppression of the myeloid immune compartment in maintaining cellular homeostasis.
- The roles of ROS and CD200 in disparate compartments of the lung.
- What factors dictate when ROS or CD200 can exert their inhibitory potential in the context of an immune response?
- The therapeutic potential or limitations of manipulating CD200 and ROS in respiratory infectious diseases.

7.1 Active suppression of the myeloid compartment

Our data shows that both ROS and CD200 actively suppress the myeloid immune compartment and maintain homeostasis in the absence of infection. This suggests that specific signals are required to maintain myeloid cells in a resting state. This concept is supported by our data showing that an absence of CD200 or the phagocyte NADPH oxidase elevates myeloid cell numbers and their activation in a naïve animal. Furthermore, a greater myeloid response is elicited to infection and potentially sustained. Therefore, important mechanisms are in place to not only maintain homeostasis in a naïve animal, but also restrict excessive myeloid driven immunity and resolve inflammation to restore homeostasis. However, the nature of suppression exerted by ROS and CD200 are distinct:

- ROS are soluble factors that are produced by phagocytes and subsequently act in an “autocrine” manner. Hence the cells are effectively restraining themselves. ROS also, however, appear to act in a “paracrine” manner to restrain neighbouring cells.
- 5 - CD200-CD200R is a cell contact dependent interaction where the inhibitory signal is imparted to the myeloid compartment by disparate cell types. Thus this inhibition is promoted in a “paracrine” manner and is dependent on other non-myeloid cells.

7.1.1 ROS mediated suppression

10 ROS are classically known for their potent anti-microbial action. However, it is now recognized that the role of such species is far more complex than originally anticipated, owing to their potential to act in a signalling capacity, and some have argued that this may even be their most significant contribution. Whilst it has been reported that ROS can be pro-inflammatory through activation of redox sensitive

15 transcription factors , our findings suggest they exhibit an important anti-inflammatory role. ROS have been implicated in oxidatively inactivating chemotactic factors , inducing anti-inflammatory cytokine production by neutrophils and macrophages and in modulation of Th1 cytokine production . However, we argue that the cells that appear most actively suppressed by ROS are those actually

20 producing them, and that this repression is, at least in part, mediated through induction of apoptosis. ROS may represent ideal mediators for imposing a level of control over myeloid cell homeostasis due to their capacity to act as second messengers. Indeed, reactive oxygen species derived from a family of superoxide-generating enzymes, termed NADPH oxidases (NOXs), that are isoforms of the

25 classical phagocyte NADPH oxidase (NOX2,) are actively produced to function in redox signalling with their primary roles focused at regulating other cell types and processes .

30 In naïve Cybb tm1 mice we observe elevated numbers of macrophages at distinct lymphoid sites, implying that, in a resting state, ROS are produced at a basal level, even at non-mucosal sites, and fulfil a role in governing myeloid cell turnover. Such

self imposed control to limit cellular numbers is seen in hematopoietic cells, as with fratricide of memory T cells. In this scenario, immunological memory depends on a self-renewing pool of antigen-specific T memory (T_m) cells and it has been postulated that T_m-cell fratricide by Fas-mediated apoptosis results in a density-dependent death rate that controls the size of the T cell pool . Microarray analysis revealed that, even in the absence of infection, significant alterations in gene expression were present in the lungs of mice lacking a functional NADPH oxidase, reiterating the important role that ROS fulfil in the redox regulation of transcription. The concept that ROS act to negatively regulate the myeloid response is supported by evidence from individuals with CGD, who exhibit aberrant inflammation with development of granulomas . Furthermore, patients with CGD exhibit heightened susceptibility to autoimmune conditions such as polyarthritis and lupus , findings that are corroborated by studies in mice . It would be of interest to infer whether the genetic variation that occurs in promoter regions of genes encoding sub-units of the NADPH oxidase correlates with susceptibility to autoimmunity and inflammation in general.

Interestingly, on this background of heightened myeloid activity, the response to infectious disease is immediate. *C. neoformans* infection is contained in granulomas, preventing dissemination to the brain, and influenza is targeted in the airways by a greater macrophage and neutrophil response. This may suggest that removing signals that restrain the myeloid compartment in a resting state and maintain homeostasis may improve immunity to infection. However, the greater myeloid driven immune response to these pathogens is also likely due to depletion of ROS induced by the actual infection. During infection, excessive production or persistence of such toxic and unstable mediators as ROS is detrimental, and thus it is plausible that they switch off the cells that are producing them. Whilst this may simply be conveyed through induction of apoptosis, the suppressive mechanisms are likely to be more complex. Whilst ROS are utilised by the immune system to destroy certain pathogenic organisms, it is important that the immune system subsequently responds to high levels of ROS to prevent unnecessary bystander injury.

7.1.2 CD200 mediated suppression

As with ROS, the absence of CD200 results in elevated myeloid cell numbers in the spleens of naïve mice, and a greater myeloid driven response to respiratory infection. It is appealing that both CD200 and its receptor are up-regulated in response to respiratory infection thus making the myeloid compartment more amenable to suppression. Indeed, the potency of CD200 induced suppression is increased with higher CD200R expression . There are now a growing family of myeloid surface proteins that mediate interactions with other cells and modulate the myeloid cell phenotype, but our understanding of the biological basis of such signals are poorly defined. Some receptors such as SIRP- β transmit a positive signal to the myeloid cell, whereas SIRP- α and CD200 lead to myeloid cell inhibition . The activity of such cells will ultimately therefore depend upon the balance between the positive and negative signals received. We and others have reported the potential of signalling through CD200R to suppress production of pro-inflammatory cytokines by myeloid cells, but there are many other facets of the myeloid response that have not been investigated, and we do not yet know why an absence of the CD200 signal leads to elevated myeloid cell numbers.

Expression of CD200 on stromal cells is interesting and could fulfil an important role in restraining myeloid cell populations in the vicinity or during migration. The potential for CD200 on endothelia to suppress neutrophils within the circulation would be an exciting and novel concept. Similarly, the high expression of CD200 on epithelia within the lung may suppress pulmonary myeloid cells at a site where tolerance or non-responsiveness is frequently desirable (Fig. 7.1A). Furthermore, the high expression of CD200R on alveolar macrophages would facilitate suppression by epithelia and support their cited immunosuppressive potential .

A second appealing concept is that acquired T and B cells actively suppress myeloid cells. It is interesting that CD200 is up-regulated on T cells upon activation thus enhancing their suppressive potential. Therefore, during infection the adaptive immune response could effectively switch off and direct the innate response (Fig 7.1). Myeloid cells have been reported to actively suppress the T cell response, through

signals such as CTLA-4, but such cellular interactions in the reciprocal direction have not been reported. It is plausible that once a selective T cell response has been induced, the T cell switches off the inflammatory phenotype of the myeloid cell to prevent unchecked inflammation. Indeed, CD200 can suppress production of pro-inflammatory cytokines by macrophages, but increases CD86 and OX40L. Thus the T cell may cause a switch in the phenotype of the myeloid cell from one that is inflammatory to one that would support antigen presentation and maintenance of T cell populations. This is the first time such a concept has been proposed. However activated T cells will express a multitude of surface proteins that can signal to macrophages and dendritic cells. As an example, if we consider OX40, we have demonstrated that signalling through OX40L on a macrophage can augment the IFN- γ induced production of pro-inflammatory cytokines such as TNF. This is the exact opposite to the effect mediated by CD200, so what would be the effect of signalling through CD200R and OX40L?

7.2 The roles of ROS and CD200 in disparate compartments of the immune response

The host's immune response to a respiratory infection must be viewed in the context of distinct compartments. The airways, lung parenchyma and draining MLN are very distinct in structure and the nature of the immune response that occurs at these sites. Whereas the MLN is a highly structured and organised lymphoid site, the lung tissue is less so and the airways are completely devoid of any structural organisation. During an immune response the airways are the most inflammatory site with high levels of inflammatory cytokines, whereas classical "inflammation" does not occur in the MLNs, but rather they exhibit controlled expansion of lymphoid populations. To this end, there appears to be a reciprocal correlation between the level of structural organisation and the potency of the inflammatory response. The lung is a functional organ and is not suited to the large scale unregulated recruitment and expansion of cells, whereas LNs are specialised for such processes, exhibiting a greater level of control over resident cells. It is important that the roles of ROS and CD200 are considered within the context of immune compartmentalisation. The relationship

between lymphoid organisation, TLR load, CD200 expression and ROS is summarised in Figure 7.2.

7.2.1 ROS and immune compartmentalisation

The elevated myeloid numbers in non-mucosal tissue, such as the spleen, of *Cybb*^{tm1} mice would imply that ROS are functioning at these sites. It is likely, however, that this will be at a very low basal level and it is their cumulative absence that gives rise to the observed phenotype. During respiratory infection, however, ROS will be induced to a much higher level in response to pathogenic burden. In this context, the presence of ROS is likely to be restricted to the lungs and airways since these are the sites where phagocytes will be stimulated. Depletion of ROS appears to lead to a more prominent response in the airways rather than the lung tissue, which may give us a clue as to the function of these species in different pulmonary sites. Influenza is primarily an infection of the upper respiratory tract and as such, myeloid cells in the airways will encounter the virus foremost. Influenza can induce the production of ROS in these cells through the phagocyte NADPH oxidase, which, as we postulate, can subsequently lead to apoptosis of the myeloid cells or exert some other inhibitory potential. In the absence of ROS, however, these cells may persist for longer and be more activated, possibly leading to enhanced containment of the virus and a more rapid and potent immune response targeted to the airways. Thus it would appear that ROS are generated by cells in the airways and fulfil an important role in regulating the myeloid response at this site.

7.2.2 CD200 and immune compartmentalisation

The suppression mediated through CD200R to the myeloid cell is distinct to that induced by ROS since it is dependent on cell contact and is thus dictated by the presence of the ligand, and the ability to form a productive interaction. Little is known regarding the nature of this interaction and what level of structural organisation is required to support such an interaction. In CD200 knock out mice, elevated myeloid numbers are seen in the spleen and mesenteric lymph nodes but not in the lung. The manner in which CD200 dictates the number of myeloid cells is unknown, but it would appear that in a naïve animal, unlike ROS, the interaction fulfils a more

significant role within organised lymphoid tissue. At these sites is an abundance of cells expressing CD200 that would be proximal to CD200R bearing myeloid cells within a highly structured environment. Furthermore, during influenza infection, the most obvious immediate phenotypic difference in CD200 knock out mice is an elevated number of myeloid cells in the MLNs, implying that once again CD200 fulfils an important role within such organised lymphoid compartments.

However, CD200 may also exert a role within the lung in the course of influenza infection since the lung may develop into an environment that is more capable of supporting an inhibitory signal through CD200R (Fig. 7.1). We see an increase in CD200 and CD200R on a per cell basis as well as an increase in the total number of cells bearing the ligand and receptor within the lung and airways, and it is plausible that this may facilitate a productive interaction. It is also possible that an increase in expression of CD200 and CD200R in the lung compartment can compensate for the lack of structural organisation at this site.

The mice utilised in these experiments have not previously encountered any previous pathogenic infection, whereas with people, some form of infection history will always be present. We see CD200R expression peaking on alveolar macrophages at day 14 in our experiments (Fig. 7.1D). Whether the elevated level of CD200R on alveolar macrophages is maintained after a primary infection is not known. If maintained, it may explain the long lived changes we and others observe within the host's immune compartment following exposure to a pathogen, which alter a subsequent response to an autologous or heterologous pathogenic challenge. We believe that the first infection modulates the response to the second by alterations in the lung environment itself or in innate immune cells. Evidence for long term modification of the innate immune compartment is provided by studies where microbial products such as CpG DNA or a modified bacterial labile toxin (LTK63) afford protection against an array of subsequent respiratory pathogens. This phenomenon has been coined "innate imprinting" and can be strictly defined as "the long term modification of a microenvironment", which will consequently lead to a reduced, but more protective,

immune phenotype to a subsequent pathogen. Myeloid inhibitory molecules such as CD200 and its receptor may contribute to this “matured” or “educated” environment.

5 A final concept that merits discussion is the potential role for CD200 in ensuring that excessive inflammation does not occur at inappropriate sites. Infection of the lung with a pathogenic organism will frequently evoke the release of pro-inflammatory cytokines, which are largely produced by myeloid cells. Conversely, at organised lymphoid organs such as localised lymph nodes there is expansion of lymphocyte populations but the environment is far less inflammatory. Myeloid cells at these sites
10 do not produce pro-inflammatory cytokines, which is most likely attributable to the absence of a high pathogen load within secondary lymphoid structures and hence less PAMP triggering of TLRs etc. It is critical that at such lymphoid sites, myeloid cells function as APCs rather than in a pro-inflammatory capacity. CD200 is likely to be pivotal in restraining myeloid cells in these organised lymphoid sites. It would be of
15 interest to examine whether myeloid cells within the MLNs produce pro-inflammatory cytokines in the absence of CD200.

7.3 The therapeutic potential of manipulating ROS and CD200 during respiratory infection

20 Immunotherapeutic strategies are now considered as a potentially important facet of our armoury to combat infectious disease. Development of vaccines and anti-microbials is, and will continue to be, the primary objective against respiratory infections, but their limitations may lead to the utilisation of immunomodulatory strategies. Vaccination strategies are hindered by the antigenic variation of the pathogen whereas anti-microbial agents are sometimes limited by efficacy and
25 increasing incidences of drug resistance. Furthermore, in infections such as influenza clinical signs of disease are only really apparent when viral titres have subsided rendering anti-virals ineffective. The significant role of immunopathology in many infections offers clear targets for manipulation. We have targeted different facets of the myeloid response to respiratory infection with influenza and *C. neoformans*.
30 Whilst we have seen clear potential for manipulation of ROS and CD200, it is

important to question the expectations and limitations of such strategies in the context of human disease.

7.3.1 Immunomodulation during influenza infection

We demonstrate that inhibiting ROS or the signal imparted through CD200R induces
5 a greater myeloid response to influenza. However, we argue that blockade of ROS is
beneficial whereas blockade of CD200R signalling is detrimental, and that a better
therapeutic strategy would be to suppress the myeloid response through
administration of CD200:Fc. These differential strategies are defined by the disparate
immune compartments in that we believe ROS and CD200 are primarily acting: ROS
10 in the airways and CD200 in the MLNs. Reducing the levels of ROS during an
influenza infection induces a more rapid and potent myeloid response in the airways
that contains influenza, reducing viral titres and inflammatory infiltrate in lung tissue,
thus protecting this fragile organ. Since ROS are also strongly implicated in oxidative
stress during influenza infection their depletion would be a significant advantage.
15 However, blockade of CD200 gives rise to a greater global myeloid response,
stemming from the MLNs, that leads to augmented inflammation in all compartments
that is ineffectively resolved. Delivery of a myeloid suppressive signal through
CD200R reduces pulmonary inflammation and clinical illness with no alteration in
viral clearance. Manipulation of ROS and CD200R therefore offer two opposing
20 immunotherapeutic strategies to combat an influenza infection in mice, but what
promise do they hold in a clinical setting?

The relevance of the murine model of influenza to human clinical disease has often
been questioned. Infection of Macaques with a genetically reconstructed human
25 influenza virus causes an uncomplicated disease that simulated symptoms observed in
humans . Gene profiling of these primates, coupled with gross and histological
analysis of pulmonary infiltrate, however, demonstrates a comparable infection and
immune phenotype to that observed in the murine model . Viral kinetics was similar
as was the considerable infiltrate into the lungs and airways. Genes that encode for
30 mediators of chemotaxis, adhesion and transmigration of immune cells (particularly
monocytes and macrophages) are strongly up-regulated in the lung and draining LNs

implying a heavy traffic of immune cells into these tissues. Early in infection, a significant neutrophil and macrophage lung infiltrate is observed, followed by the recruitment of activated Th1 skewed T cells by day 7 of infection . Due to similarities between the models we therefore feel that our approaches may be translatable to higher organisms. Furthermore, a number of genes encoding protective enzymes against oxidative damage are strongly up-regulated in the influenza infected macaques, suggesting that oxidative stress is a concern and that our strategy to target ROS may be advantageous.

With the recent concerns regarding avian influenza and a potential pandemic, it is prudent to consider the potential of our immunomodulatory strategies against more virulent and pandemic strains of influenza. Avian H5N1 influenza causes severe disease in humans and a combination of *in vitro* and animal studies have indicated that high and disseminated viral replication is important in disease pathogenesis . However, *in vitro* and animal studies have also implicated cytokine dysregulation in H5N1 pathogenesis, with a significant induction of pro-inflammatory cytokines and chemokines , thus making immuno-modulatory strategies a plausible therapeutic alternative. Furthermore, studies with virulent strains of influenza demonstrate that the innate immune response is a major contributing factor in the clinical course of disease . Virological and immunological studies in individuals infected with H5N1 recently show that a high viral load and resulting intense inflammatory responses are central to influenza H5N1 pathogenesis . High levels of IP-10, MIG and MCP-1 are observed from bronchial epithelial cells and alveolar macrophages, which are strong chemoattractants for monocytes/macrophages. Elevated IL-8 is also observed, which is fundamental to recruitment of neutrophils. Other post-mortem studies in H5N1 infected individuals show that the pulmonary infiltrate consists primarily of macrophages rather than lymphocytes . Furthermore, mice infected with an adapted influenza virus recombined with the surface HA and NA proteins of the highly virulent 1918 pandemic strain of influenza exhibit a significant neutrophilic recruitment, which causes excessive tissue damage, most likely through release of ROS . Thus it would appear that myeloid cells are a suitable target to manipulate during infection with highly virulent influenza.

Influenza A has been shown to accelerate apoptosis of neutrophils *in vitro*, potentially through induction of ROS, which may facilitate spread of virus. It can thus be seen why the absence of ROS, as seen in our experiments, may lead to elevated neutrophil numbers in the airways and reduced lung viral titres. Our strategy to deplete ROS may reduce neutrophil apoptosis, preventing spread of virus, and concomitantly prevent oxidative stress caused by persistence of neutrophils within the inflamed lung. However, the pleiotropic roles of ROS in homeostasis and immune regulation would make manipulation of their levels in a clinical setting very complex but still demands further investigation. It may be that CD200 manipulation can confer a more targeted strategy than manipulating ROS. As described previously, significant pulmonary recruitment of macrophages is elicited by H5N1 influenza, and we have demonstrated the potency of signalling through CD200R to suppress the activity of this myeloid cell. Whilst we have not investigated the potential of CD200 to manipulate neutrophil function, similar suppression of this cell type may also be anticipated, and it would be of interest to assess the ability of CD200 to limit production of ROS. It is interesting that individuals infected with H5N1 influenza exhibit elevated levels of IL-6 and IFN- γ in their serum, both of which were reduced by administration of CD200:Fc in our murine model. A company has recently been established with Professor Tracy Hussell and Imperial College (named "Stormbio") that will seek to translate some of our murine based immunotherapeutic studies into a clinical setting, and manipulation of ROS and CD200 are currently being investigated with a murine pandemic influenza model by the Southern Research Institute at the University of Alabama.

A general complication with influenza is the occurrence of bacterial co-infections. It is often anticipated that immunotherapeutic strategies that dampen inflammation may render the host more susceptible to a bacterial co-infection including commensals from the upper respiratory tract. However, a large proportion of deaths with pandemic strains of influenza are attributable to overwhelming primary viral pneumonia rather than bacterial secondary pneumonia. Nonetheless, it is very important to consider the effect that manipulating facets of the immune response would have on the

establishment of bacterial co-infections. Indeed, utilisation of anti-TNF therapy during arthritis causes reactivation of latent tuberculosis. Signalling through CD200R reduces pro-inflammatory cytokine production by macrophages, which may be critical for the clearance of bacterial infections. However, we and others have demonstrated that signalling through CD200R is incapable of suppressing pro-inflammatory cytokine production induced by the bacterial product LPS. It is therefore conceivable that administration of CD200:Fc would selectively suppress influenza induced cytokine production by myeloid cells, without compromising the capacity of the host to respond to any subsequent bacterial infection. Also, signalling through CD200R would not affect all cell types capable of secreting inflammatory cytokines that may be protective against a bacterial co-infection. With this respect, manipulation of CD200 signalling would hold a significant advantage over more global treatment strategies such as neutralisation of TNF. Furthermore, it is anticipated that bacteria gain a foothold within the lung following influenza infection owing to heightened lung damage. We have shown that ligation of CD200R reduces the inflammatory infiltrate that is responsible for much of the pathology following influenza infection.

A major concern with depletion of ROS is compromised clearance of a bacterial co-infection, especially since absence of a functional phagocyte oxidase leads to enhanced susceptibility to certain bacterial pathogens such as *S. aureus*. However, as discussed, there may be reduced viral-induced and immuno-pathology by such treatment, which may diminish the potential of bacteria to establish themselves within the lung. Furthermore, it is anticipated that another reason why bacterial co-infections occur is that influenza induces apoptosis of neutrophils, which is mediated through ROS, the absence of which would leave this important bactericidal activity unscathed. We have recently developed an influenza and bacterial co-infection model within our laboratory to address such issues.

7.3.2 Immunomodulation during *C. neoformans* infection

We demonstrate in this thesis that the removal of suppressive signals to the myeloid compartment boosts immunity to *C. neoformans*. Depletion of ROS leads to a greater myeloid response with ensuing Th1 bias and improved control of the pathogen.

Preliminary studies into the blockade of the inhibitory signal imparted through CD200 gives rise to a greater myeloid response and prevention of cryptococcal dissemination. Whilst *C. neoformans* is capable of causing significant pulmonary disease in immune-competent individuals, this pathogen predominantly causes morbidity and mortality in the immuno-compromised, especially HIV-infected individuals. CD4⁺ T cells are the primary targets of HIV replication and numbers are reduced more than five-fold during infection. Boosting the myeloid immune response would be anticipated to promote immunity to *C. neoformans* in both immune-competent and immuno-compromised individuals, since myeloid cells would likely be unaffected by the immune disorder in question. Furthermore, with our strategies we observe reduced dissemination to the brain, which represents a significant clinical problem of fungal meningitis in immuno-compromised individuals.

Through depletion of ROS, a greater Th1 response was induced along with a preferential bias for promoting CD8⁺ T cells. CD8⁺ T cells have an established role in control of HIV viraemia, and thus we may not only boost immunity to *C. neoformans* but also potentially HIV. It will be important to establish whether the improved immunity to *C. neoformans* and the elevated CD8⁺ T cell response in the absence of ROS will persist independently of CD4⁺ T cell help – a hypothesis easily testable through depletion of this T cell subset. One concern, however, is that whilst depletion of ROS ultimately proves beneficial during a *C. neoformans* infection, generation of ROS is frequently an important effector mechanism against a number of pathogens. Accordingly, such a strategy may be wrought with danger in immuno-compromised individuals who frequently succumb to multiple infections.

7.4 Conclusion

In this thesis, I show that CD200 and ROS may actively suppress the myeloid immune compartment and fulfil an important homeostatic role. Furthermore, the myeloid response to respiratory infection may be modulated through the manipulation of ROS and CD200 inhibition. Through promotion of the myeloid compartment we are able to promote immunity to infection, whereas inhibition of myeloid cells can alleviate immunopathology. These results provide valuable insights into the potential

of immune manipulation as a treatment strategy for respiratory infections, and further our understanding of the role of ROS and CD200 in myeloid homeostasis and in the fight against infectious disease.

Claims:

1. A therapeutic composition for use in treating, ameliorating or preventing an infection in a subject, comprising a compound modulative of reactive oxygen species level; or
5 CD200 level; or
CD200R level; or
a combination of two or more of said levels
in said subject.
2. A therapeutic composition as recited in claim 1, wherein said infection is a
10 pulmonary infection.
3. A therapeutic composition as recited in claim 1 or claim 2, wherein a causative agent of said infection includes a viral agent or a fungal agent or a virus agent and a fungal agent.
4. A therapeutic composition as recited in claim 3, wherein said viral agent
15 comprises influenza A or pandemic influenza.
5. A therapeutic composition as recited in claim 3, wherein said fungal agent comprises *Cryptococcus neoformans*.
6. A therapeutic composition as recited in any preceding claim, wherein said compound comprises CD200.
- 20 7. A therapeutic composition as recited in any one of claims 1-5, wherein said compound comprises a CD200 fusion protein, a soluble CD200, an agonistic antibody to CD200R, a blocking antibody to CD200, or a combination thereof.
8. A therapeutic composition as recited in any one of claims 1-5, wherein said compound comprises CD200R.
- 25 9. A method for treating, ameliorating or preventing a microbial infection in a mammalian host comprising administering to said host a compound modulative of reactive oxygen species level; or
CD200 level; or
CD200R level; or

a combination of two or more of said levels
in said subject.

10. A method as recited in claim 9, wherein said infection is a pulmonary infection.

5 11. A method as recited in claim 9 or claim 10, wherein a causative agent of said infection includes a viral agent or a fungal agent or a virus agent and a fungal agent.

12. A method as recited in claim 11, wherein said viral agent comprises influenza A or pandemic influenza.

10 13. A method as recited in claim 11, wherein said fungal agent comprises *Cryptococcus neoformans*.

14. A method as recited in any one of claims 9-13, wherein said compound comprises CD200.

15 15. A method as recited in any one of claims 9-13, wherein said compound comprises a CD200 fusion protein, a soluble CD200, an agonistic antibody to CD200R, a blocking antibody to CD200, or a combination thereof.

16. A method as recited in any one of claims 9-13, wherein said compound comprises CD200R.

17. A therapeutic composition for use in treating, ameliorating or preventing an infection in a subject, comprising a compound modulative of

20 CD200 level; or

CD200R level; or

a combination of said CD200 level and said CD200R level
in said subject.

25 18. A therapeutic composition as recited in claim 17, wherein said infection is a pulmonary infection.

19. A therapeutic composition as recited in claim 17 or claim 18, wherein a causative agent of said infection includes a viral agent or a fungal agent or a virus agent and a fungal agent.

20. A therapeutic composition as recited in claim 19, wherein said viral agent comprises influenza A or pandemic influenza.
21. A therapeutic composition as recited in claim 19, wherein said fungal agent comprises *Cryptococcus neoformans*.
- 5 22. A therapeutic composition as recited in any one of claims 17-21, wherein said compound comprises CD200.
23. A therapeutic composition as recited in any one of claims 17-21, wherein said compound comprises a CD200 fusion protein, a soluble CD200, an agonistic antibody to CD200R, a blocking antibody to CD200, or a combination thereof.
- 10 24. A therapeutic composition as recited in any one of claims 17-21, wherein said compound comprises CD200R.
25. A method for treating, ameliorating or preventing a microbial infection in a mammalian host comprising administering to said host a compound modulative of
- 15 CD200 level; or
CD200R level; or
a combination of said CD200 level and said CD200R level
in said subject.
26. A method as recited in claim 25, wherein said infection is a pulmonary infection.
- 20 27. A method as recited in claim 25 or claim 26, wherein a causative agent of said infection includes a viral agent or a fungal agent or a virus agent and a fungal agent.
28. A method as recited in claim 27, wherein said viral agent comprises influenza A or pandemic influenza.
29. A method as recited in claim 27, wherein said fungal agent comprises
- 25 *Cryptococcus neoformans*.
30. A method as recited in any one of claims 25-29, wherein said compound comprises CD200.

31. A method as recited in any one of claims 25-29, wherein said compound comprises a CD200 fusion protein, a soluble CD200, an agonistic antibody to CD200R, a blocking antibody to CD200, or a combination thereof.

32. A method as recited in any one of claims 25-29, wherein said compound
5 comprises CD200R.

33. A therapeutic composition for use in treating, ameliorating or preventing an inflammatory pulmonary infection in a mammalian host having a CD200-CD200R axis comprising a compound modulative of said axis.

34. A therapeutic composition as recited in claim 33, wherein the compound
10 modulative of the CD200-CD200R axis is a compound modulative of
CD200 level; or
CD200R level; or
a combination of said CD200 level and said CD200R level
in said subject.

35. A therapeutic composition as recited in claim 33 or claim 34, wherein a
15 causative agent of said infection includes a viral agent or a fungal agent or a virus agent and a fungal agent.

36. A therapeutic composition as recited in claim 35, wherein said viral agent comprises influenza A or pandemic influenza.

37. A therapeutic composition as recited in claim 35, wherein said fungal agent
20 comprises *Cryptococcus neoformans*.

38. A therapeutic composition of any one of claims 33-37, wherein said compound comprises CD200.

39. A therapeutic composition of any one of claims 33-37, wherein said
25 compound comprises a CD200 fusion protein, a soluble CD200, an agonistic antibody to CD200R, a blocking antibody to CD200, or a combination thereof.

40. A therapeutic composition of any one of claims 33-37, wherein said compound comprises CD200R.

41. A method for treating, ameliorating or preventing an inflammatory pulmonary infection in a mammalian host having a CD200-CD200R axis comprising administering to said host a compound modulative of said axis.

42. A method as recited in claim 41, wherein the compound modulative of the CD200-CD200R axis is a compound modulative of

CD200 level; or

CD200R level; or

a combination of said CD200 level and said CD200R level

in said subject.

43. A method as recited in claim 41 or claim 42, wherein a causative agent of said infection includes a viral agent or a fungal agent or a virus agent and a fungal agent.

44. A method as recited in claim 43, wherein said viral agent comprises influenza A or pandemic influenza.

45. A method as recited in claim 43, wherein said fungal agent comprises *Cryptococcus neoformans*.

46. A method of any one of claims 41-45, wherein said compound comprises CD200.

47. A method of any one of claims 41-45, wherein said compound comprises a CD200 fusion protein, a soluble CD200, an agonistic antibody to CD200R, a blocking antibody to CD200, or a combination thereof.

48. A method of any one of claims 41-45, wherein said compound comprises CD200R.

49. A therapeutic composition for use in treating, ameliorating or preventing a microbial infection in a subject comprising a compound mimetic of superoxide dismutase.

50. A therapeutic composition as recited in claim 49, wherein said compound comprises a metalloporphyrin.

51. A therapeutic composition as recited in claim 49, wherein said compound comprises a manganic porphyrin.

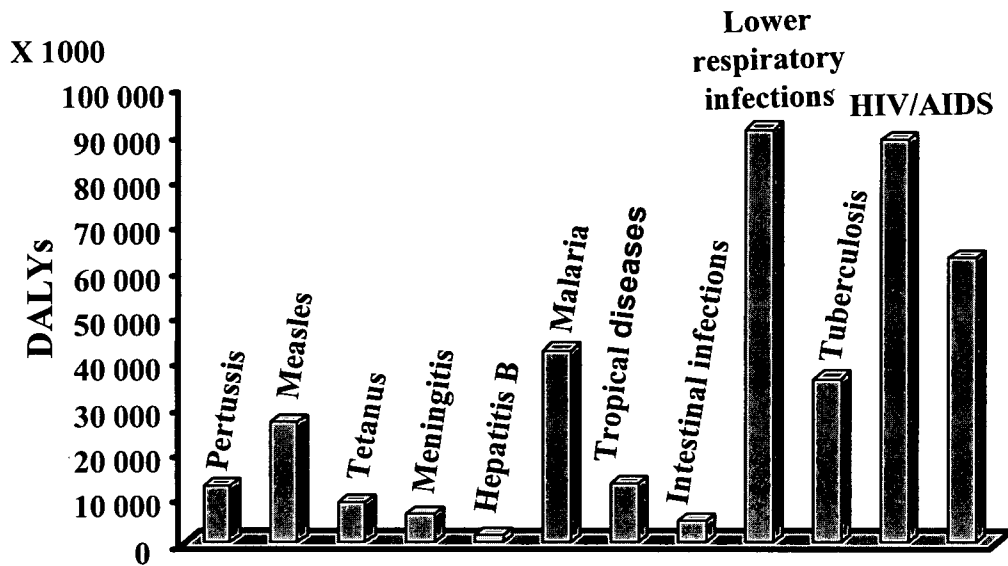
52. A therapeutic composition as recited in claim 49, wherein said compound comprises manganese (III) tetrakis (N-ethyl pyridinium-2-yl) porpyhrin.
53. A therapeutic composition as recited in any one of claims 49-52, wherein said microbial infection comprises a fungal infection.
- 5 54. A therapeutic composition as recited in claim 53, wherein said fungal infection comprises a *Cryptococcus neoformans* infection.
55. A therapeutic composition for use in treating, ameliorating or preventing pulmonary inflammation in a subject in which a microbe is a causative agent comprising a compound mimetic of superoxide dismutase.
- 10 56. A therapeutic composition as recited in claim 55, wherein said compound comprises a metalloporphyrin.
57. A therapeutic composition as recited in claim 55, wherein said compound comprises a manganic porphyrin.
58. A therapeutic composition as recited in claim 55, wherein said compound
15 comprises manganese (III) tetrakis (N-ethyl pyridinium-2-yl) porpyhrin.
59. A therapeutic composition as recited in any one of claims 55-58, wherein said microbe is a fungus.
60. A therapeutic composition as recited in claim 59, wherein said fungus is *Cryptococcus neoformans*.
- 20 61. A method for treating, ameliorating or preventing a microbial infection in a mammalian host comprising administering to a subject a compound mimetic of superoxide dismutase.
62. A method as recited in claim 61, wherein said compound comprises a metalloporphyrin.
- 25 63. A method as recited in claim 61, wherein said compound comprises a manganic porphyrin.
64. A method as recited in claim 61, wherein said compound comprises manganese (III) tetrakis (N-ethyl pyridinium-2-yl) porpyhrin.

65. A method as recited in any one of claims 61 -64, wherein said microbial infection comprises a fungal infection.
66. A method as recited in claim 65, wherein said fungal infection comprises a *Cryptococcus neoformans* infection.
- 5 67. A method for treating, ameliorating or preventing pulmonary inflammation in a subject in which a microbe is a causative agent comprising administering to a subject a compound mimetic of superoxide dismutase.
68. A method as recited in claim 67, wherein said compound comprises a metalloporphyrin.
- 10 69. A method as recited in claim 67, wherein said compound comprises a manganic porphyrin.
70. A method as recited in claim 67, wherein said compound comprises manganese (III) tetrakis (N-ethyl pyridinium-2-yl) porpyhrin.
71. A method as recited in any one of claims 67 -70, wherein said microbe is a
15 fungus.
72. A method as recited in claim 71, wherein said fungus is *Cryptococcus neoformans*.

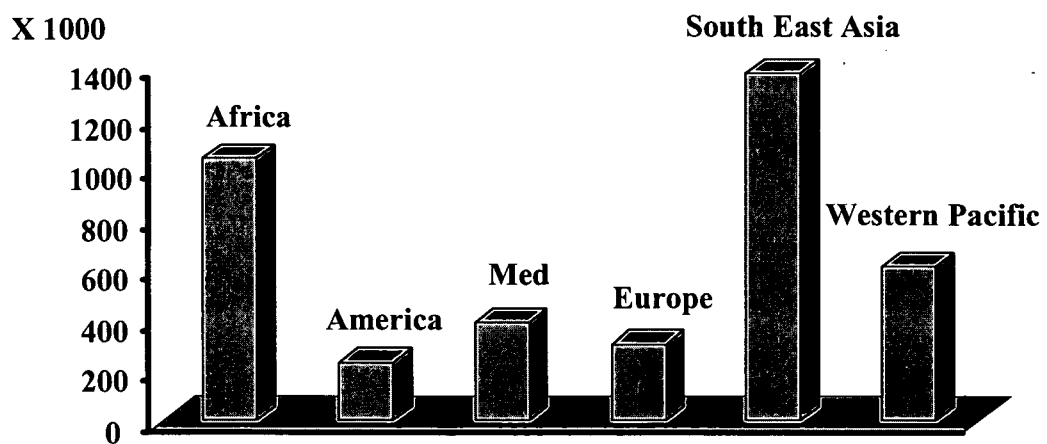
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Figure 1.1

a. **Burden of disease (DALYs)
according to the WHO for 2001**

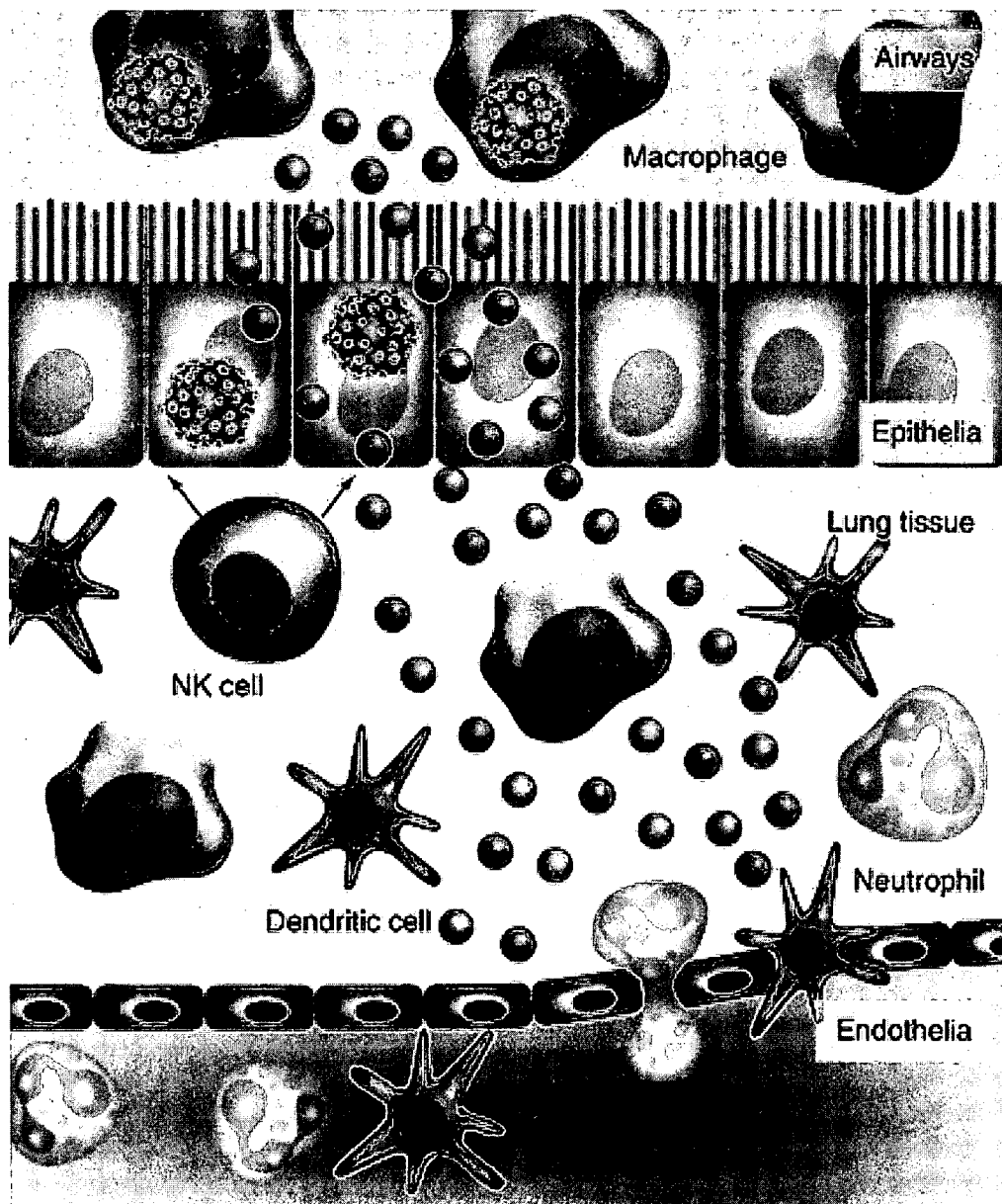


b. **Death and disability by WHO region**



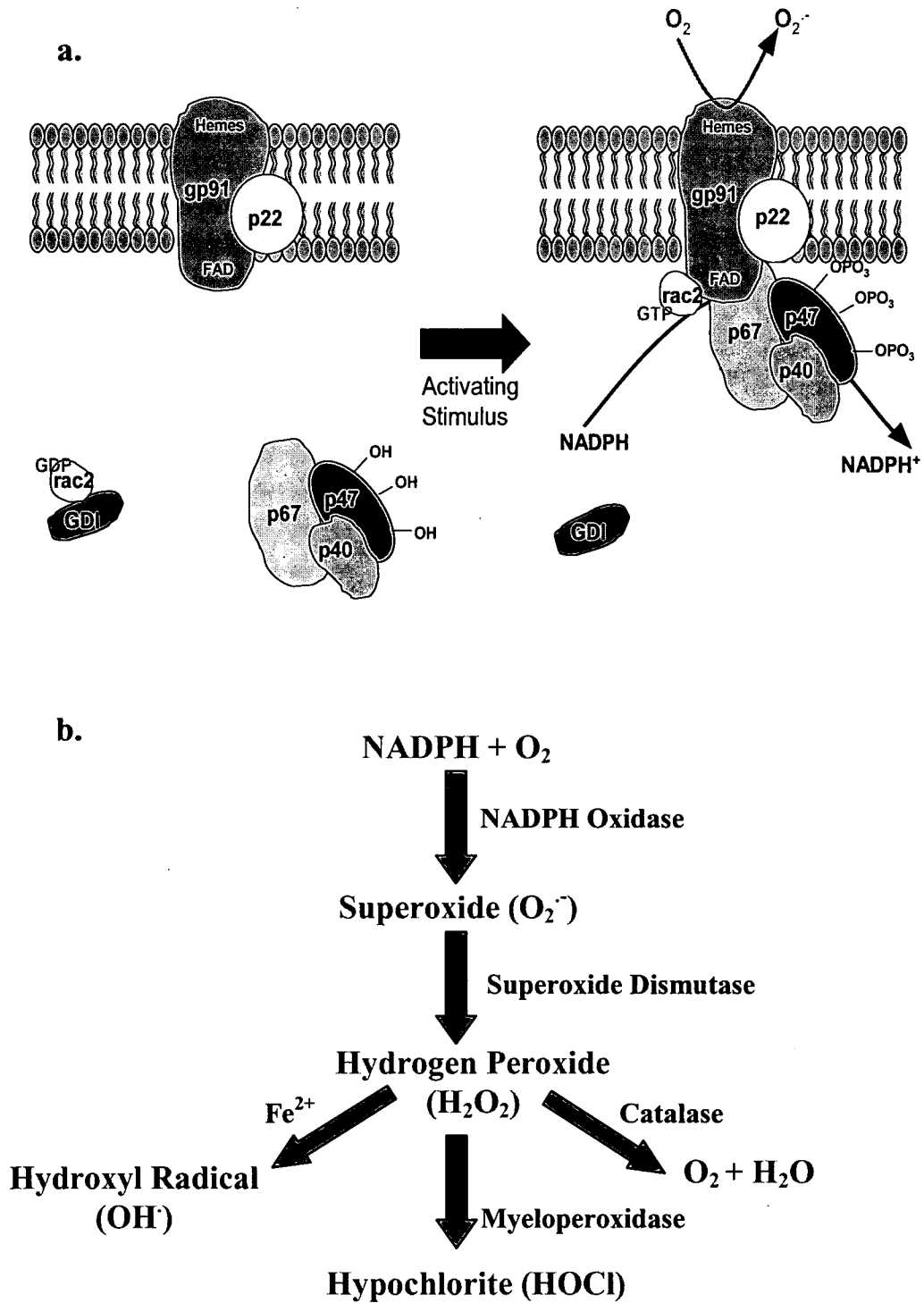
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Figure 1.2



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Figure 1.3



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Figure 1.4

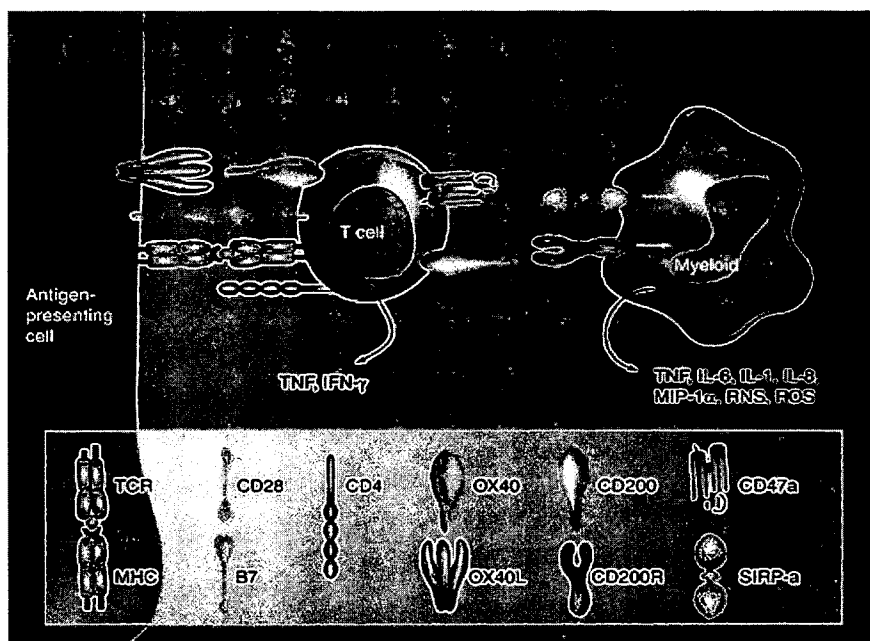
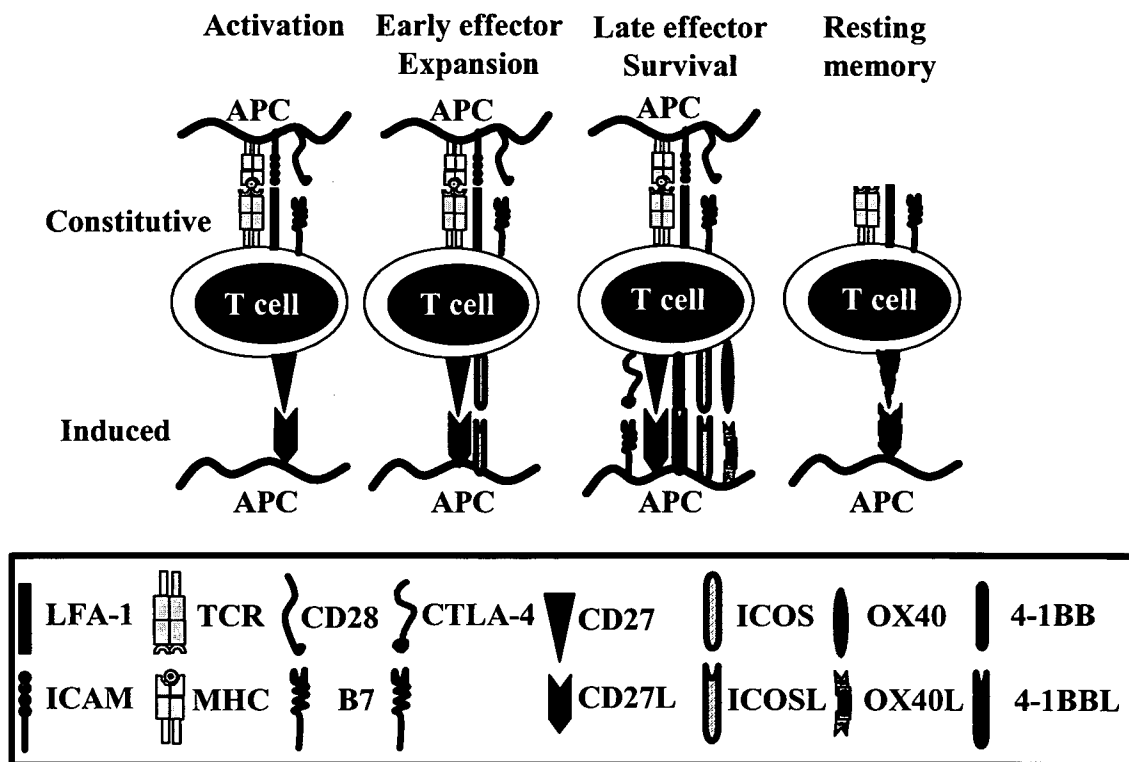


Table 1.2

Cell type	CD200	CD200R
T cell	-/+	-/+
Activated T cell	+	-/+
Thymocyte	+	-
B cell	+	-
Mon./Macrophage	-	+
Dendritic cell	-/+	+
Neutrophil	-	+
Mast cell	-	+
Basophil	-	+
Endotheliim	+	-
Epithelium	-/+	-
Neuron	+	-

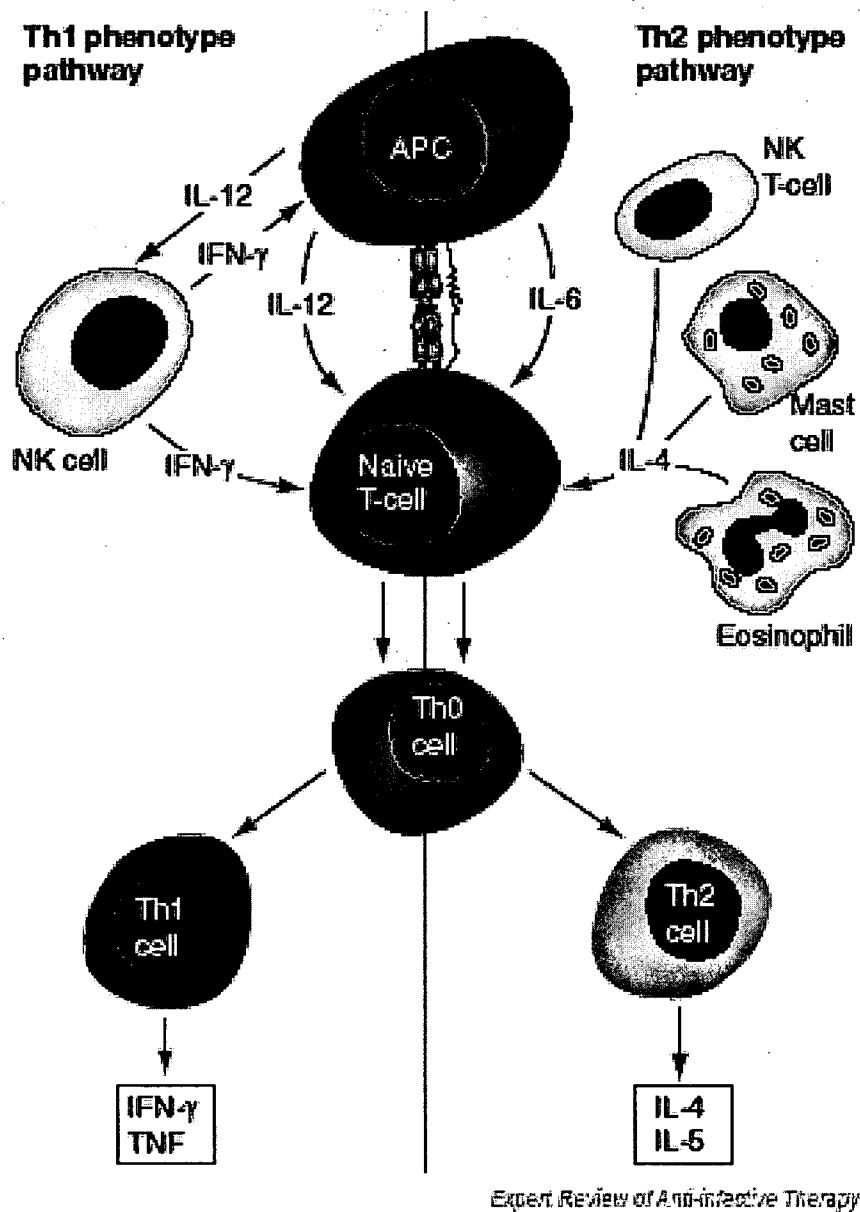
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Figure 1.5



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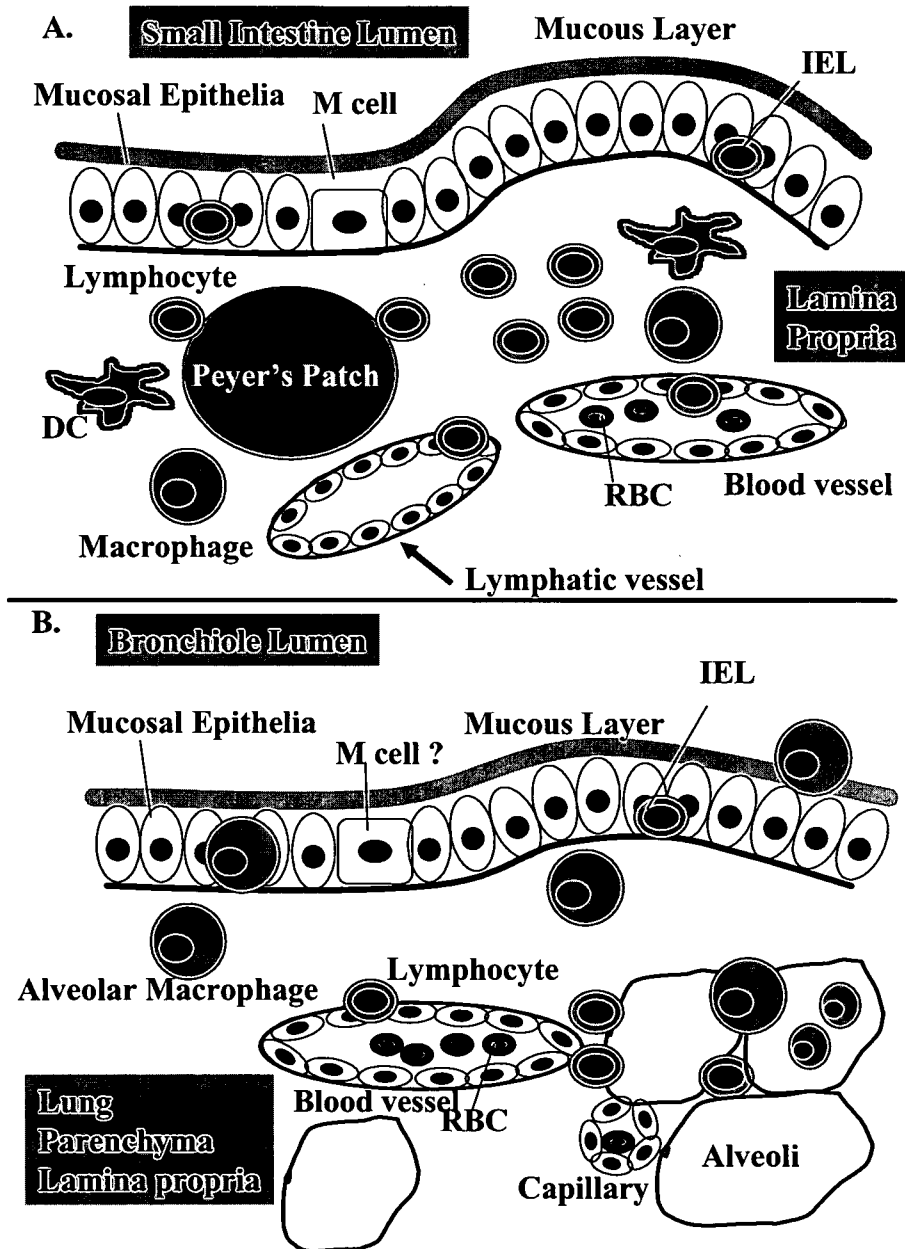
Figure 1.6



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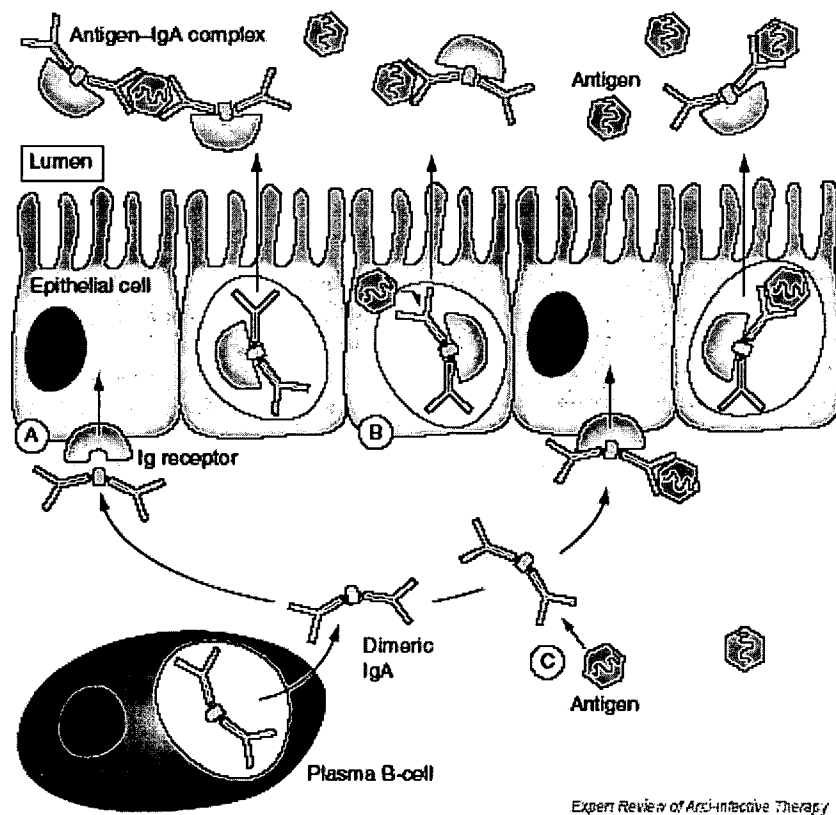
Figure 1.7

Figure 2: Snelgrove et al

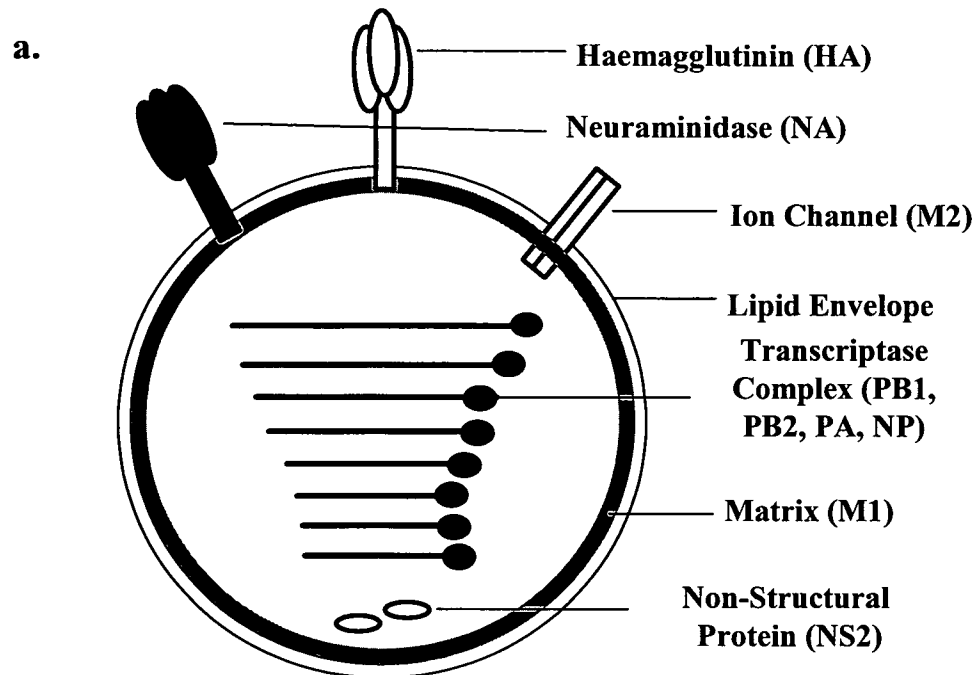
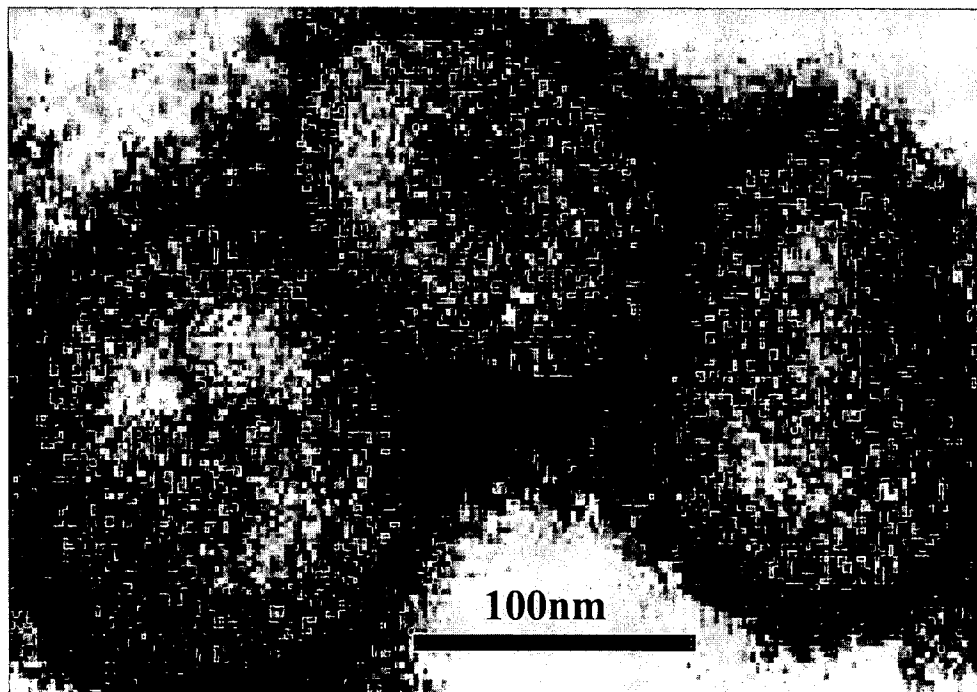


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Figure 1.8

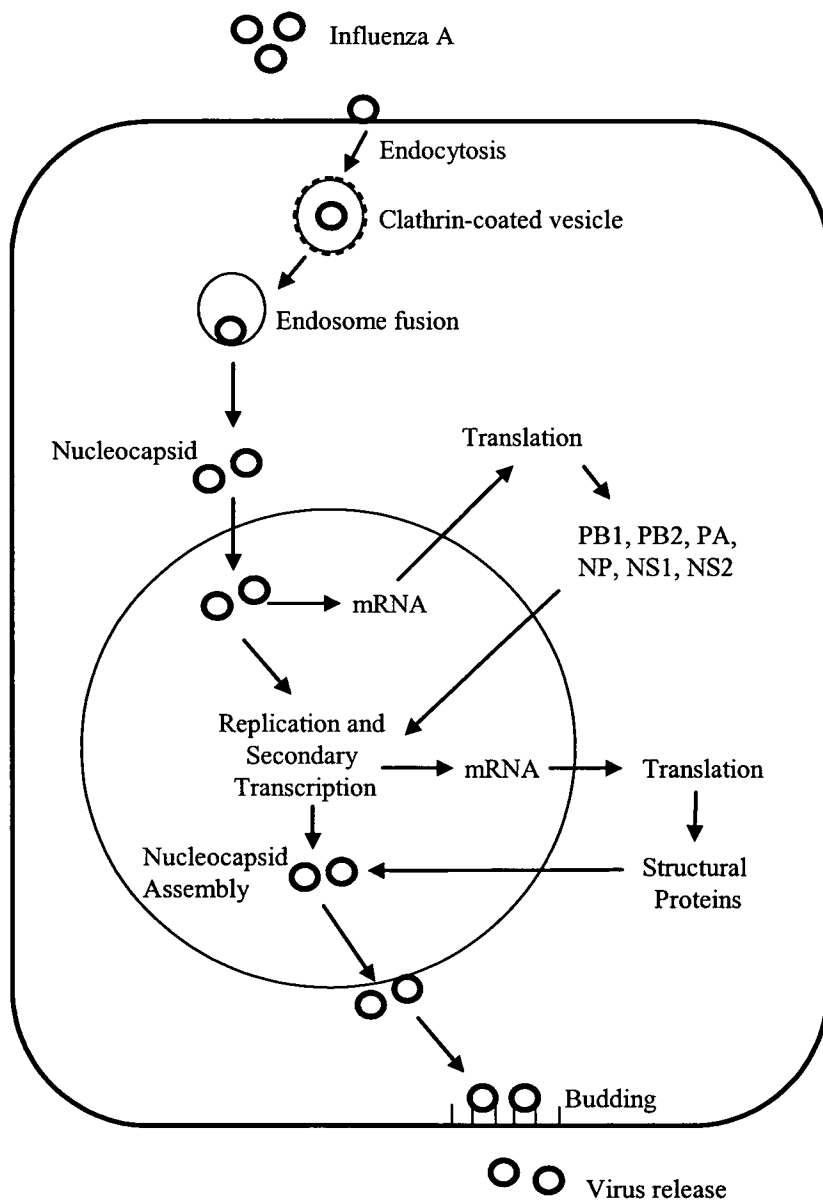


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Figure 1.9**b.**

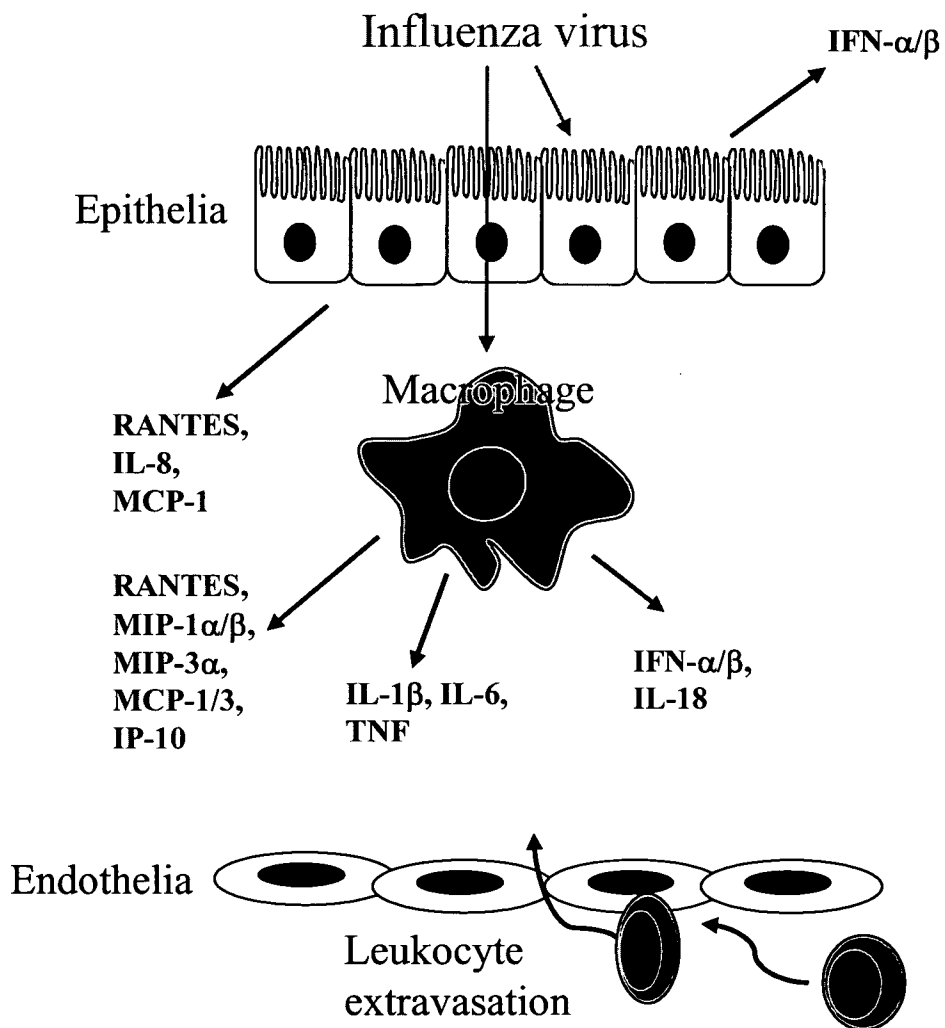
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Figure 1.10



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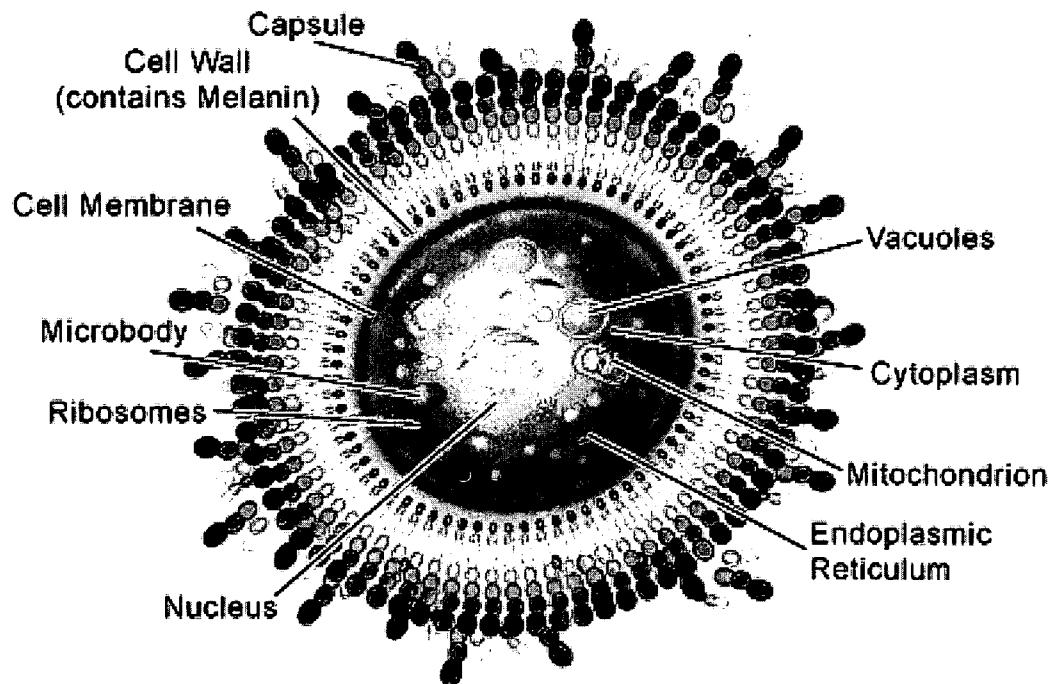
Figure 1.11



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Figure 1.12

a.



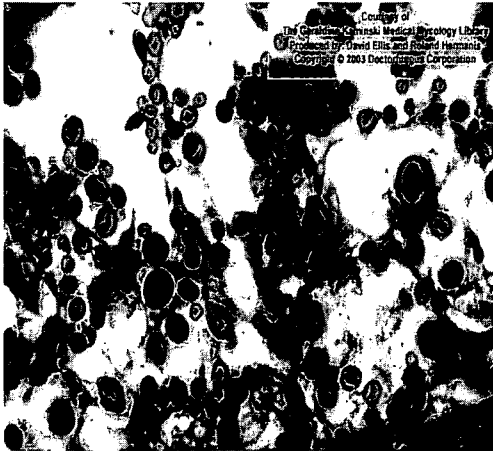
b.



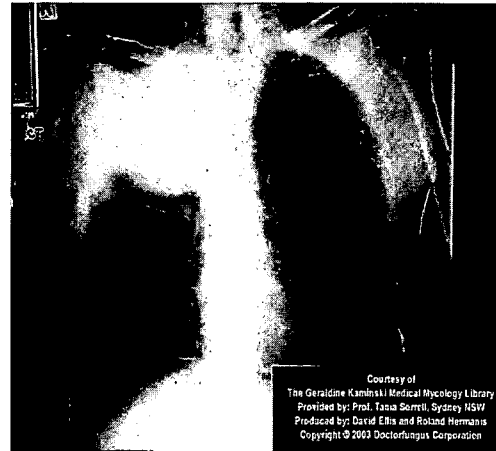
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Figure 1.13

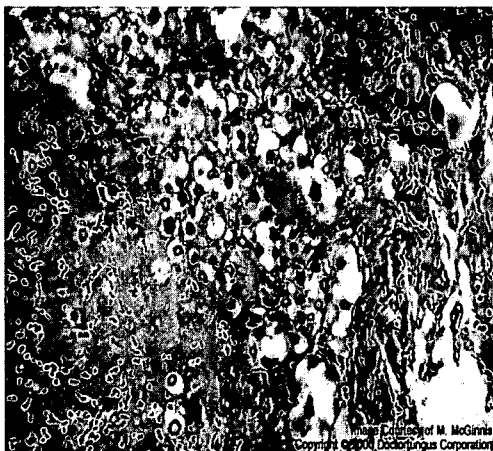
a.



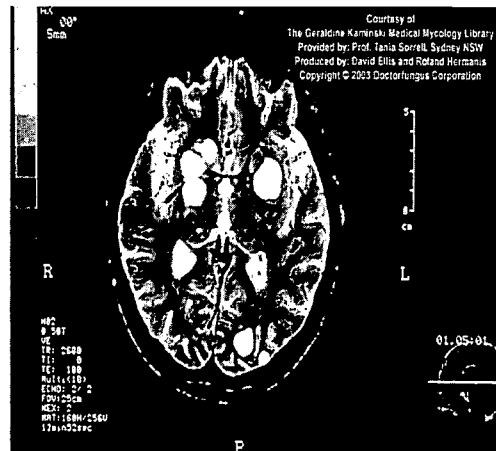
b.



c.

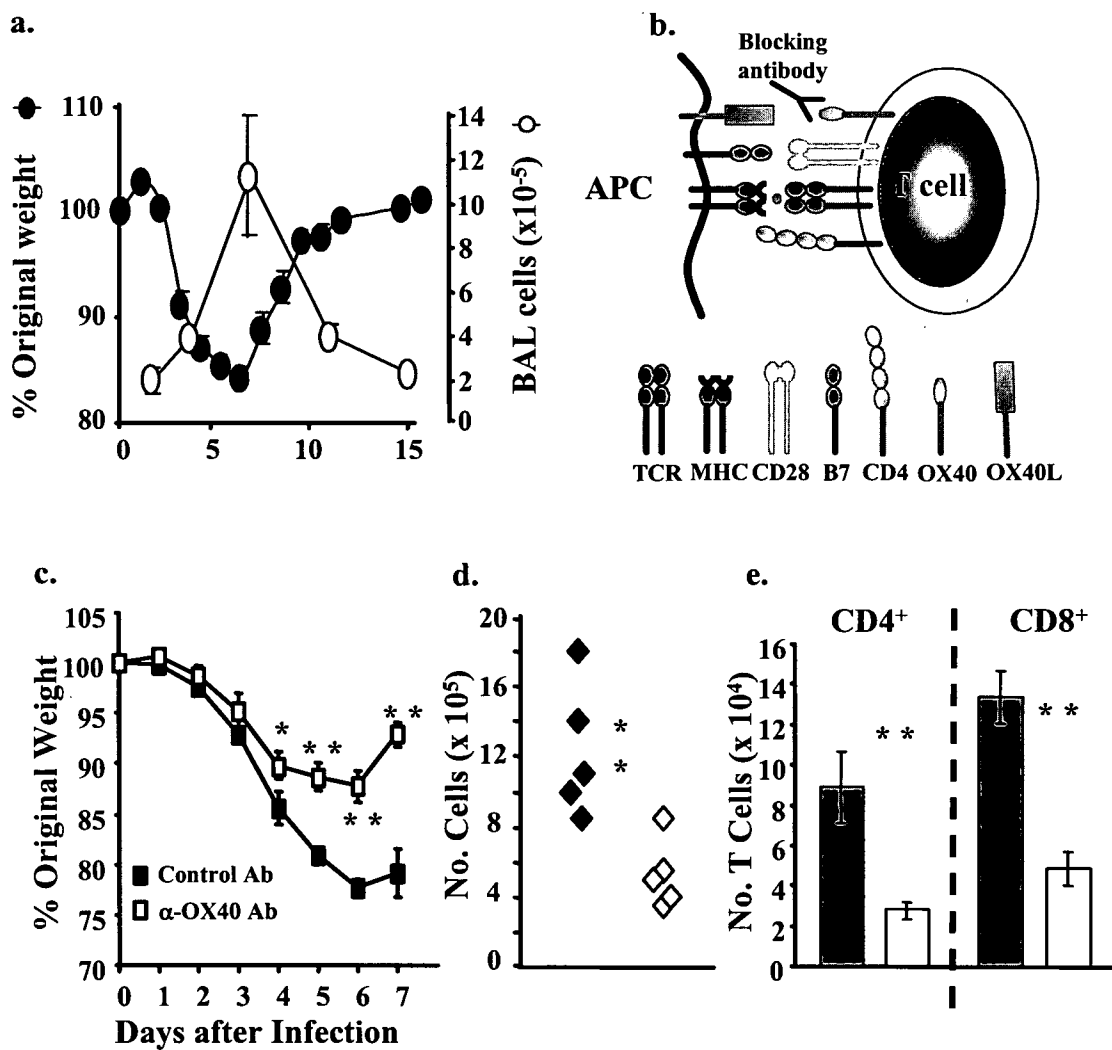


d.



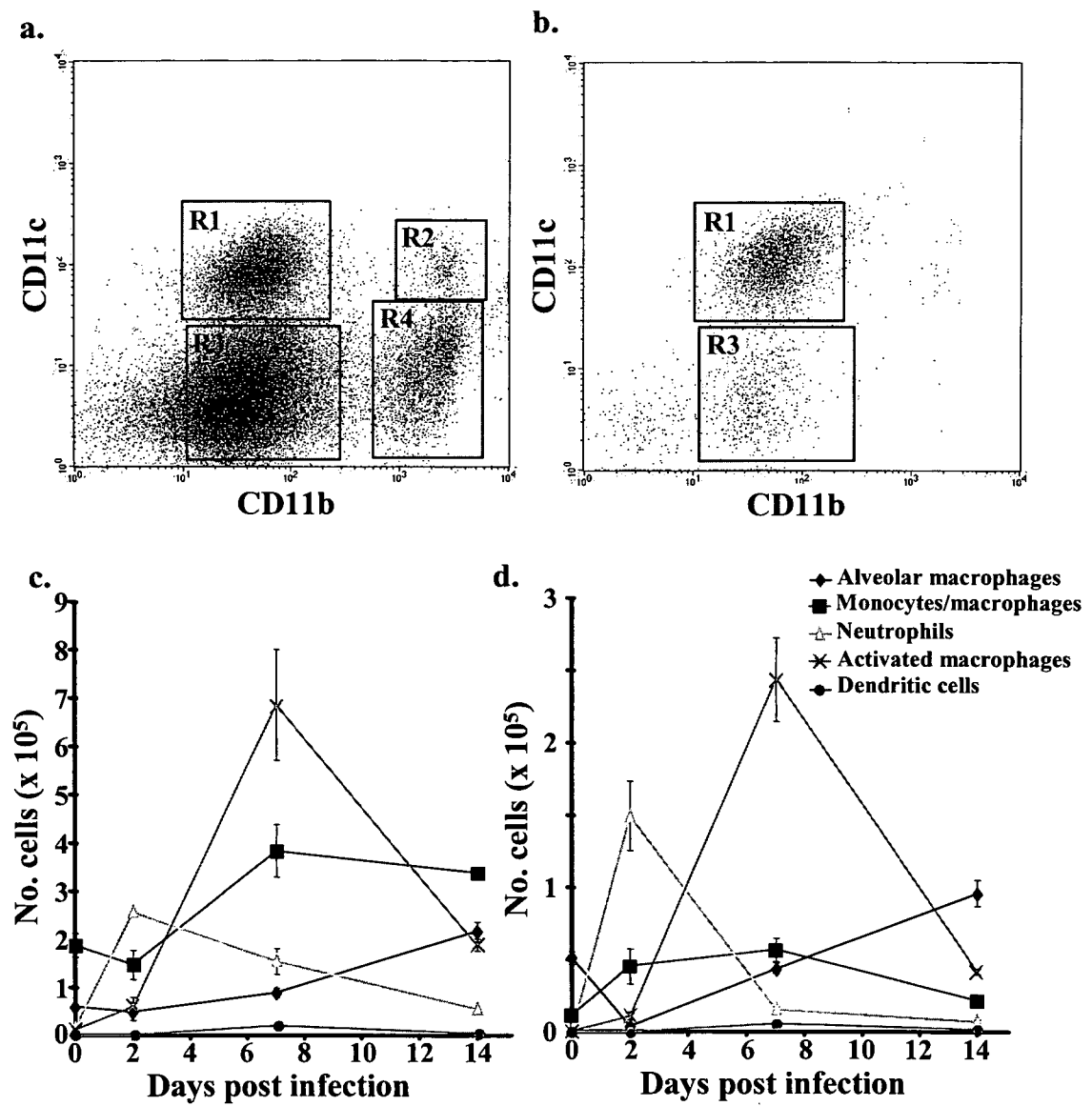
14/100

Figure 3.1



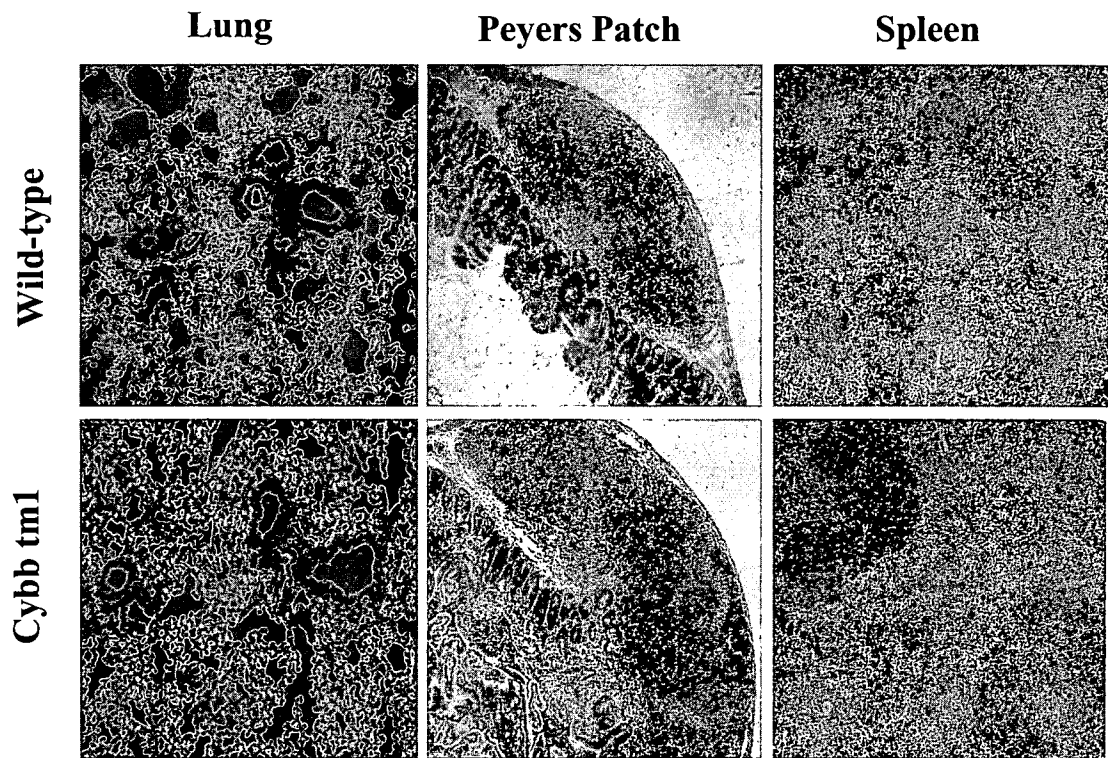
15/100

Figure 3.2



16/100

Figure 3.3



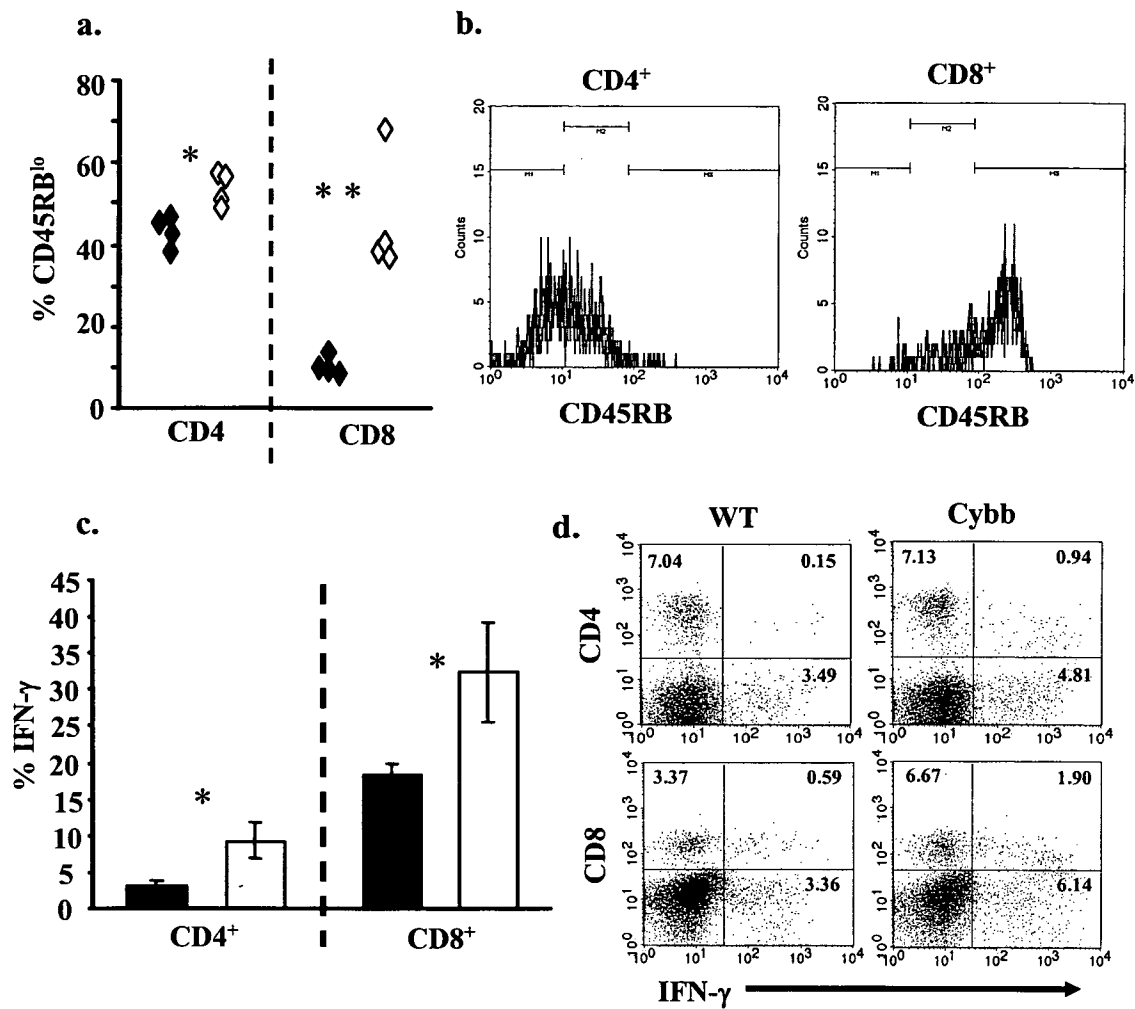
17/100

Table 3.1

	Lung	Spleen	Peyers Patch
Total cell no.	2.78x10 ⁵ +/-0.75 * 8.20x10 ⁵ +/-3.22	1.67x10 ⁷ +/-0.17 * * 5.58x10 ⁷ +/-0.31	4.00x10 ⁶ +/-1.70 * 7.97x10 ⁶ +/-0.07
CD4 ⁺ T cells	0.48x10 ⁴ +/-0.02 * 4.55x10 ⁴ +/-1.02	2.75x10 ⁶ +/-0.29 * * 8.1x10 ⁶ +/-0.24	4.18x10 ⁵ +/-1.40 * 6.07x10 ⁵ +/-0.63
CD8 ⁺ T cells	0.47x10 ⁴ +/-0.01 * 1.02x10 ⁴ +/-0.32	1.41x10 ⁶ +/-0.15 * * 5.27x10 ⁶ +/-0.17	1.16x10 ⁵ +/-0.28 * 2.78x10 ⁵ +/-0.22
CD11b ⁺ Mac.	0.50x10 ⁴ +/-0.09 * * 2.87x10 ⁴ +/-1.25	3.09x10 ⁵ +/-0.75 * * 22.26x10 ⁵ +/-5.40	2.25x10 ⁴ +/-0.55 * * 14.7x10 ⁴ +/-2.65

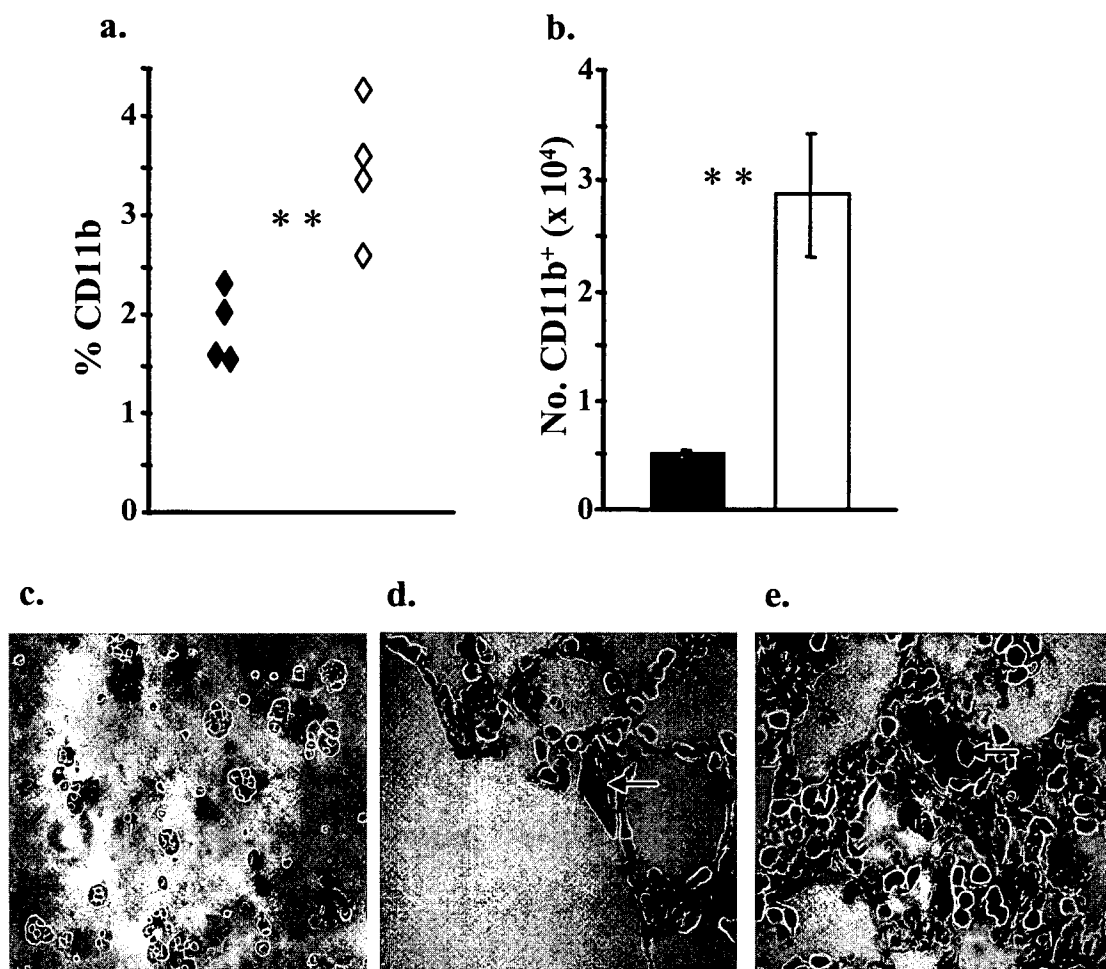
18/100

Figure 3.4

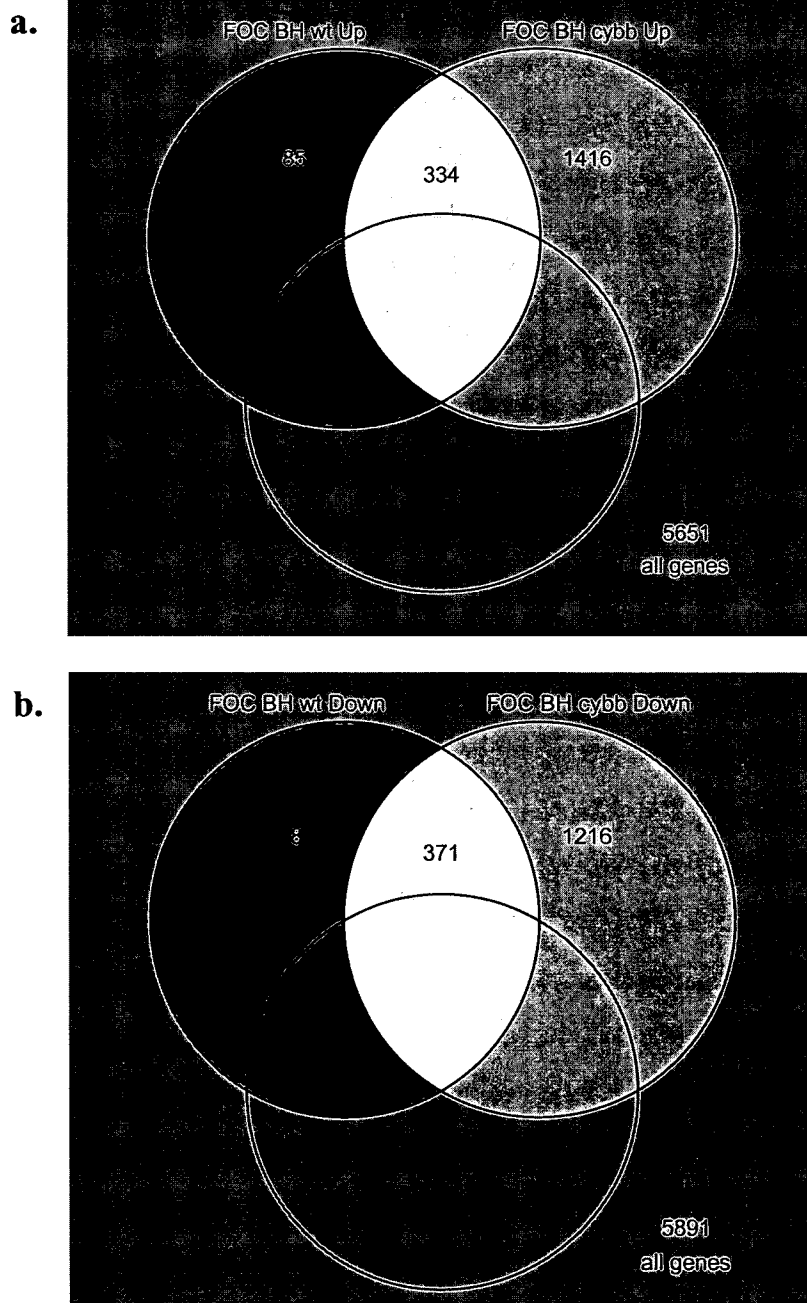


19/100

Figure 3.5



20/100

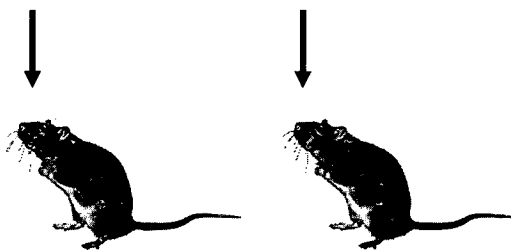
Figure 3.6

21/100

Figure 3.7

Day 0

50 HA Influenza A x31 (i.n)

Wild-type
C57BL/6Cybb^{tm1}**Days 1,
3 and 7****BAL:** - Surface Staining (flow cytometry)

- Cytocentrifugation

- Cytokines (ELISA)

Lung: - Surface/intracellular cytokine staining

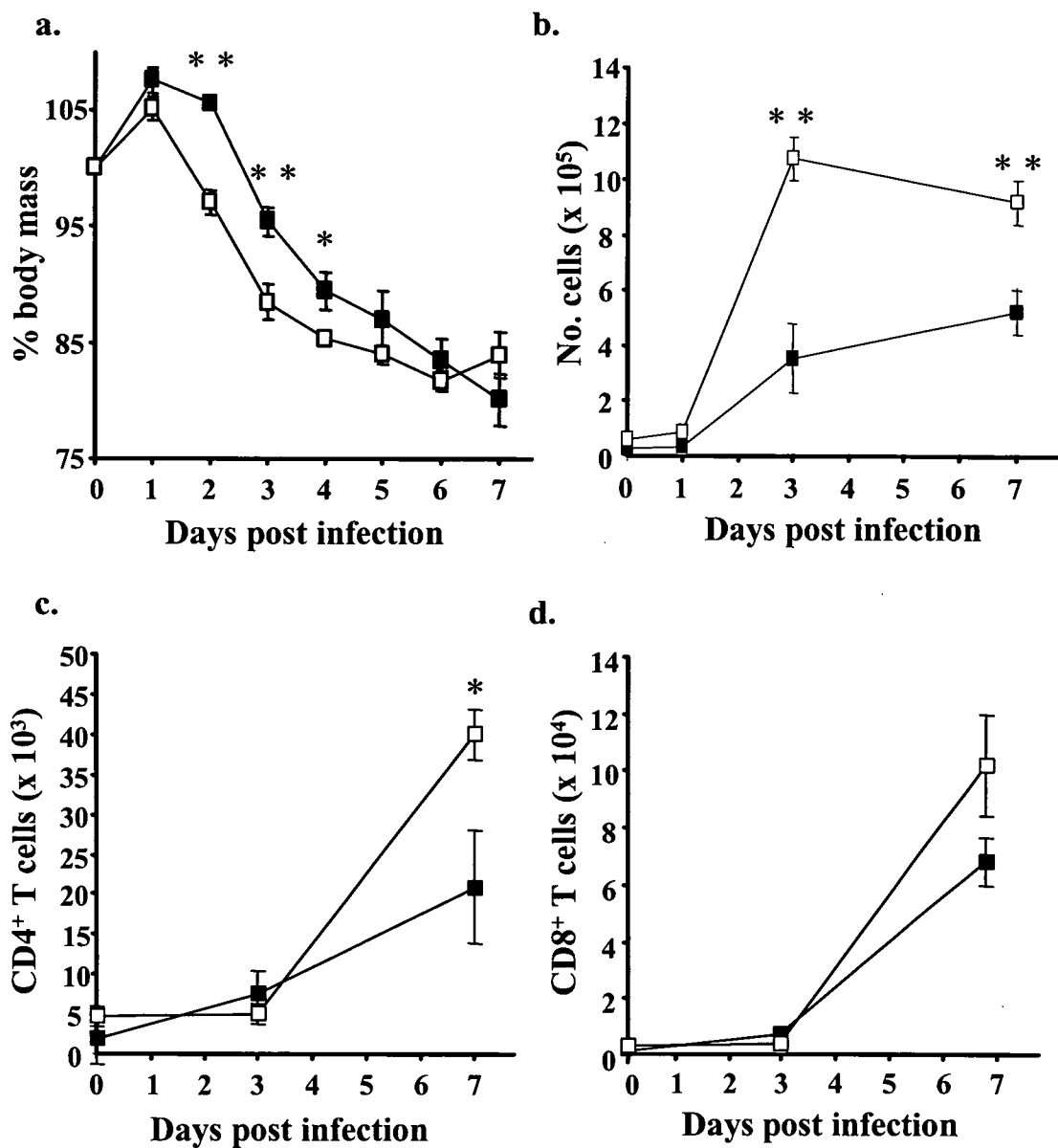
- Influenza titres

- Histology (H/E)

Serum: - Influenza-specific antibody, TNF (ELISA)**Nasal Washes:** - Influenza-specific IgA

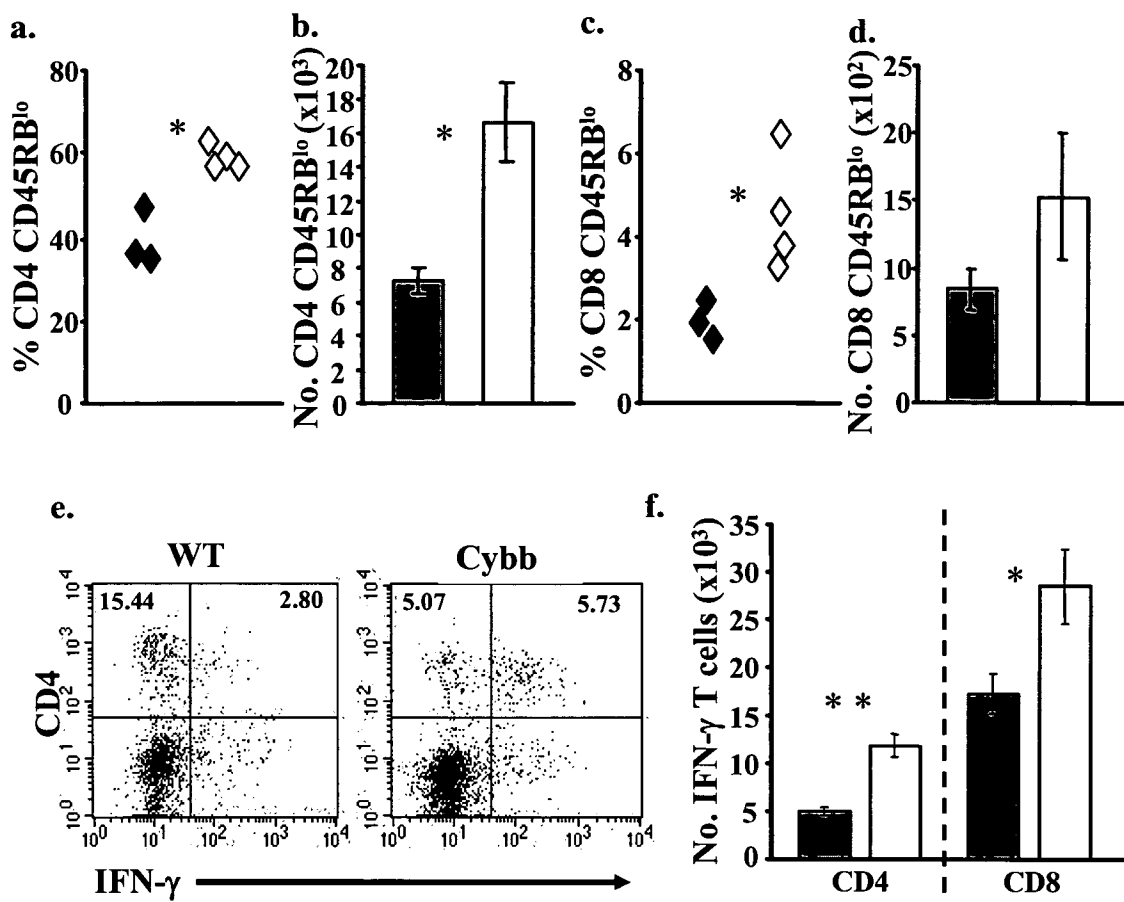
22/100

Figure 3.8



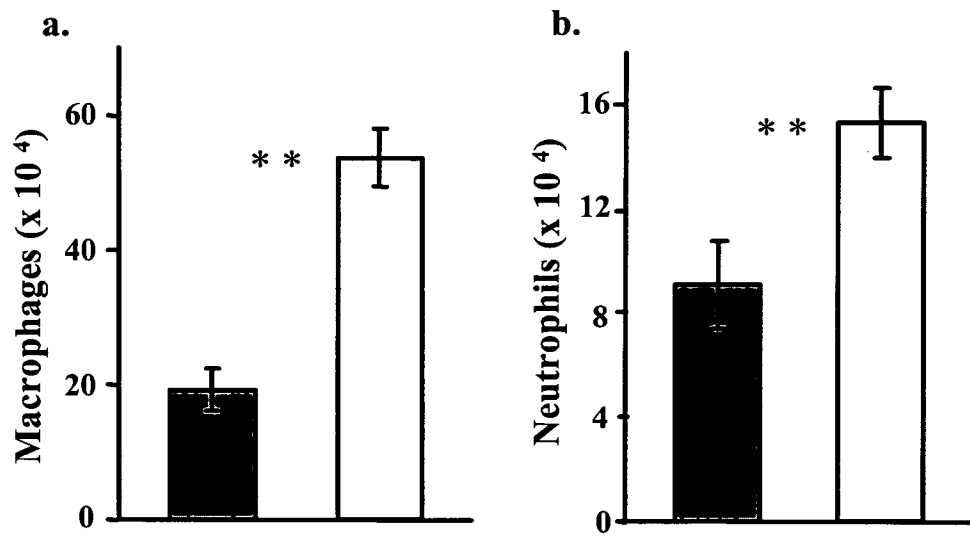
23/100

Figure 3.9



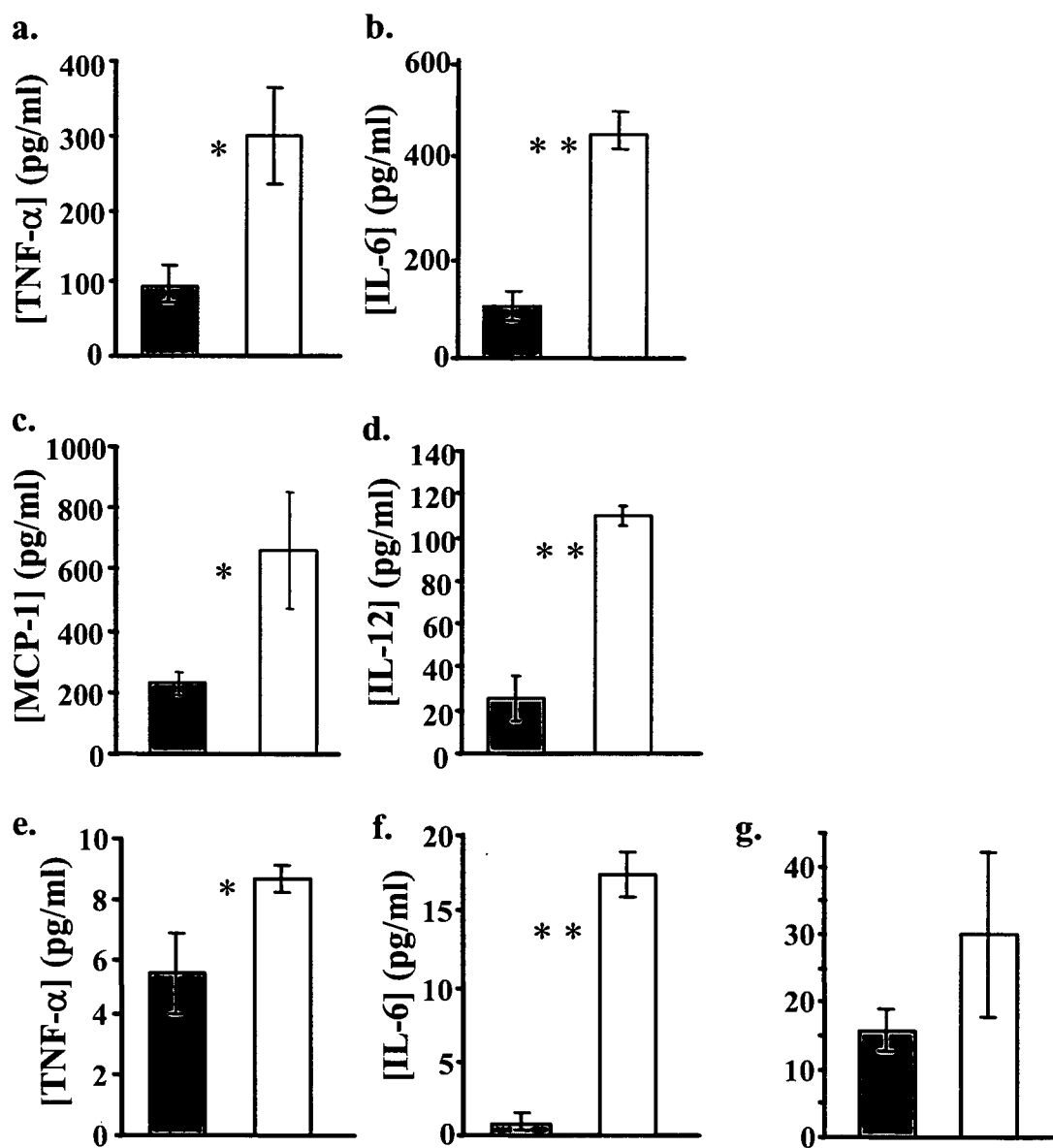
24/100

Figure 3.10



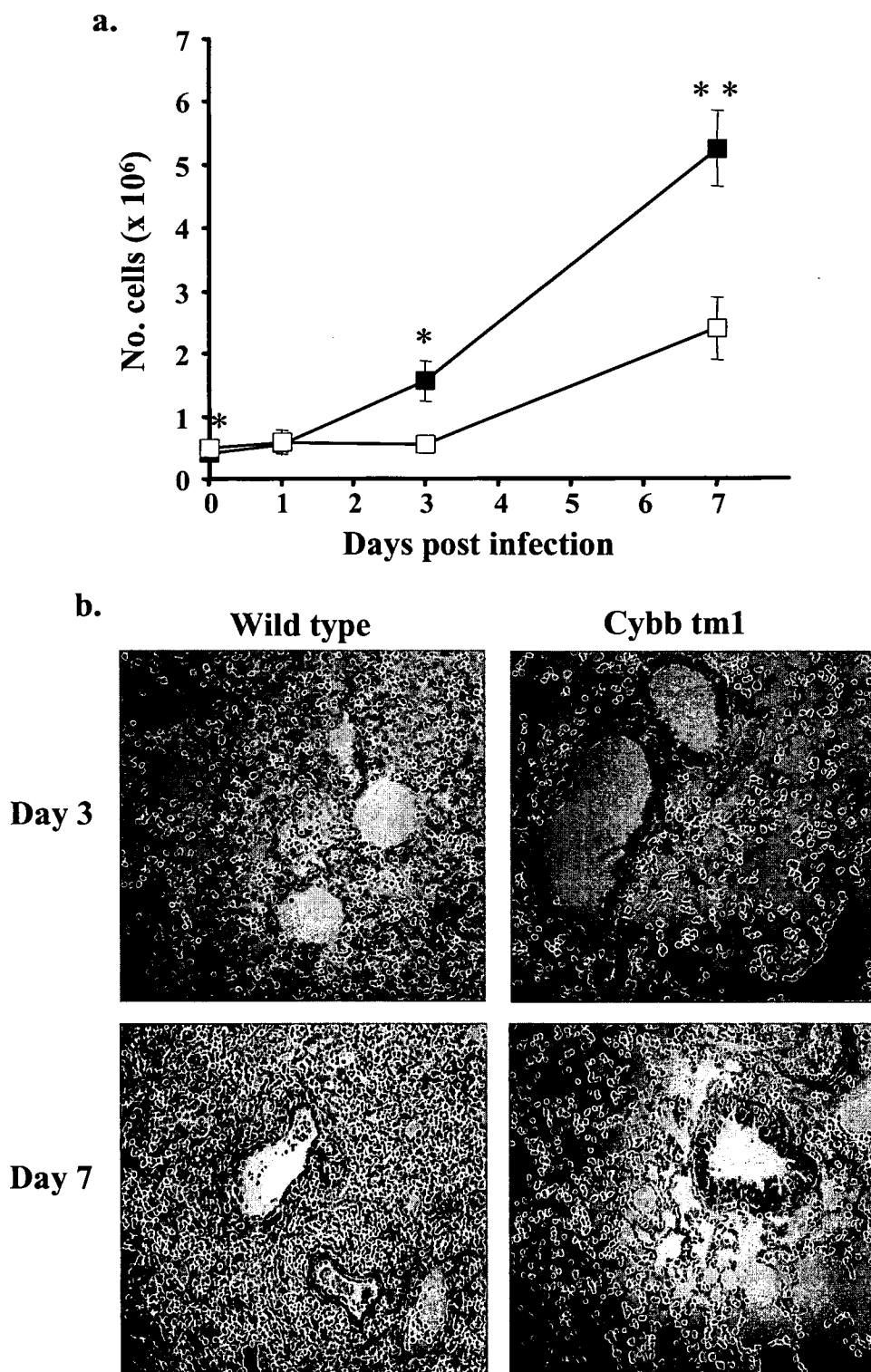
25/100

Figure 3.11



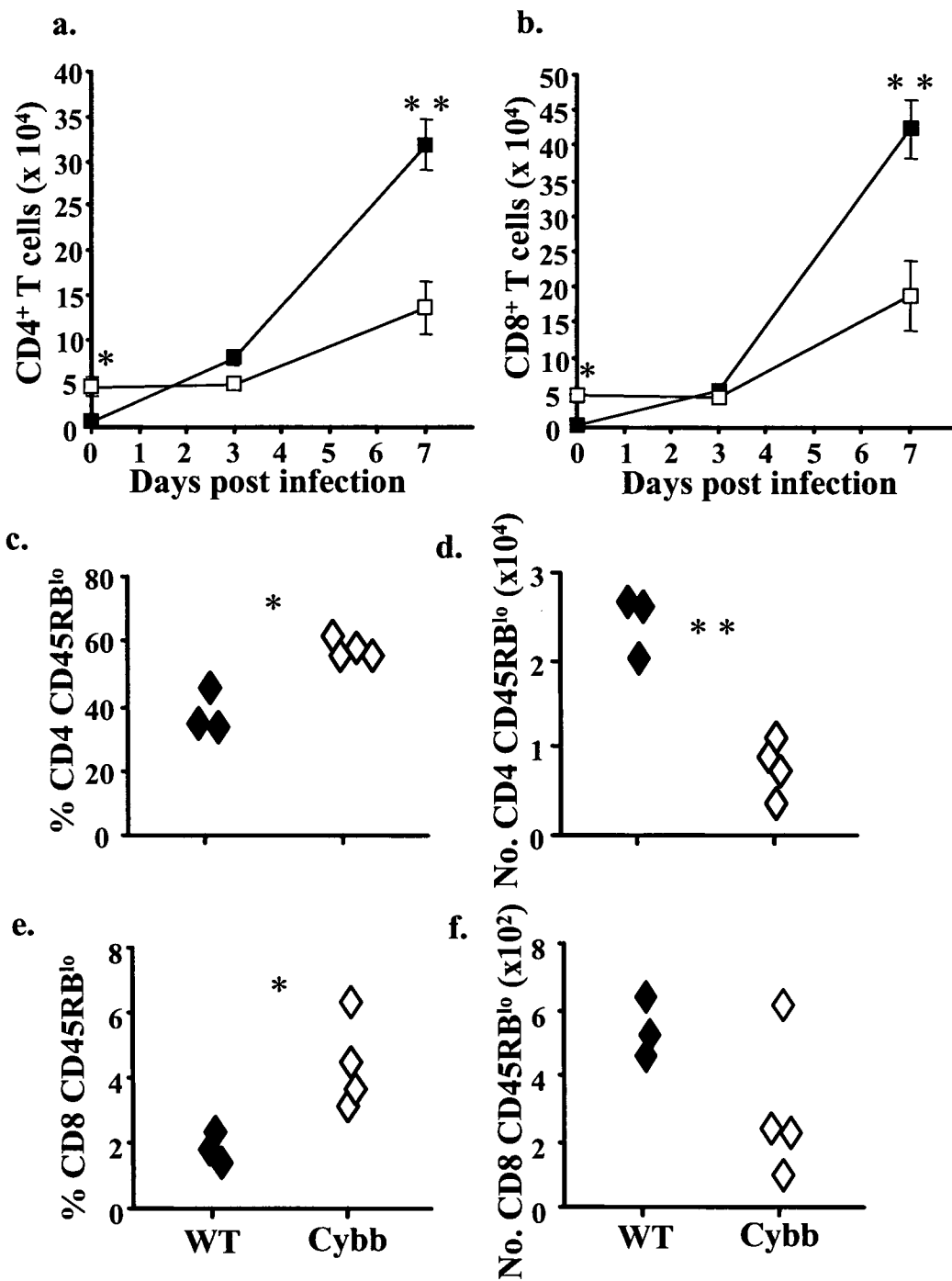
26/100

Figure 3.12



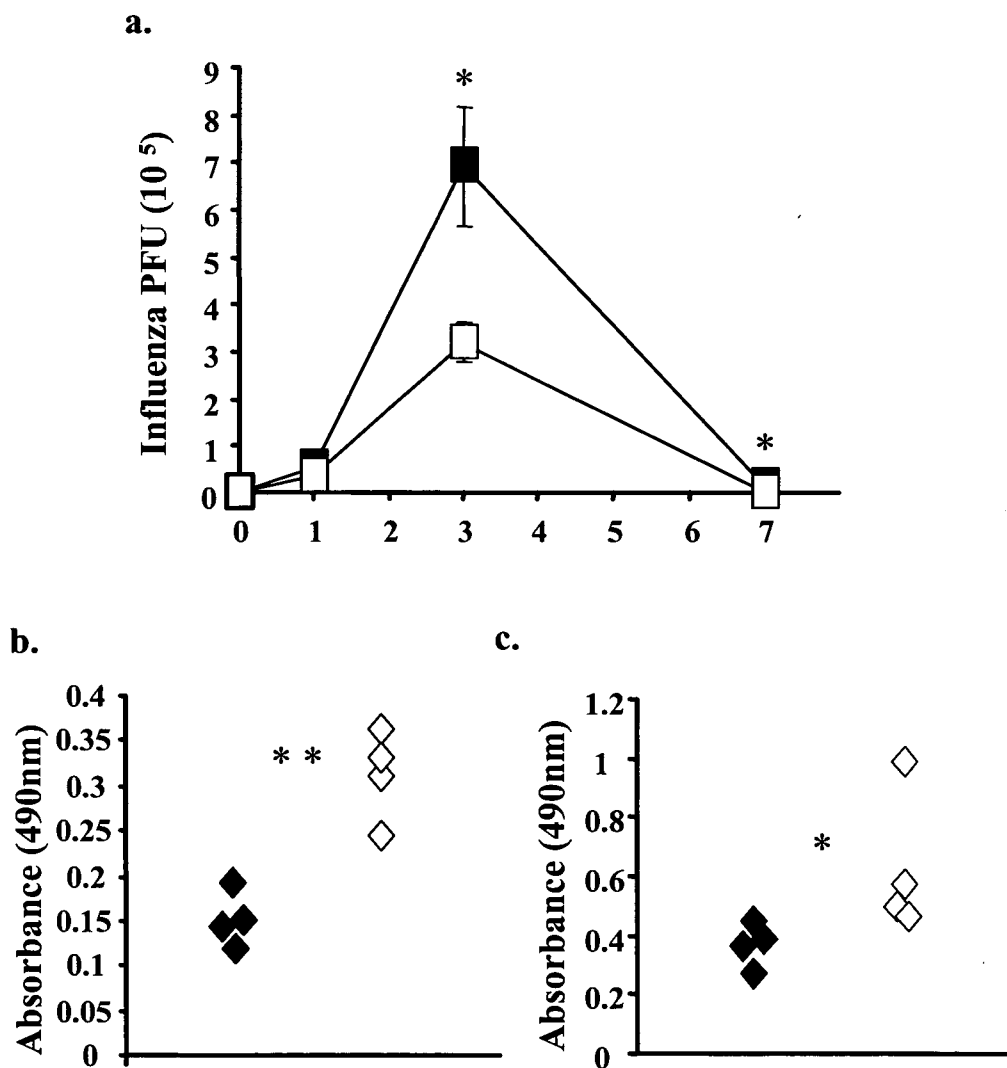
27/100

Figure 3.13



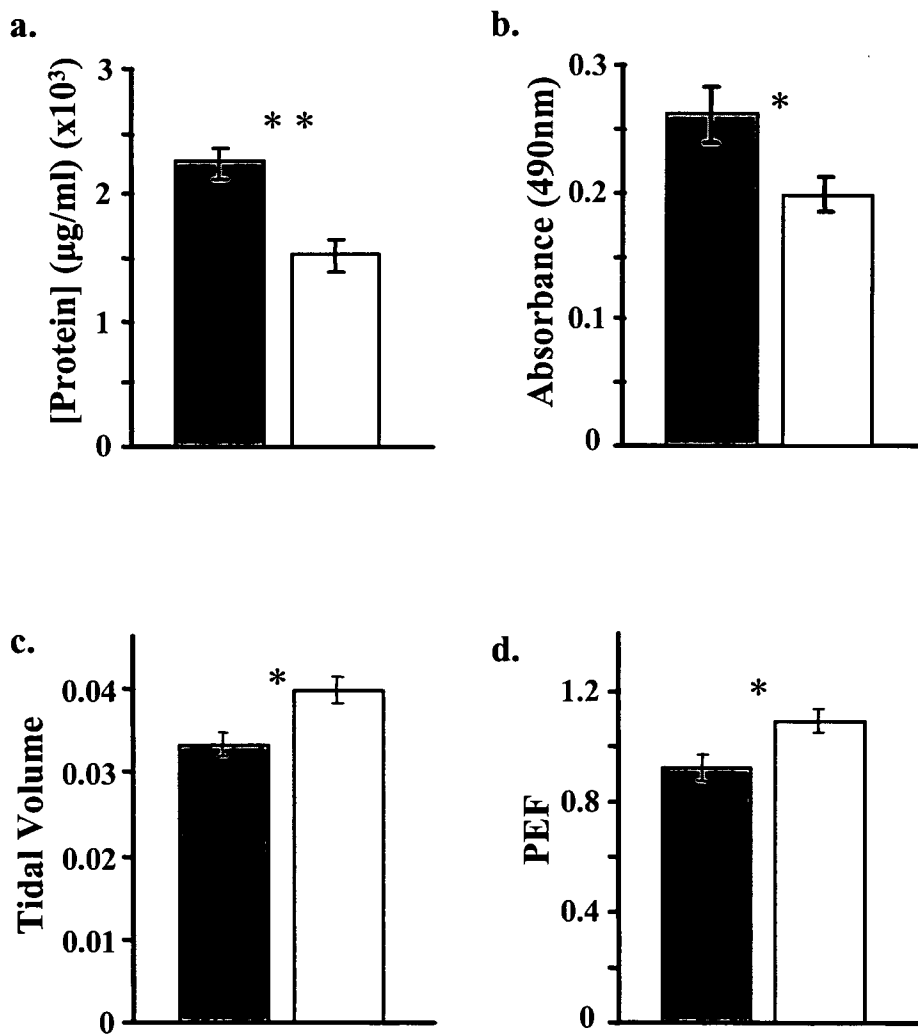
28/100

Figure 3.14



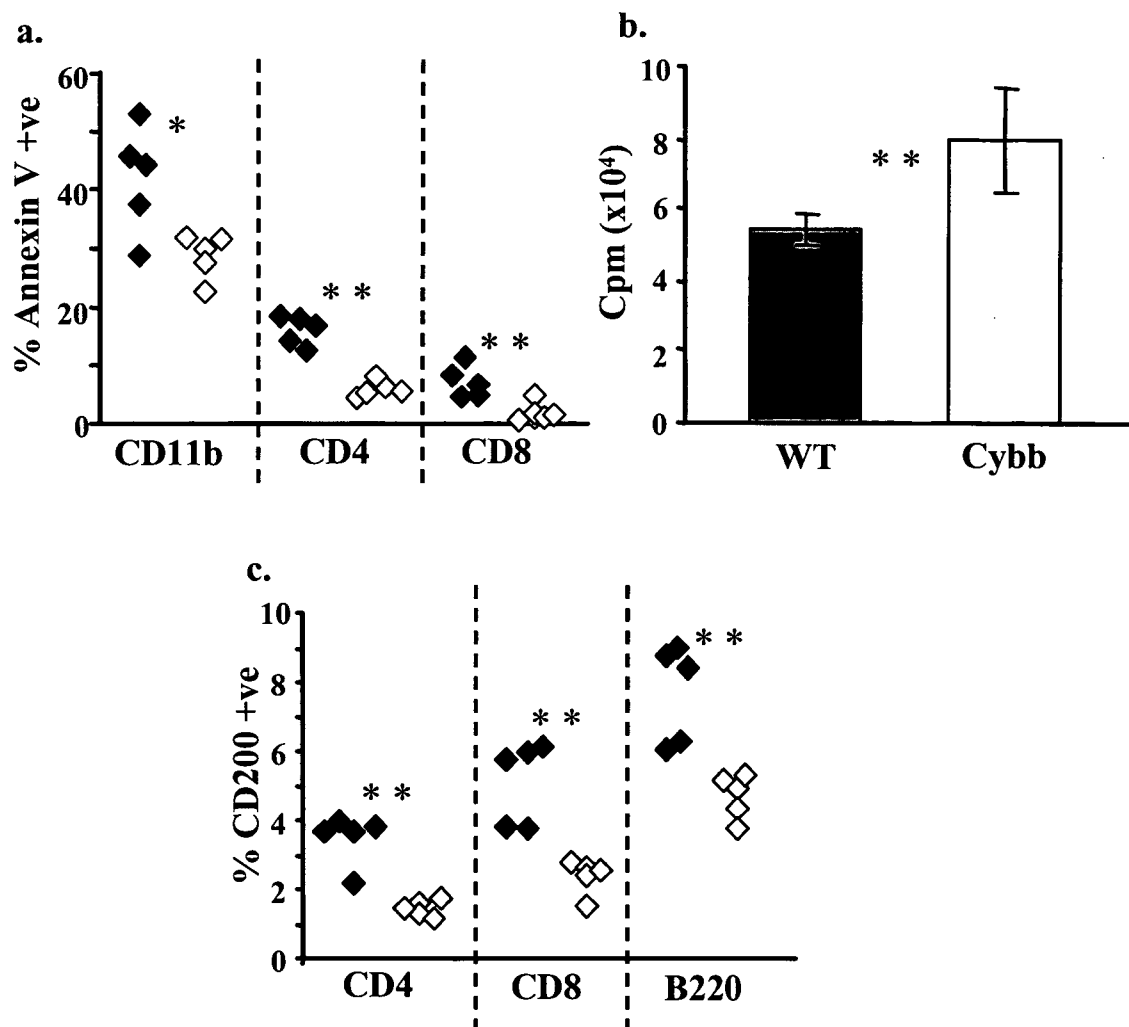
29/100

Figure 3.15



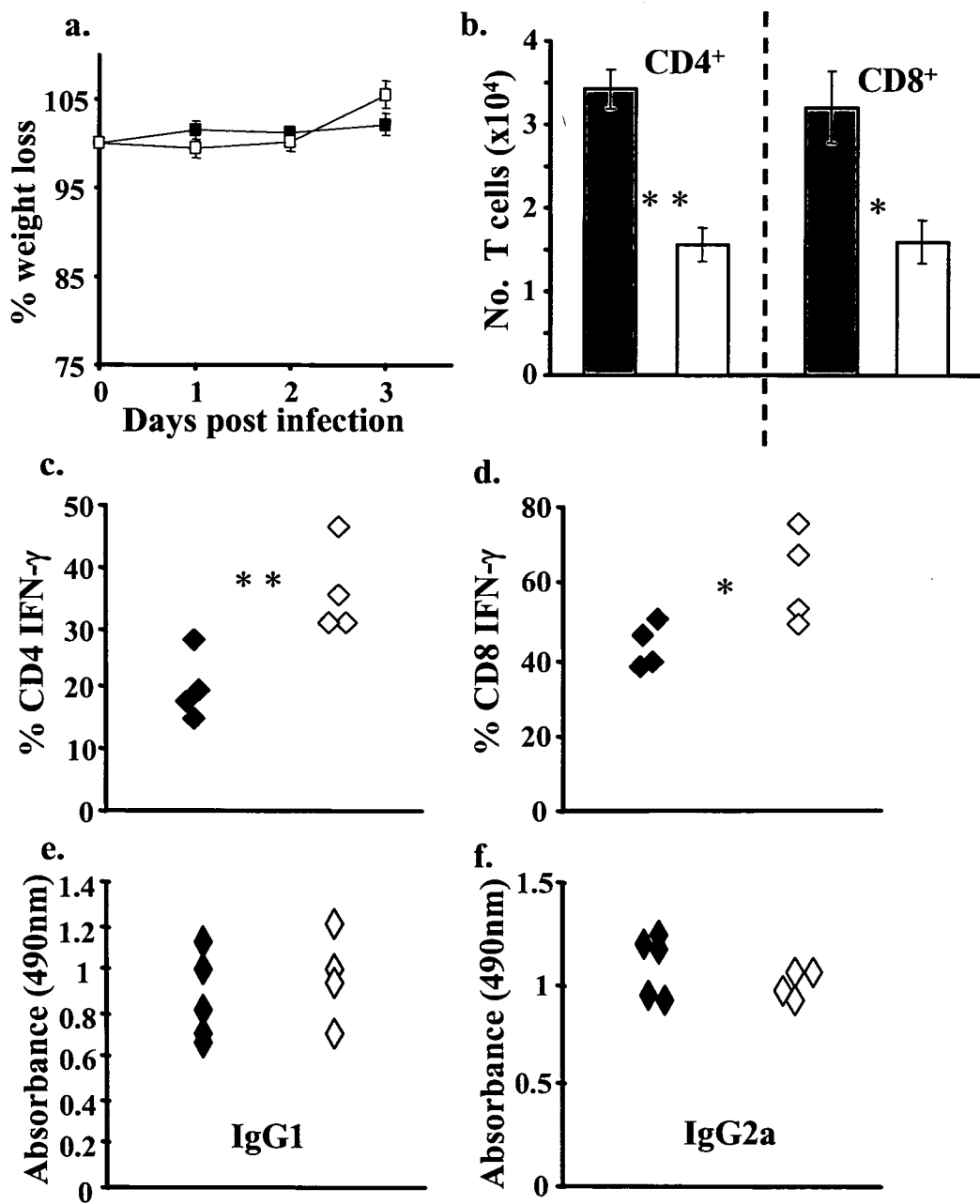
30/100

Figure 3.16



31/100

Figure 3.17

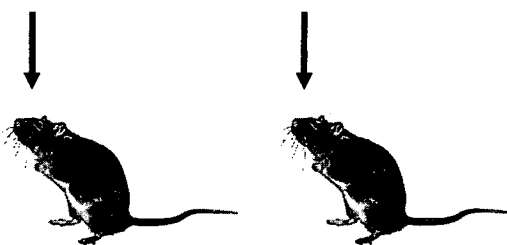


32/100

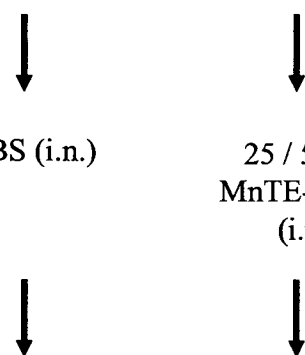
Figure 3.18

Day 0

50 HA Influenza A x31 (i.n)

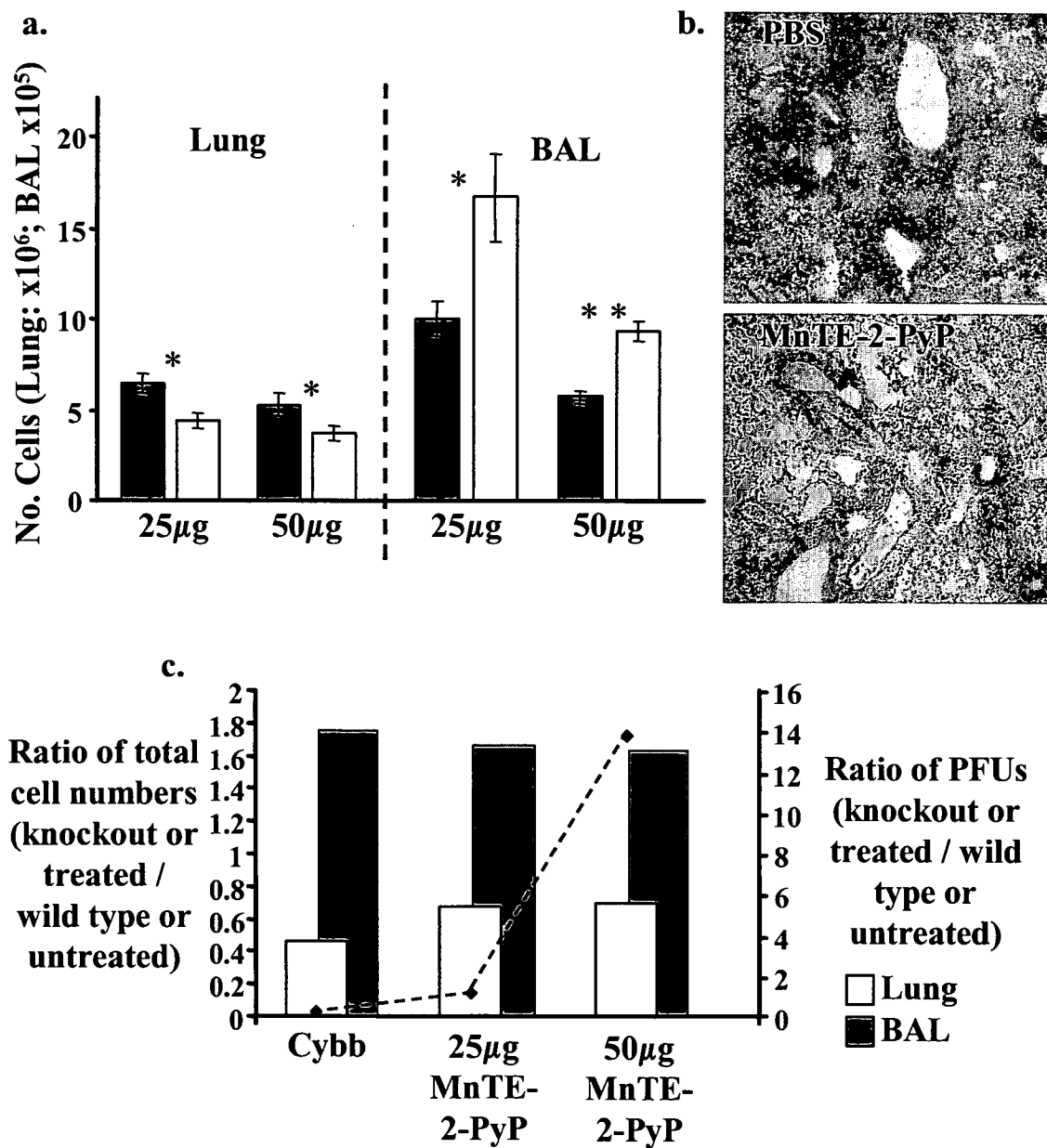
**Day 0, 2,
4 and 6**

PBS (i.n.)

25 / 50 μ g
MnTE-2-PyP
(i.n)**Day 7****BAL:** - Surface Staining (flow cytometry)
- Cytokines (ELISA)**Lung:** - Surface/intracellular cytokine staining
- Influenza titres
- Histology (H/E)**Serum:-** Influenza-specific antibody
- Cytokines (ELISA)**Nasal Washes:** - Influenza-specific IgA

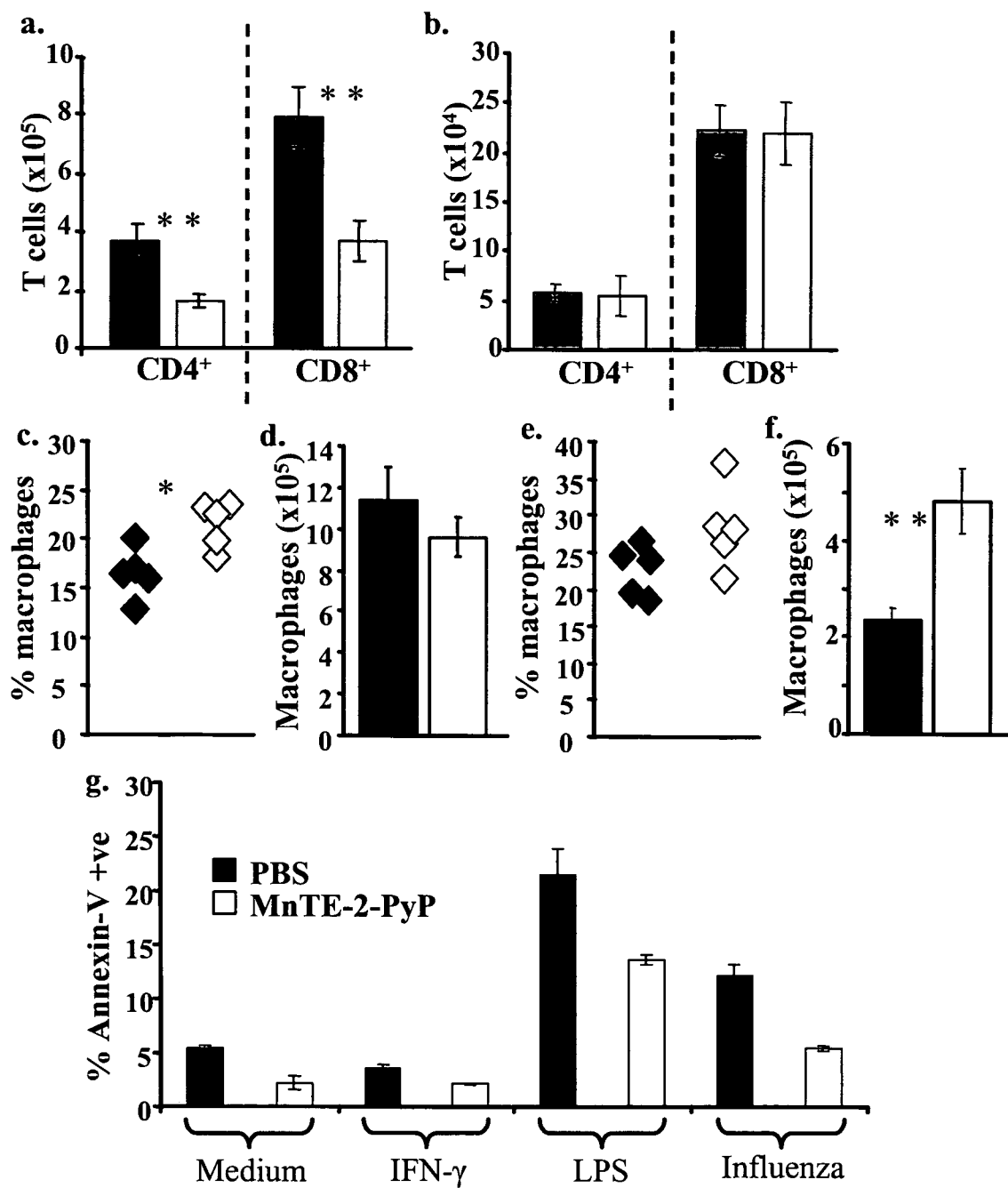
33/100

Figure 3.19



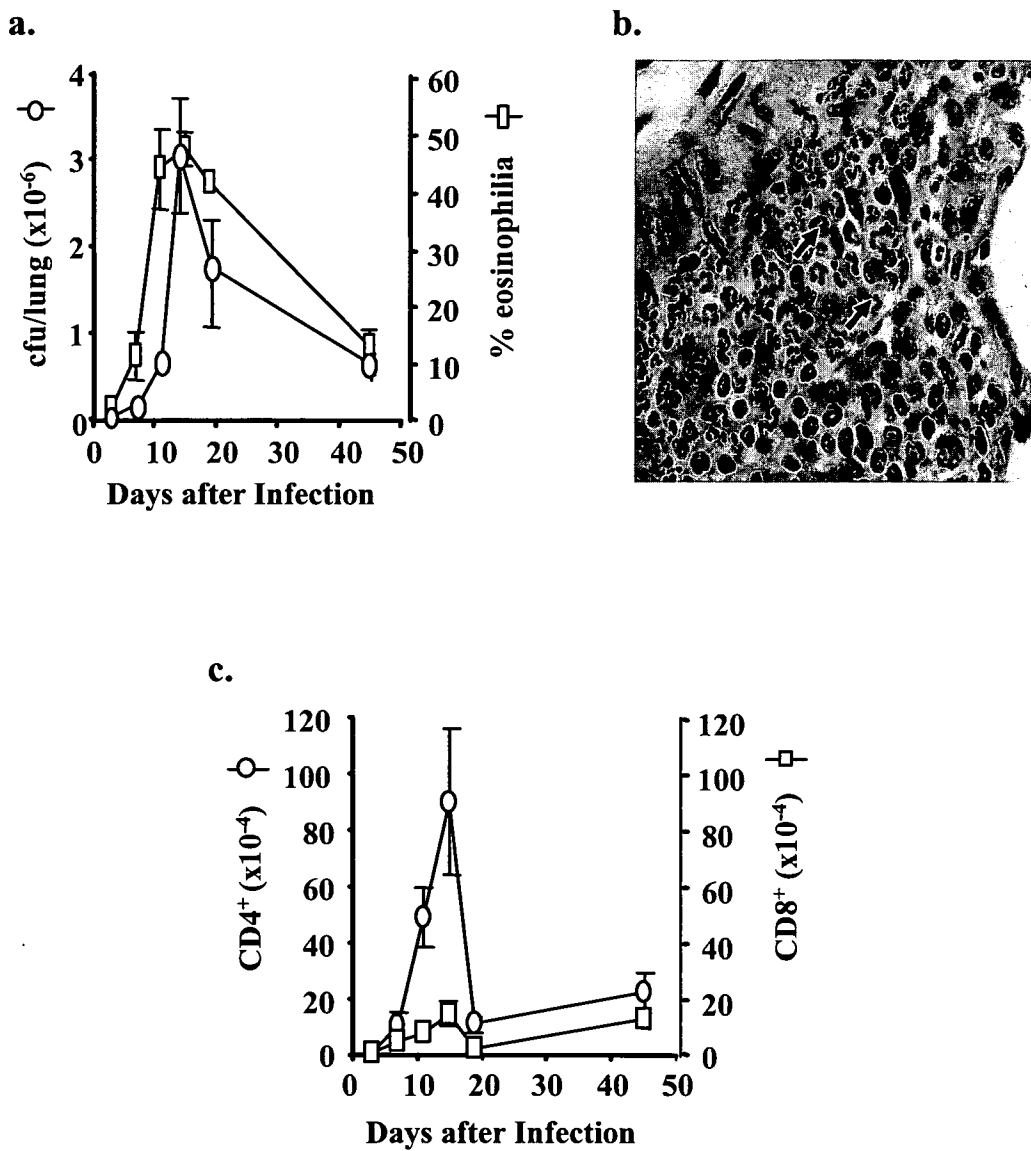
34/100

Figure 3.20



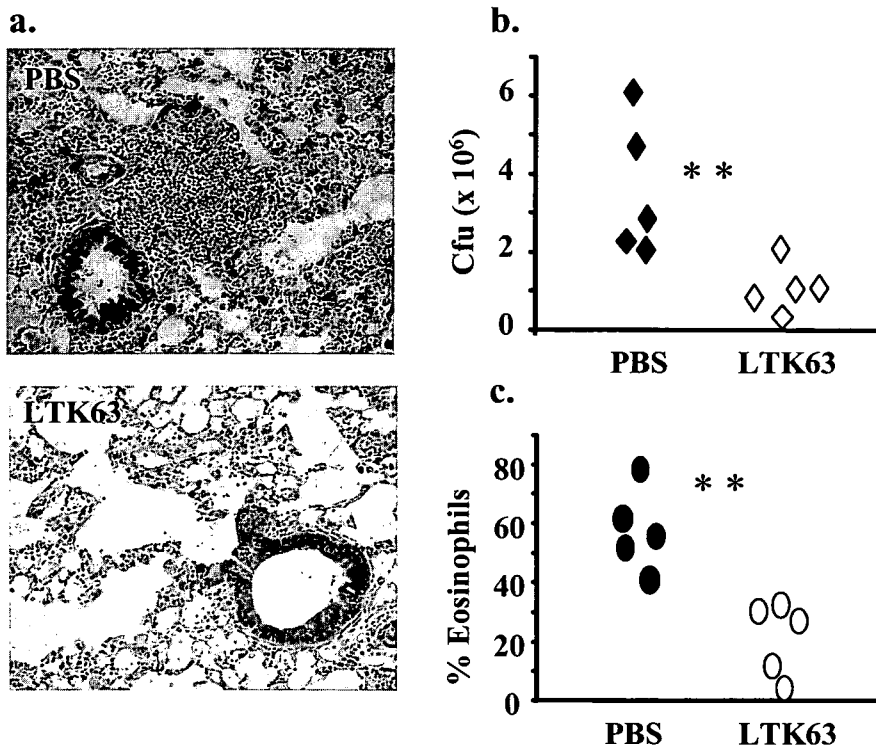
35/100

Figure 4.1



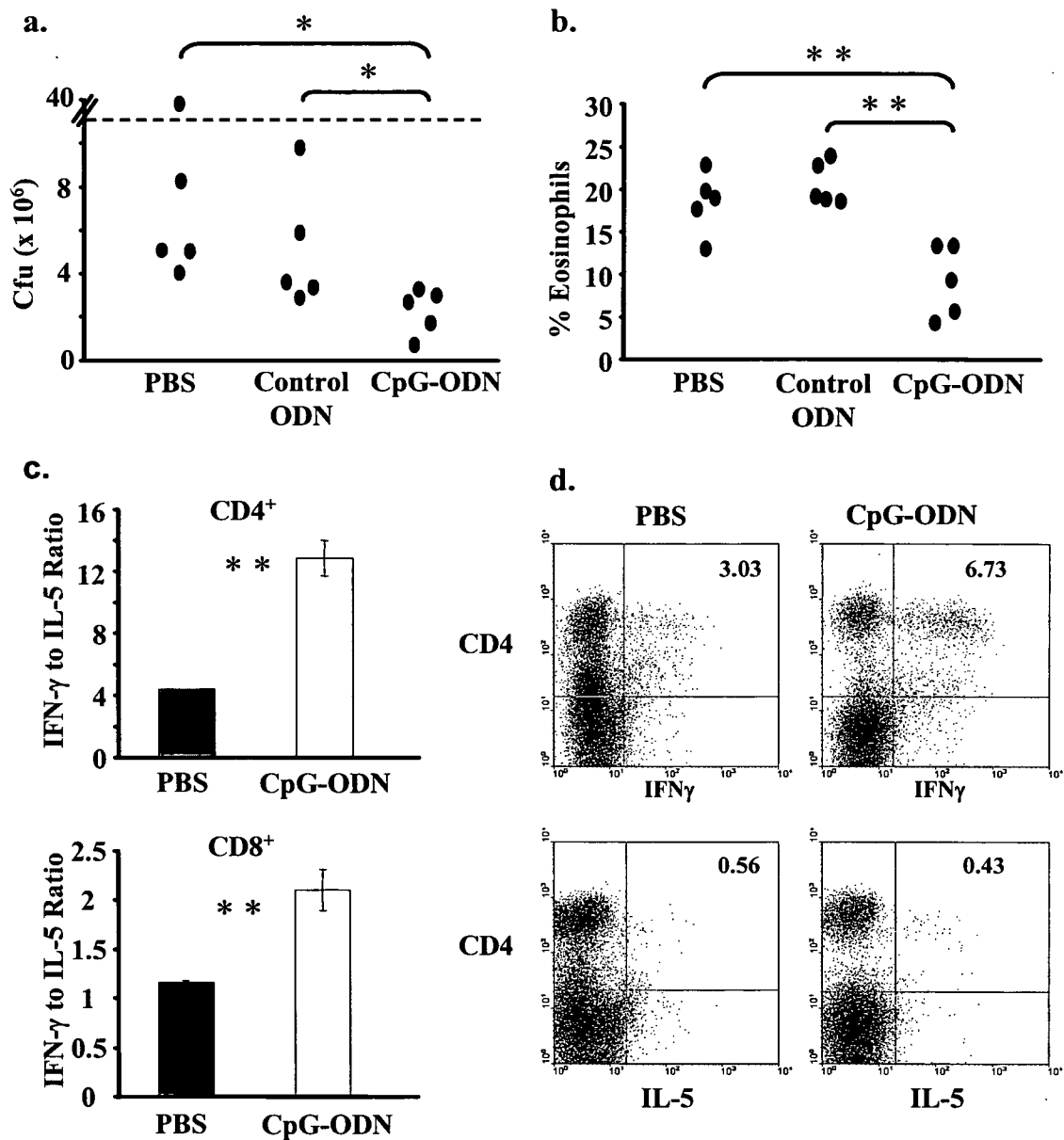
36/100

Figure 4.2



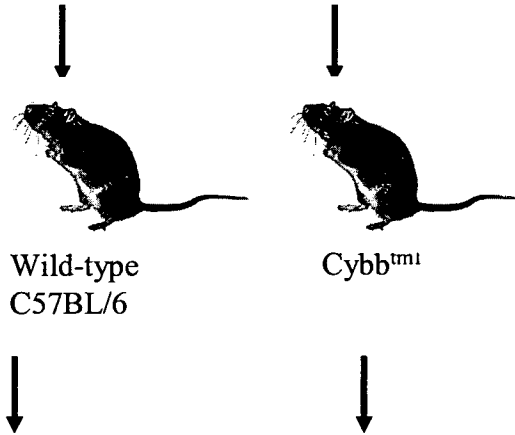
37/100

Figure 4.3



38/100

Figure 4.4

Day 0 1×10^4 *C. neoformans* (i.n)**Day 5, 8,
12 or 35**

BAL: - Surface Staining (flow cytometry)
 - Cytoentrifugation
 - Cytokines (ELISA)
 - *C. neoformans* CFUs

Lung: - Surface/intracellular cytokine staining
 - *C. neoformans* CFUs
 - Histology (H/E)

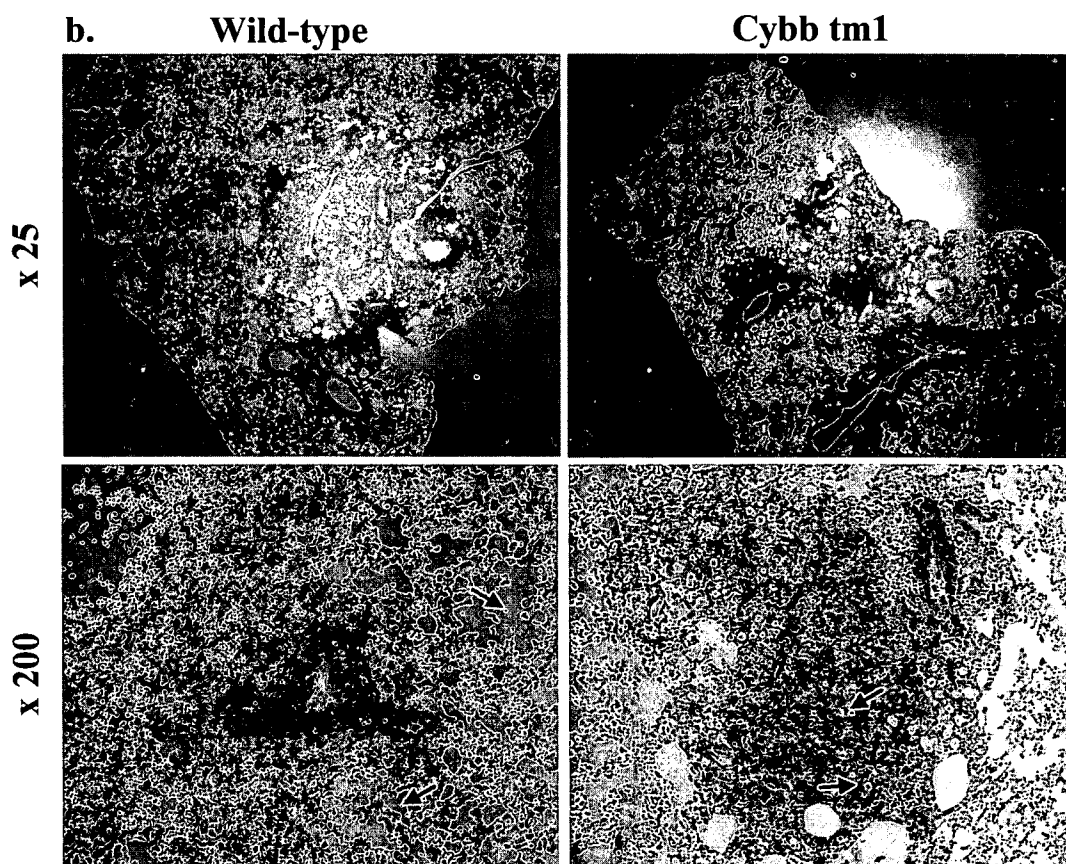
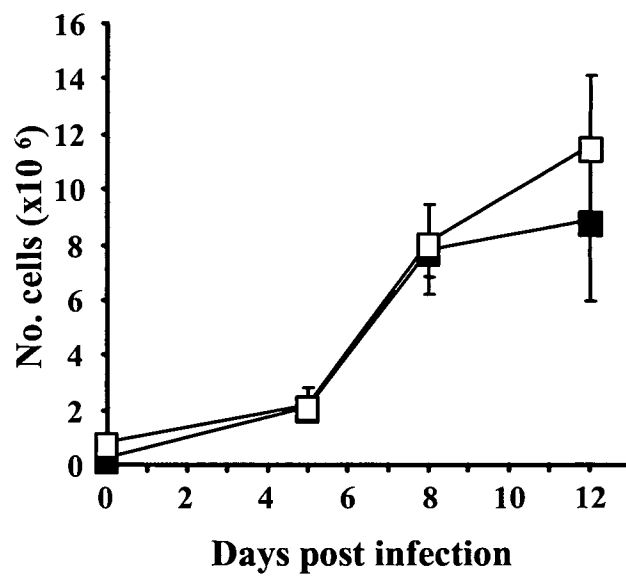
Brain / Spleen:
 - *C. neoformans* CFUs

Serum:- Cryptococcus-specific antibody

Nasal Washes: - IgE

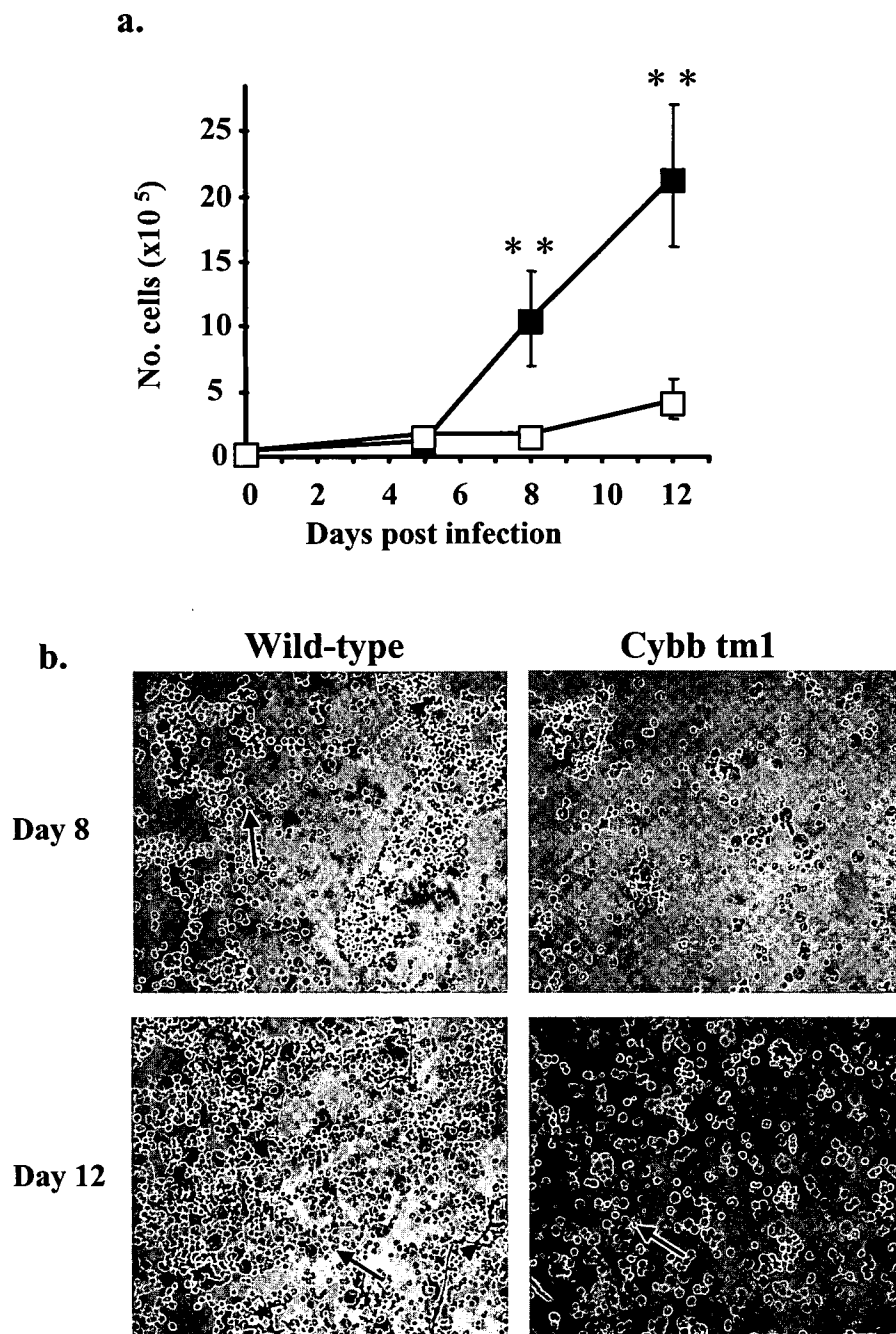
39/100

Figure 4.5



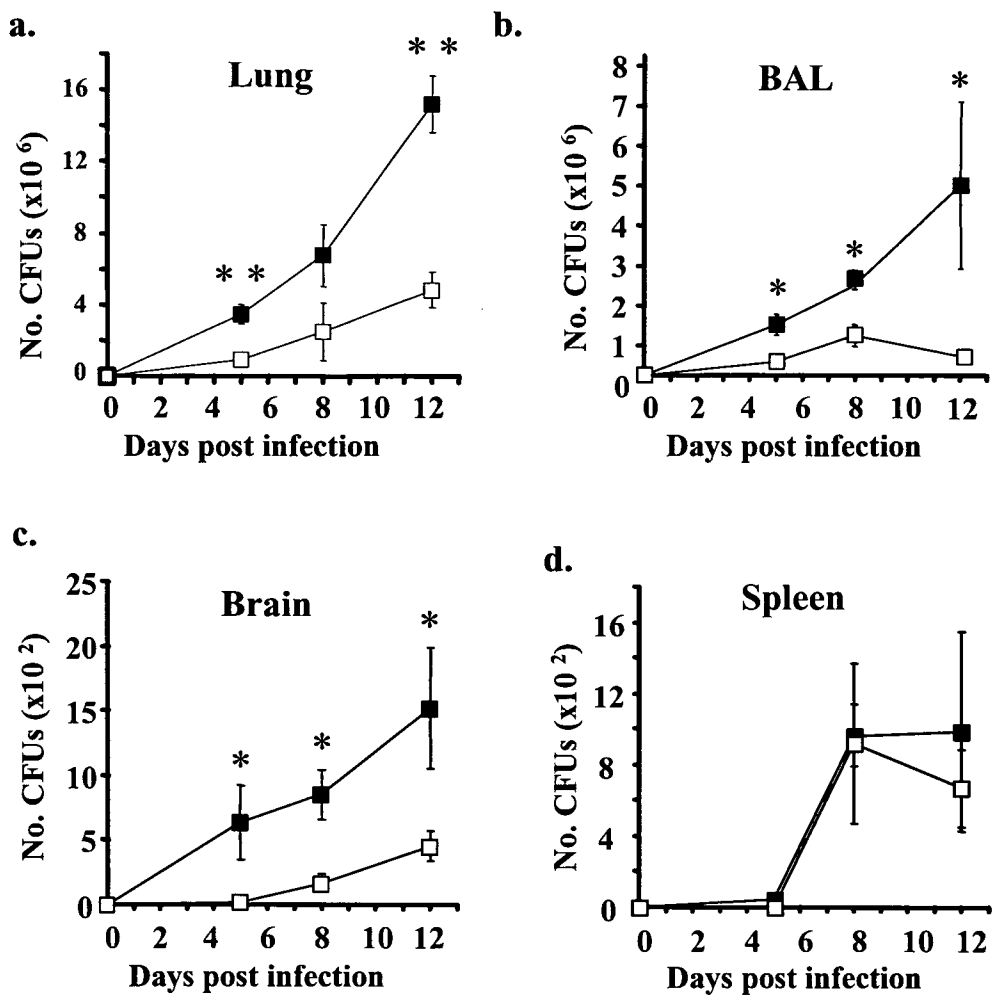
40/100

Figure 4.6



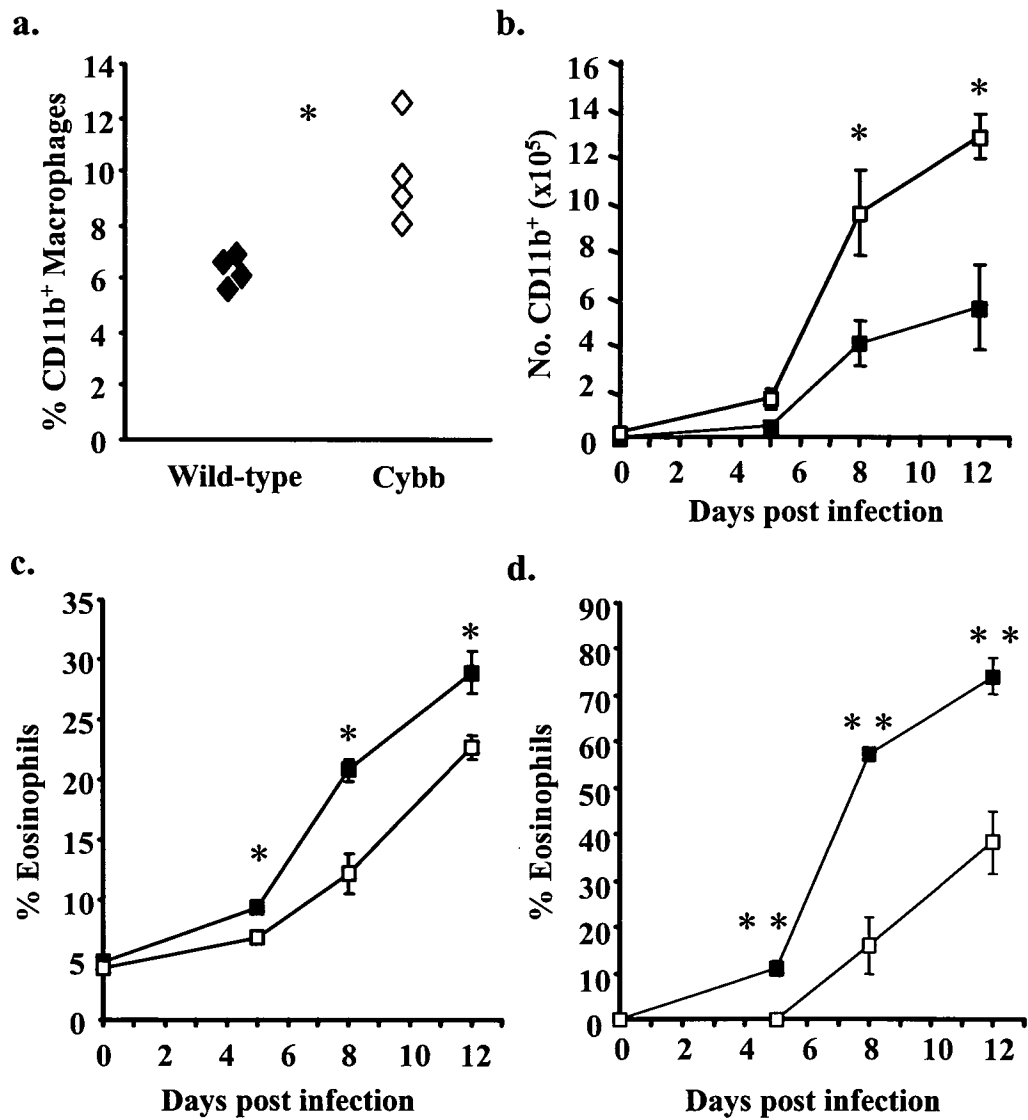
41/100

Figure 4.7



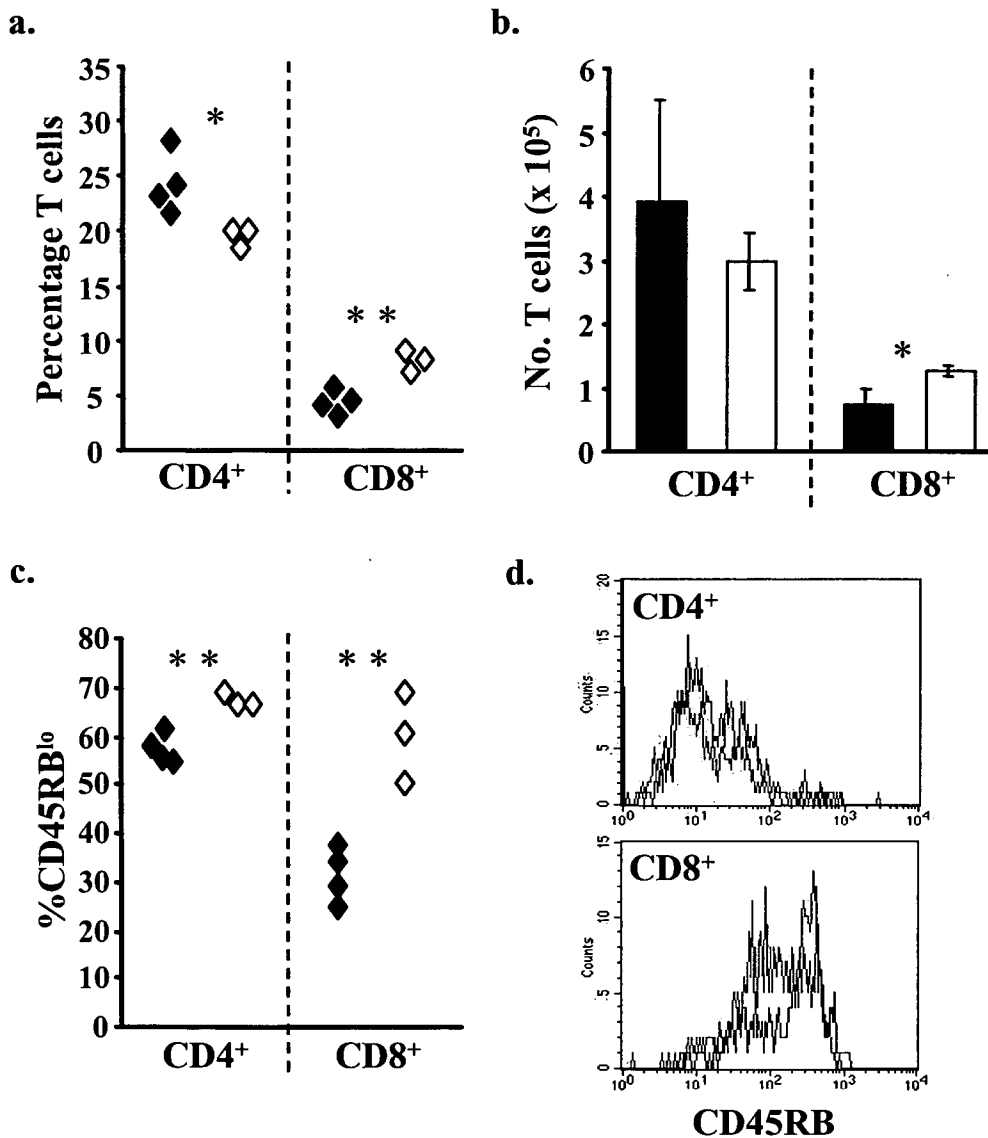
42/100

Figure 4.8



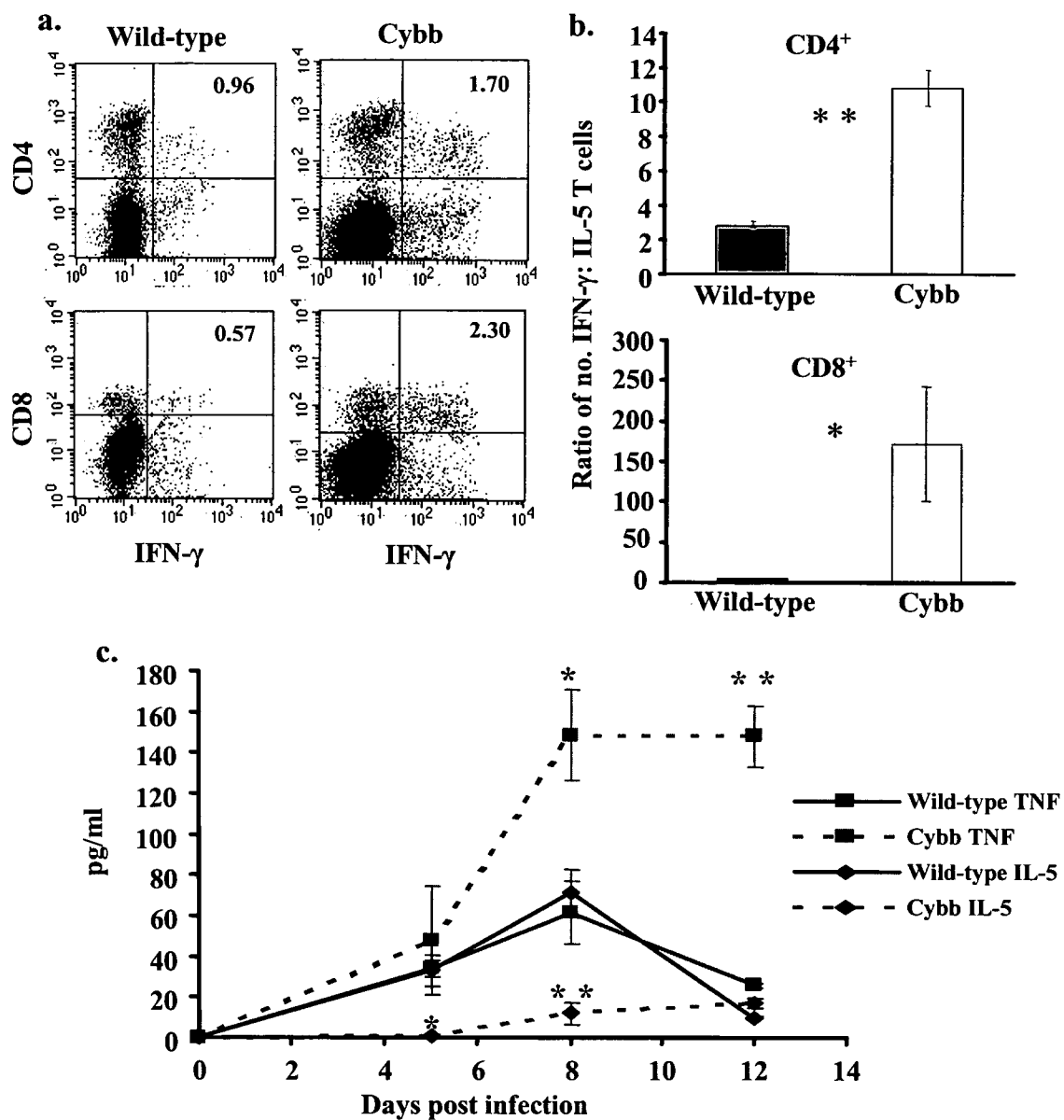
43/100

Figure 4.9



44/100

Figure 4.10

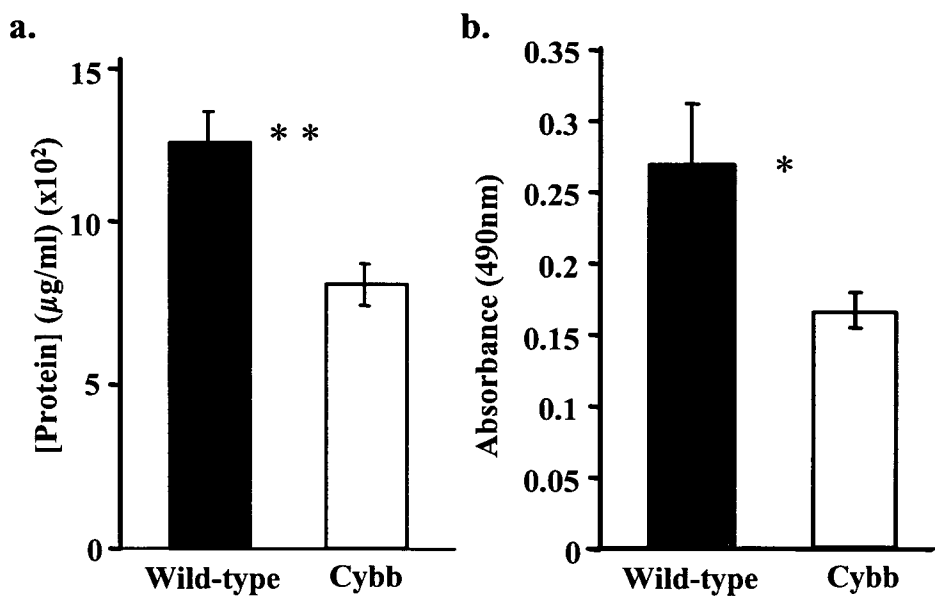


45/100

Table 4.1

Group	% B220	Total Antibody	IgG1	IgE
Wild-type	46.1 +/- 2.1	0.59 +/- 1.7	0.11 +/- 0.009	0.35 +/- 0.056
Cybb	28.3 +/- 4.0	0.13 +/- 0.02	0.09 +/- 0.003	0.087 +/- 0.021

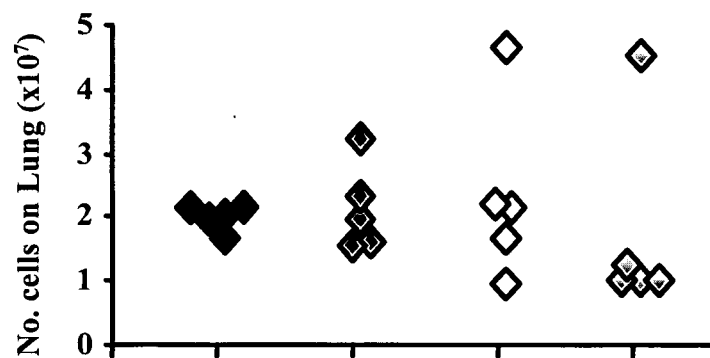
Figure 4.11



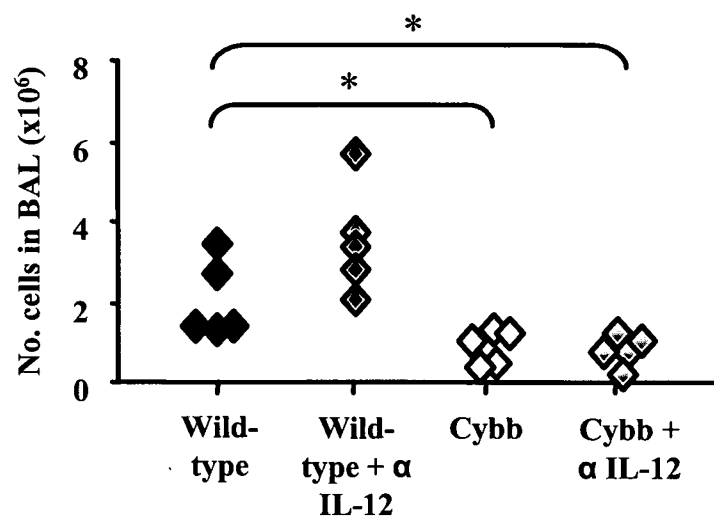
46/100

Figure 4.12

a.

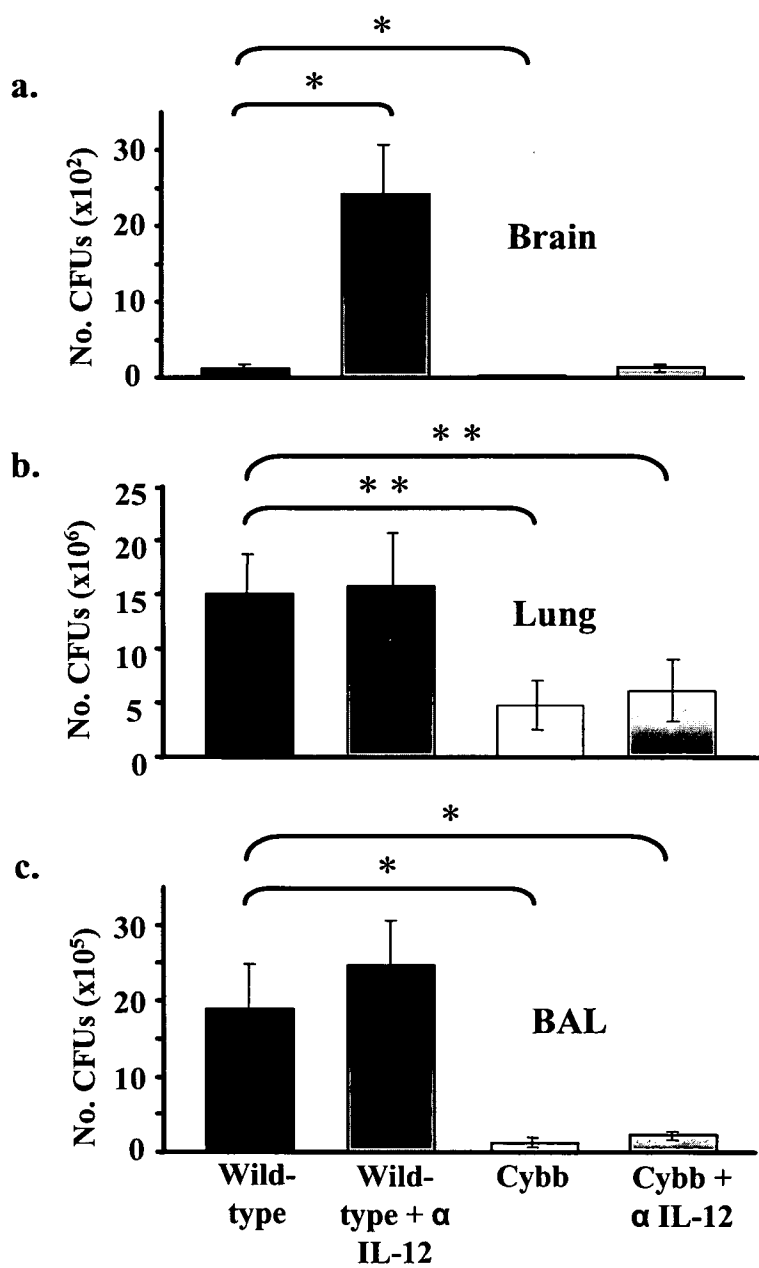


b.



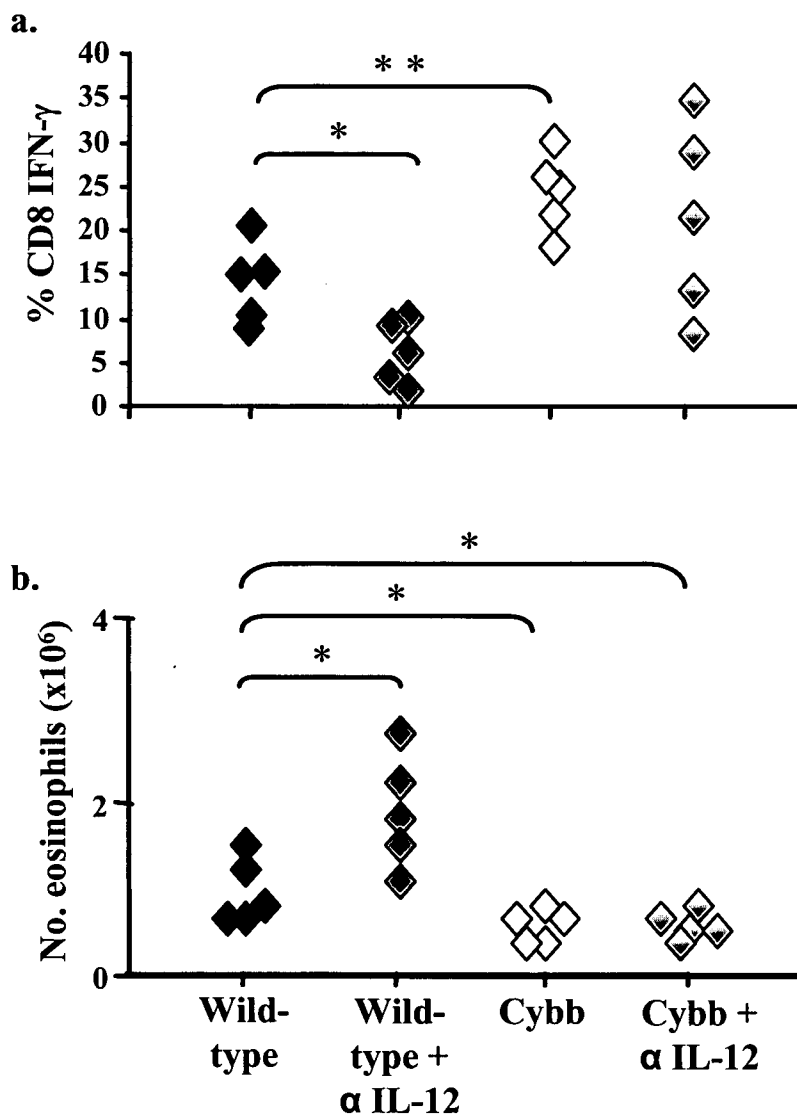
47/100

Figure 4.13



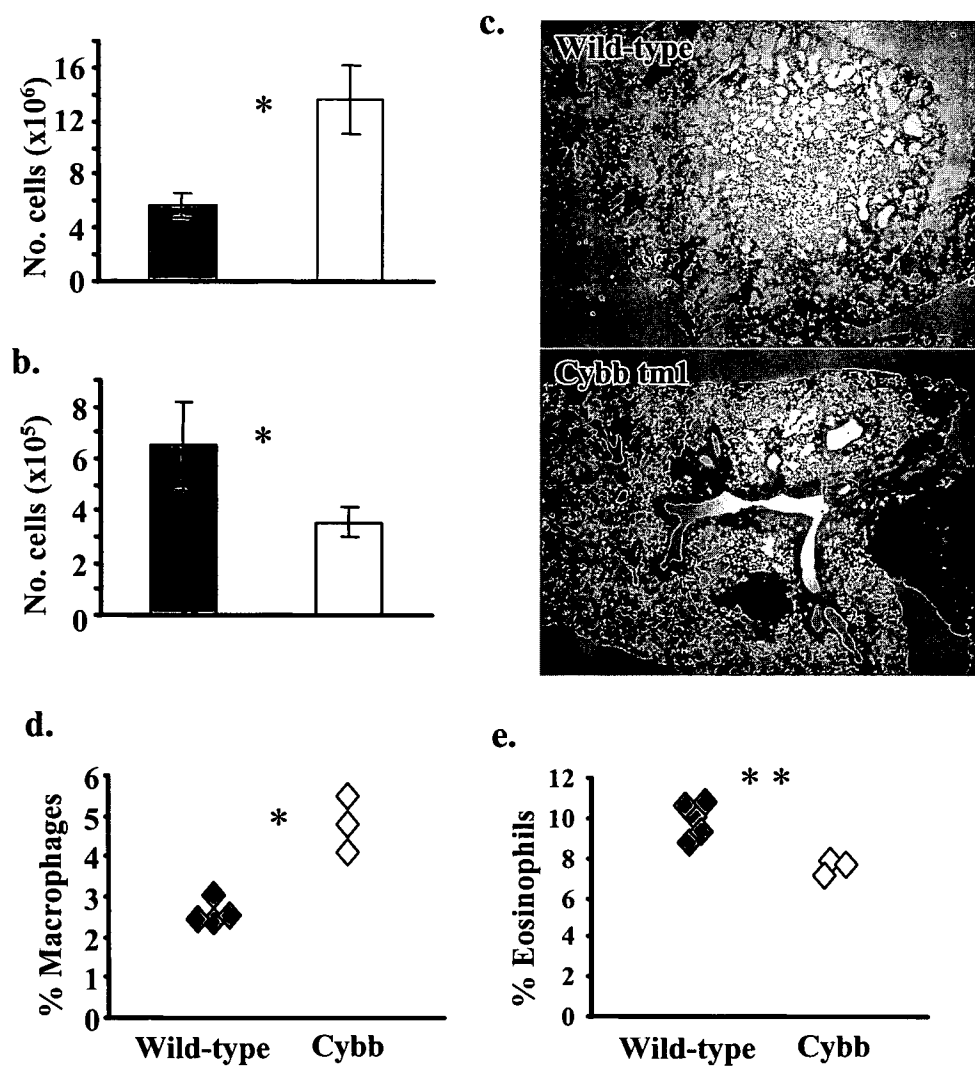
48/100

Figure 4.14



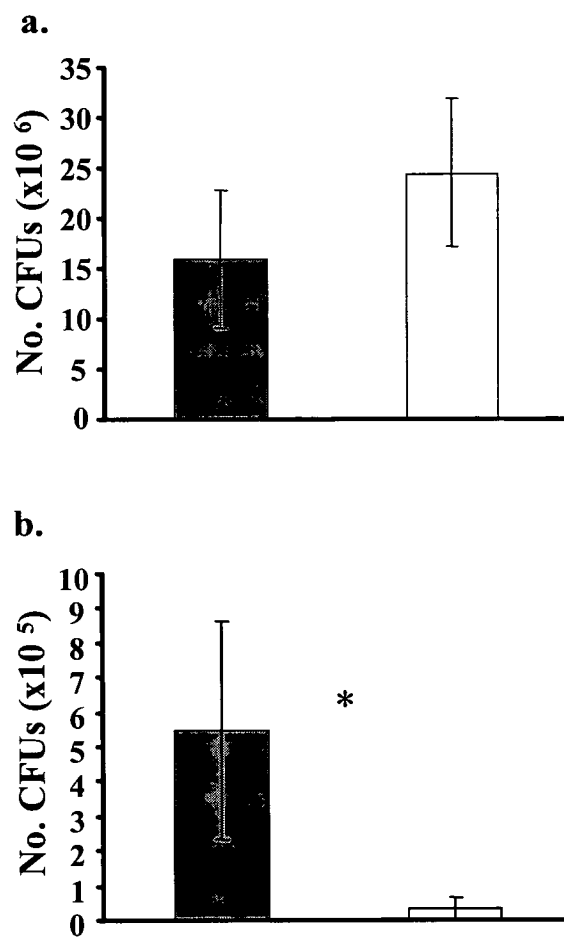
49/100

Figure 4.15



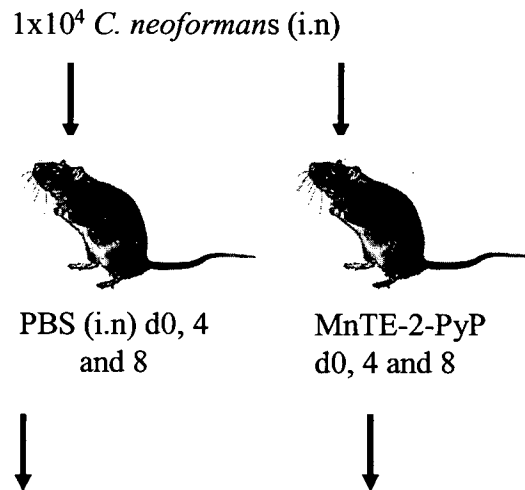
50/100

Figure 50



51/100

Figure 4.16

Day 0**Day 12****BAL:** - Surface Staining (flow cytometry)

- Cytoentrifugation

- Cytokines (ELISA)

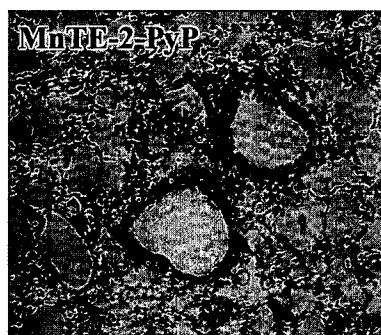
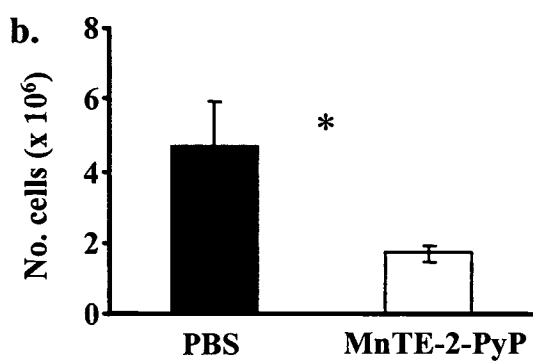
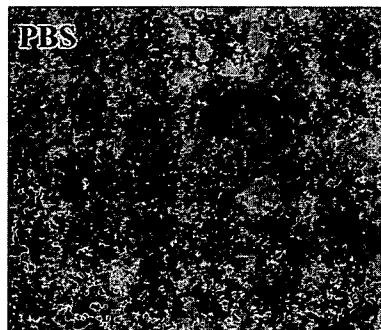
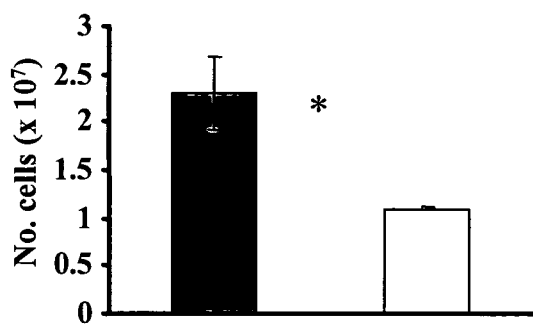
- *C. neoformans* CFUs**Lung:** - Surface/intracellular cytokine staining- *C. neoformans* CFUs

- Histology (H/E)

Brain / Spleen:- *C. neoformans* CFUs**Serum:-** Cryptococcus-specific antibody**Nasal Washes:** - IgE

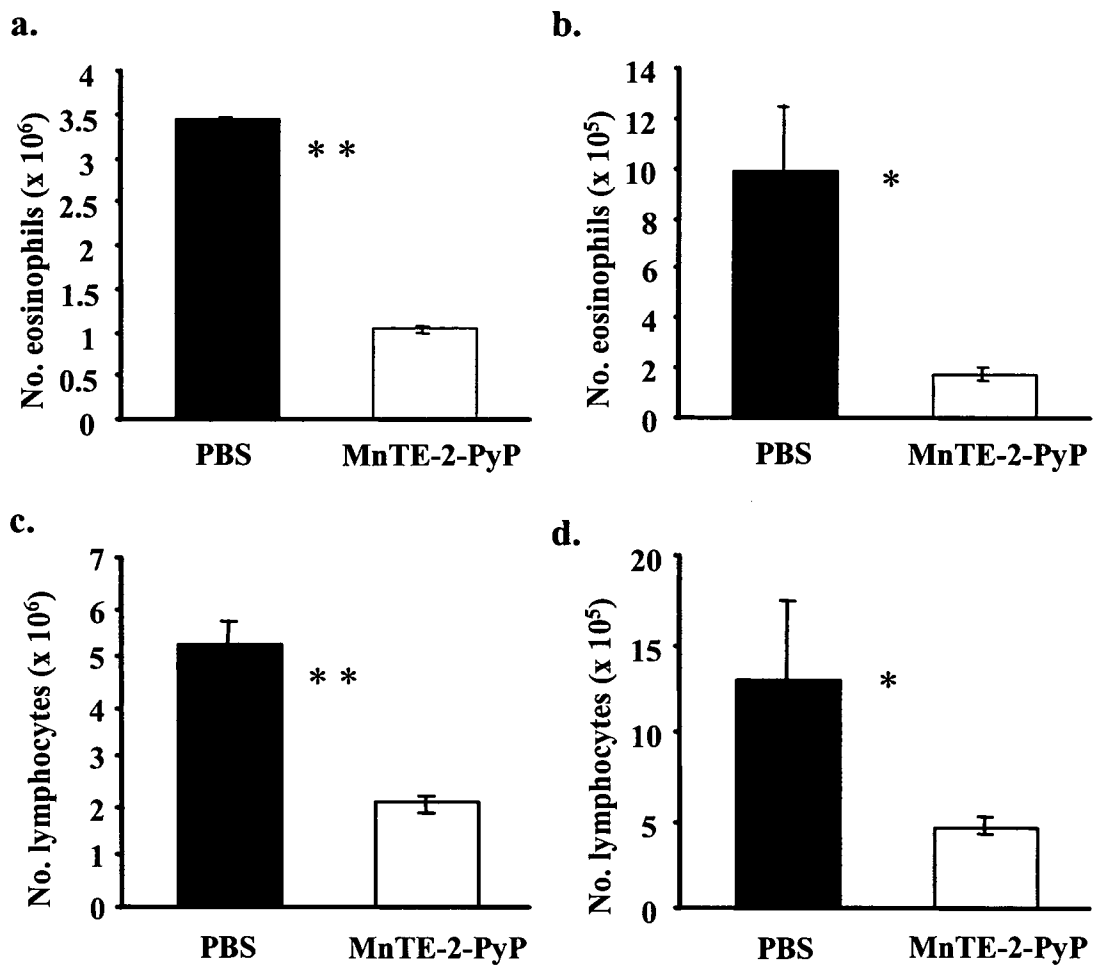
52/100

Figure 4.18



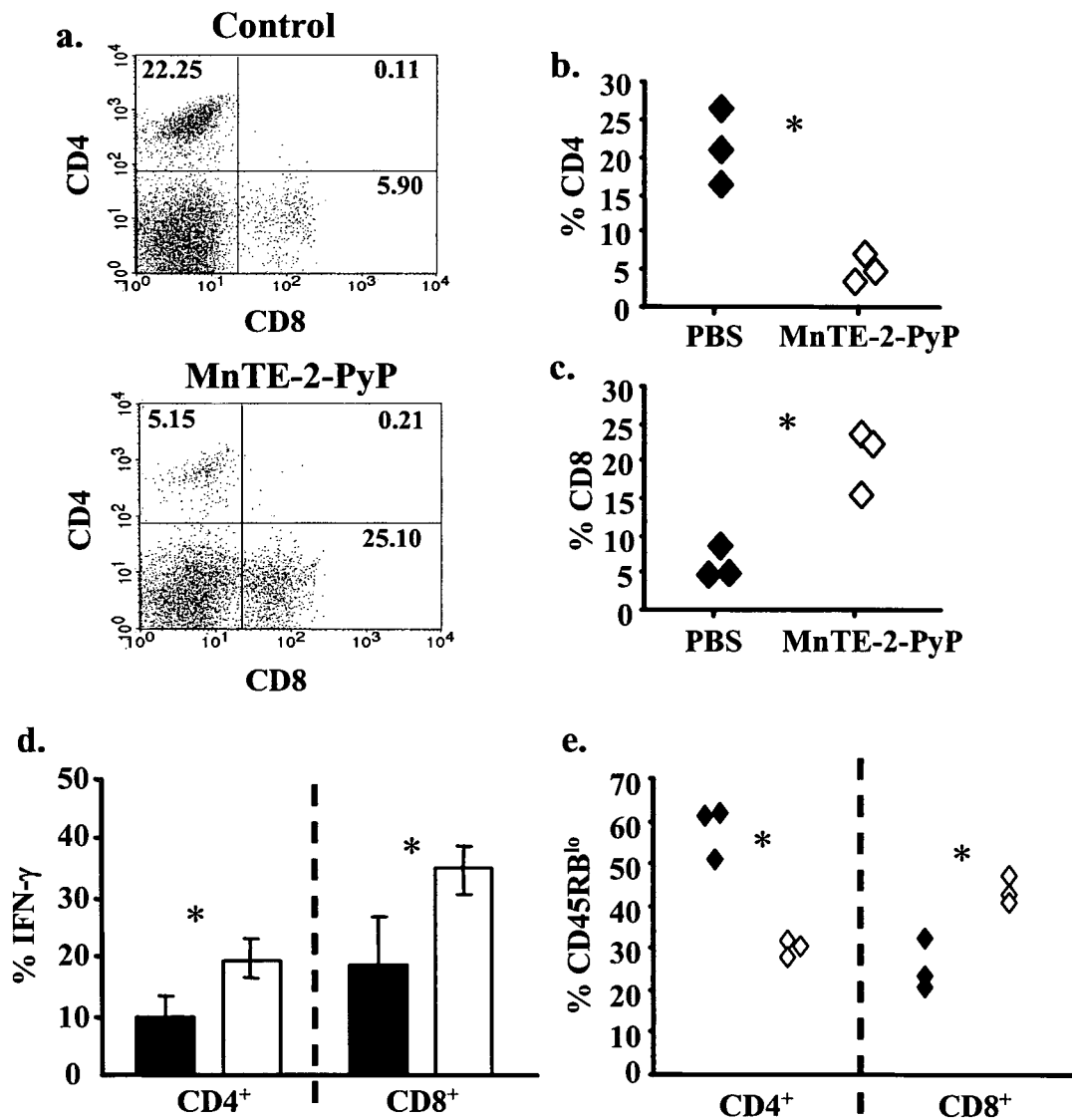
53/100

Figure 4.19



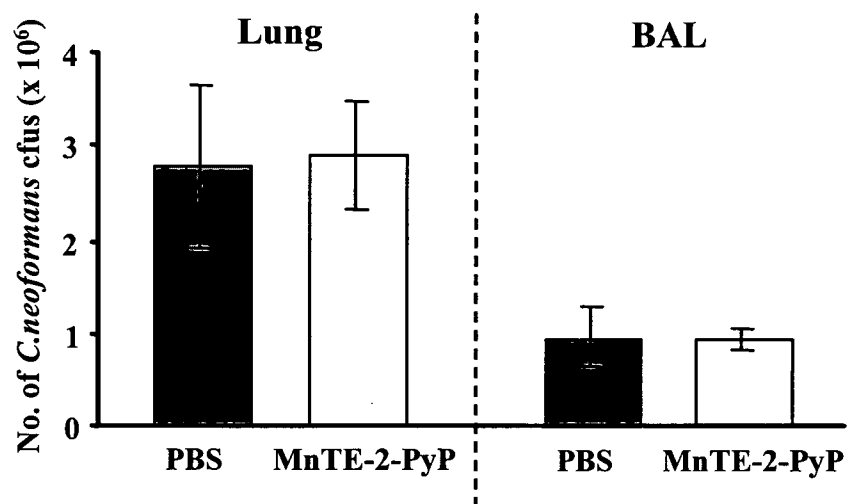
54/100

Figure 4.20

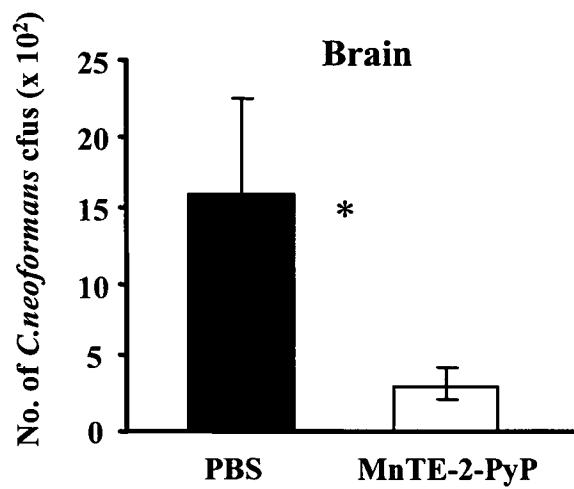


55/100

Figure 4.21

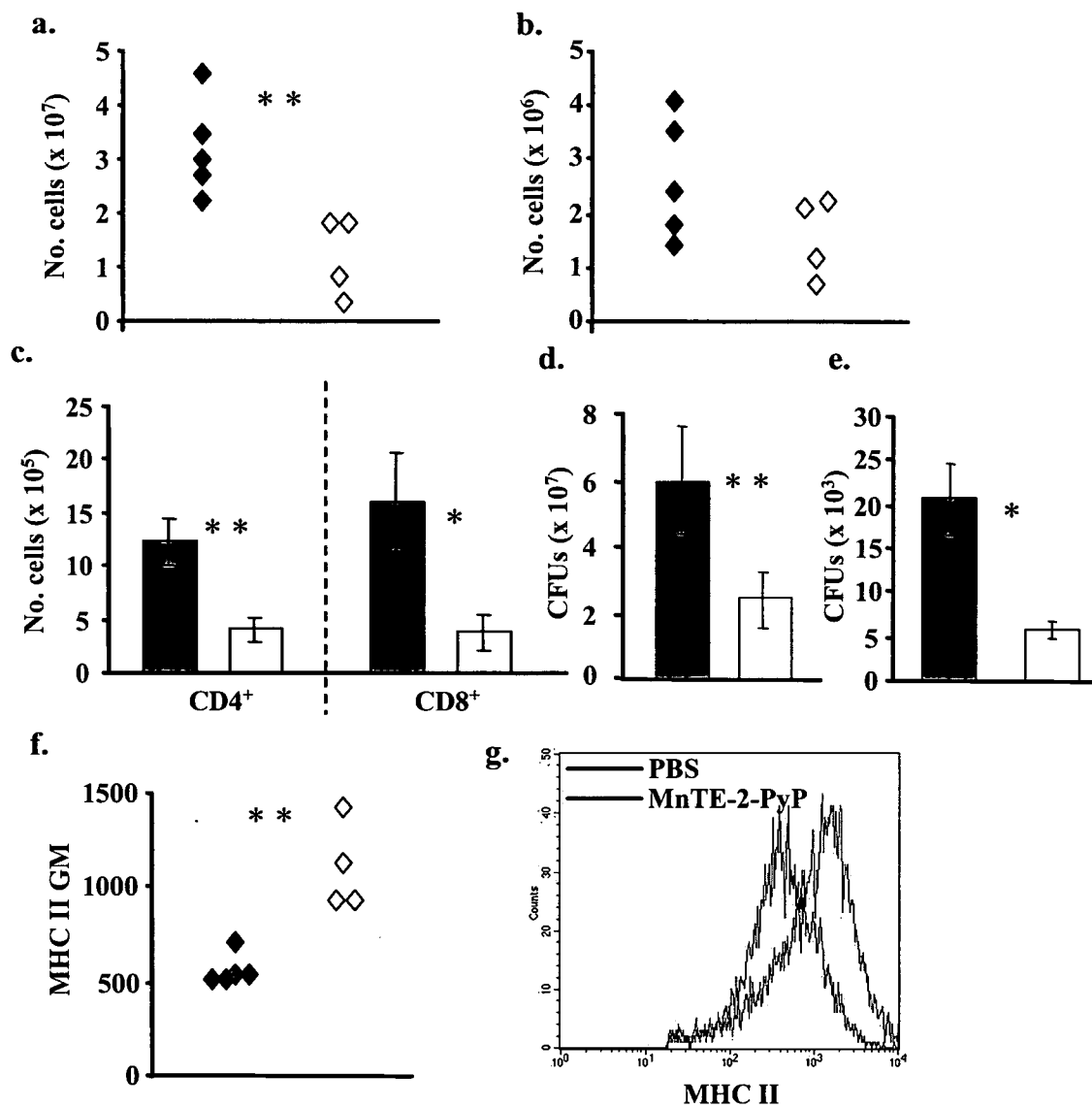


b.



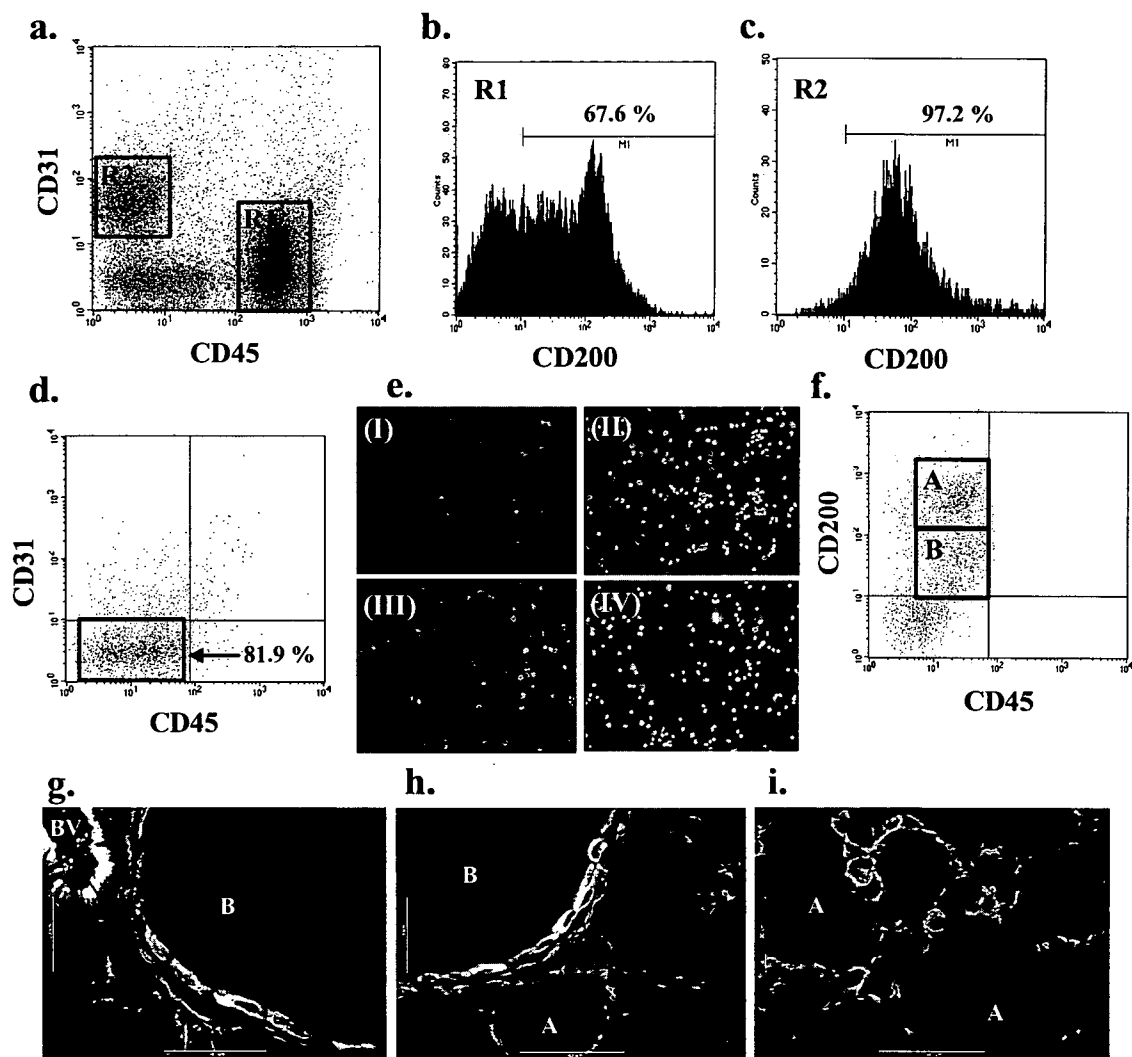
56/100

Figure 4.22



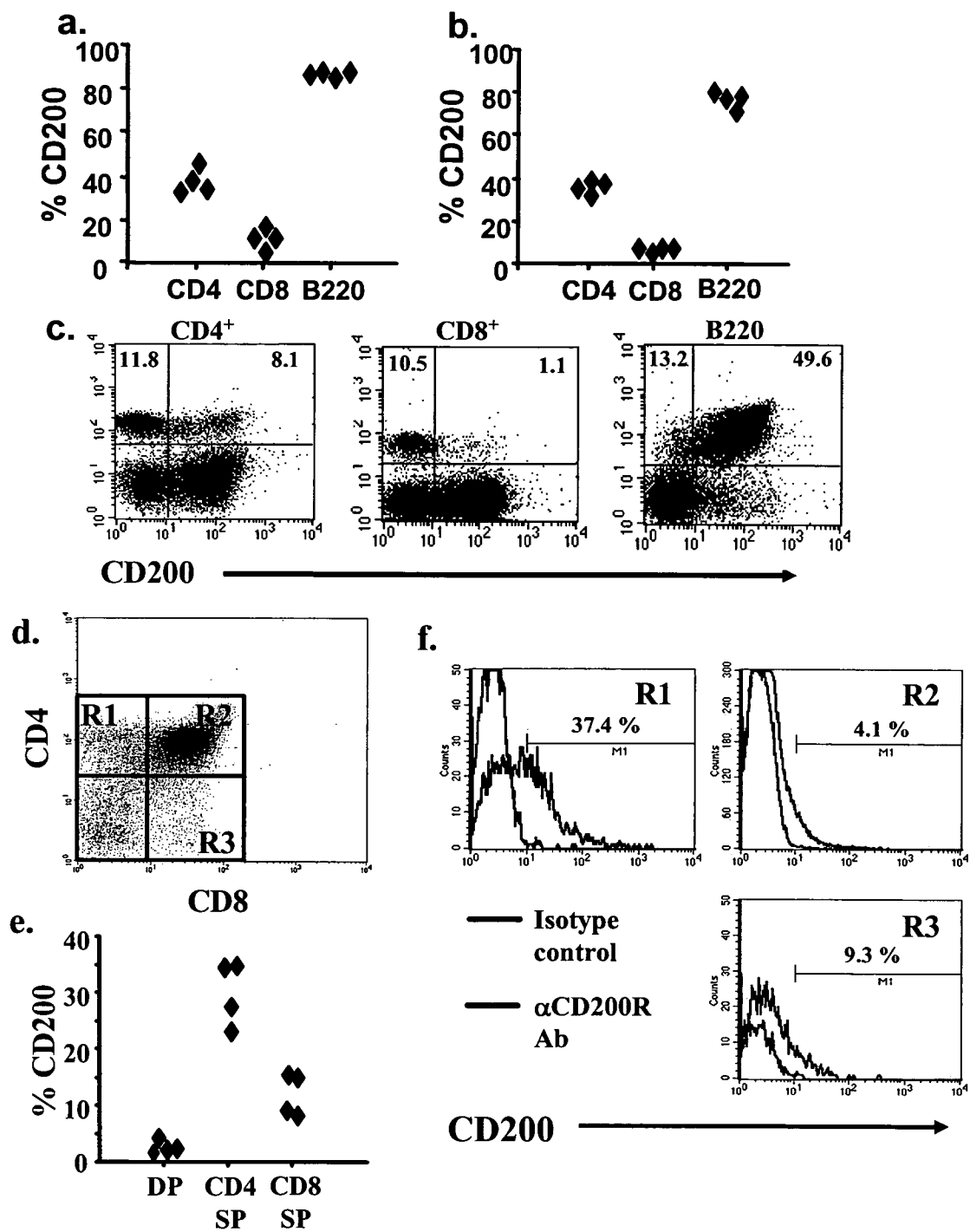
57/100

Figure 5.1



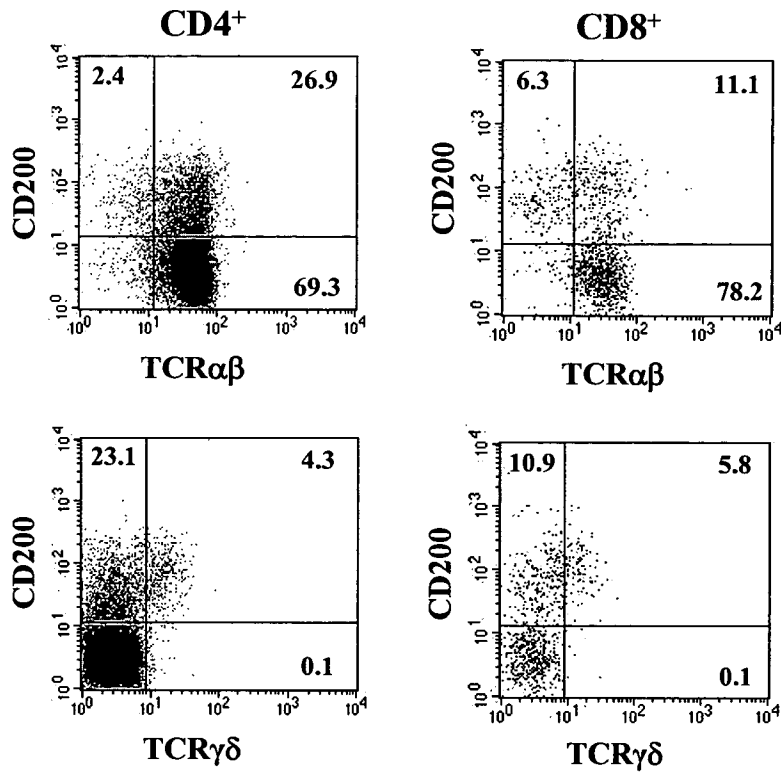
58/100

Figure 5.2



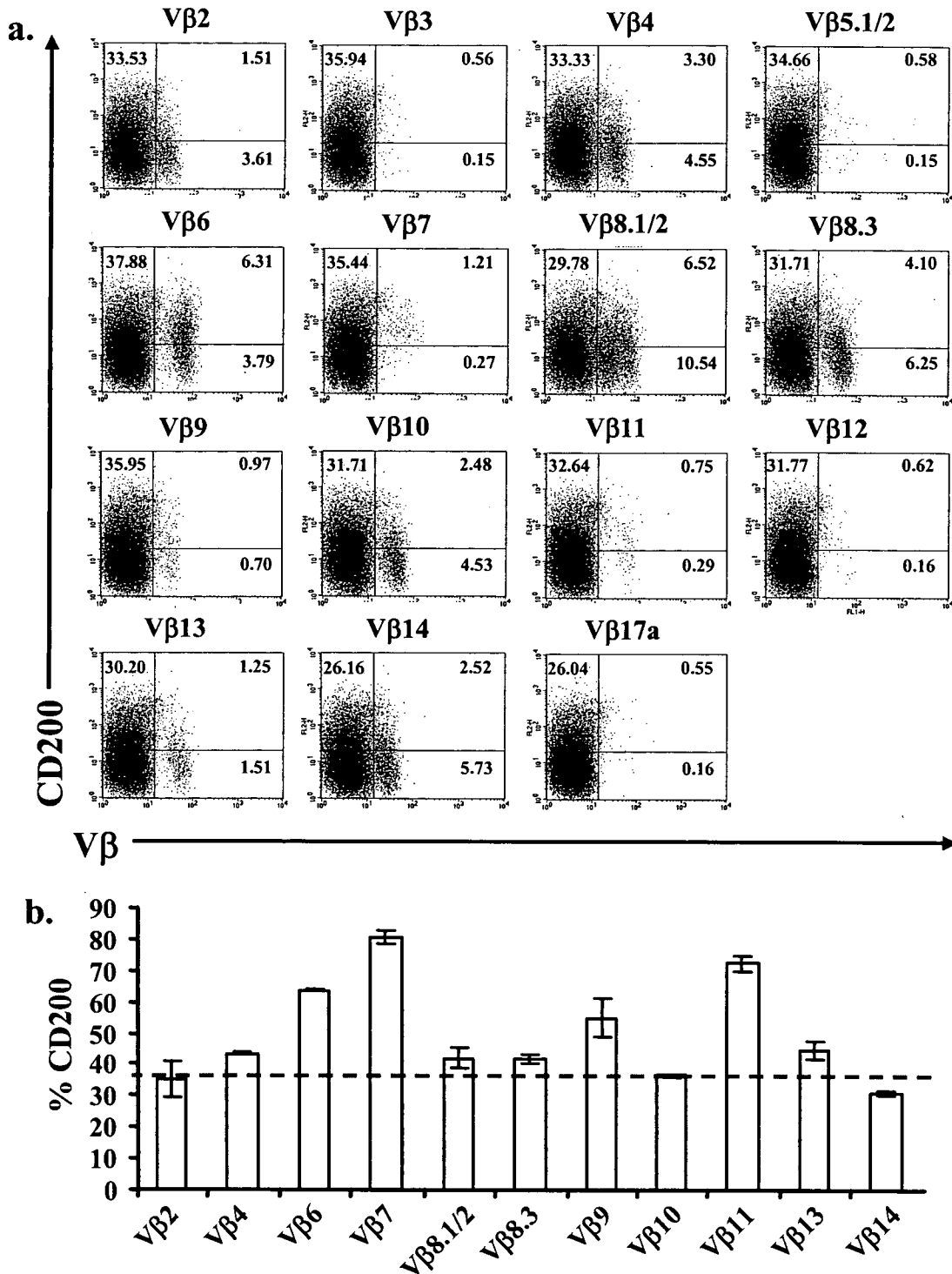
59/100

Figure 5.3



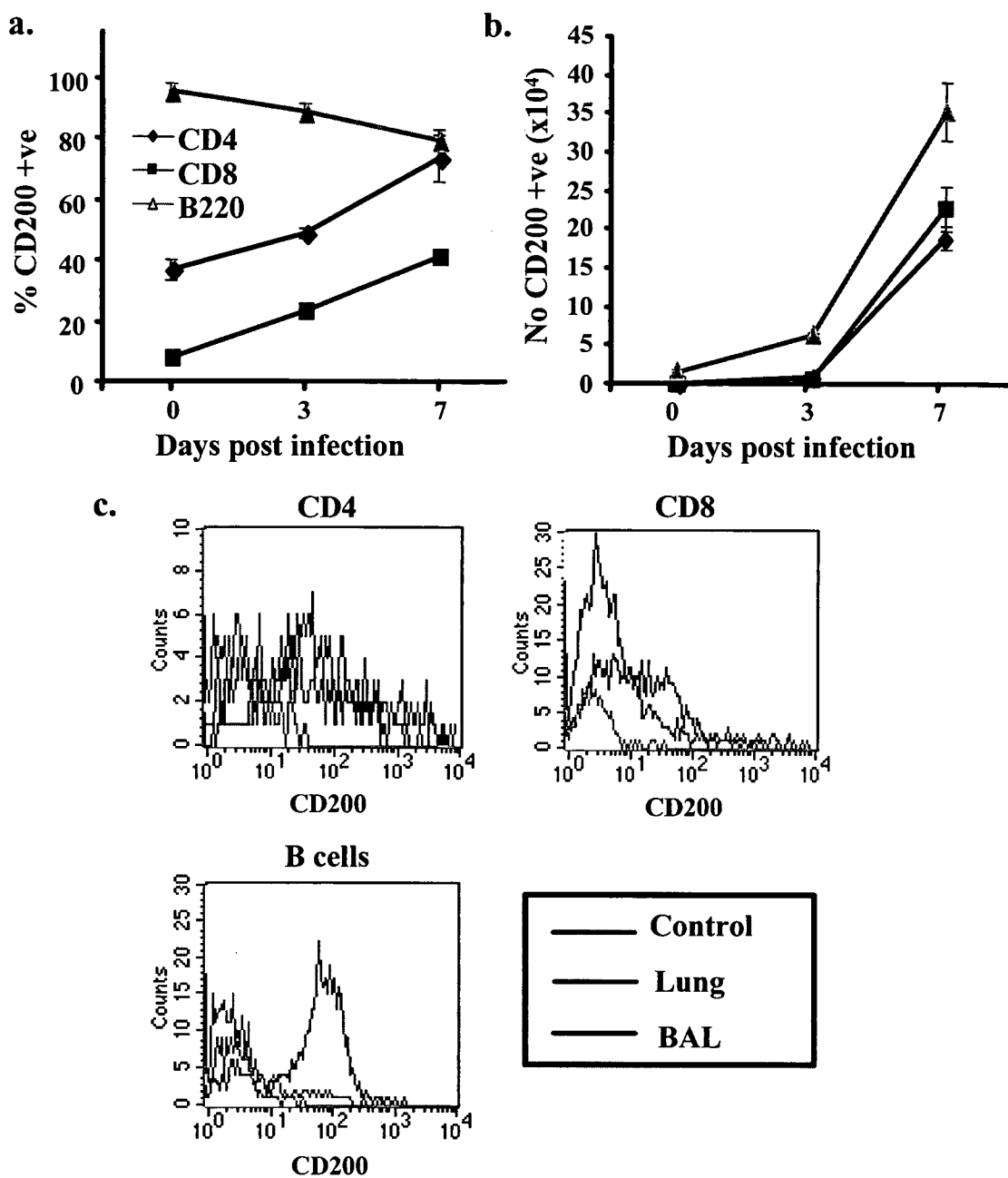
60/100

Figure 5.4



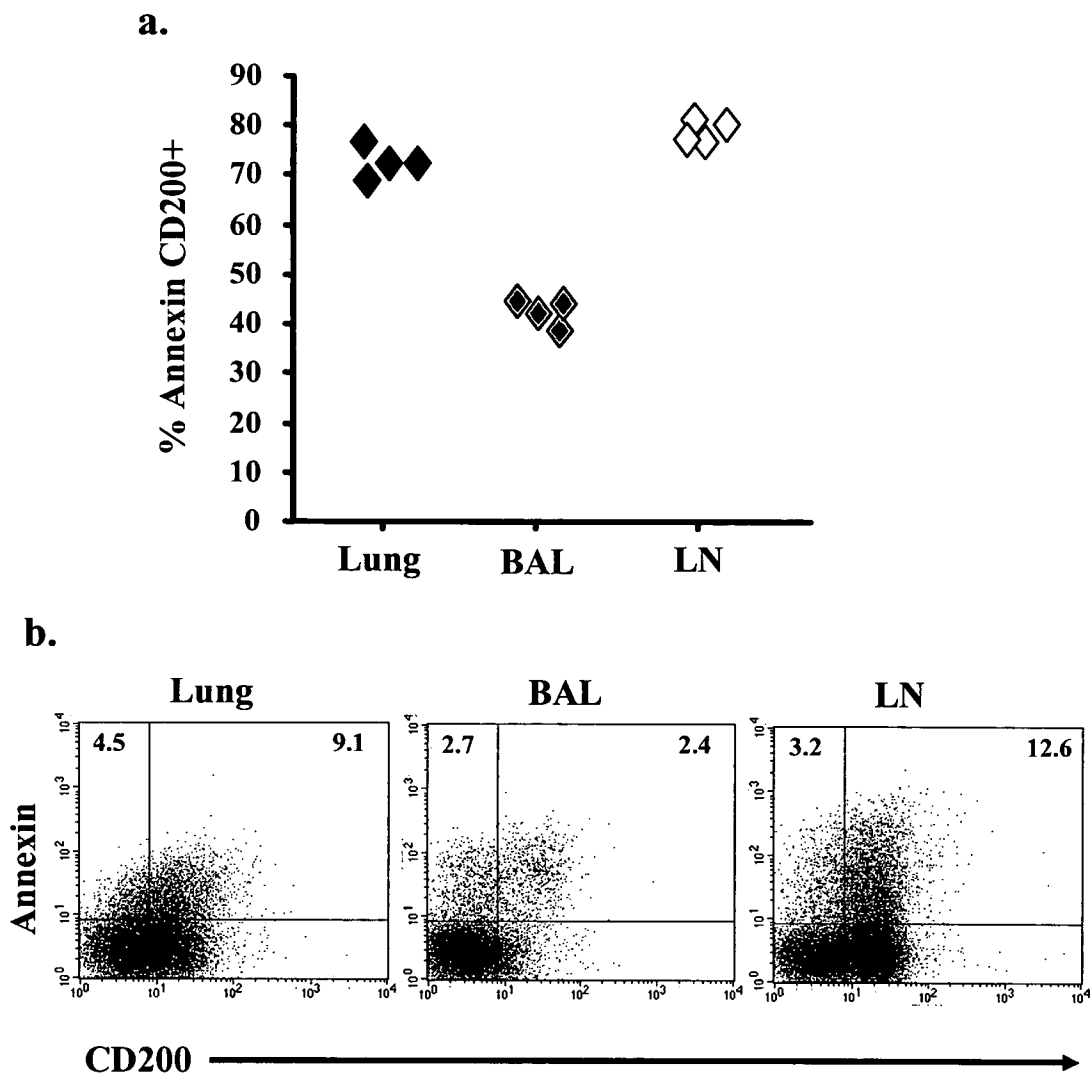
61/100

Figure 5.5



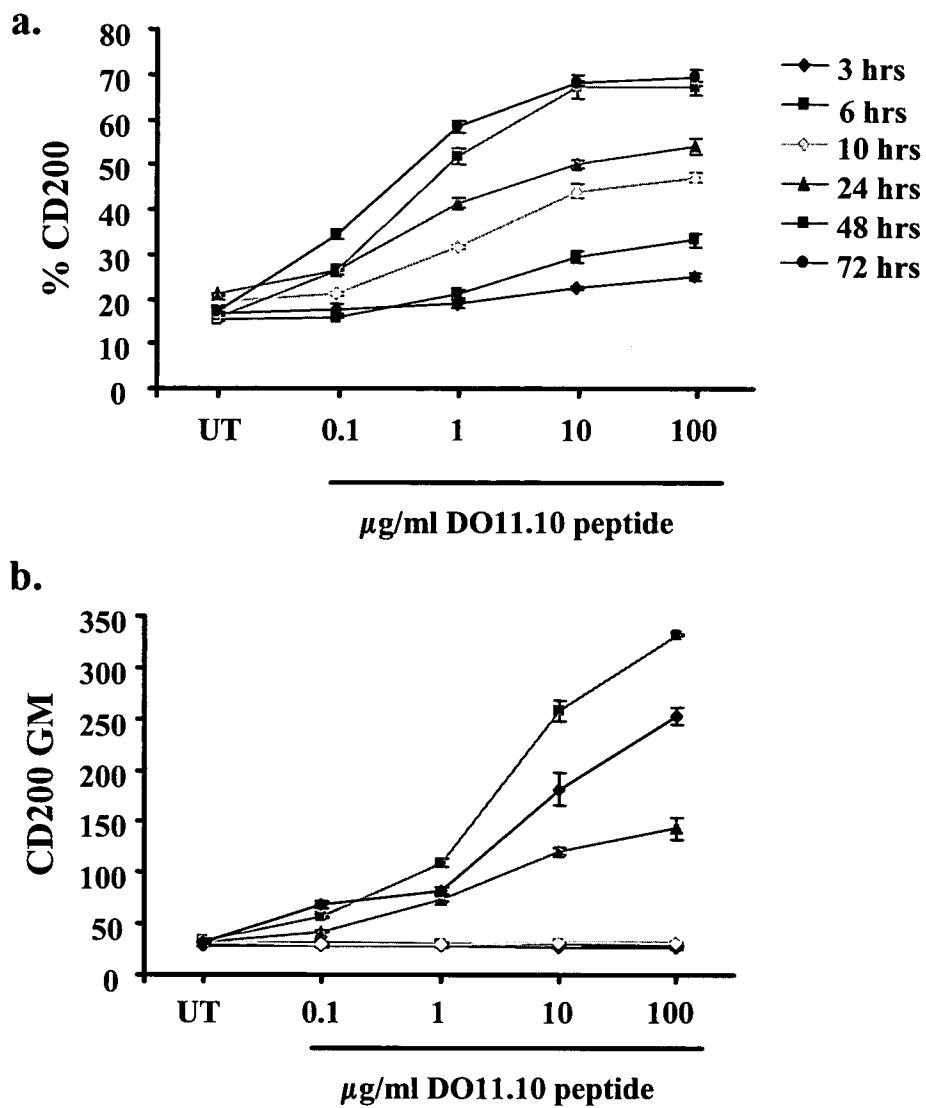
62/100

Figure 5.6



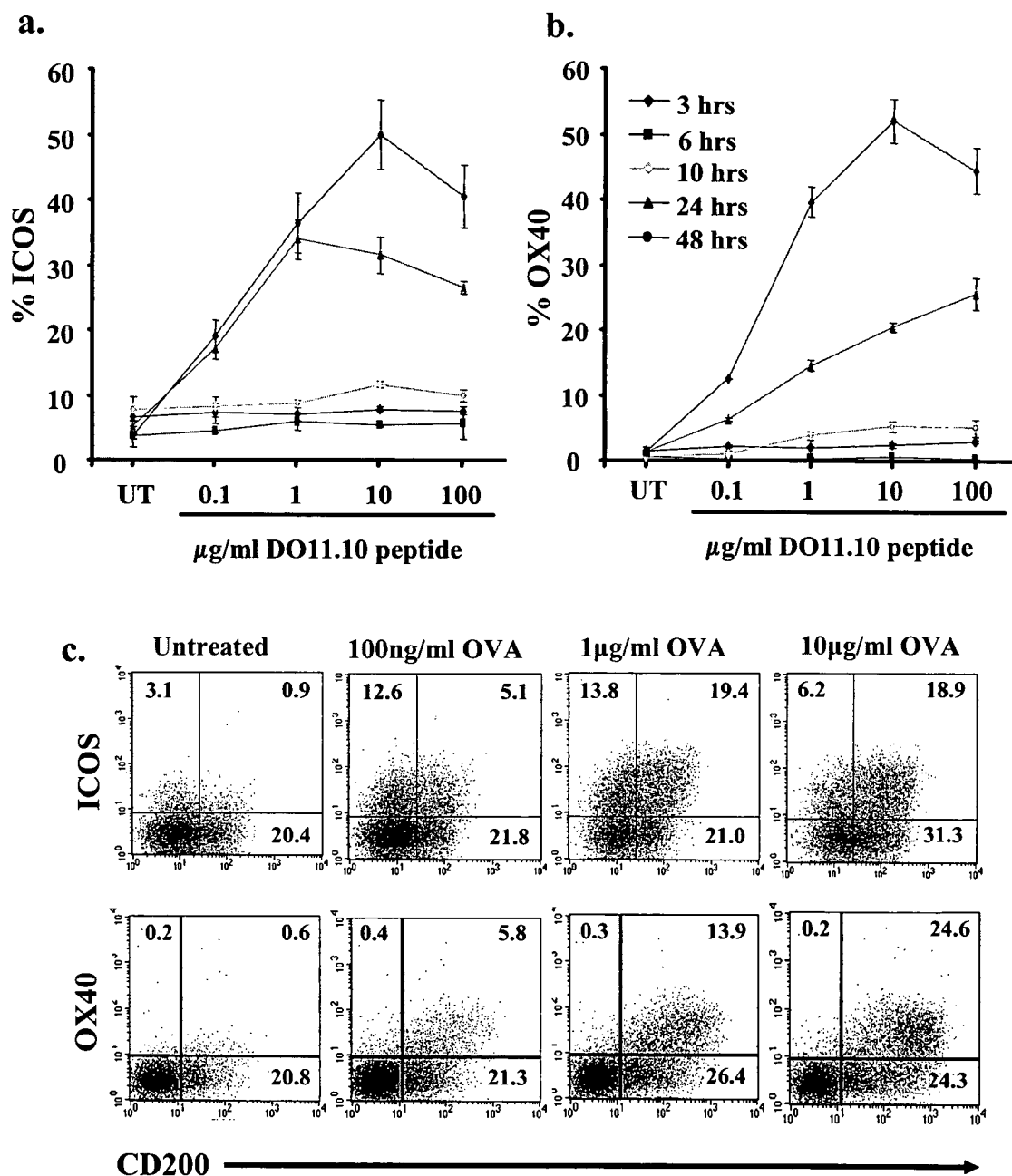
63/100

Figure 5.7



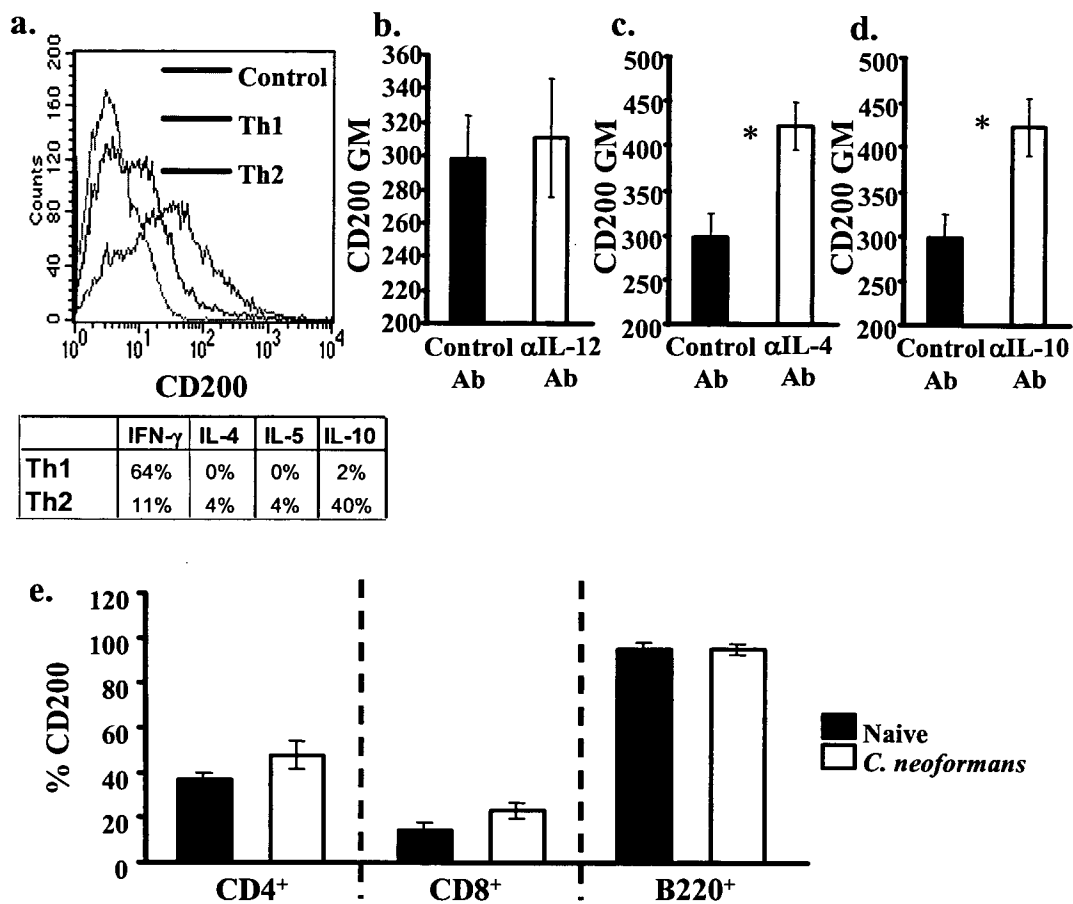
64/100

Figure 5.8



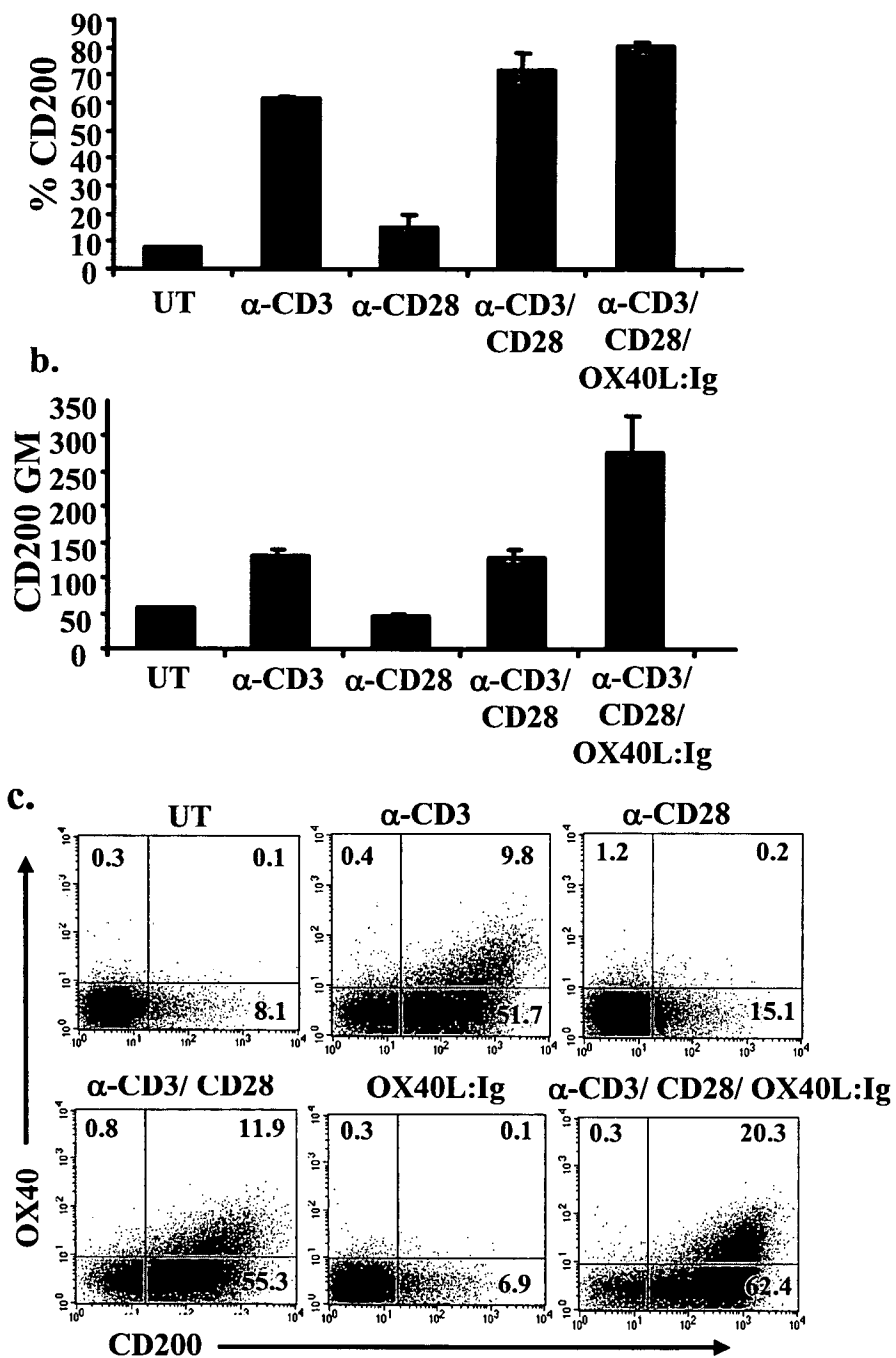
65/100

Figure 5.9



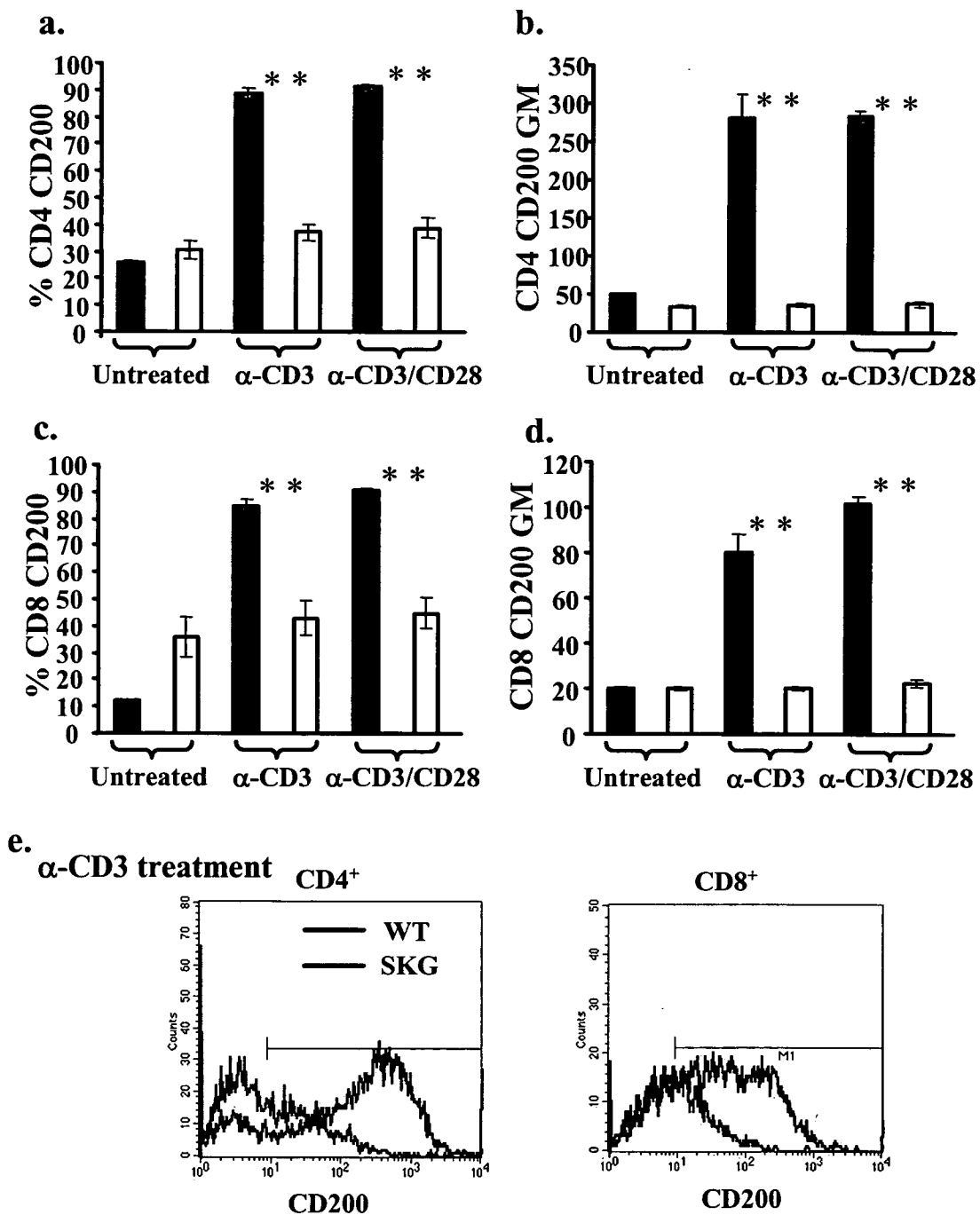
66/100

Figure 5.10



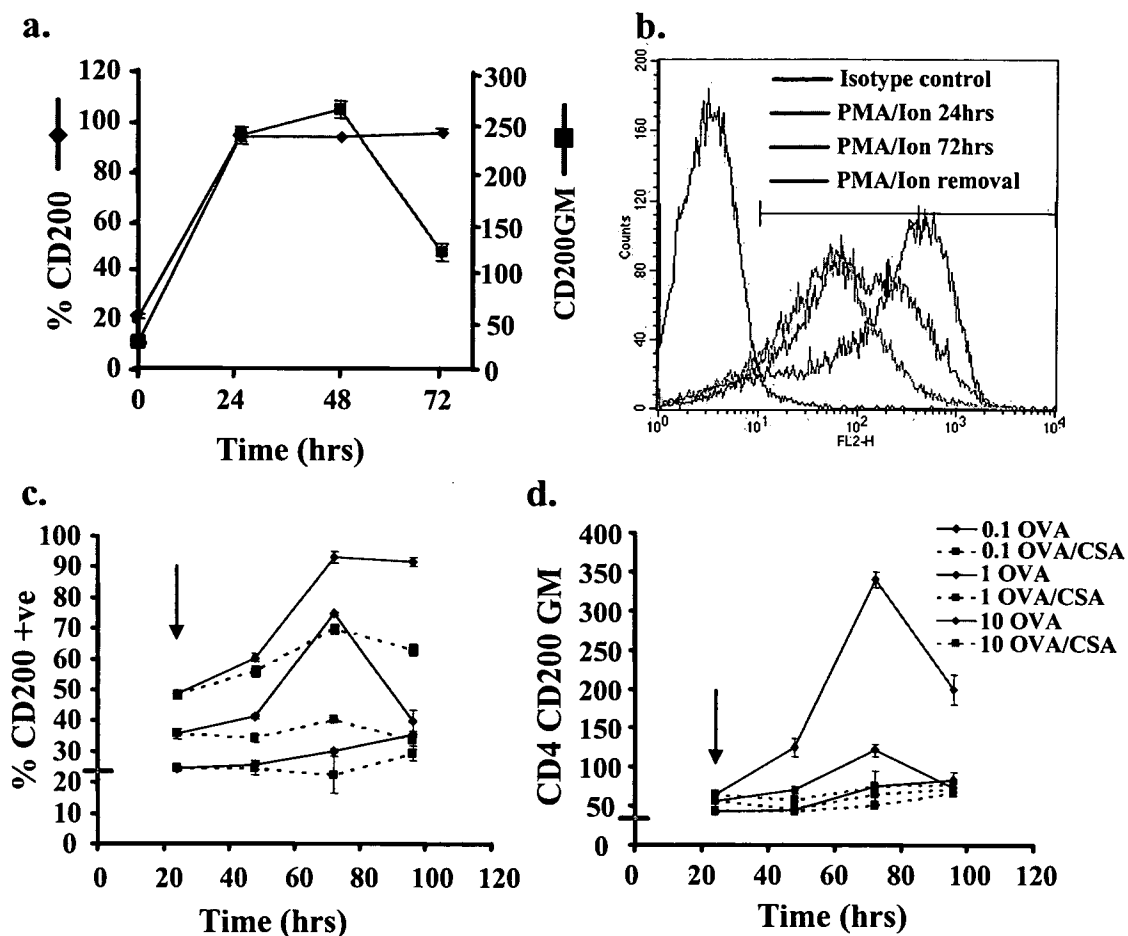
67/100

Figure 5.11



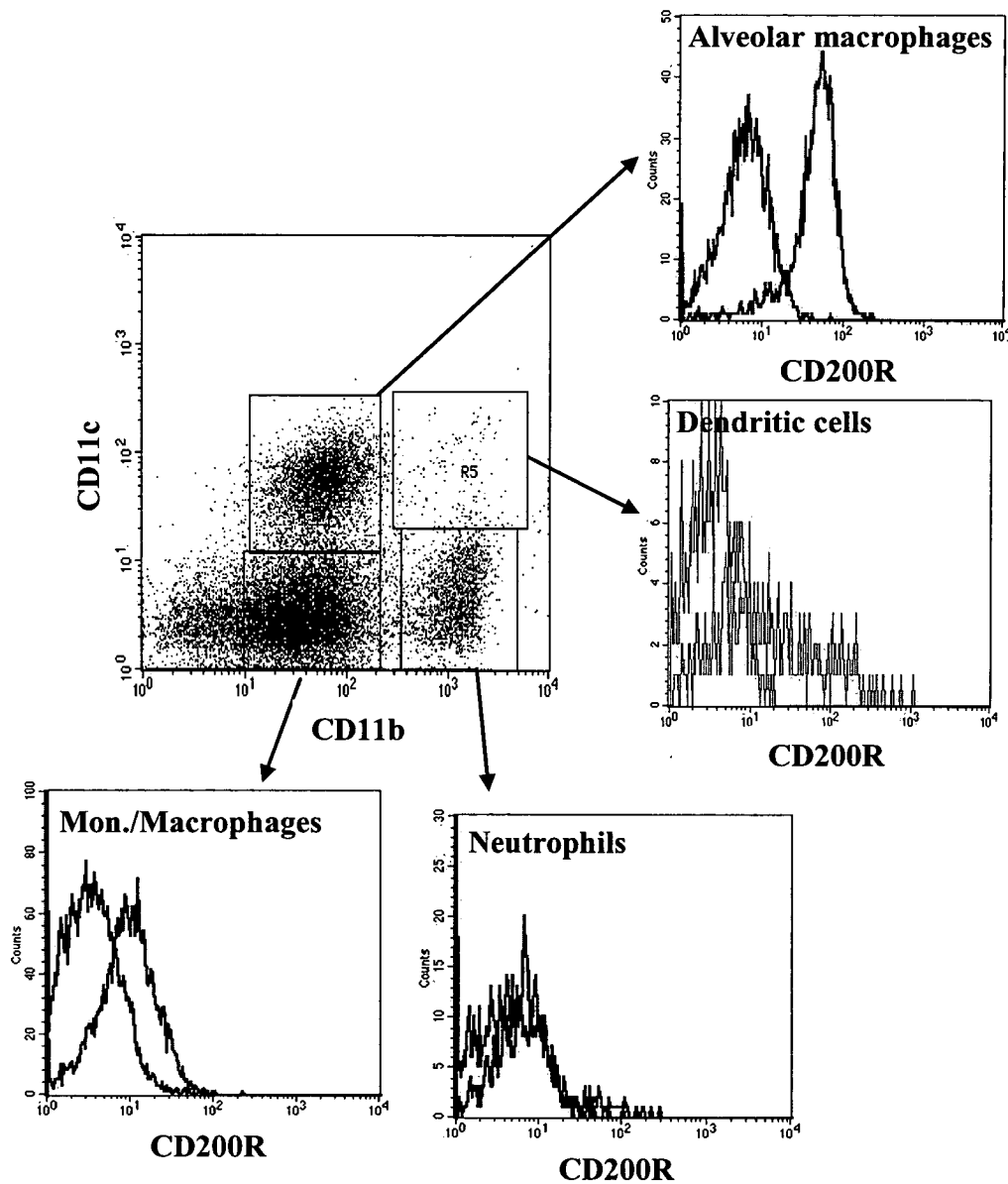
68/100

Figure 5.12



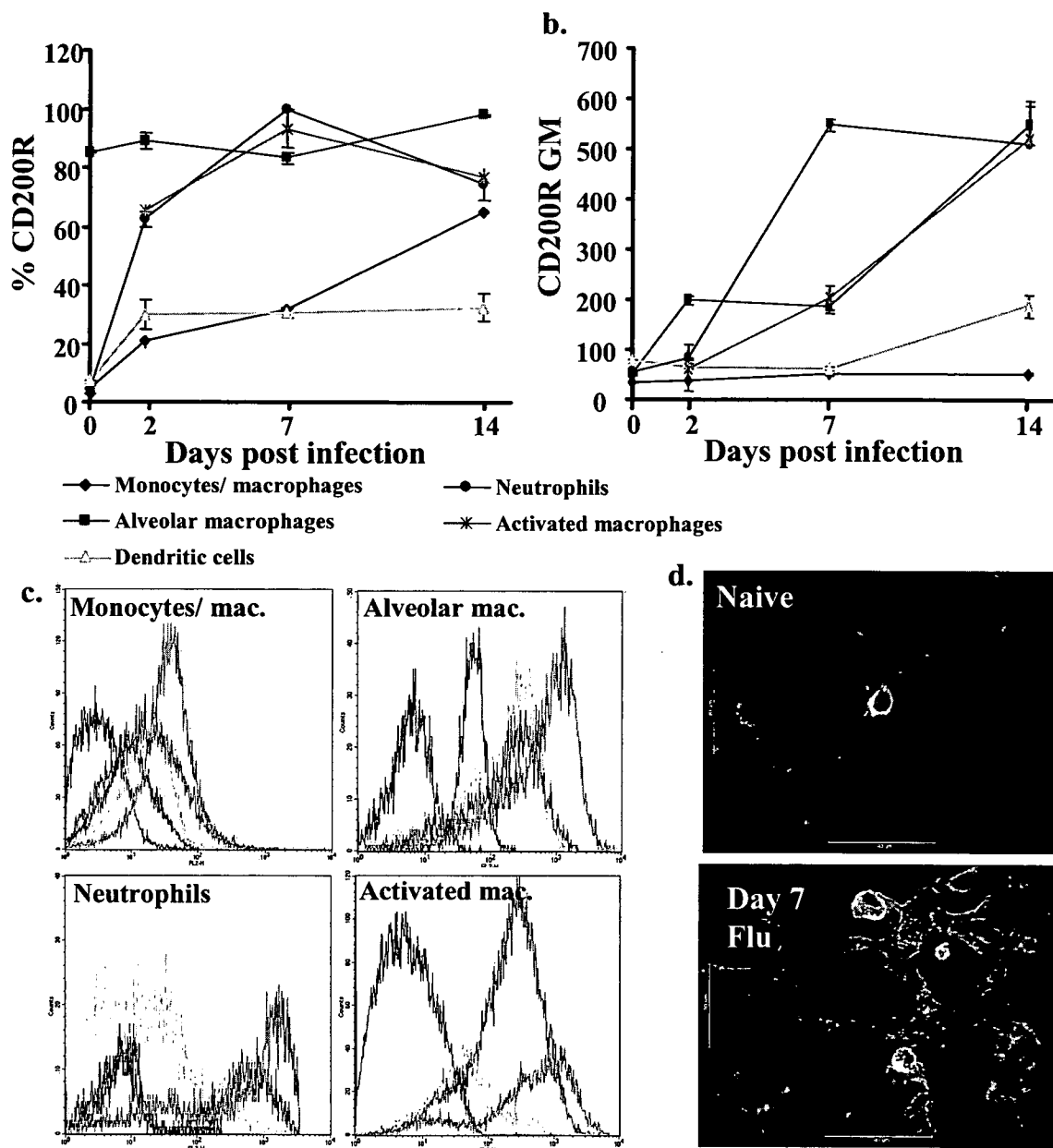
69/100

Figure 5.13



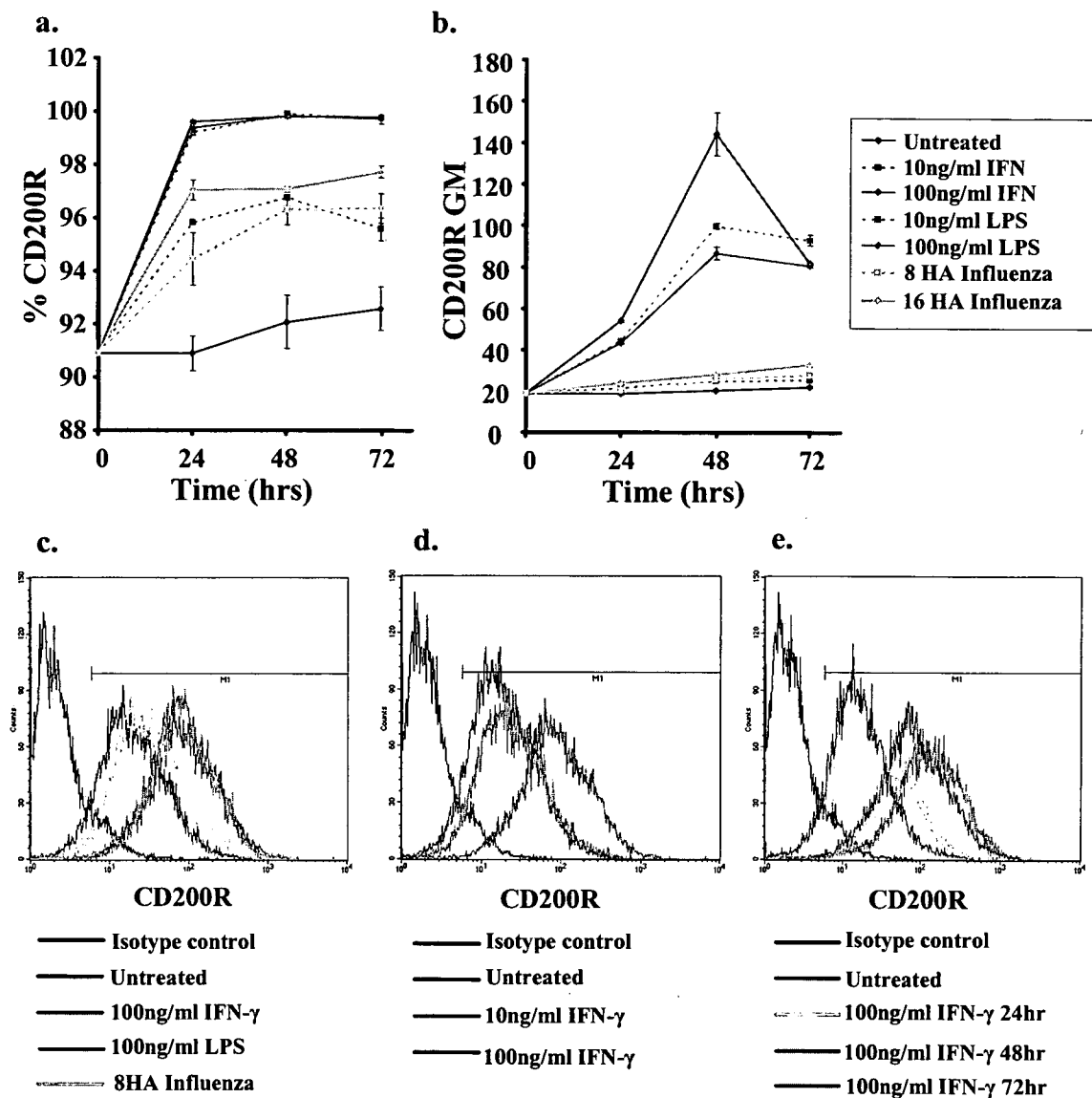
70/100

Figure 5.14



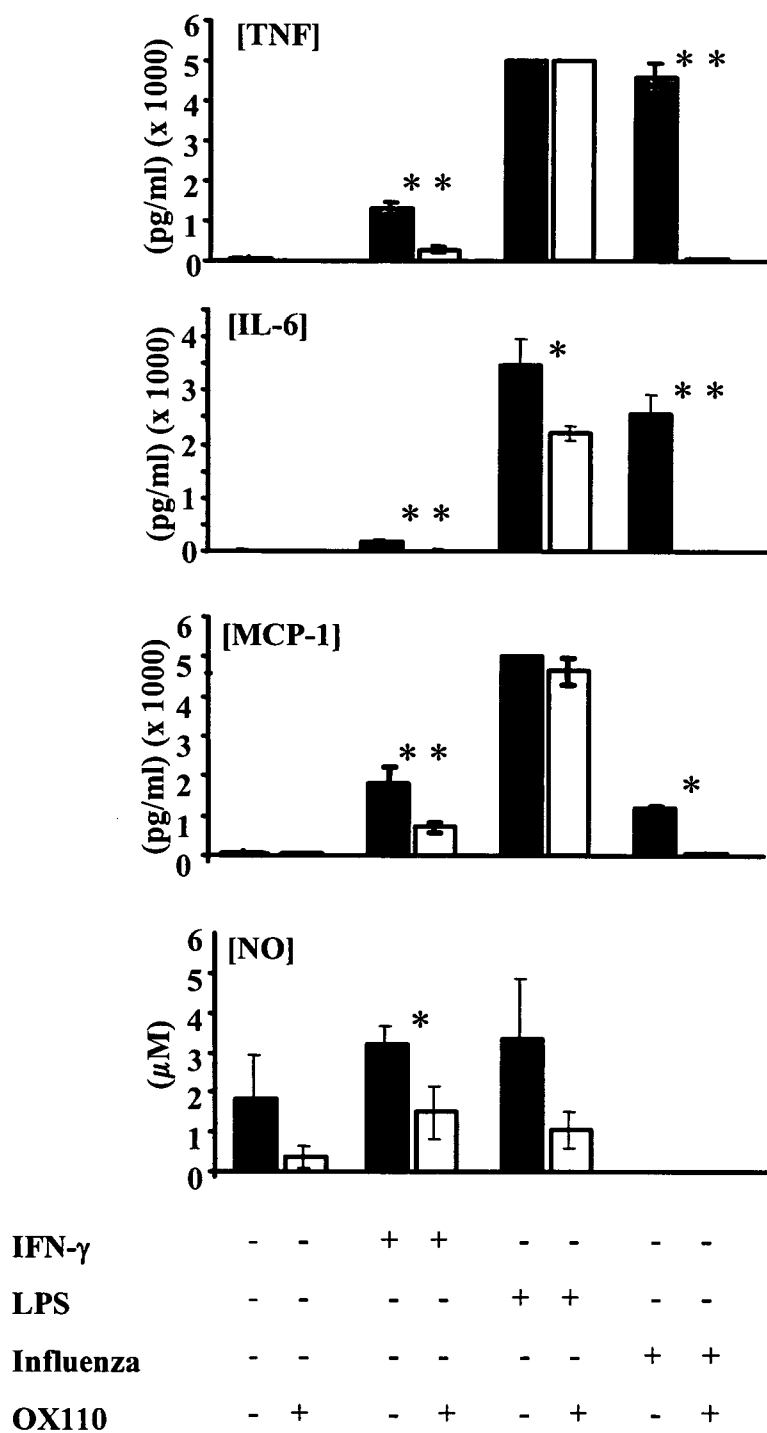
71/100

Figure 5.15



72/100

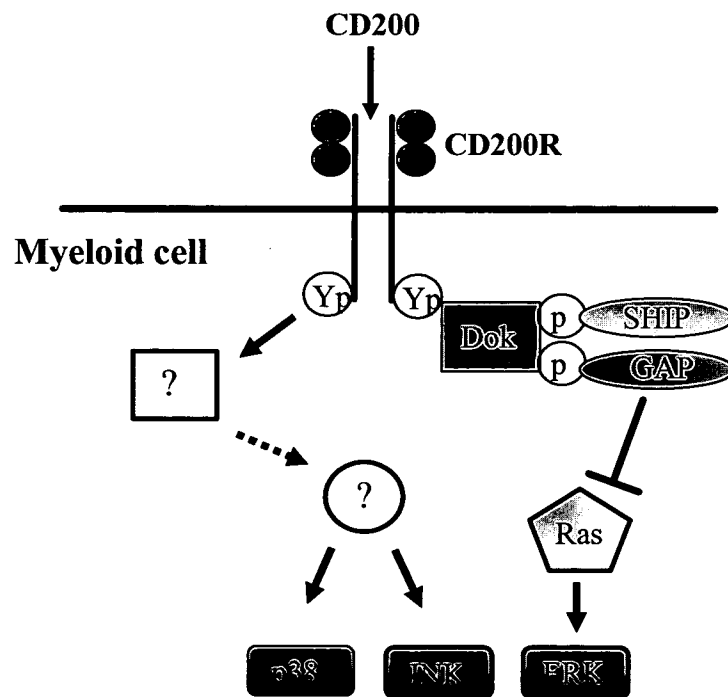
Figure 5.16



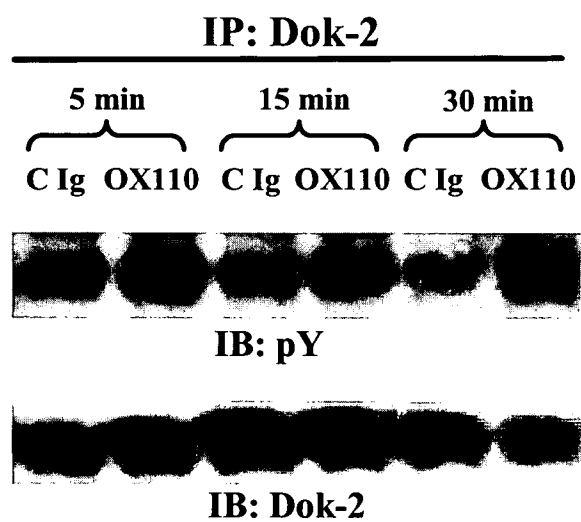
73/100

Figure 5.17

a.

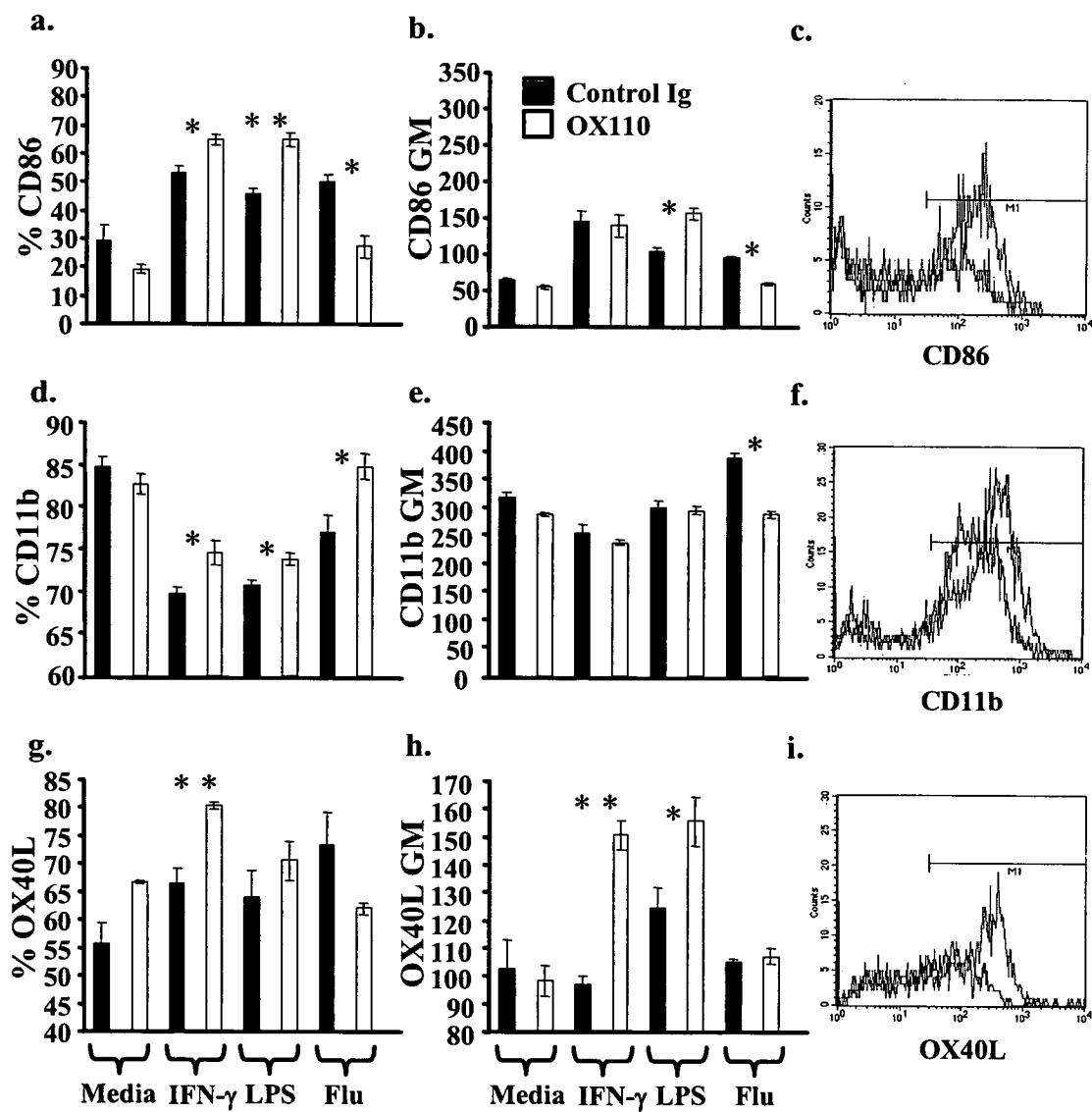


b.



74/100

Figure 5.18



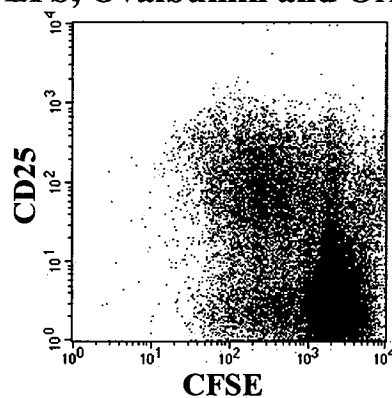
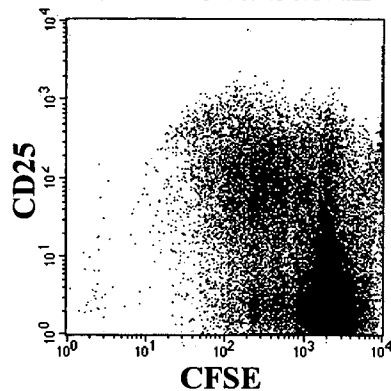
75/100

Figure 5.19

a.

LPS and Ovalbumin

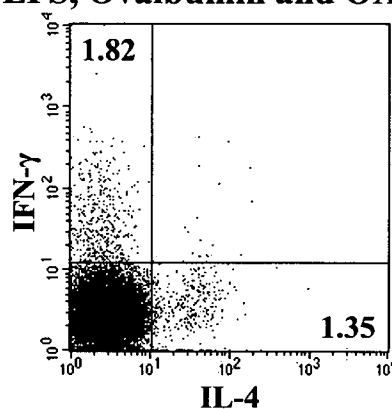
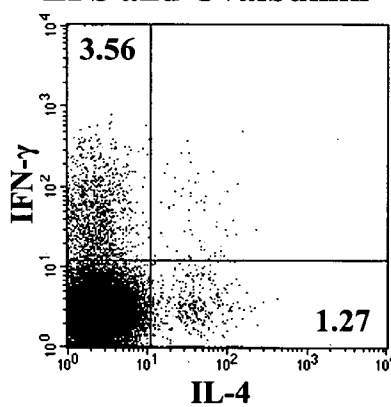
LPS, Ovalbumin and OX110



b.

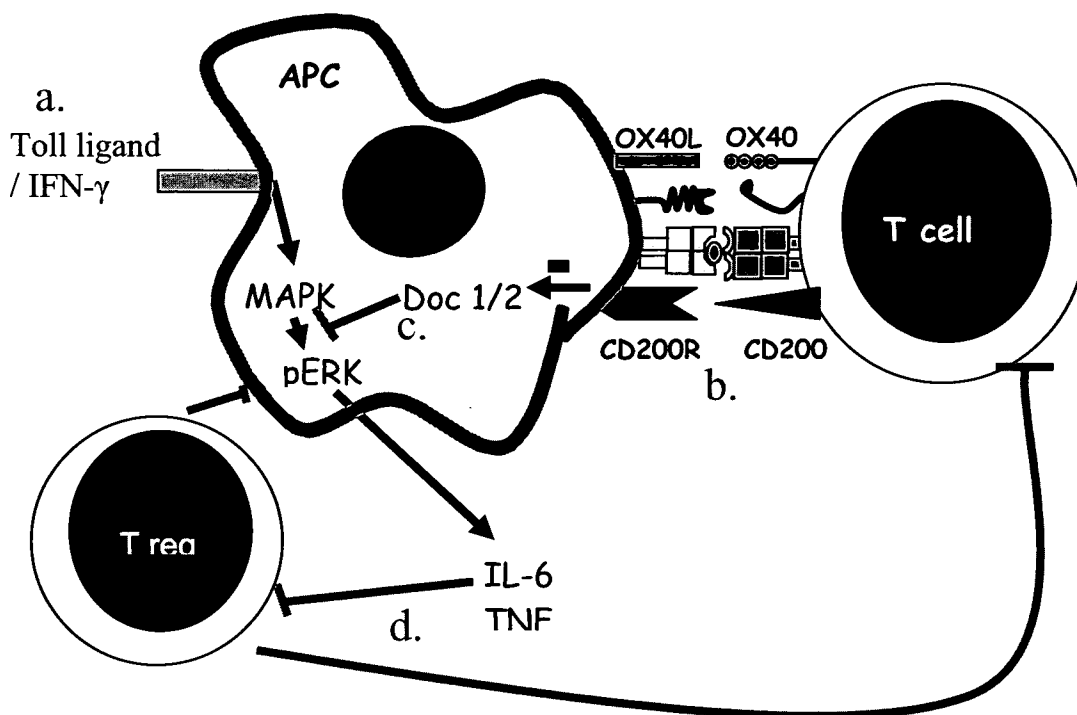
LPS and Ovalbumin

LPS, Ovalbumin and OX110



76/100

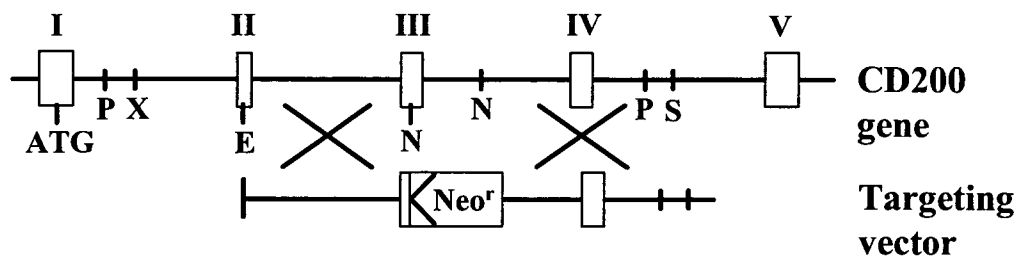
Figure 5.20



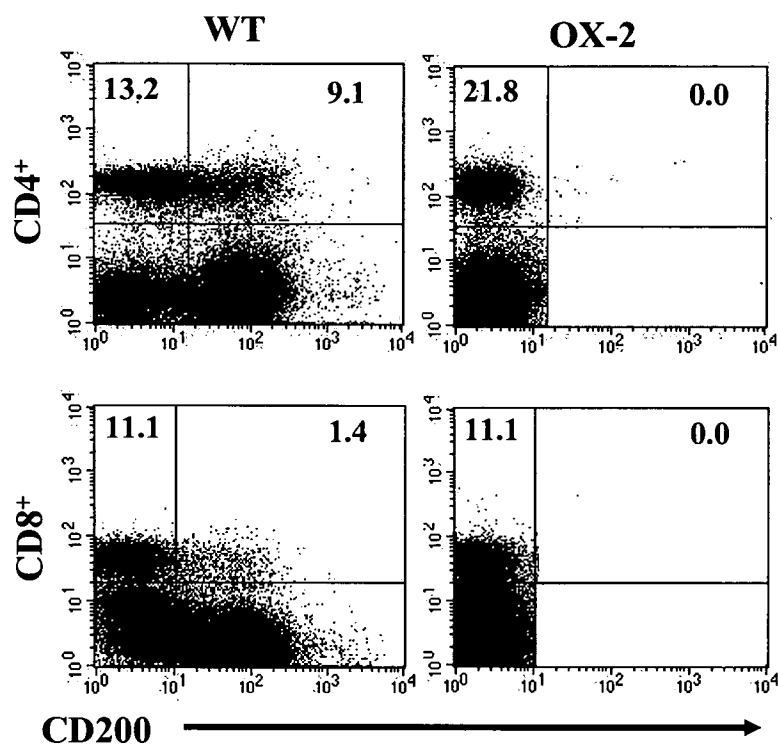
77/100

Figure 6.1

a.



b.

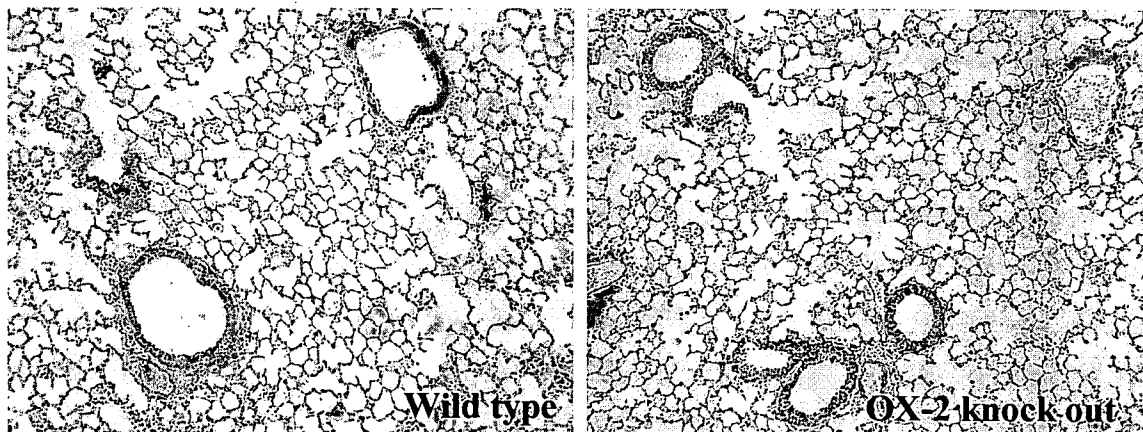


78/100

Table 6.1

	Lung	BAL	Spleen
Total cell no.	1.03x10 ⁶ +/-0.24 0.60x10 ⁶ +/-0.08	0.90x10 ⁵ +/-0.10 1.62x10 ⁵ +/-0.58	6.92x10 ⁷ +/-0.61 8.68x10 ⁷ +/-1.81
CD4 ⁺ T cells	3.88x10 ⁴ +/-1.87 2.01x10 ⁴ +/-0.43	- -	1.88x10 ⁶ +/-0.10 2.07x10 ⁶ +/-0.27
CD8 ⁺ T cells	2.24x10 ⁴ +/-1.31 1.06x10 ⁴ +/-0.22	- -	0.91x10 ⁶ +/-0.05 0.99x10 ⁶ +/-0.08
CD11b ⁺ Myeloid	2.35x10 ⁵ +/-0.64 1.99x10 ⁵ +/-0.32	7.74x10 ⁴ +/-1.74 9.02x10 ⁴ +/-0.60	3.15x10 ⁶ +/-0.38* 6.20x10 ⁶ +/-1.45

Figure 6.2

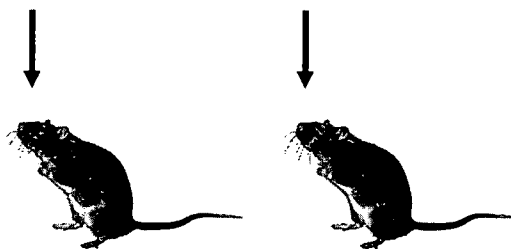


79/100

Figure 6.3

Day 0

25 / 50 HA Influenza A x31 (i.n)

Wild-type
C57BL/6

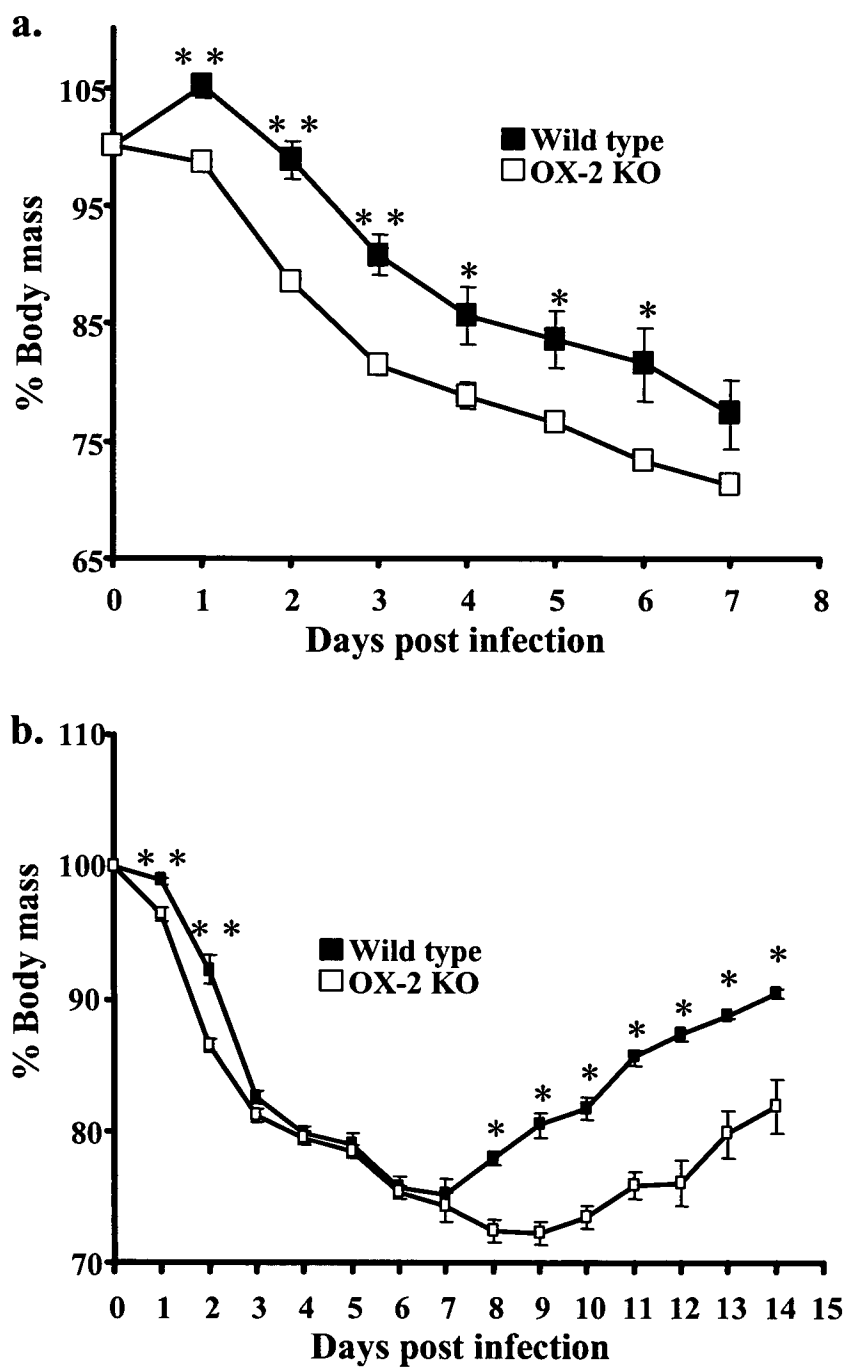
OX-2 KO mice

**Days 2,
7 and 14**

- BAL:** - Surface Staining (flow cytometry)
- Cytoentrifugation
- Cytokines/chemokines (Luminex)
- Lung:** - Surface/intracellular cytokine staining
- Influenza titres
- Histology (H/E)
- Node:** - Surface Staining (flow cytometry)
- Serum:** - Influenza-specific antibody, TNF (ELISA)
- Nasal Washes:** - Influenza-specific IgA

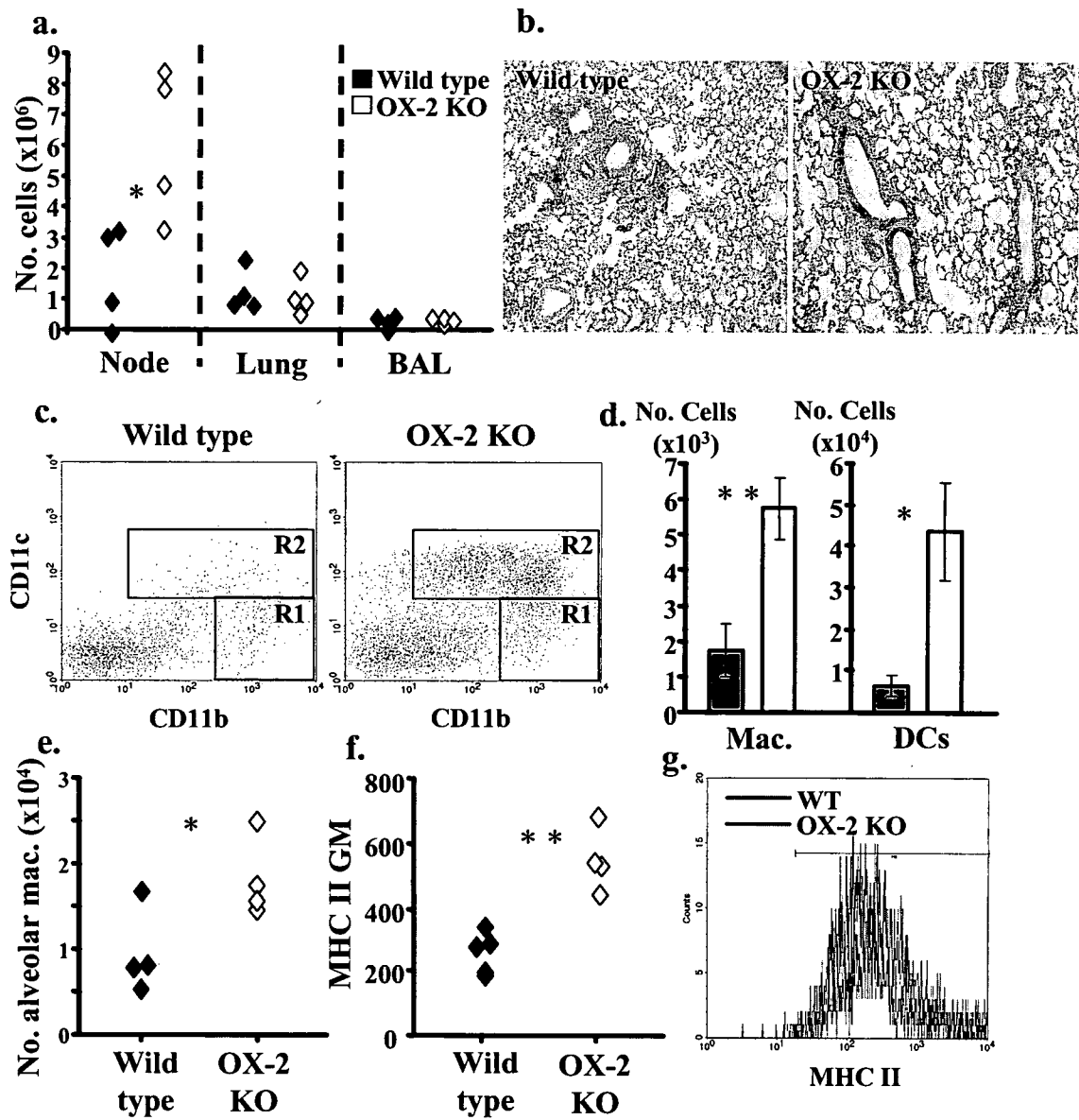
80/100

Figure 6.4



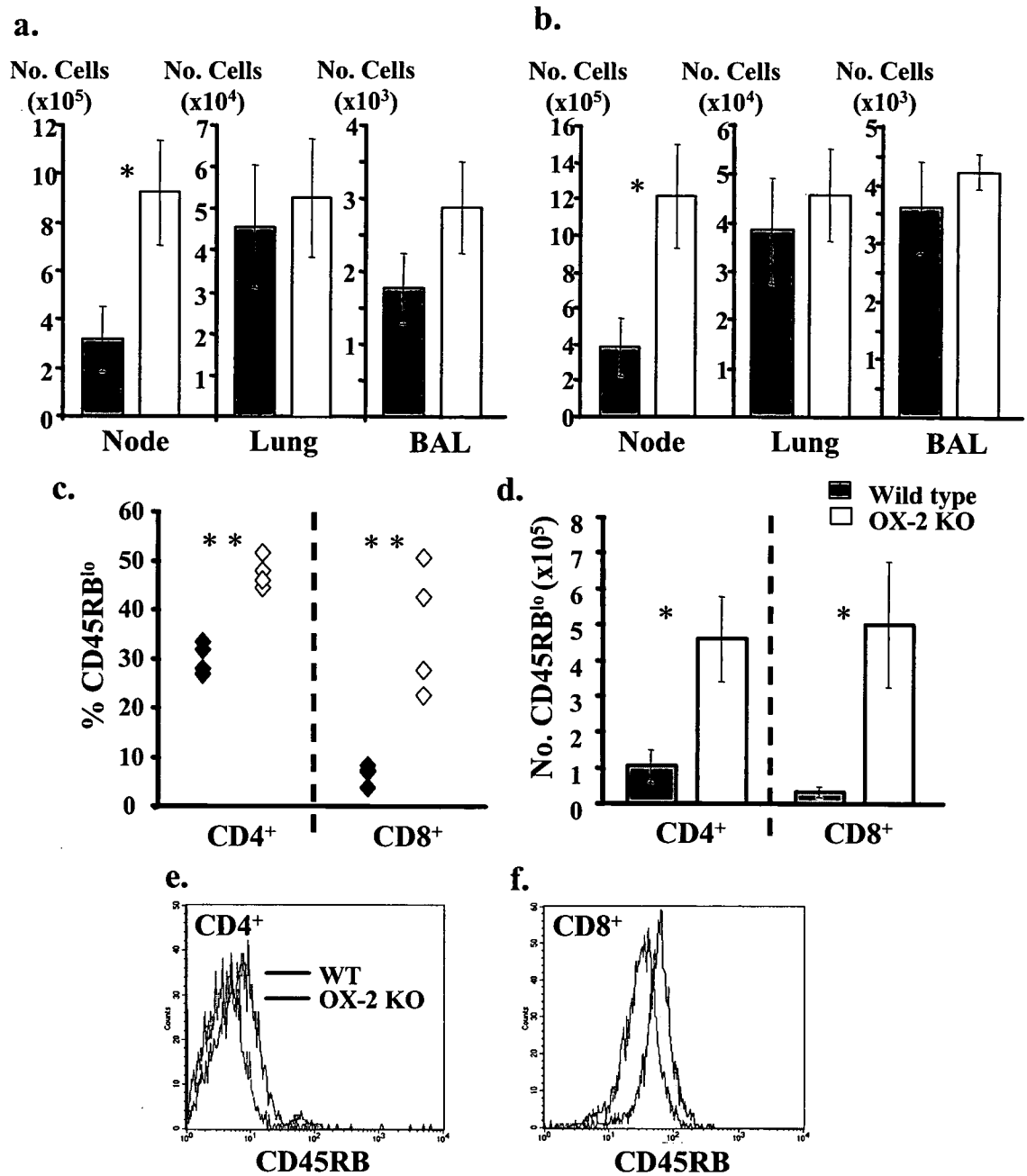
81/100

Figure 6.5



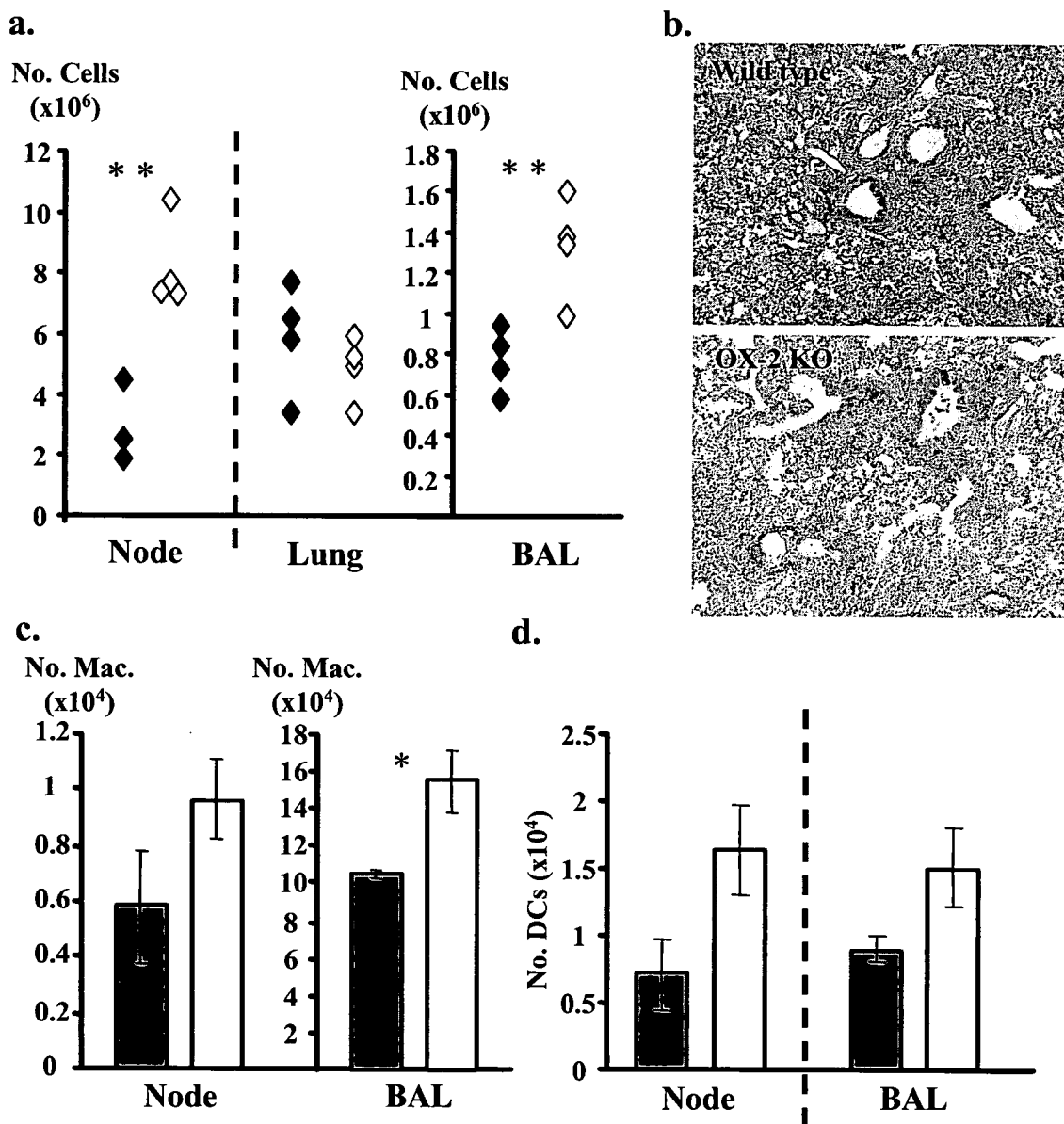
82/100

Figure 6.6



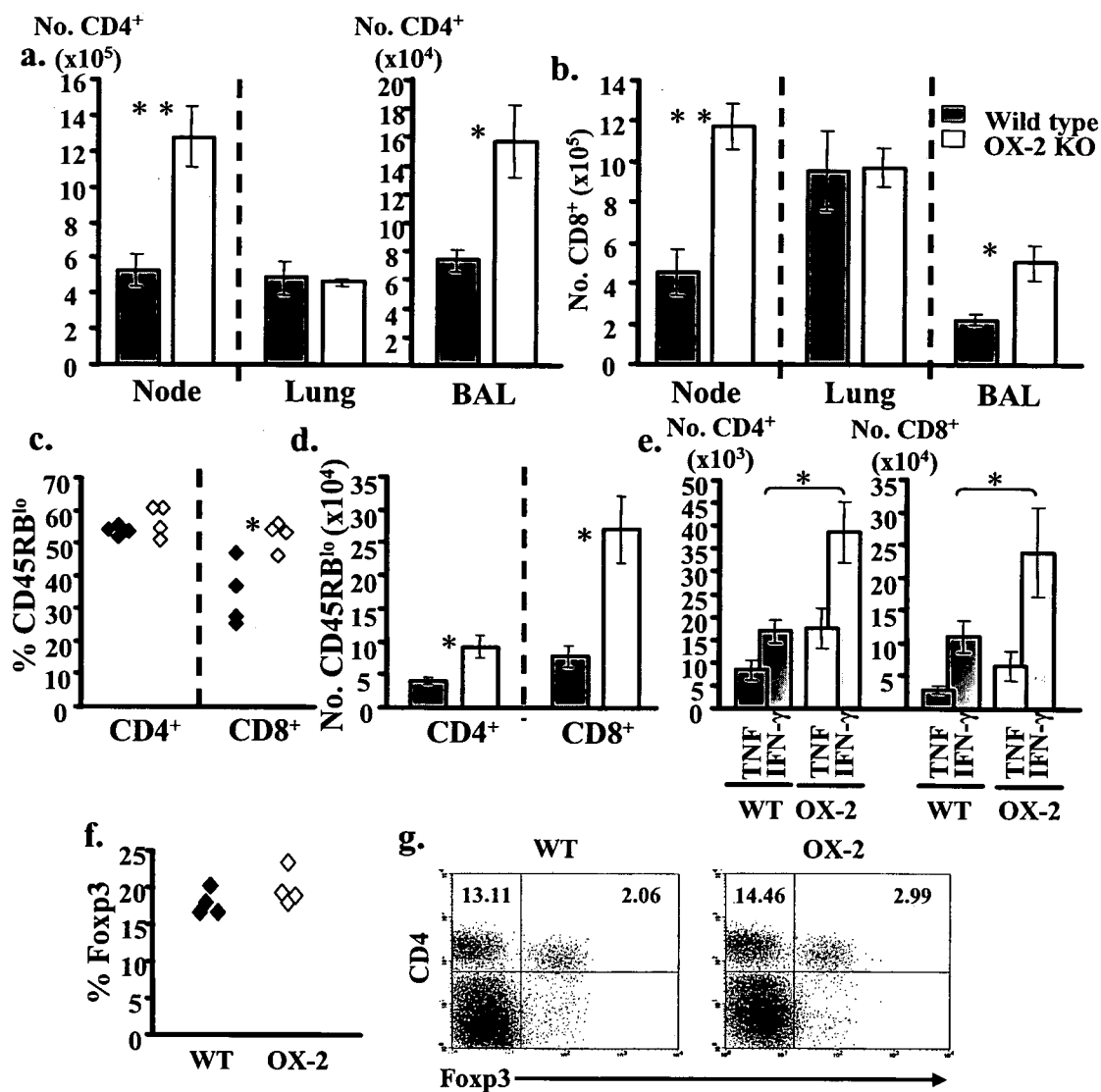
83/100

Figure 6.7



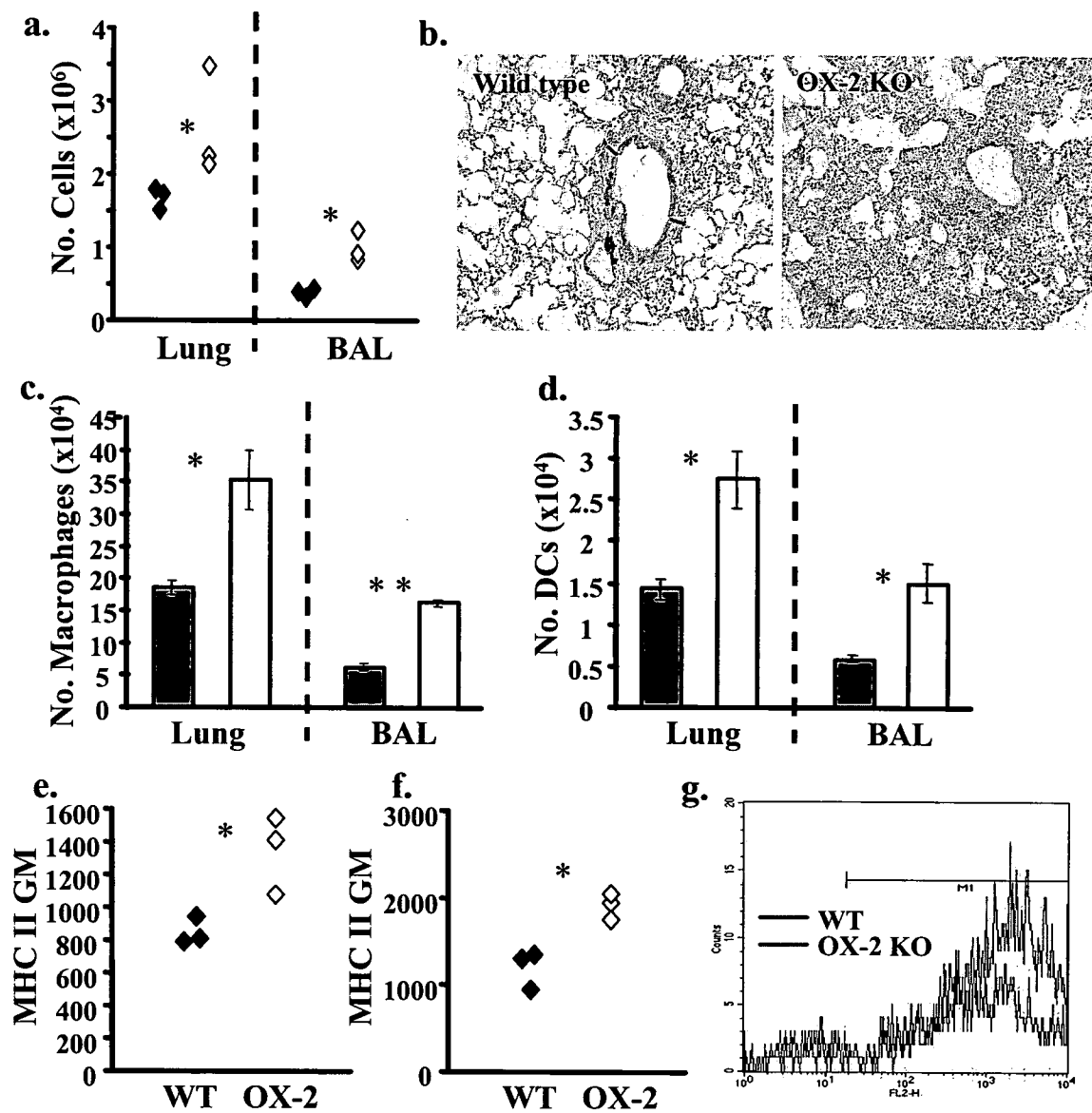
84/100

Figure 6.8



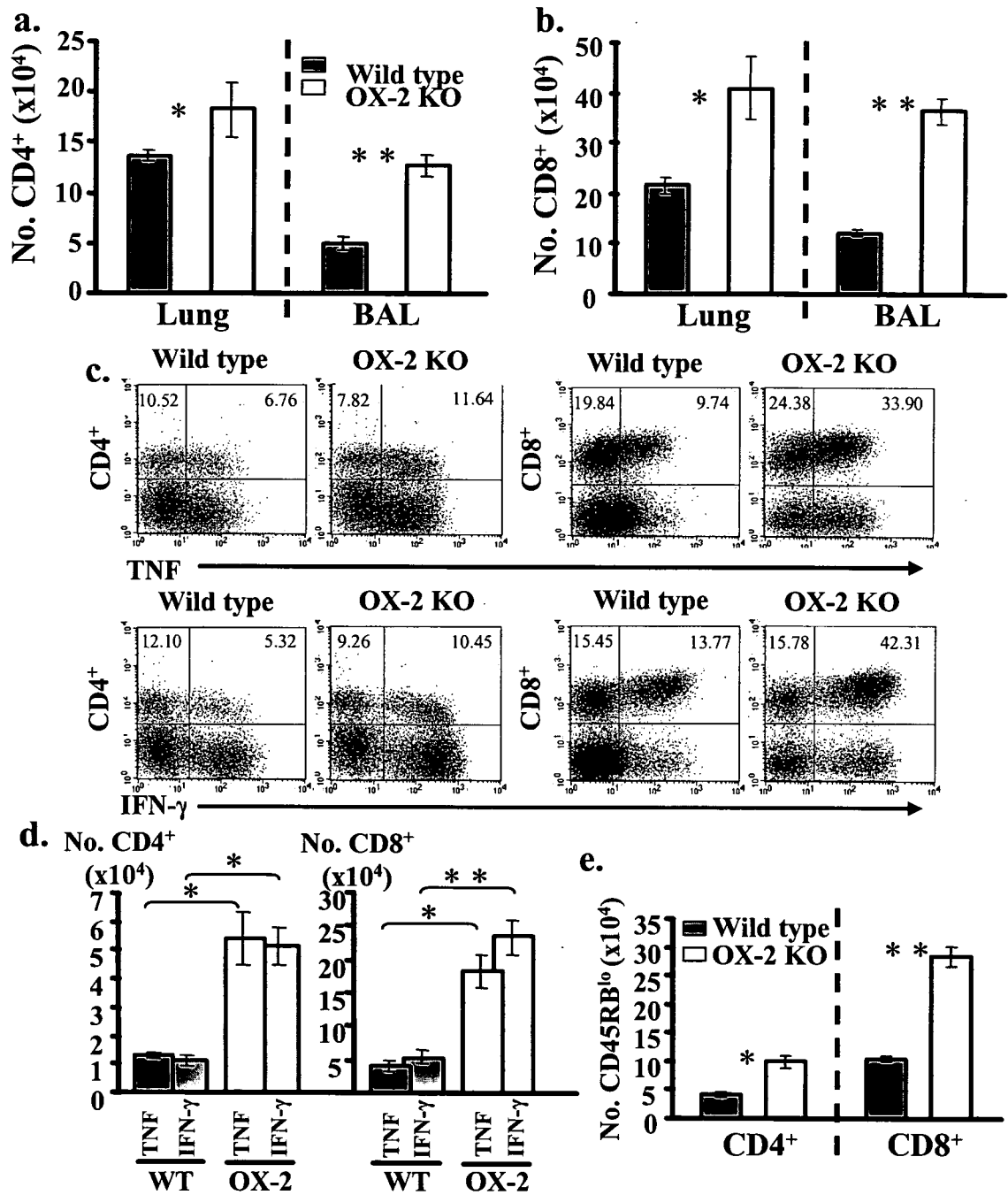
85/100

Figure 6.9



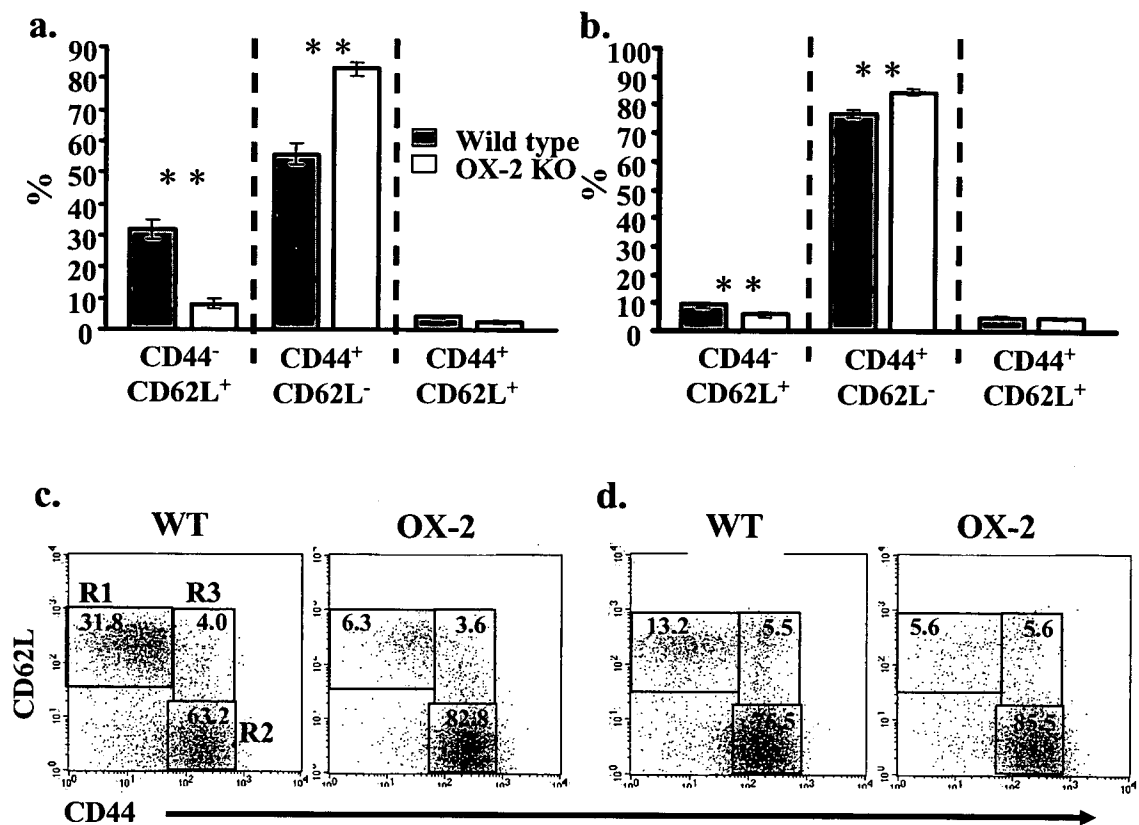
86/100

Figure 6.10



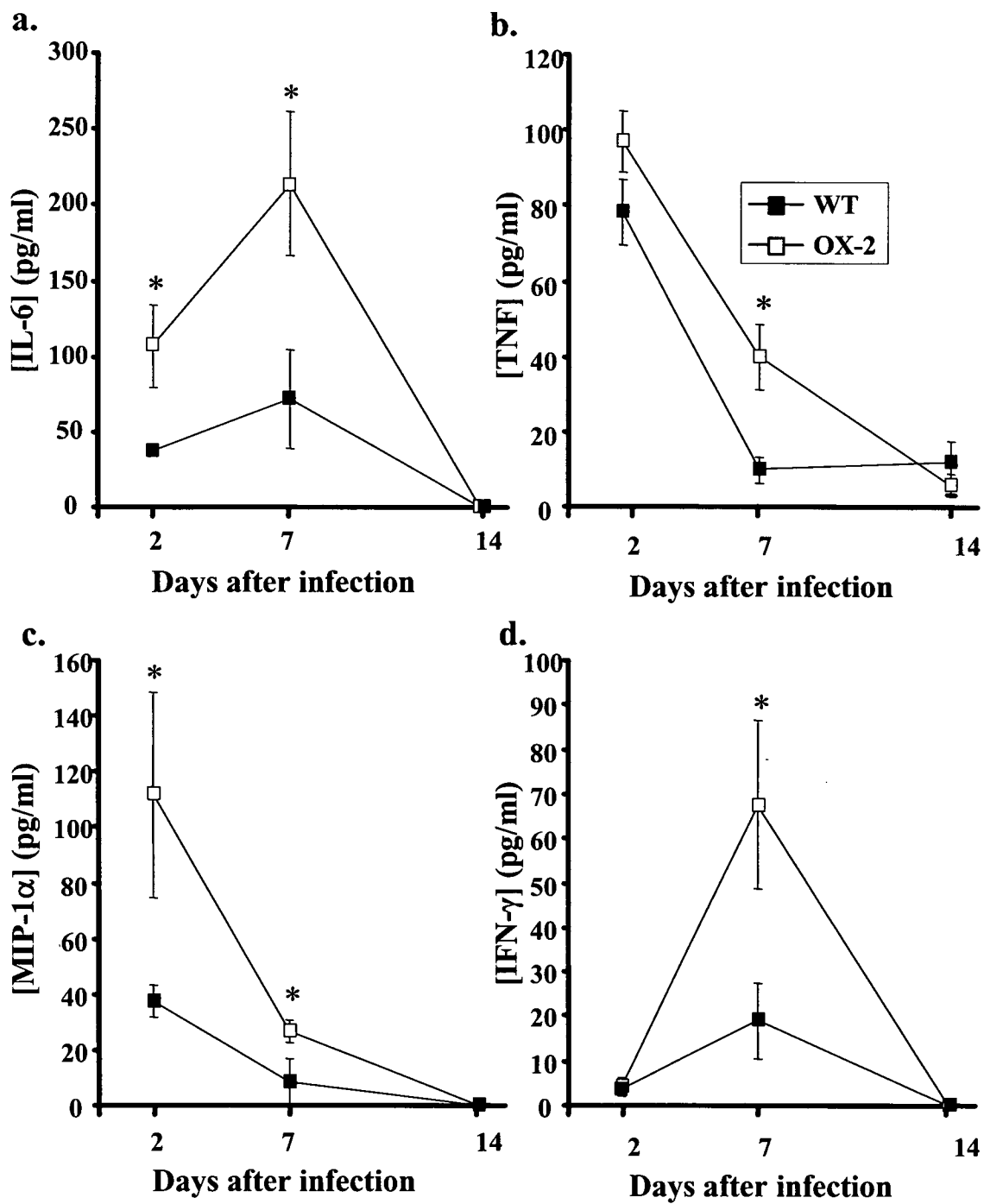
87/100

Figure 6.11



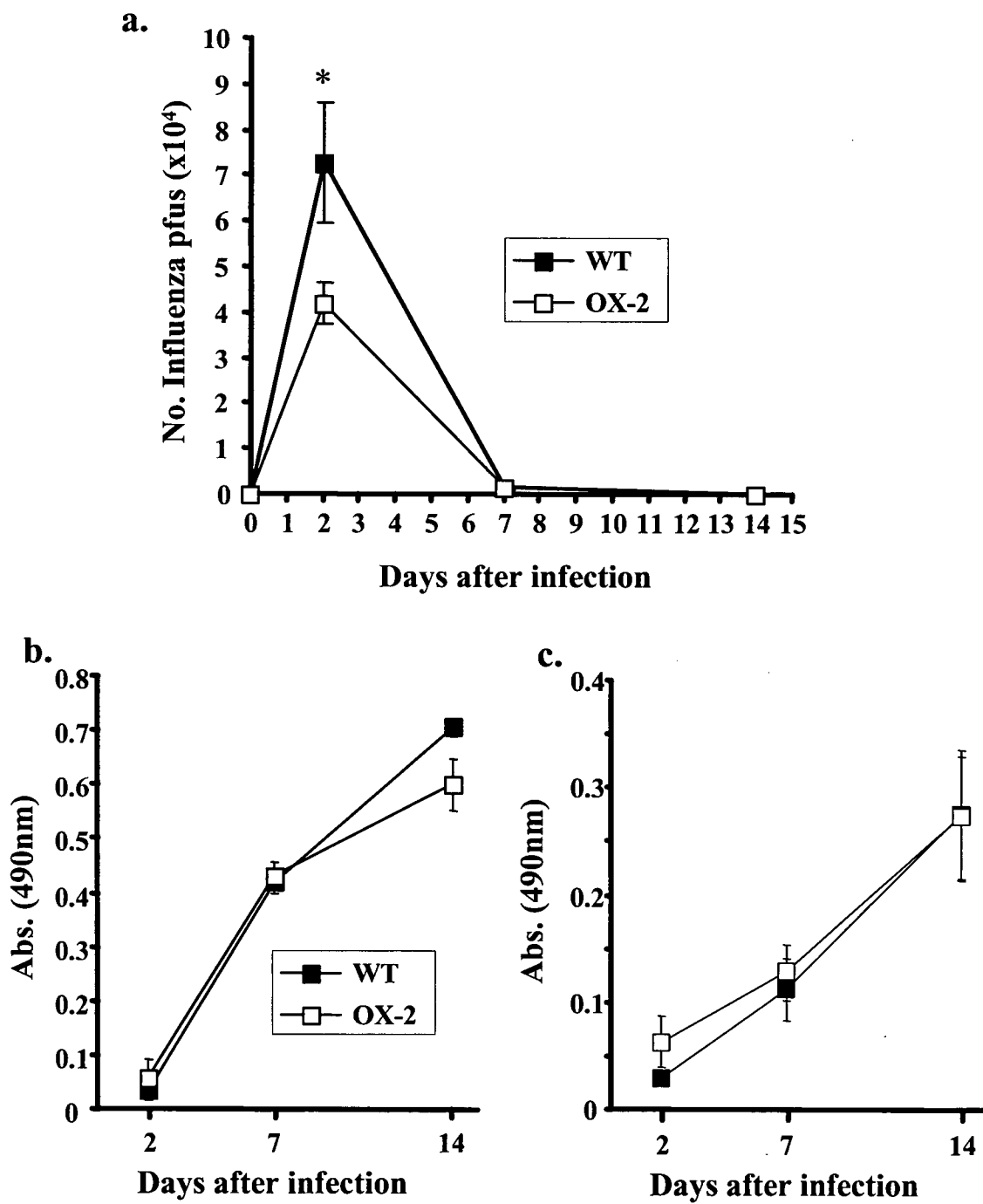
88/100

Figure 6.12



89/100

Figure 6.13

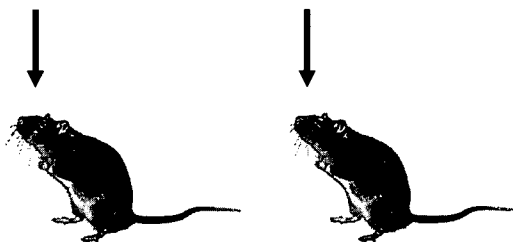


90/100

Figure 6.14

Day 0

50HA Influenza A x31 (i.n)

**Day 0, 2,
4 and 6**

10µg IgG (i.p.)

10µg CD200:Fc
(i.p.)**Day 7**

BAL: - Surface Staining (flow cytometry)
- Cytokines (CBA)

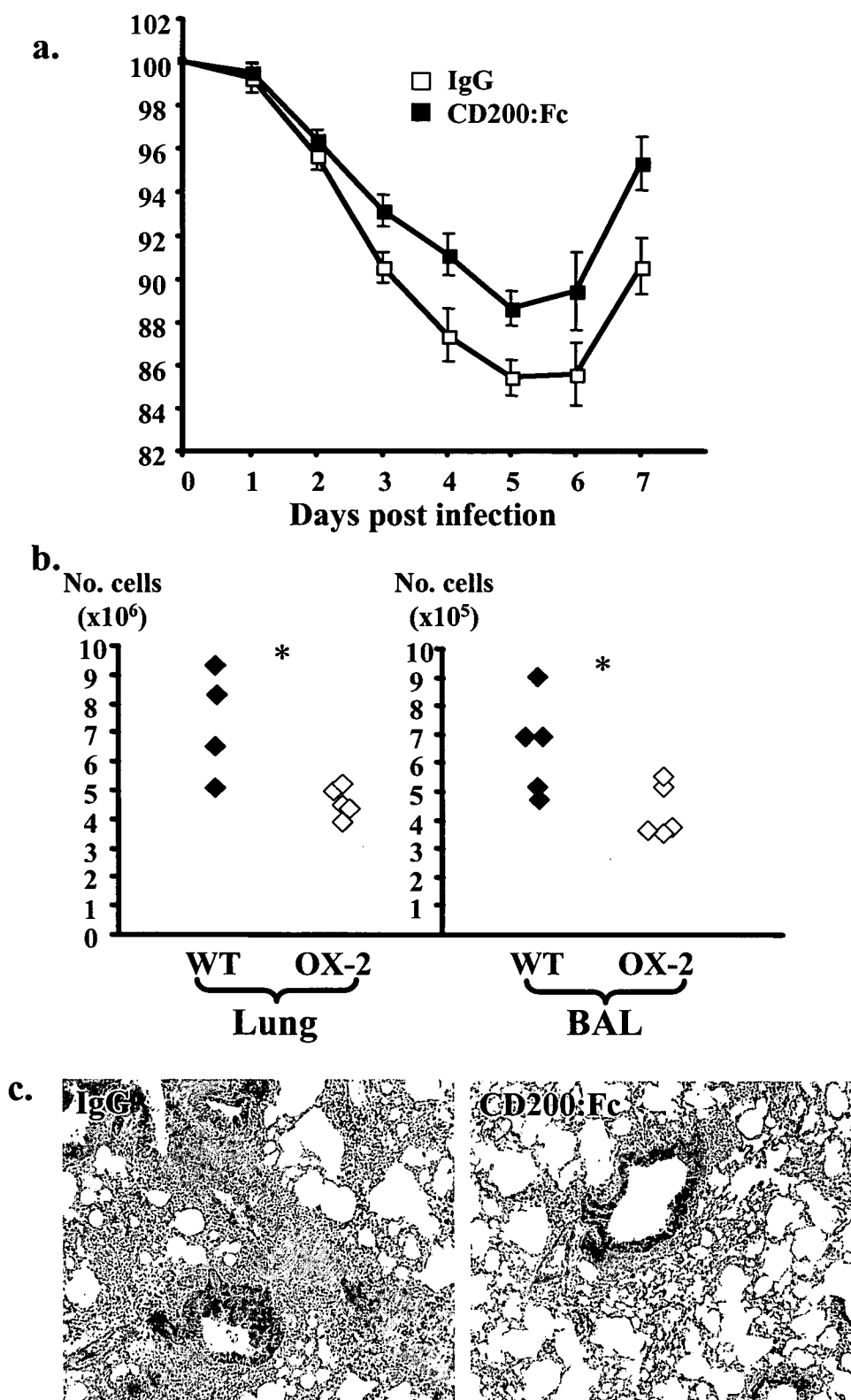
Lung: - Surface/intracellular cytokine staining
- Influenza titres
- Histology (H/E)

Serum: - Influenza-specific antibody
- Cytokines (CBA)

Nasal Washes: - Influenza-specific IgA

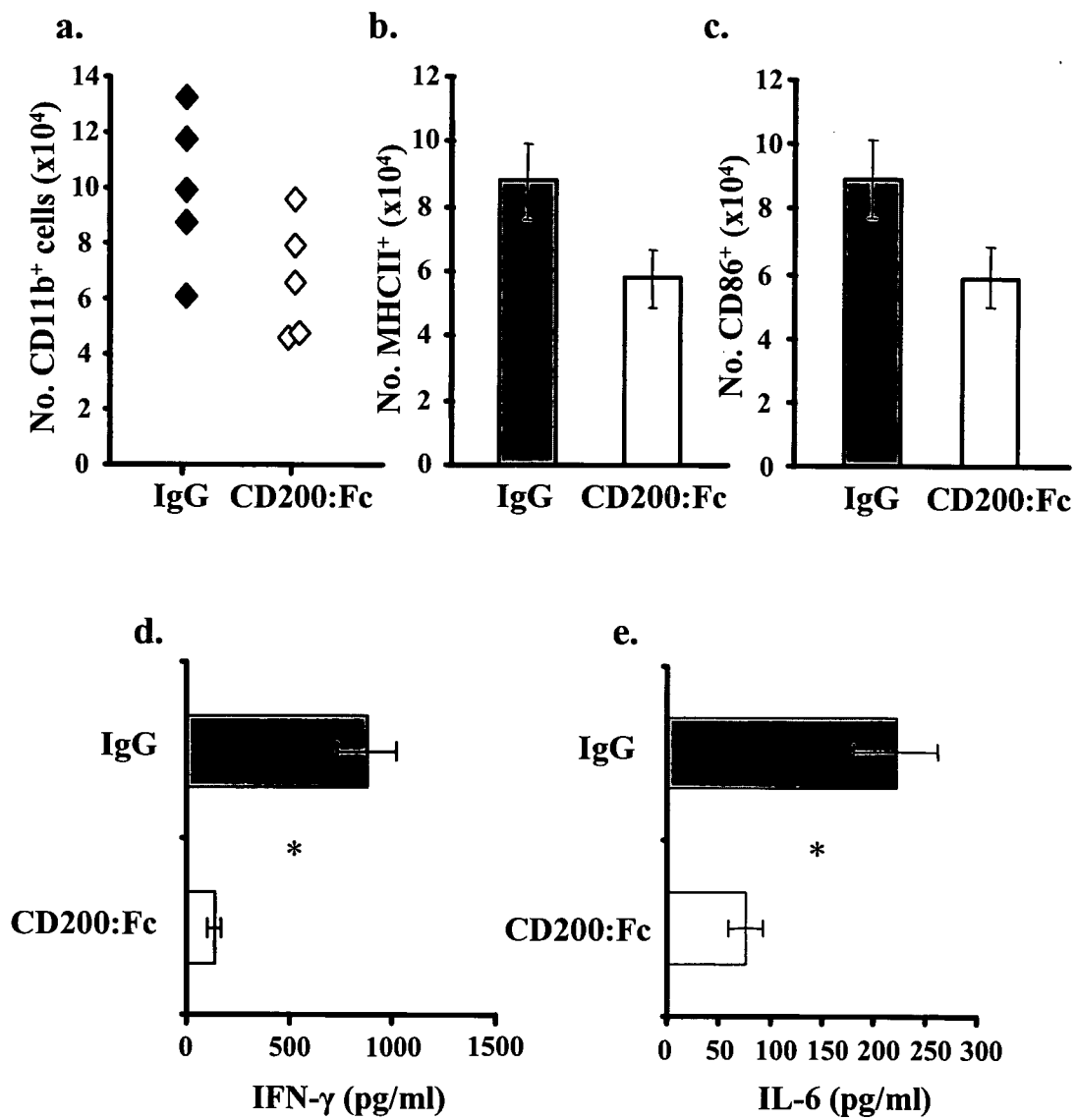
91/100

Figure 6.15



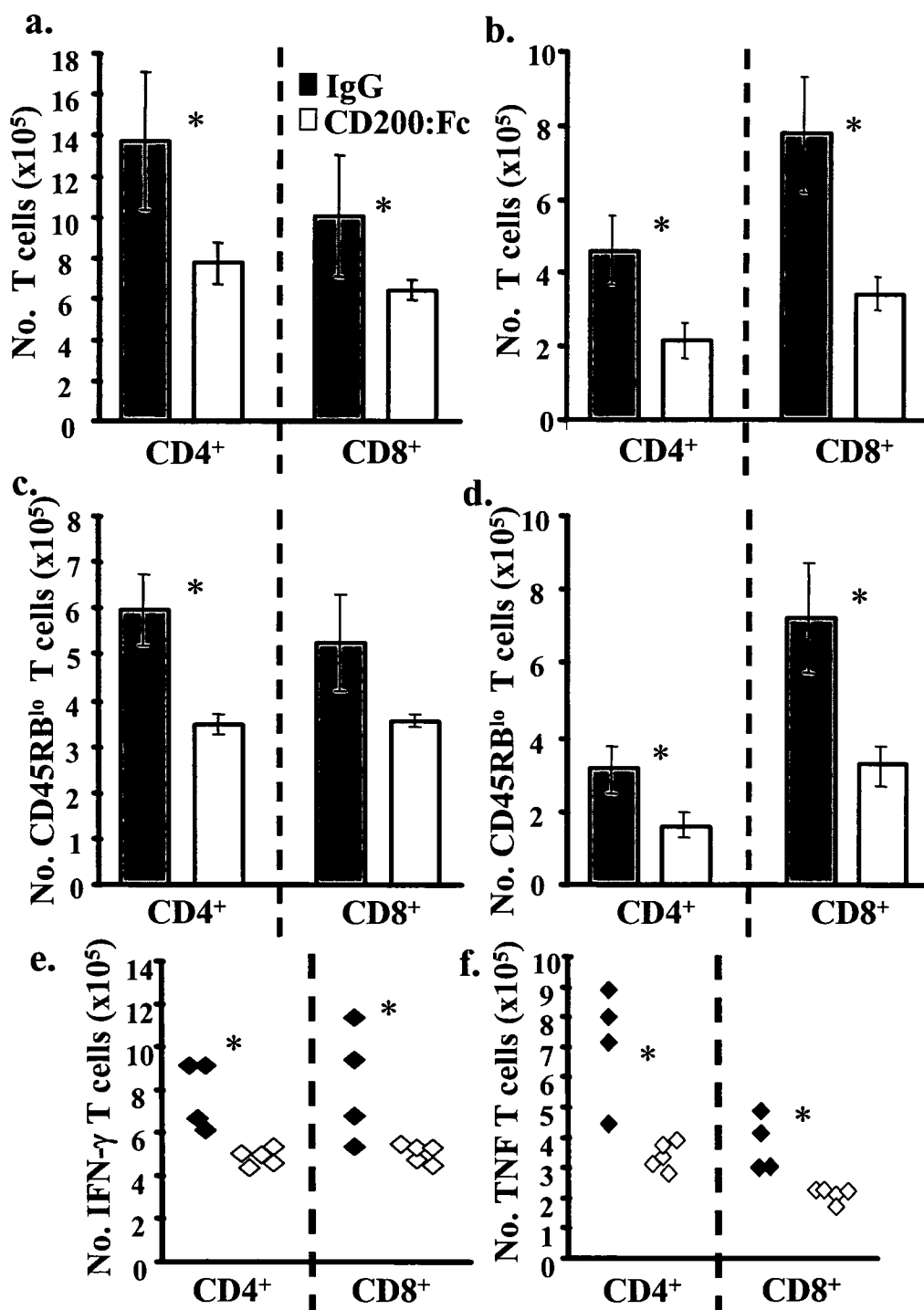
92/100

Figure 6.16



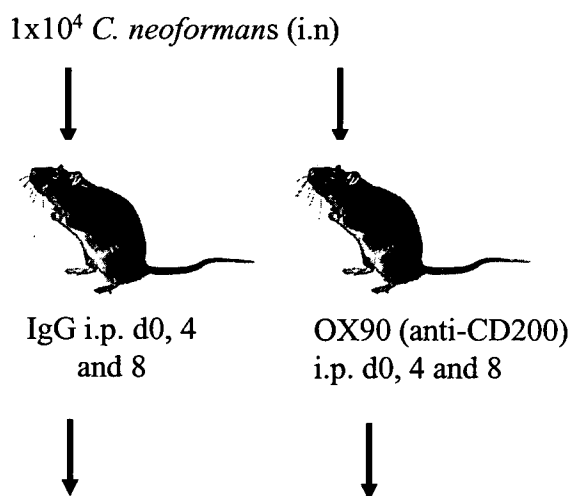
93/100

Figure 6.17



94/100

Figure 6.18

Day 0**Day 12****BAL:** - Surface Staining (flow cytometry)

- Cytoentrifugation

- Cytokines (ELISA)

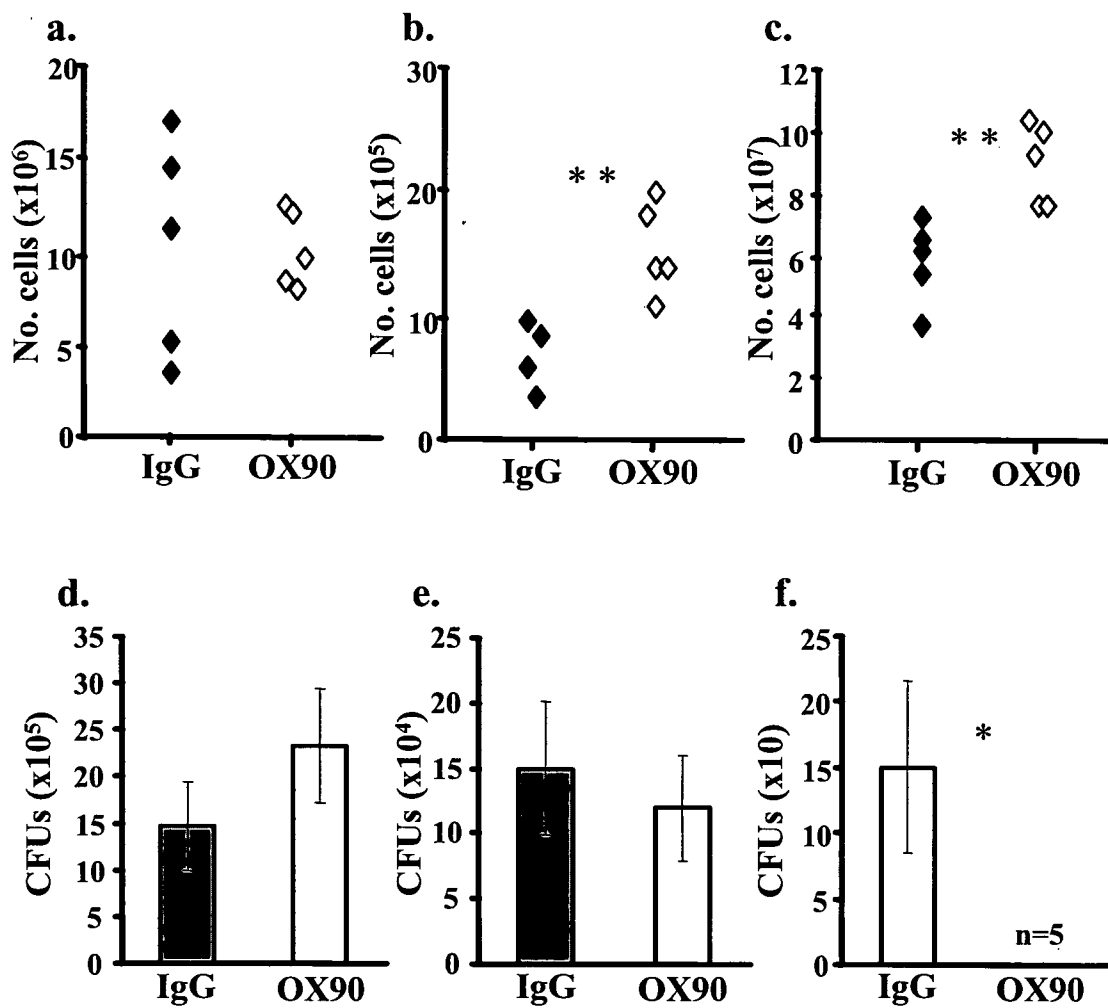
- *C. neoformans* CFUs**Lung:** - Surface/intracellular cytokine staining- *C. neoformans* CFUs

- Histology (H/E)

Brain / Spleen:- *C. neoformans* CFUs**Serum:**- Cryptococcus-specific antibody**Nasal Washes:** - IgE

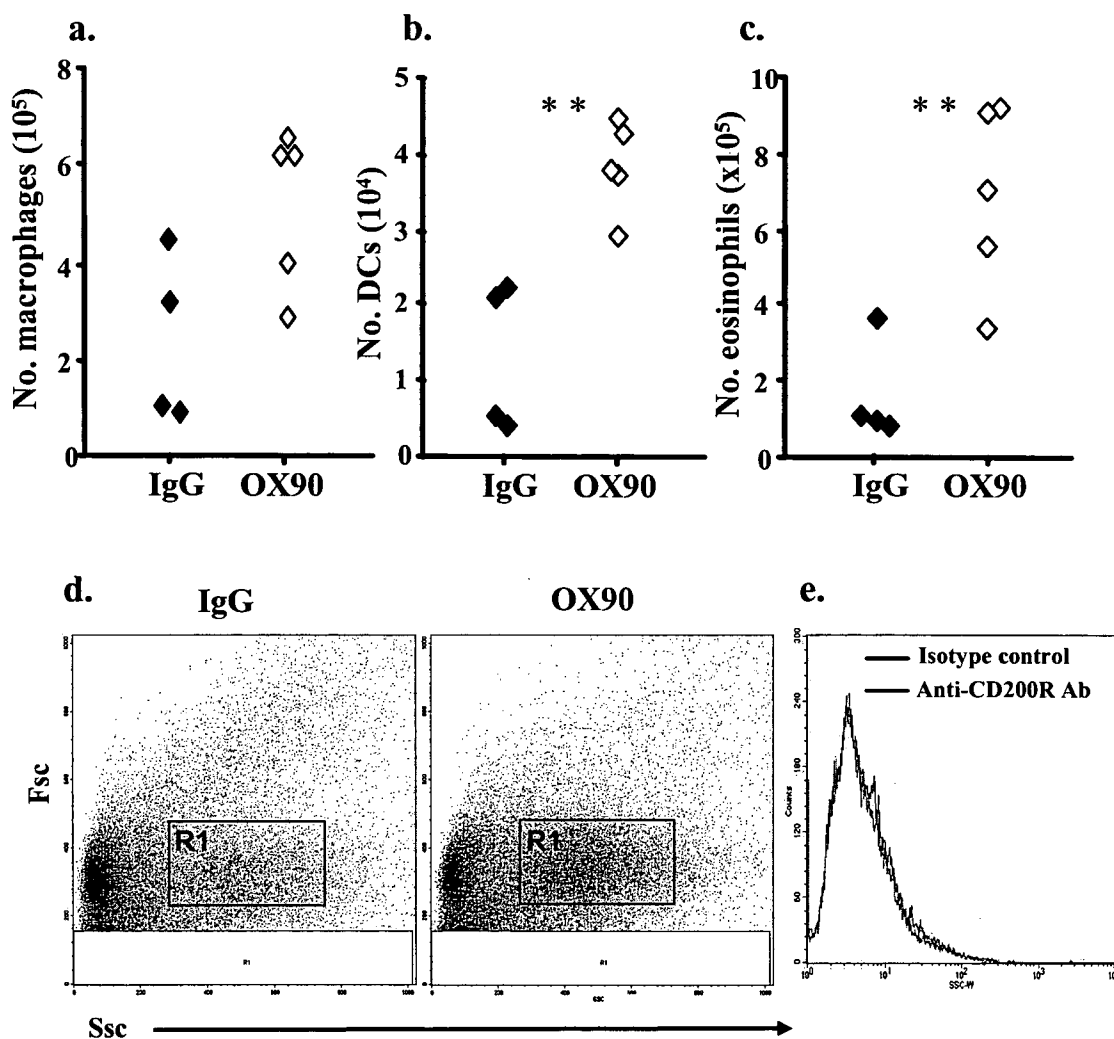
95/100

Figure 6.19



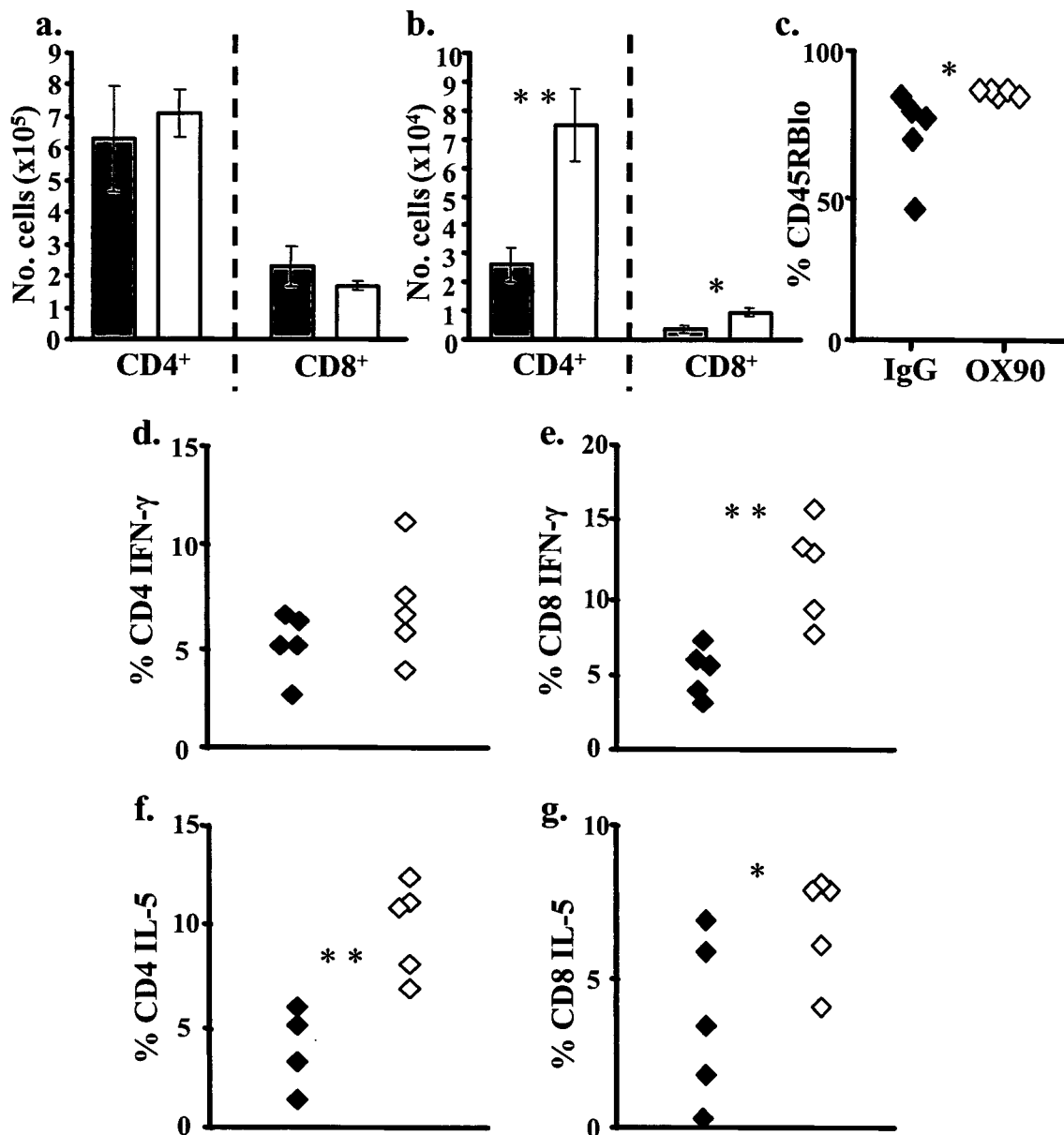
96/100

Figure 6.20



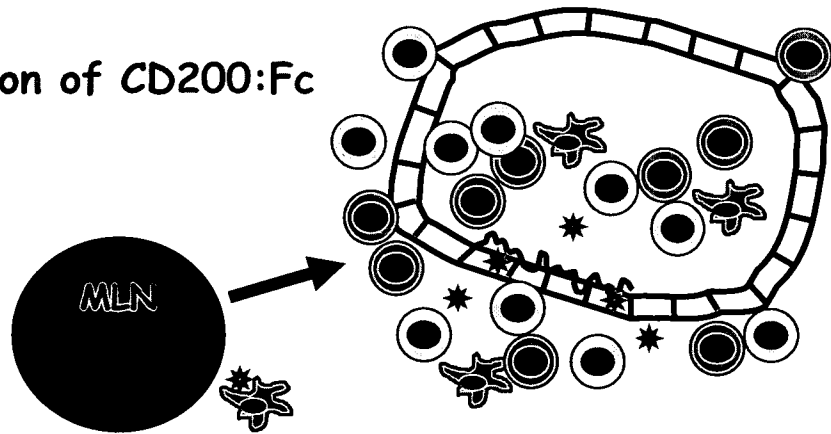
97/100

Figure 6.21



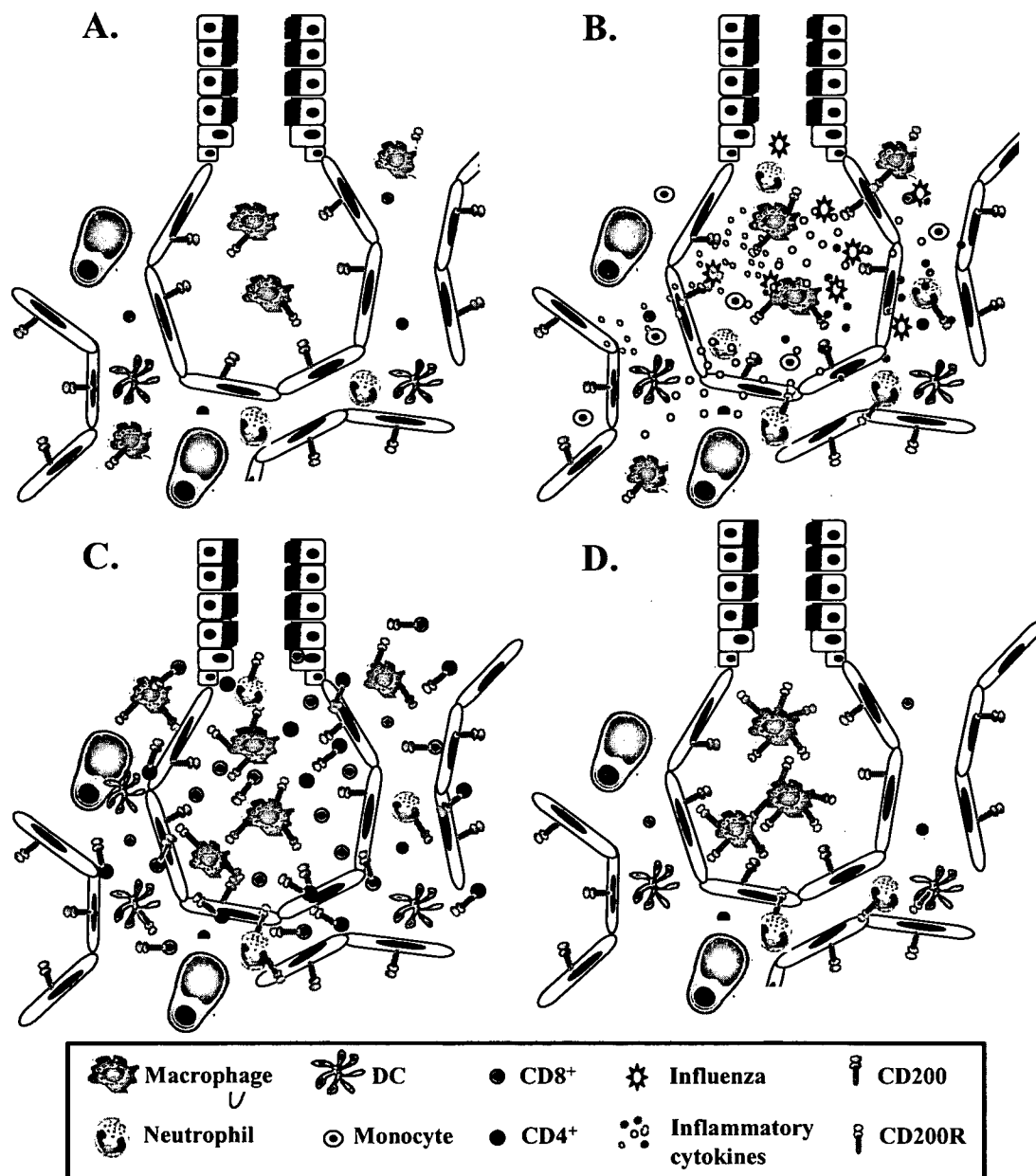
98/100

Figure 6.22

Mode of action of CD200:Fc**Phagocytosis of pathogen****Production of inflammatory signals****Migration of APC to the node****Maturation of the APC into a "professional"****Stimulation of migration competent T cells in node**

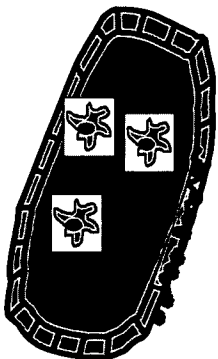
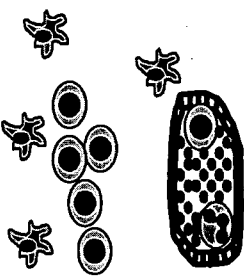
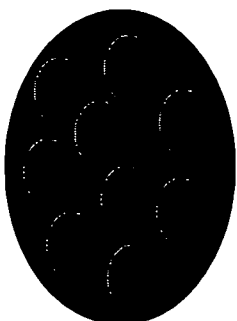
99/100

Figure 7.1



100/100

Figure 7.2

	Airway	Lung parenchyma	Lymph node
			
	No organisation	Scattered organisation	Organised
Inflammation	++++	++	(-)
TLR load/ ligand	++++	++	(-)?
CD200	+	+++	++++
ROS	+++	++	-
Late co- stimulatory molecules	++++	++	-
Effector function	++++	++	-