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A MURINE MODEL OF PULMONARY INFLAMMATION

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ABSTRACT

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Asthma is the commonest treatable chronic pulmonary disease affecting all ages and affecting up to 2-3 million each year in the UK. Yet despite improvements in the potency and selectivity anti-asthmatic medication and a greater understanding of the pathophysiology of the disease there is clear evidence to indicate that the incidence and severity of asthma is now reaching epidemic proportions.

Over the last twenty years it has become apparent that asthma is a chronic inflammatory disease driven by a Th-2 subpopulation of lymphocytes. Consequently a number of Th-2 driven models of allergic inflammation have been developed in animals. The two most commonly employed strategies to provoke a Th-2 response, particularly in rodents, are infection with gastrointestinal nematode Nippostrongylus brasiliensis (N.b.) or nebulisation of ovalbumin (OA) to the airways of OA-sensitised animals. However, many OA models do not mimic the chronic inflammation of the airways so typical of asthma in humans and until only recently they did not induce airway obstruction at all.

In this thesis we examined systemic and pulmonary response to N.b. in wild-type and genetically manipulated mice to determine how closely this mimicked allergic inflammation seen in humans. Our results suggest that there are a number of similarities in the cellular response in the lungs and elsewhere between N.b. infection and the allergic inflammation seen in asthmatic airways. Specifically, N.b. infection provoked an early and late inflammatory response, which culminated in the selective recruitment from the microvasculature of eosinophils and T cells expressing a restricted range of cell adhesion molecules. These eosinophils were shown to be small, mature, functionally active and recruited in much greater numbers that seen in OA-induced models. Furthermore, N.b. was shown to override genetically manipulated obstructions to the development of a Th-2 response. The similarity in the histopathology, bronchoalveolar lavage and effector cell function, between this model and allergic inflammation of the airways suggests that infection with N.b. may confer a number of specific advantages over OA-induced models of asthma and therefore represent a useful investigative tool in the fight against this common disease.

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LIST OF ABBREVIATIONS

AHR	:	Airway Hyperresponsiveness
APC	:	Antigen Presenting Cell
BAL	:	Bronchoalveolar Lavage
BHR	:	Bronchial Hyperresponsiveness
BSA	:	Bovine Serum Albumin
c	:	Circulating
cAMP	:	Cyclic Adenosine Monophosphate
CC16	:	Clara Cell Protein
CC	:	Chemokine
CCR3	:	Eotaxin Receptor
CD	:	Cluster Differentiation
CD18H	:	Cluster Differentiation 18 Hypomorph
$CD4^+$	:	T-Helper Cell
CTL	:	Cytotoxic T Lymphocytes
DP	:	Prostaglandin D Receptor
EDN	:	Eosinophil -Derived Neurotoxin
EGF	:	Epidermal Growth Factor
EGFR	:	Epidermal Growth Factor Receptors
FBS	:	Fetal Bovine Serum
FcεRI	:	High Affinity Ige Receptor
FcεRII	:	Low Affinity Ige Receptor
GMCSF	:	Granulocyte Macrophage Colony-Stimulating Factor
h	:	Hour
HBSS	:	Hanks Balanced Salt Solution
HDM	:	House Dust Mite
HEL	:	Hen Egg Lysozyme
hRV	:	Human Rhinovirus
ICAM	:	Intercellular Adhesion Molecule
ICOS	:	Inducible Co-Stimulator Protein
IFN	:	Interferon
Ig	:	Immunoglobulin
IL	:	Interleukin
ip	:	Intra Peritoneal
ISH	:	*In Situ* Hybridization
L3	:	Third-Stage *Nippostrongylus Brasiliensis* Larvae
LCMV	:	Lymphocytic Choriomeningitis Virus
LFA	:	Lymphocyte Function Associated Antigen
LT	:	Leukotriene
Mac-1	:	Macrophage Antigen-1
MAdCAM	:	Mucosal Addressing Adhesion Molecule
MCD	:	Mast Cell Deficient
MCP	:	Mast Cell Protease
MMP-9	:	Mucosal Mast Cell Protease 9
MoAbs	:	Monoclonal Antibodies
MHC	:	Major Histocompatability Complex

N.b.	:	*Nippostrongylus Brasiliensis*
NES	:	*Nippostrongylus Brasiliensis* Excretory-Secretory Protein
NF-κB	:	Nuclear Factor-Kappa Beta
NK	:	Natural Killer cells
OA	:	Ovalbumin
O2-	:	Superoxide Anion
PAF	:	Platelet-Activating Factor
PAF-AH	:	Platelet-Activating Factor Acetylhydrolase
PBS	:	Phosphate Buffered Saline
PDEs	:	Phosphodiesterases
PG	:	Prostaglandin
PCR	:	Polymerase Chain Reaction
pi	:	Post Infection
PPD	:	Purified Protein Derivatives
PPNK	:	Preproenkephalin
r	:	Recombinant
RBC	:	Red Blood Cell
RSV	:	Respiratory Syncytial Virus
RT	:	Room Temperature
s	:	Soluble
sc	:	Subcutaneously
SD	:	Sprague-Dawley
SMNC	:	Splenic Mononuclear Cells
STAT	:	Signal Transducers And Activators Of Transcription
TcR	:	T-Cell Receptor
T cell	:	Thymocyte Cell
TNF	:	Tumour Necrosis Factor
TW	:	Tween
VCAM	:	Vascular Cell Adhesion Molecule
VSV	:	Vesicular Stomatitis Virus
WCC	:	White Cell Count
WT	:	Wild Type

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CHAPTER 1

Introduction

1.1 THE RISING TIDE OF ASTHMA

Asthma is the commonest treatable chronic pulmonary disease affecting all age groups with a prevalence of up to 10% in some populations (Levy et al 1984). This translates into 2-3 million people suffering asthmatic symptoms each year in the UK. Yet despite improvements in the potency and selectivity of the drugs used to treat the disease, asthma still imposes a significant medical, social and financial burden on society. For example, a survey of 7,000 asthmatics reported that 40% are woken every night by their asthma, and 70% reported being woken at least once a week (Turner-Warwick et al 1989). Similarly, 25% of all asthmatics reported feeling breathless on most days of the week and at least a quarter require at least one emergency hospital admission each year (Gellert 1990 et al; Anderson et al 1983). It has been estimated that asthma management alone currently imposes a financial burden on the NHS of about £400 million per annum. In addition it costs the Department of Social Security £60 million in sickness benefit and the nation £350 million in terms of lost productivity (Glaxo 1990). A large proportion of the NHS bill is due to pharmaceutical costs; with the total number of prescriptions for asthmatic drugs doubling during the 1980s, and prescriptions for inhaled corticosteroids increasing fourfold.

Despite the increasing use of highly efficacious drugs and a greater understanding of the pathophysiology of the disease there is clear evidence to indicate that the incidence and severity of asthma in western society has risen steadily throughout this century (Burney 1990 et al, Holgate et al 1993). Some authors have suggested it is now reaching epidemic proportions (Holgate ST at al 1999b). Furthermore the death rate remains unchanged (Berrill et al1993).

Key factors driving these rising trends are the increased expression of atopy, the adoption of a western life style, increased exposure to sensitising allergens and reduced stimulation of the immune system during critical periods of development (Holgate ST et al 1999b, Seaton et al 1994). In allergic disease, there is a polarisation of the T-lymphocyte response, and enhanced secretion of inflammatory cytokines involved in regulation of immunoglobulin E (IgE), mast cells, basophils and eosinophils, ultimately leading to inflammation and disease. A clear understanding of the cellular and molecular mechanisms of allergic disease and the

complex interplay between genetic and environmental factors will undoubtedly create new opportunities for public health and therapeutic interventions.

1.2 GENETIC FACTORS

It has been estimated that 40-60% of asthma is genetically determined, and the single biggest risk factor for the development of asthma is atopy (Holgate et al 1997a). Atopy increases the risk of asthma by 10-20 times in atopic individuals compared to non-atopics.

Candidate and genome-wide searches for the "asthma gene" have identified a wide variety of potential gene targets (see Table 1-1)(Ono SJ et al 2000, Holgate at al 1997b, Daniels et al 1996). Some genes may play a non-specific role in increasing the risk of asthma. For example, 11q13, on the short arm of chromosome 11 close to the centromere, is thought to play a role in determining total IgE levels (Cookson et al 1988). Others may play a more specific role. Thus the genes encoding the HLA complex on chromosome 6p, specifically HLA DR and DP promote IgE hyperresponsiveness to house dust mite (HDM)(Ansari et al 1989). Similarly the genes encoding the TCR on chromosome 14q may play an important role (Burastero et al 1995).

Various genes, on chromosomes 4 and 7, have been implicated in determining airway hyperresponsiveness (Drazen JM et al 1999, Daniels et al 1996; Cookson et al 1988). Of course IgE and bronchial hyperresponsiveness may themselves be related. For example, the β-chain of the high affinity IgE receptor (FcεRI) has been specifically implicated in hyperresponsiveness particularly when inherited through the mother (Sandford et al 1993). However, several groups have failed to confirm any such association (Shirakawa et al1994) and the FcεRIβ explanation is unlikely to hold true for the majority of individuals that have specific IgE to common allergens. Alternatively, genes may code for the numerous proteins and peptides implicated in the inflammatory process such as 5q31 and 6p 21.3. The picture is further complicated by the variety of environmental factors that may affect the numerous steps in gene translation.

An alternative genetic hypothesis centres around the fact that many of the cytokines implicated in allergic inflammation, namely interleukin-4 (IL-4), IL-3, IL-5 IL-6, IL-9, IL-12, granulocyte macrophage colony-stimulating factor (GM-CSF), interferon (IFN) regulatory factor (a transcription factor which inhibits IFN-γ expression), the β_2-adrenergic and corticosteroid receptors, are all encoded on the long arm of chromosome 5q (Meyers et al 1994). The potential relationship between the IL-4 gene cluster, the β_2-adrenoreceptor, IgE and asthma is still unclear. In addition, chromosomes 4, 5, 7, 11 and chromosome 6 have been implicated in their linkage to eosinophil counts (Daniels et al 1996).

The genetics of antigen presentation must also be considered. Thus, specific sequences of the T-cell receptor (TcR) α-chain have been associated with atopy (Moffatt et al 1994). This may influence the efficiency of HDM or other aeroallergen epitope presentation in the cleft of the major histocompatability complex (MHC) class II to the TcR.

It would be fair to conclude that the genetic basis of asthma is so complex that identifying single genetic targets for therapeutic intervention is a questionable strategy for preventing the disease (Anderson GG et al 1999). Similarly, because some asthmatics have no inherited genetic predisposition (Panhuysen et al 1995), while others may have one or any of the above predisposing genetic factors then full genetic screening would have to be performed on all asthmatics if gene targeting was to prove successful in reducing disease expression. Furthermore, it is unlikely that genetic factors can explain the increasing prevalence of asthma and allergic disease within the population. Therefore we have to look to environmental factors to explain the rising tide of asthma and much of the pathogenesis of the disease.

Table 1-1. Candidate genes and chromosomal regions in asthma for which positive linkage or association has been suggested (adapted from Holgate et al 1997).

Chromosomes & candidate genes	**Coding for**	**Function**
5q31	IL-3, IL-4, IL-5, IL_9, IL-13, GM-CSF	Cytokines upregulating eosinophil, mast cell and basophil function
5q32	β_2-adrenoreceptor	bronchodilation
6p	HLA complex	antigen presentation
6p21.3	TNFα polymorphism	inflammatory cytokine
11q13	FcεRI polymorphism	Transduces signal on mast cell, basophil and dendritic cell
12q	IFN-γ	inhibits Th-2 cells
12q	A form of NOS & mast cell growth factor	promotes inflammation
14q	TCR α/δ complex	T cell activation

IL = interleukin. GM-CSF = granulocyte colony stimulating factor. TNF = tumour necrosis factor. FcεRI = high affinity receptor for C3 domain of IgE. IFN = interferon. Th2 = T helper 2 cell. NOS = nitric oxide synthetase. TCR = T cell receptor.

1.3 ENVIRONMENTAL FACTORS

A wide variety of environmental factors have been implicated in the development, progression and death from asthma. These environmental factors may be specific to the development of asthma or non-specific factors that alter general immunity (Tarlo SM et al 2000, Watkins et al 1996). Both may be at play simultaneously and both may begin *in utero*. The specific factors affecting the development of asthma are discussed below.

1.4 FACTORS AFFECTING ASTHMA-SPECIFIC IMMUNITY

The allergen exposure may occur prior to birth. For example, it has been suggested that the physicochemical nature of the antigens encountered *in utero* may lock the immune response into permanent allergen hyperresponsiveness (Piccinni et al 1993; Holtet al 1994). This is supported by evidence demonstrating that T-lymphocytes isolated from the cord blood of babies that subsequently developed allergic disease showed greater allergen responsiveness, with greater IL-4 and lower IFN-γ production in response to common allergens (Warneret al 1994).

Subsequent allergen exposure to HDM allergens in the first year of life has then been shown to predict whether a genetically predisposed child develops asthma by the age of 11 years. Furthermore, the level of exposure to one of the major HDM allergens, Der p1, predicted the onset of wheezing, with a greater than 10 pg of Der p1 per gram of dust during the first year of life increasing the risk of asthma fivefold (Sporik et al 1990). The importance of allergen exposure is supported by the finding that allergen avoidance dramatically reduces the risk of asthma in genetically "at risk" children (Ashrad et al 1993).

The proteolytic nature of many of the inhaled allergens favours their penetration through the disrupted airway epithelium (Stewart et al 1989), resulting in the production of allergen specific IgE antibodies (Arshad et al 1992). Whether the respiratory tract can be sensitised in the absence of a systemic IgE response is unclear. Moreover, sensitisation of the respiratory tract appears to be favoured when allergens are presented in association with adjuvant factors, such as inhaled irritants, air pollution including cigarette smoke and viral infections (Holt et al 1989).

1.4.1 Respiratory tract infections

In children, respiratory infections are associated with wheezing (Martinez et al 1995), and many first episodes of wheezing are associated with infection (Horn et al 1979). Infections are important precipitants of acute asthmatic attacks in children (Johnston et al 1995) and there is some evidence to suggest that infection with respiratory syncytial virus (RSV) predispose infants to IgE-mediated hyperresponsiveness and asthma (Russi et al 1993). Furthermore, specific RSV proteins (designated F and G), that determine virulence, have been shown to drive the immune response towards a T helper (Th)-1 (F-protein) or a Th-2-dominated picture (G-protein)(Alwan et al 1993).

1.4.2 Smoking

It is clear that foetal lung development *in utero* is critical to subsequent risk of developing respiratory disease (Barker et al 1991). Maternal smoking also seems to be important factor. Thus children born to women who smoked during pregnancy have reduced lung function and raised cord IgE levels (Royal College of Physicians 1992; Magnusson et al 1986). Similarly, children passively exposed to their parent's cigarette smoke have increased IgE levels (Ronchetti et al 1990), although a number of studies have reported sex differences in this effect (Martinez et al 1988). Maternal smoking has been shown to double the risk of asthma (Ehrlich et al 1992), but this is an effect that decreases with age (Lewis et al 1995).

Exposure to tobacco as an adult increases IgE levels in adults and accelerates the decline in lung function with age (Tager et al 1988; Jensen et al 1992). However, many of the studies examining the relationship between smoking and IgE failed to fully account for potential confounding variables and the overall effect of smoking on IgE levels is likely to be small (Friedhoff et al 1986). Smoking may have an effect through sensitising individuals to occupational allergens (Sunyer et al 1992). Conversely, smoking may, in some way, protect against hay fever (Bakke et al 1990; Jarvis et al 1995), although this may be due to people with hay fever avoiding smoking (Becklake et al 1990).

Overall, smoking seems to be an important environmental factor. There is little doubt that adults that smoke have more respiratory disease than non-smokers, but there is still much debate as to whether there is an increase in asthma among smokers. The prevalence of cough, wheeze and bronchial hyperresponsiveness is certainly increased in smokers but whether this is "asthma" or a proxy is still disputed.

1.4.3 Pollution

Environmental factors clearly interact in a complex way. For example, smoke and pollution may lower resistance to virus infection (Rose et al 1989), and enhance the sensitivity of the airways to allergens (Devalia et al 1994) as well as causing direct epithelial damage (Holt et al 1990). However, pollution is probably not a major factor in the rising tide of asthma, with some data indicating that the prevalence of asthma and the percentage of positive skin prick test responses are lower in polluted areas compared to relatively "clean" areas (Von Mutius et al 1992; Wichmann et al 1989). Most of the reviews of the data are contradictory with some suggesting that increased pollution is associated with increases in respiratory mortality,

particularly in the elderly and infirm (Dockery et al 1994), while other reviews suggest that the pollution effects only occurs during the winter months (Walters et al 1994).

Some studies have demonstrated specific links between certain pollutants, such as diesel exhaust particle emissions or airborne endotoxins, and specific IgE production or the gene expression of Th-2 cytokines in humans (Wan GH et al 2000, Casillas AM et al 1997). Others have shown that repeated exposure of very young mice to allergen alone or pollutant alone had no effect on airway hyperresponsiveness (AHR) and did not cause inflammation or allergen-specific antibody production. However, similar exposures of adult mice to either allergen alone or to allergen and pollutants did result in AHR (Hamada K et al 2000).

Recently an extensive review of the relationship between asthma and outdoor air pollution in the UK concluded that most asthmatic patients were unlikely to be affected by exposure to the levels of non-biological pollution currently encountered (Committee on the Medical Effects of Air Pollution, 1995).

1.4.4 Foetal nutrition

Animal and human studies have demonstrated that poor foetal nutrition can have lifelong effects on physiology and metabolism. Specifically, a rapidly growing foetus experiences a sudden growth retardation if exposed to undernutrition, while slowly growing babies are unaffected (Chandra et al 1991). The sudden change in nutritional level results in the activation of a "brain-sparing reflex" with blood being shunted away from the body, a disproportionately large head circumference and a wasted thymus (Harding et al 1991). The significance of the wasted thymus is unclear, but it has been suggested that this may disrupt lymphocyte development and an alteration in T-helper ($CD4^+$) subset ratio. Specifically, sudden malnutrition is believed to increase the Th-2:Th-1 ratio and, since Th-2 cells have been implicated as key players in IgE-mediated allergic inflammation, increase the risk of developing asthma (Godfrey et al 1994). The disproportionate effect of malnutrition on Th-1 cells is supported by evidence suggesting that Th-1 cells may be more sensitive to environmental stress (Ricci et al 1993).

1.5 AIRWAY MUCOSAL INFLAMMATION

The last twenty years have witnessed a considerable increase in our understanding of complex interplay between the numerous environmental and genetic factors involved in the aetiology of asthma. At the same time substantial evidence has been gathered using, modern, molecular biological techniques, such as the polymerase chain reaction (PCR), immunohistochemistry and *in situ* hybridization (ISH) to suggest that asthma is an inflammatory disease of the airways involving almost all the cells of the immune system (Bjornsdottir US et al 1999, Pearlman DS et al 1999).

The inflammatory cell infiltrate, which consists predominantly of degranulated mast cells and activated T lymphocytes and eosinophils, exacerbates the airway smooth muscle contraction, excessive mucus secretion and mucosal oedema and leads to the airflow obstruction characteristic of patients with asthma (Redington, et al 1994; Djukanovic et al 1990; Azzawi et al 1990). Furthermore, it has been shown that the degree of cellular infiltration is closely correlated with disease activity (Robinson et al 1993a; Wardlaw et al 1988).

Current opinion suggests that this cellular infiltration is brought about by the sequential interaction of pro-inflammatory cytokines and cell adhesion molecules (Pilewski et al 1995). These cytokines may be derived from a number of cell types, including the Th-2 sub-population of $CD4^+$ lymphocytes, which have been implicated as the major orchestrator of the inflammatory process (see below).

1.5.1 T Lymphocytes

Up until the mid 1980s there was a general consensus that the mast cell was the major player in airway mucosal inflammation. Asthma was seen as a type I immediate hypersensitivity reaction dependent on IgE-mediated mast cell mediator release. This explained atopic asthma, while non-atopic asthma was thought to be due to non-IgE-mediated mast cell degranulation. In support of this theory sodium cromoglycate, which was widely used to treat asthma, was thought to work by stabilising the mast cell membrane. However, there were a number of facts that did not fit with the mast cell hypothesis. Firstly, corticosteroids, the mainstay in asthma management since the 1950s, had no effect on mast cell histamine release. Secondly only a proportion of individuals with atopy and "unstable" mast cells developed asthma. Perhaps the most important piece of evidence to destroy the mast cell hypothesis was the demonstration

that between acute attacks asthmatics were still hyperresponsive to non-specific stimuli. The idea of asthma as a chronic inflammatory disease was born (Austen et al 1984).

The use of fibre-optic bronchoscopy to obtain bronchoalveolar lavage (BAL) and bronchial biopsies enabled the cellular infiltration of the airways to be investigated in more detail. These techniques rapidly established that eosinophils and mononuclear cells infiltrated the airways even in the absence of clinical symptoms and were important in asthma pathophysiology (American Thoracic Society 1987).

The idea that the T lymphocyte was the major co-ordinator of airway inflammation evolved out of an increased understanding of the immunology of allergic responses in general as well as the knowledge that T cells are known to be exquisitely sensitive to corticosteroids. Thus, evidence accumulated to suggest that T cells orchestrated the function of B cells and eosinophils through specific cytokine signals (Corrigan et al 1990) and promoted a B-cell isotype switch to IgE production (Kopf et al 1993; Kuhn et al 1991; Tepper et al 1990).

T lymphocytes were seen as crucial to allergic inflammation because they are the only cellular population capable of allergen-specific responses. These allergen-specific responses were believed to occur in regional lymphoid nodes following professional MHC restricted antigen presentation by dendritic cells to specific T cell receptors (TCR). Specifically γ/δ TCR have been implicated as important in this pulmonary homeostasis independent of α/β TCRs (Lahn M et al 1999). These allergen-specific T cells then undergo selective clonal expansion and are recruited to the airway by leucocyte-endothelial adhesion mechanisms and chemotaxis. It is these chronically activated T memory cells that are believed to "drive" chronic airway inflammation.

Support for the T cell hypothesis came from studies demonstrating that the numbers of activated T cells expressing activation markers in the blood, BAL and bronchial biopsies of asthmatics were increased in comparison with normal subjects (Corrigan et al 1988). Furthermore there was a direct correlation between the number of activated peripheral blood T cells and lung function (Corrigan et al 1990). Double immunofluorescence studies of BAL cells demonstrated the activated T cells, expressing the CD25 (IL-2R) marker, were primarily from the $CD4^+$ subset (Robinson et al 1993b).

1.5.2 The Th-1, Th-2 continuum

In 1986 Mossman defined two distinct subsets of $CD4^+$ cells, namely Th-1 and Th-2 based on their cytokine expression (Mossman TR et al 1986). This discovery was a turning point in the understanding of allergic inflammation. Th-2 cells were shown to predominantly express IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 and GM-CSF while Th-1 cells predominantly express IL-2, IL-12, IFN-γ and tumour necrosis factor (TNF)-β. These two sub-populations were shown to be mutually inhibitory via their cytokines, IL-10 and IFN-γ (Fiorentino et al 1989).

The Th-2 population supports antibody production by B cells and stimulates maturation and activation of eosinophils, while the Th-1 population promotes cell-mediated responses through its ability to activate cytotoxic and phagocytic cells (Kapsenberg et al 1991). An increasing volume of research implicated the Th-2 population as the primary orchestrators of allergic inflammation of the airways in animals (Coffman et al 1989; Finkelman et al 1988).

Initially the existence of distinct T helper sub-populations of lymphocytes in humans was questioned. However, a number of recent studies have now confirmed a pivotal role played by the IL-4 gene cluster encoded by Th-2-like T cells in human allergic disease. For example, T cell clones from atopic individuals can be stimulated to produce Th-2 cytokines in response to antigens (Parronchi et al 1992), T cell mitogens (Field et al 1993), or allergens (Wierenga et al 1991; Parronchi et al 1991), including house dust mite (O'Hehir et al 1993). In addition, late-phase allergic reactions are associated with increased gene transcription for IL-3, IL-4, IL-5, and GM-CSF (Robinson et al 1992; Del Prete et al 1993). Furthermore, these cytokines have been shown to be involved in the recruitment and activation of mast cells and eosinophils following the upregulation of adhesion molecule expression (Hamelmann E et al 1999).

However, there are still many debates as to the degree of polarisation of the immune response with some populations of T lymphocytes showing great heterogeneity, particularly in humans (Abbas et al 1996).

1.5.3 Factors involved in the development of a Th1 or Th2 response

I. Antigens

The mechanisms that induce the differentiation of naïve T cells into Th-2 cells remain a source of great interest. As indicated earlier the nature of the antigens encountered *in utero* or infancy may lock the T cell response into a permanent Th-1-like or Th-2-like pattern (Piccinni et al 1991; Warner et al 1994; Holt et al 1994; Sporik et al1990).

Murine experiments have demonstrated that the generation of an IFN-γ or an IL-4-predominant response is dependent on the type and duration of antigenic stimulation. For example, adult antigens from *Nippostrongylus brasiliensis* (N.b.) promote a Th-2 response, while BCG purified protein derivatives (PPD) or *Brucella abortus* antigens promote Th-1 responses, both *in vitro* (Saito et al 1994), and *in vivo* (Street 1990). Similar findings have been reported in humans using *Toxocara canis* (Del Prete et al 1991). Factors intrinsic to the antigen seem to stimulate Th-2 differentiation. Thus an alteration in the antigenic structure switched the response from Th-2 to Th-1 with the production of IFN-γ and IgG2a antibodies (Gieni et al 1993).

In addition to the antigenic structure an antigen's intrinsic enzymatic activity may be an important determinant of Th-2 development (Thomas et al 1993). There is also some evidence to suggest that antigen load is a factor in determining the balance between Th-1 and Th-2 subsets, with low antigen loads favouring Th-1 responses whereas high loads or repeated exposure favours a Th-2 response (Hosken et al 1995). This may be because the cells used to present antigen at low concentrations promote a Th-1 phenotype through the production of IL-12, a Th-1 cytokine, whereas at higher concentrations IL-12 is not produced thereby favouring a Th-2 phenotype (Abbas et al 1996).

II. Antigen presenting cells (APCs).

$CD4^+$ lymphocytes respond to antigenic epitopes presented in the peptide cleft of the MHC Class II molecule on APCs (Harding et al 1996). While B lymphocytes can act as APCs under some circumstances (Morris SC et al 1994), the professional APCs in the airways are the dendritic cells that primarily reside in the peripheral non-lymphoid tissue (Lanzavecchia et al 1996). These cells are up to 100 times more efficient at stimulating T cell proliferation than alveolar macrophages (Hance et al 1993). Having taken up the antigen or allergen they

migrate to draining lymph nodes where they lose their ability to engulf antigen but increase their ability to stimulate naïve T cells (Lanzavecchia et al 1996).

The APC T-cell interaction and the subsequent phenotypic development of T cells seem to be critically dependent on the co-stimulatory signals provided by APCs. Langerhans cells, which are present in abundance in the upper airways, seem to preferentially promote the development of the Th-2 phenotype (Hauser et al 1989). The best-described co-stimulatory system is the coupling of B7-1 and B7-2 with its T cell partner, CD28. The B7 ligand plays an important role in T-B cell interaction. In addition, B7-1 and B7-2 differentially promote the development of the Th phenotypes, which may determine the degree of inflammation (Lenschow et al 1996).

Thus, administration of anti-B7-2 mAb to ovalbumin (OA)-sensitised mice abolished allergen-induced AHR, pulmonary eosinophilia, and elevations in serum IgG1 and IgE levels. Anti-B7-2 treatment of OA-sensitised mice also reduced total lung IL-4 and IL-5 mRNA and BAL fluid IL-4 and IL-5 protein levels with no significant changes in IFN-γ message or protein levels. In contrast, treatment with anti-B7-1 mAbs had no effect on allergen-induced airway hyperresponsiveness, IgE production or cytokine production. However, it significantly suppressed pulmonary eosinophilia.

Therefore B7-2 appears to be necessary for the development of in vivo allergic responses to inhaled allergen while B7-1 is less critical (Keane-Myers et al AM 1998). Some authors have suggested that the ability of B7-2 to turn on a Th-2 response is antigen-specific (Gause WC et al 1997). These findings were in contrast to earlier studies, which demonstrated that either B7-1 or B7-2 could provide the required co-stimulatory signals to switch on a Th-2 response in vivo (Greenwald RJ et al 1997, Gause WC et al 1995). More recently it has been suggested that the signal provided by B7-2 may be confined to the later stages of the Th-2 response (Gause WC et al 1999). The ability of B7-2 to turn on a Th-2 response is influenced by CD40. With certain aspects of the Th-2 response, such as cytokine production, being CD40-dependent while other aspects, such as effector cell function being CD40-independent (Lu P et al 1996).

In contrast to this clear role for B7-2 in promoting a Th-2 response the role of CD28 remains unclear. For example, CD28-deficient mice can still generate significant Th-2

responses to some infectious pathogens. This suggests that molecules other than CD28 can respond to the B7-2 signal. One candidate for this role is the inducible co-stimulator protein (ICOS), which is structurally and functionally related to CD28.

Blocking ICOS only partially impairs the Th-1 and Th-2 response to infection with lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), and N.b. in normal mice. But blocking ICOS in CD28 deficient mice significantly impaired both Th-1 and Th-2 responses to these pathogens. In contrast, blocking ICOS in CD28 deficient mice did not alter the CTL response to LCMV and VSV.

Therefore it appears that ICOS and CD28 may be interchangeable in their ability to respond to B7-2 and switch on a Th-1 or Th-2 response. In contrast, cytotoxic T lymphocytes (CTL) responses are activated independent of ICOS or CD28. Put another way ICOS may regulate both CD28-dependent and CD28-independent Th-1 and Th-2 responses, but has no affect on CTL responses in vivo (Kopf M et al 2000).

III. Cytokine production

- **Interleukin (IL)-4**

Numerous in vitro and in vivo studies, in both animal models and human asthmatics, have implicated IL-4 as an important pro-inflammatory molecule driving the Th-2 response and isotype switching to IgE. For example, IL-4 deficient mice generate significantly attenuated, but not absent, Th-2 responses. Similarly, blockade of IL-4R has been shown to significantly reduce antigen-induced AHR, eosinophil recruitment to the airways and goblet cell metaplasia (Gavett SH et al 1997). In addition, it has been shown that IL-4 causes a dose dependent, FcγRII/FcγRIII and T cell independent redistribution of B cells to the spleen in addition to modestly increasing B cell life span. It has been suggested that these latter two effects may promote antibody responses to a systemic antigen challenge (Mori M et al 2000).

Recently, a more sophisticated understanding of the role of IL-4 in allergic inflammation is beginning to emerge. For example, evidence has accumulated to suggest that Th-2 cells can develop in the absence of IL-4 (Kropf P et al 1999), and exposure to IL-4, during T cell activation can promote deletion of responding $CD4^+$ T cells (Morris SC et al 2000). Furthermore, novel cellular sources, such as NK cells, have been identified that produce IL-

4 even in IL-4R deficient mice (Noben-Trauth N et al 1997). In addition, recent data have suggested that, under certain circumstances, IL-4 can inhibit inflammation. Thus the administration of exogenous soluble IL-4 to mice significantly reduces late phase pulmonary inflammation, blocks airway eosinophil infiltration, VCAM-1 expression, and mucus hypersecretion. Thus soluble IL-4 and IL-4 receptor may have anti-inflammatory effects in asthmatic patients (Henderson WR Jr et al 2000).

- **IL-5**

Just as IL-4 has been implicated in driving the Th-2 response and IgE synthesis, IL-5, which is normally produced by T-cells, mast cells and eosinophils, has been strongly implicated in the generation of airway eosinophilia (Hamelmann E et al 1999), IL-5-regulated pulmonary eosinophilia and airways dysfunction can occur independently of IL-4 (Hogan SP et al 1997b). Therefore IL-5 has been the target for therapeutic attack using a variety of strategies such as antisense oligonucleotides and anti-IL-5 monoclonal antibodies (MoAbs). Anti-IL-5 monoclonal antibodies have been administered systemically or more recently inhaled intranasally (Shardonofsky FR et al 1999). Such approaches have also been shown to inhibit antigen-induced AHR (Karras JG et al 2000). In fact IL-5 has been more strongly implicated in the induction of airway symptoms than IL-4 (Hofstra CL et al 1999).

More recently the critical role IL-5 plays in the recruitment eosinophil to the airways has been confirmed in patients with mild asthma. In a double-blind randomised placebo-controlled trial, a single intravenous infusion of humanised monoclonal antibody to IL-5 lowered the mean blood eosinophil and prevented the blood eosinophilia that follows allergen challenge for up to 16 weeks. In addition, anti-IL5 almost completely abolished the sputum eosinophilia and this effect persisted for up to 30 days. However, anti-IL5 had no effect on the late asthmatic response or on airway hyper-responsiveness to histamine (Leckie MJ et al 2000). This suggests that while anti-IL-5 antibody is very effective at inhibiting blood and airway eosinophilia it may not be very helpful in symptomatic asthma (Barnes PJ et al 2002).

Such evidence confirms the work of earlier authors who demonstrated that mice genetically manipulated to overexpress IL-5 and develop permanent airway eosinophilia did not develop the normal respiratory distress or pathology on exposure to aeroallergens or helminths (Dent LA et al 1997).

- **IL-9**

Recent evidence has suggested that IL-9 is a key cytokine increasing the susceptibility to asthma. Specifically, IL-9, which is produced by Th-2 cells, has a wide range of biological functions on many cell types. For example, selective expression of IL-9 in the lungs of transgenic mice has been shown to cause massive airway inflammation with eosinophils and lymphocytes and elevated serum IgE levels.

In addition, these mice also express a number of other impressive pathologic changes in the airways, such as mast cell accumulation, epithelial cell hypertrophy associated with accumulation of mucus-like material within non-ciliated cells and increased subepithelial deposition of collagen. These IL-9 mice had normal baseline airway resistance and markedly increased airway hyperresponsiveness to inhaled methacholine (Temann UA et al 1998). Even intratracheal instillation of IL-9 in naïve C57BL/6 mice, which express very low levels of IL-9, increased BAL fluid eosinophilia and significantly increased serum total IgE levels. In addition, IL-9 was found to induce IL-5Rα in vivo and in vitro, suggesting a potential mechanism for the novel actions of IL-9 on eosinophils (Levitt RC et al 1999).

Furthermore, IL-9 induced eosinophil chemotaxis has been shown to be dependent on the up-regulation of CC-chemokine expression in the airway (Dong Q et al 1999). Recently bone marrow-derived mast cells have been shown to be very potent producers of IL-9 particularly when activated with a combination of ionomycin, IL-1 and IgE with additional stimulation provided by IL-10, a cytokine normally produced by bronchial epithelial cells (Stassen M et al 2000).

- **IL-10**

It has become abundantly clear that not only do IL-4, IL-5 and IL-9 induce airway inflammation but also IL-10 and IL-12 play a crucial role. Although IL-10 is a Th-2 cytokine, particularly in the mouse, it is believed to play a predominantly anti-inflammatory role inhibiting Th-1 and Th-2 cells. For example studies using IL-10 knockout mice demonstrated that these mice developed greater eosinophilic airway inflammation but comparable levels of allergen-specific serum IgE compared to the WT mice after allergen challenge (Tournoy KG et al 2000b).

Similarly, direct intratracheal instillation of 25 ng of recombinant murine IL-10 increased BAL fluid IL-10 concentration beyond that seen following antigen exposure but decreased BAL fluid IL-4, IL-5, and IFN-γ levels by 40-85% and eosinophil numbers by 70%. IL-10 treatment also increased airway reactivity to methacholine but only in sensitised and antigen challenged mice. Thus, in some models, IL-10 can reduce Th-2 cytokine levels and eosinophilic inflammation but augment airway hyperreactivity (van Scott MR et al 2000).

This ability of IL-10 to play an anti-inflammatory role while increasing airway reactivity was confirmed in other studies with IL-10 knockout mice. For example, despite developing pulmonary inflammatory in response to aeroallergens IL-10 knockout mice failed to develop AHR in both in vitro and in vivo. Reconstitution of IL-10 deficient mice with the IL-10 gene fully restored the development of airway hyperresponsiveness comparable to control mice (Makela MJ et al 2000). Therefore IL-10 may play a more important role in regulating airway tone than regulating pulmonary inflammation.

In contrast to the above reports some authors have demonstrated that IL-10 gene knockout mice have significantly reduced eosinophilic inflammation, mucus production and IL-5 production with no changes in IL-4 and IgE responses. These authors also demonstrated significantly greater production of the Th-1 cytokine IFN-γ and IL-12 in IL-10 knockout mice (Yang X et al 2000). The results suggest that endogenous IL-10 may play an important role in promoting pulmonary eosinophilic inflammatory reaction and mucus production in some models and suppressing inflammation in others. Furthermore the data suggest that IL-10 may be more influential in the development of IL-5-producing Th-2 cells than Th-2 cells, which produce both IL-4 and IL-5. The reasons for the contradictions in the literature are not yet clear.

- **IL-12**

The role of IL-12 in allergic inflammation has been the focus of much attention. For example, administration of IL-12 to mice at the time of antigen challenge has been shown to abolish airway hyperresponsiveness and pulmonary eosinophilia in addition to increasing IFN-γ and decreasing IL-4 and IL-5 expression. The effects of IL-12 were partially dependent on IFN-γ. Thus concurrent treatment with anti-IFN-γ MoAbs reversed the IL-12 induced inhibition of airway hyperresponsiveness and eosinophilia. Treatment of mice with

IL-12 at the time of a second antigen challenge also prevented airway hyperresponsiveness and significantly reduced numbers of BAL inflammatory cells, reflecting the ability of IL-12 to inhibit responses associated with ongoing antigen-induced pulmonary inflammation (Gavett SH et al 1995).

The main source of IL-12 is macrophages, dendritic cells, and other monocytes. In addition to inhibiting IL-4 production and Th-2 responses IL-12 has been shown to be important in driving a strong Th-1 response by stimulating the production of IFN-γ from natural killer and Th-0 cells. Consequently, IL-12 has been shown to play an important role in resistance to a number of intracellular pathogens, including Listeria and Leishmania as well as prolonging the survival of the N.b. in the gut. IL-12 has also been proposed as an anti-tumor agent and as a potential therapeutic option in the treatment of HIV. Conversely, IL-12 has been shown to accelerate autoimmunity (Bancroft AJ et al 1997).

The effects of IL-12 on Th-2 responses have received particularly close attention. For example IL-12 has been shown to stimulate IFN-γ and IL-10 gene expression during primary and secondary N.b. infections. In addition IL-12 inhibited IL-3, IL-4, IL-5, and IL-9 gene expression during primary infections but had little inhibitory effect during secondary infections. IL-12 also inhibited IgE, mucosal mast cell, and blood and tissue eosinophil responses during primary infections, but only eosinophil responses during secondary infections. Anti-IFN-γ antibodies inhibited the effects of IL-12 on IgE secretion and mast cell recruitment but did not affect IL-12 inhibition of eosinophilia.

These studies indicate that while IL-12 may provide a novel therapeutic approach to the treatment of allergic inflammation it has to be administered during the early phase of inflammation to effect an isotype switch from Th-2 to a Th-1 response. If IL-12 treatment is not administered until after Th-2 cytokines have been produced it is much less effective (Finkelman FD et al 1994). More recently it has been suggested that the administration of inhibitory cytokines, such as IL-10, interferons, and IL-12 may not be as promising as hoped because systemic delivery produces intolerable side effects. In addition, because so many cytokines are involved in asthma, drugs that inhibit the synthesis of multiple cytokines may prove to be more useful (Barnes PJ et al 2002).

- **IL-13**

In addition to IL-4, IL-5, IL-6, IL-9 and IL-10 Th-2 cells also secrete IL-13. The production of IL-4 has been shown to play a central role in resolving nematode infection where its synthesis is helpful but its production is unhelpful in the airways where it drives an inflammatory cascade. However, IL-4 deficient mice still expel nematode parasites suggesting IL-4 is not the only cytokine involved in the resolution of such infections. Recent studies using transgenic mice and exogenously administered cytokines has suggested that IL-13 is also important. Thus IL-13 deficient mice failed to expel nematodes or develop goblet cell hyperplasia until IL-13 was administered exogenously. These results suggest that IL-13 and IL-4 are not redundant but may share some common features. (McKenzie GJ et al 1998).

For example, there is now compelling evidence that IL-4 and IL-13 share receptor components, including IL-4Rα and IL-13Rα1. In fact there is evidence to suggest that the IL-13 regulation of the Th-2 response to nematode infection requires IL-4Rα (Barner M et al 1998).

The exact role of IL-4 and IL-13 in the induction of Th-2 responses was investigated in a recent series of experiments. These studies, using a pulmonary granuloma model, compared mice with a dual IL-4 and IL-13 deficiency to those with just IL-4 or IL-13 deficiency.

These experiments demonstrated that eosinophil infiltration, IgE and IL-5 production were reduced in both the IL-4 and IL-13-deficient mice. In contrast, eosinophil infiltration, IgE and IL-5 production were completely abolished in the mice with the combined deficiency of both cytokines. Furthermore, mice with combined IL-4/13-deficiency were unable to expel gastrointestinal nematodes and developed a Th-1-like response characterised by the expression of IFN-γ and the production of IgG2a and IgG2b. These studies also found that mice lacking either IL-4 or IL-13 appeared to have developed compensatory mechanisms since the mice expressed elevated levels of IL-5 and airway eosinophilia, although serum IgE remained undetectable (McKenzie GJ et al 1999). Therefore these more recent studies support the view that IL-4 and IL-13 perform a synergistic role in initiating a rapid Th-2 response. This differing role for IL-4 and IL-13 was emphasised by new research

demonstrating that B7 costimulation is required to induce IL-4, but not IL-13 responses (Urban J et al 2000b).

- **IL-16**

IL-16 is a pro-inflammatory cytokine synthesised predominantly by the airway epithelium as well as mast cells, $CD8^+$ and $CD4^+$ cells and eosinophils. It is a precursor molecule that is processed by cleavage of the C-terminal and uses CD4 to induce early lymphocyte, particularly CD4, chemotaxis as well as IL-2R and HLA-DR expression (Center DM et al 1997).

IL-16 has been identified in bronchial biopsies of atopic asthmatics. The cellular source of IL-16 was identified as epithelial cells and non-epithelial inflammatory cells, 60% of which were T cells and 17% were eosinophils. Mast cells have also been shown to be a source of IL-16 in asthmatic airways (Laberge S et al 1999).

- **IL-18**

IL-18 is secreted by the airway epithelium, activated macrophages, basophils and mast cells stimulated with IL-3. IL-18 shares some of its biologic activities with the Th-1 cytokine IL-12 (Cameron LA et al 1999). Both IL-18 and IL-12 have been shown to induce IFN-γ production by Th-1 cells and B cells but its exact role in allergic inflammation remains unclear (Yoshimoto T et al 1997).

Intrapulmonary administrations of IL-18 to naïve or sensitised mice has been shown to promote a Th-2 response with an increased BAL eosinophilia, serum levels of specific IgE and IgG1, and the production of IL-4 and IL-5 by cultured splenocytes and lung cells. However, these effects were not robust and could be reversed by manipulating the experimental protocol (Wild JS et al 2000). Although IL-18 enhanced antigen-induced airway eosinophilia, IL-18 did not affect antigen-induced AHR in sensitised mice (Kumano K et al 1999). The enhancement of Th-2 responses was thought to be due, in part, to increasing TNF-α production.

In contrast, some studies have reported that in vivo administration of IL-18 not only inhibited antigen-specific Th-2 responses (Yoshimoto T et al 1997), and eosinophil

recruitment but also affected apoptosis through Fas-Fas ligand interactions (Kodama T et al 2000). Whether IL-18 promotes or suppresses Th-2 responses may depend on the cytokine environment, particularly the co-production of IL-12 (Yoshimoto T et al 1999).

- **Interferon Gamma (IFN-γ)**

Interferon gamma (IFN-γ) is only produced by $CD4^+$, $CD8^+$ and NK cells and is involved in the regulation of many phases of the inflammatory response. IFN-γ activates macrophages and modulates MHC class I and II expression. This enables macrophages and dendritic cells to function as antigen presenting cells. But perhaps most importantly IFN-γ has an immunoregulatory role inhibiting Th-2 cells and their cytokine production. It is also a powerful inhibitor of IL-4-induced IgE synthesis. The production of IFN-γ has been shown to block airway eosinophilia and mucus production through inhibitory pathways that are activated downstream of Th-2 cytokine secretion (Cohn L et al 1999).

The peripheral blood lymphocytes of atopics are less able to generate IFN-γ than non-atopics. However $CD8^+$ cells from atopics generate more IFN-γ than $CD8^+$ cells harvested from non-atopics. The number of IFN-γ producing $CD8^+$ T cells in atopics is related to asthma severity, AHR, blood eosinophilia and IL-12 production (Magnan A O et al 2000).

IFN-γ has also been shown to play an important role in protecting the airway from viral infection. For example, a recent study explored the role of IFN-γ on human rhinovirus (hRV) infection, which is a major cause of upper respiratory tract infections and can exacerbate existing airway inflammation. This virus attaches to ICAM-1 expressed on nasal and bronchial epithelial cells. In contrast to TNF-α, IL-1β and the IL-8, which are released soon after viral infection, IFN-γ decreased ICAM-1 expression on hRV-infected epithelial cells. This effect of IFN-γ was mirrored by a reduction in viral titres (Sethi SK et al 1997).

Despite these encouraging studies the use of recombinant (r) IFN-γ as a therapeutic option has not been shown to be very effective. Thus an early study demonstrated that while rIFN-γ may produce a 30% reduction in the number of circulating eosinophils it produced little clinical improvement (Boguniewicz M et al 1993).

- **IL-11, IL-6 and TNF-α**

Unravelling the complexity and redundancy in cytokine expression by immune cells should shed some light on the pathophysiology of allergic inflammation. For example, recent studies examining the expression of two closely related cytokines, IL-11 and IL-6 demonstrated that these cytokines have opposing effects in the mouse lung.

Specifically, IL-11 caused airways obstruction and increased airway reactivity to methacholine whereas IL-6 did not. Both transgenic strains showed similar emphysema-like airspace enlargement, nodular peribronchiolar collections of mononuclear cells, thickening of airway walls, and subepithelial airway fibrosis. But when compared with littermate control mice, the IL-6 mice showed an approximately 50% increase in airway calibre and an increase in airway wall thickness. In contrast, the remodelling response was more robust in the IL-11 transgenic mice (Kuhn C 3rd 2000). The effects of IL-6 overlap to some extent those of tumour necrosis factor (TNF) alpha.

TNF-α originates principally from macrophages unlike TNF-β, which is produced predominantly by T lymphocytes. However, a number of other sources have now been identified. Apart from its obvious effects inducing cachexia, fever and acute phase protein production by the liver, TNF-α upregulates the expression of many adhesion molecules on the vascular endothelium facilitating the recruitment of neutrophils and eosinophils to the airways.

TNF-α has also been shown to strongly enhance IL-8 production, a major neutrophil chemoattractant, by human airway smooth-muscle cells in a dose dependent manner. Dexamethasone and fluticasone but not β2-agonists markedly inhibit this effect of TNF-α. Interestingly, a combination of both the steroid and β2-agonists was even more effective at inhibiting the effects of TNF-α (Pang L et al 2000). In addition, TNF-α has been shown to potentiate the effect of IFN-γ in inducing both necrosis and apoptosis in the airway epithelium, possibly influencing epithelial shedding and remodelling in airway inflammation (Kampf C 1999). Not surprisingly TNF-α has been found in airway biopsies and bronchoalveolar lavage fluids from asthmatic patients and has been associated with increased risk for atopy (Castro J 2000).

IV. Chemokine Production

Chemokines comprise four supergene families. Two of these families (the CC and CXC chemokine groups) are quite large and contain over 50 identified ligands and at least 14 individual receptors. Two additional chemokine families (C, CXXXC chemokines) are small and contain lymphotactin and fractalkine, respectively, as their members. In addition to their originally identified chemotactic activity, chemokines possess a variety of biological activities, ranging from immunomodulating leukocyte activation to suppressing HIV infection. The latter effect is due to the ability of specific chemokine receptors to serve as co-receptor for HIV entry into specific leukocyte sub-populations. A number of in vitro and in vivo studies have underscored the importance of chemokine biology in the progression of both acute and chronic lung diseases (Kunkel SL et al 1999).

For example, a number of studies have investigated the role of chemokines such as eotaxin and monocyte chemoattractant protein (MCP)-1 and RANTES, in allergic inflammation. It is now apparent that chemokines, through their influence on endothelial cells, alveolar cells, neutrophils, eosinophils, basophils, mast cells, monocytes, and lymphocytes, play an important role in leukocyte recruitment, cellular activation, histamine and serotonin release and the regulation of acute and chronic homeostatic immunity (Rothenberg ME et al 1999, Conti P et al 1999). However, a number of studies have begun to suggest that certain chemokines might be beneficial in preventing allergic response. For example, Met-RANTES, a modified antagonist of RANTES, and eotaxin receptor (CCR3) antagonists have shown promise as novel therapeutic agents potentially useful in atopic disorders. Therefore blanket suppression of chemokine activity may be detrimental. Whether chemokines are actually essential for an allergic response awaits confirmation with gene knockout animal experiments.

V. Signal transducers and activators of transcription (STATs)

Recently there has been considerable focus on the role of signal transducers and activators of transcription (STATs) and nuclear factor-kappaB (NF-kappa B) in allergic inflammation (Wong WS et al 2000). NF-κB is a family of DNA-binding protein factors that are required for transcription of most proinflammatory molecules, including adhesion molecules, enzymes, cytokines, and chemokines. NF-κB activation seems to be a key early event in airway inflammation (Christman JW et al 2000).

Thus the NF-κB family of transcription factors induces many genes involved in immune and inflammatory responses. Mice with deletions of individual NF-κB related subunits express different phenotypes, suggesting that the NF-κB transcription factors have different functions. For example, c-Rel, which is expressed in lymphoid cells and is important for lymphocyte activation, has been shown to be important in allergen-induced pulmonary inflammation and airway hyperresponsiveness. Thus, a recent study demonstrated that c-Rel-deficient mice did not develop pulmonary inflammation, bronchoalveolar lavage (BAL) fluid eosinophilia, or an increase in total serum IgE in response to allergen provocation. C Rel deficiency also prevented the induction of airway hyperresponsiveness and chemokine expression. Specifically, monocyte chemoattractant protein-1, which is regulated by NF κB, was decreased in allergen-treated c-Rel-deficient mice relative to wild-type controls. These results suggested that NF-κB/Rel transcription factors are important in allergen-induced pulmonary inflammation and airway hyperresponsiveness (Donovan CE et al 1999).

VI. Platelet-activating factor (PAF)

Platelet-activating factor (PAF) was first discovered in 1972 as a soluble factor released by IgE stimulated basophils. It is a potent lipid mediator produced by a number of cells and has been implicated in variety inflammatory processes such as airway constriction, eosinophil and neutrophil recruitment, airway oedema, and mucus accumulation. For example, PAF has been shown to increase vascular permeability and stimulate the extravasation of protein through a variety of different mechanisms in different tissues (Mathison R et al 1992). PAF is degraded to biologically inactive lyso-PAF by cellular and secreted PAF-acetylhydrolase (PAF-AH). However, treatment with recombinant PAF-AH did not significantly reduce either the early- or late-asthmatic response, sputum eosinophil or neutrophil cell counts nor did such treatment alter sputum ECP or tryptase levels (Henig NR et al 2000).

The importance of PAF in allergic inflammation remains uncertain to this day. Studies evaluating PAF receptor antagonists have generated mixed results and have only been consistently successful in inhibiting PAF induced bronchoconstiction (Christie PE et al 2002).

1.5.4 Eosinophils

Eosinophils are well recognised as the central effector cell in allergic inflammation (Gleich GJ et al 2000). Eosinophils release toxic basic proteins and lipid mediators such as cysteinyl-leukotrienes that cause bronchial epithelial damage and airflow obstruction. Eosinophils, and their pro-inflammatory mediators, are found in increased numbers in the peripheral blood, sputum, bronchial biopsies and BAL fluid of patients with asthma (Beasley et al 1989). They are particularly prominent in the post-mortem airways in asthma deaths (Dunnill et al 1960 &1969). However recent evidence has suggested that they may not be critical to AHR. Thus, studies using C57BL/6 mice immunised with purified Der p1 and exposed daily 30-min aerosol of different concentrations of house Der p1 extract developed AHR, eosinophil infiltration of the airways and allergen-specific IgE. However, lowering the concentration of the Der p1 aerosol also induced AHR and IgE without apparent eosinophil influx into the airways suggesting that AHR is not related to either eosinophil influx or allergen-specific serum IgE (Tournoy Kget al 2000).

1.5.5 Mast Cells

Mast cells are ideally placed, in the intraepithelial layer, to respond to inhaled antigens. During airway inflammation they release a wide variety of pro-inflammatory mediators including histamine, proteases, prostaglandins, heparin sulphate and leukotrienes as well as a spectrum of cytokines and chemokines that are involved in leucocyte recruitment and activation. For example, mast cells release prostaglandin (PG) D2. Prostaglandin D2 is a major lipid mediator released from mast cells. Mice overexpressing PGD2 showed a substantially increased expression of PGD2, but not PGE2, in the lungs and a significantly greater recruitment of eosinophils and lymphocytes to the BAL fluid. In addition mice overexpressing PGD2 generated much greater levels of Th2 cytokines in the lung. These results suggest that PGD2 plays an important role in late phase allergic reactions in the pathophysiology of bronchial asthma (Fujitani Y et al 2002).

Mast cells have also been shown to synthesise many of the key Th-2 cytokines, such as IL-4, IL-5, IL-9 and IL-10 (Bradding P et al 1992; Wilson SJ et al 2000; Stassen M et al 2000), and their central role in maintaining airway dysfunction in asthma is underpinned by the efficacy of interventions that interfere with mast cell function or activation (Holgate ST et al 2000, Marquardt DL et al 2000).

Recently mast cells have been implicated in the increased morbidity and mortality in elderly onset asthma (Atsuta R et al 1999) and this has sharpened the attention given to mast cells as therapeutic targets (Holgate ST et al 2000).

In addition, the last five years have seen a more sophisticated and complex role for mast cells in the inflammatory process emerge. For example, it has been suggested that mast cells secrete an equivalent volume of cytokines as T cells. In addition, mast cells have been implicated in IgE synthesis (Yoshikawa T et al 2001), and have been shown to be capable of antigen recognition and presentation (Abraham SN et al 1997). Furthermore, the evidence indicating that mast cells can change their phenotype in situ (Tomita M et al 1999, Arizono N 1987) has highlighted the complex immunoregulatory role played by these cells in airway inflammation. Therefore, mast cells have returned to centre stage in the investigation of chronic allergic inflammation (Williams CM 2000).

1.5.6 Immunoglobulin (Ig)-E

The importance of IgE in airway inflammation and development of AHR has been well known for some time. The switch from IgM through IgG1 to IgE production by B lymphocytes is induced by Th-2 cells (Mandler R et al 1993). This isotype switch of B cells into IgE-secreting plasma cells is a complex cascade of events in which cytokines play a crucial role (Kanowith-Klein S 1986). For example, both IL-4 and IL-13 induce IgE synthesis whereas IFN-γ and IL-12 inhibit IgE synthesis (Yssel H et al 1998). IL-4 in particular was, until recently, considered to be an absolute prerequisite for isotype switching. However, recent data have revealed a novel IL-4-independent pathway for IgE switching in the mouse that is strongly activated in retroviral infection (Morawetz RA et al 1996).

However, in most models IL-4 is required to drive isotype switching. The Th-2 cytokines, including IL-4, which drive the switch, are not only synthesised by T cells they are also produced by basophils and mast cells. Therefore T cells, mast cells and basophils may all be involved in IgE synthesis, in addition to $CD8^+$ T cells which have previously been shown to regulate IgE synthesis (Holmes BJ 1996). IgE production by B cells not only requires the presence of IL-4 or IL-13, but it also requires a physical interaction between T and B cells,

involving a number of surface and adhesion molecules such as CD40-CD40L and CD28/CD80 (Yssel H et al 1998). In addition to the above factors gender has been shown to influence IgE synthesis (Chen XJ et al 1996).

Once IgE has been produced it binds to high affinity (FcεRI) or low affinity (FcεRII or CD23) receptors on various immune cells. Binding to high affinity IgE receptors (FcεRI) expressed on the surface of mast cells induces mast cell activation. This activation occurs through the cross-linking of tetrameric IgE receptors each containing single alpha- and beta- and two gamma-subunits (Shaikh N et al 1997).

Mast cells then secrete, upon subsequent exposure to specific antigen, a panel of pro-inflammatory mediators, including a number of immunoregulatory cytokines. This IgE- and antigen-specific mast cell activation and mediator production is thought to be critical to the pathogenesis of allergic inflammation, and also contributes to host defence against parasites.

While there is a genetic polymorphism in FcεRI expression, recent evidence suggests that this polymorphism does not affect mast cell activation (Furumoto Y et al 2000). Conversely, it has been suggested by some (Payet ME et al 1999), but not all authors (Texido G et al 1994) that binding to CD23, the low affinity IgE receptor, modulates IgE-mediated disease.

IgE itself increases the expression of the high affinity IgE receptor on mouse mast cells in vitro and in vivo at least 30-fold. This positive feedback loop and increase in mast cell FcεRI expression has two components: an early cycloheximide-insensitive phase, followed by a later and more sustained cycloheximide-dependent phase. IgE-driven increases in FcεRI expression significantly enhance the ability of mouse mast cells to release serotonin, IL-6 and IL-4 in response to challenge with specific antigen (Yamaguchi M et al 1997).

The exact role of IgE in allergic inflammation has been the focus of much research. For example, the link between total IgE and airway inflammation and AHR has been explored. It has been suggested that IgE plays an important role in the development of airway inflammation and AHR when there is little IL-5-induced airway eosinophilia. In contrast, IgE

plays a much less important role when there is significant eosinophilic infiltration of the airways. This suggests that anti-IgE therapy (see below) may be more relevant to allergic rhinitis and conjunctivitis, rather than to atopic asthma where anti-IL-5 therapy may be more beneficial (Hamelmann E et al 1999b).

More recently IgE has been shown to be present in airway secretions from humans with allergic rhinitis and bronchial asthma. The importance of this local IgE production to overall airway inflammation is not well understood. However, it has been suggested that antigen-specific IgE can capture airborne antigens and form immune complexes. These immune complexes may then function as potent inducers of immune responses in the lung, contributing to the perpetuation of airway inflammation (Zuberi RI et al 2000).

The use of IgE as an antigen receptor is critically dependent on the transmembrane and cytoplasmic domains of IgE. In mice lacking most of the cytoplasmic tail of IgE, serum IgE levels are reduced by 50 percent and specific responses are reduced by 40 to 80 percent, without a clear secondary response (Achatz G et al 1997).

1.5.7 Neutrophils

Acute severe asthma is often associated with neutrophilic infiltration of the airways and this neutrophil recruitment has been implicated in early asthmatic death following an acute asthmatic attack (Sur 1993). In addition, an early airway neutrophilia has been reported after local allergen bronchoprovocation (Montefort et al 1994), and neutrophils have been shown to be present in high numbers in the airways of patients with chronic severe asthma. The functional significance of the early influx of neutrophils into the airways is unclear. However, it has been suggested that the production of eosinophil chemotactic factors by neutrophils enhanced the response to low levels of antigen challenge (Katy et al 1976; Czarnetzki et al 1978).

Like all other leukocytes their recruitment to the airways is dependent on cell adhesion molecule expression and is critically regulated by the autonomic nervous system (Mathison R et al 1993). Leukotriene B4 and cytokines such as IL-8, GM-CSF, and TNF-α have been shown to chemoattract neutrophils and reduce neutrophil apoptosis, and selective agents directed against these may prevent neutrophil influx and accumulation. Paradoxically, in

vitro, corticosteroids enhance neutrophil survival by reducing apoptosis, so corticosteroid therapy may exacerbate neutrophil activity in vivo (Sampson AP et al 2000).

Recent evidence has suggested that the secretion of elastase, a potent secretagogue, by neutrophils promotes goblet cell degranulation and this may partly explain the mucus hypersecretion that is a common characteristic of asthma. In an OA-induced model of allergic inflammation in sensitized guinea pigs drugs that inhibit the early recruitment of neutrophils, identified by the expression of CD16 and 3,3'- diaminobenzidine staining, also inhibited goblet cell degranulation (Agusti C et al 1998).

1.5.8 Epithelium

While asthma is an inflammatory disorder of the airways involving mediator release from mast cells and eosinophils and orchestrated by T cells, inflammation alone is insufficient to explain the chronic nature of the disease and its progression. There is evidence to suggest that the epithelium is fundamentally disordered in chronic asthma.

Thus there is increased epithelial fragility, and an altered phenotype to one that secretes mucus, mediators, cytokines, chemokines and growth factors. Epithelial injury is mediated by exogenous factors such as air pollutants, viruses and allergens as well as by endogenous factors including the release of proteolytic enzymes from mast cells (tryptase, chymase) and eosinophils (MMP-9). Following injury, the normal epithelium should respond with increased proliferation driven by epidermal growth factor (EGF). The epithelial response in asthma appears to be impaired despite upregulation of CD44 capable of enhancing presentation of EGF stimulants to epidermal growth factor receptors (EGFR). Because the epithelium is fixed in this "repair phenotype", it becomes a continuous source of proinflammatory products as well as growth factors that drive airway wall remodelling (Holgate ST et al 1999).

However, airway wall remodelling does not only involve the epithelium it also involves a number of changes in the airway vasculature including increased vascularity, vasodilation and microvascular leakage. Evidence suggests that the number and size of bronchial vessels is increased in patients with asthma compared with normal controls. In particular, there may be increased numbers of vessels in patients with fatal asthma. One of the effects of inhaled corticosteroid treatment is to reduce this vascularity.

Bronchial vessels may undergo proliferation in response to inflammatory stimuli. Many factors can induce angiogenesis including a range of mediators and growth factors. Others can cause a vascular response by inducing vasodilation, microvascular leakage and airway wall oedema. Mast cells may play a key role in modulating these vascular remodelling changes by releasing cytokines and growth factors. This angiogenesis, vasodilation and microvascular leakage may be the target for novel therapeutic strategies in the future (Wilson J et al 2000).

Another player in airway remodelling is the non-ciliated bronchiolar Clara cells. These cells secrete Clara cell protein (CC16), a 15.8-kDa homodimeric protein, which appears to protect the respiratory tract against oxidative stress and inflammation. In vitro, CC16 has been shown to modulate the production and/or the activity of various mediators of the inflammatory response including, IFN-γ and TNF-α. CC16 has also been found to inhibit fibroblast migration. This protective role is confirmed by studies on transgenic mice, which have demonstrated that CC16 deficiency is associated with an increased susceptibility of the lung to viral infections and oxidative stress. In humans, a polymorphism of the CC16 gene, localized to a region linked to airway diseases, has recently been discovered in association with an increased risk of developing childhood asthma. Finally, CC16 has been suggested to be a peripheral marker for assessing the integrity of the lung epithelium. The determination of CC16 in serum is a new non-invasive test to detect Clara cell damage or increased epithelial permeability in various acute and chronic lung disorders (Broeckaert F et al 2000).

1.6 ANIMAL MODELS OF ALLERGIC DISEASE

The investigation of the pathogenesis and evolution of human disease has, over the years, relied heavily upon the design, development and analysis of animal models of those diseases. Animal models have enabled scientists to probe the cellular mechanisms of disease in a way that is not technically possible in humans. The information gathered has not only shed considerable light on the cellular mechanisms of disease states but has also provided novel leads for therapeutic intervention and a robust test bed for drug discovery programmes and the testing of pharmacological compounds. In investigating allergic inflammation of the airways a

number of different animals models have been employed, each has their champions and each confers different experimental advantages. However, despite considerable scientific endeavour, there is no "ideal" animal model and prudent research initiatives will employ a variety of different models to address different experimental questions.

Guinea pigs were one of the earliest laboratory animals used to study allergic disease. The guinea pig model has various advantages over other animal models. Guinea pig airways are easily sensitised to foreign protein and there are a number of histological similarities between the lungs of antigen-exposed guinea pigs and asthmatics (Kallos et al 1984). There has been a considerable amount of work done to establish appropriate sensitisation regimes that will generate acute and chronic inflammatory changes in the guinea-pig airway. Airway sensitivity can be passively transferred to naïve guinea pigs using antigen-specific mouse IgE antibodies (Desquand et al 1989).

However, the greater availability of immunological tools to probe the inflammatory response of mice has shifted the balance away from the guinea pig and made the humble mouse the animal of choice for probing airway inflammation (Vargaftig BB et al 1999). This switch to murine models has been reinforced by two factors. First of all a number of research centres have now developed sophisticated techniques for accurately detecting subtle changes in airway calibre in the mouse. Secondly sophisticated sensitisation regimes that have recently been able to induce airway hyperreactivity and obstruction in the mouse (Neuhaus-Steinmetz U et al 2000),

1.7 NOVEL THERAPEUTIC OPTIONS

Corticosteroids have been the mainstay of asthma therapy since the 1950s, however their side effects on childhood growth has invigorated research into other anti-inflammatory therapies in the management of airways inflammation (Schleimer RP et al 1997). For example, many new therapeutic agents have been developed either to attenuate the pro-inflammatory processes in asthma or to augment the host anti-inflammatory mechanisms. Thus, an increase in cyclic adenosine monophosphate (cAMP) levels generally suppresses the activities of immune and inflammatory cells, and a family of phosphodiesterases (PDEs) closely regulates the level of cAMP. So drugs that inhibit PDEs have been developed. An

alternative therapeutic target has been heparin, a glycosaminoglycan released exclusively from mast cells, since this possesses anti-inflammatory actions. Agents that affect tyrosine kinases, and NF-κB, as well as antibodies and soluble receptors directed against IgE, IL-4, and IL-5 have also been developed in recent years (Wong WS et al 2000).

One such novel therapeutic approach has been the development of monoclonal antibodies against IgE. IgE plays a pivotal role in the cascade of biochemical events leading to the allergic inflammation and nearly all asthmatics have higher than normal IgE levels in their serum following adjustment for age and sex. Therefore MoAbs that can eliminate IgE may be of use in the treatment of allergic disease. Recently a variety of MoAbs directed against IgE, such as rhuMAb-E25 and CGP 56901, have undergone clinical testing.

The murine MoAb rhuMAb-E25, which recognises the specific Fcε III portion of circulating IgE that binds to the high-affinity IgE receptor, was tested in allergic asthma and seasonal allergic rhinitis. The rhuMAb-E25, was humanised to avoid the problems of antigenicity, and entered into a series of phase I clinical trials and shown to be well tolerated. Subsequent phase II studies suggested that this approach may have value since the MoAb reduced serum free IgE concentrations in a dose-dependent manner (Jardieu PM 1999). This reduction in IgE was shown to significantly reduce the early and late asthmatic response. In addition the phase II trials demonstrated a significant improvement in asthmatic symptoms, reduced bronchodilator and corticosteroid use, decreased asthma exacerbations and improved quality of life (Fick RB Jr 1999). Other strategies for decreasing IgE levels include IFN-γ, IL-4 antibodies, IL-4 receptor antibodies and soluble IL-4 receptors (Fahy JV et al 2000).

But despite the availability of such highly efficacious drugs many asthmatics continue to suffer. One approach to this continued suffering is to continue to develop models of allergic inflammation that will enable us to probe more deeply the different aspects of allergic inflammation, and potentially open up new fronts in the fight against this common problem.

1.8 SPECIFIC AIMS AND OBJECTIVES

The main aim of this thesis was to develop an animal model of allergic inflammation of the airways in a department that, at the time, did not possess a viable animal model. Such an animal model could then be dissected to shed light on the role of various cells and mediators in a way that is not possible in human subjects. Obviously one of the key markers of success in the development of any animal model is that it bears sufficient resemblance to the pathophysiology of the disease it is designed to model. So each chapter presented in this thesis aims to either refine the model in terms of its mimickery of allergic disease or aims to investigate the immunological process at play in airway inflammation.

1.8.1 Specific Chapter Hypotheses & Aims

- Chapter 1: There is significant evidence to indicate that asthma is an inflammatory process involving multiple cells and effector molecules. This chapter aims to review the evidence suggesting that asthma is an inflammatory disease and highlight the fact that no one animal model is ideal and a variety of research strategies are required to shed light on the pathophysiology of asthma.

- Chapter 2: The histopathological response in the lungs to infection with N.b. larvae bears sufficient similarity to allergic inflammation in the lungs to make N.b. infection a worthwhile alternative strategy to OA-driven models. This chapter aims to characterise the lung histology following helminth infection and compare it the disease being modelled, namely asthma.

- Chapter 3: The cellular provenance of the pulmonary response to N.b. antigen mimics that seen in allergic inflammation of the airways and therefore an N.b. -driven model is a viable alternative to an OA-driven model. This chapter also aims to characterise the basic qualities of an N.b.-driven model and explore the recruitment strategies and functionality of these cells, contrasting this to what is seen in asthma.

- Chapter 4: The N.b. infective burden can be substantially reduced yet still produce a significant peripheral eosinophilia and pulmonary inflammation. Such a reduced

response would make a reduced larval burden model more amenable to therapeutic manipulation and provide a range in the severity of pulmonary inflammation.

- Chapter 5: Adult antigens are more important than larval antigens in the generation of the pulmonary inflammatory response. The identification of the key N.b. antigens could help identify what antigens could be isolated from N.b. to drive future developments of the N.b. model. For example, N.b. antigens could be nebulised directly into the airways in a manner similar to OA-driven models.

- Chapter 6: Balb/c mice are the optimum murine strain in which to build the N.b.-driven model of pulmonary inflammation.

- Chapter 7: Knocking out ICAM-1 would significantly reduce the pulmonary inflammatory response to N.b. antigens.

- Chapter 8: Disruption of CD18-driven cellular recruitment would significantly reduce the pulmonary inflammatory response to N.b. antigens.

- Chapter 9: Mast cell deficiency would significantly reduce the pulmonary inflammatory response to N.b. antigens.

- Chapter 10: Infection of C57BL/6 mice with N.b. antigens is a viable model of pulmonary inflammation and offers some advantages over OA-driven models. Potentially an N.b.-driven model can offer a second antigenic sensitisation regime that may help elucidate the pathophysiological mechanisms at play in inflammation of the airways.

CHAPTER 2

The Histopathological Response to Nippostrongylus Brasiliensis

2.1 INTRODUCTION

The use of animals to model human disease has been a cornerstone of scientific enquiry for many years. In fact such approaches have shed considerable light on the pathogenesis of a wide variety of chronic inflammatory conditions particularly allergic inflammation of the airways. They have identified many new therapeutic targets and have also been a rich test bed for a wide variety of therapeutic interventions. Over the years animal models of airway inflammation have employed a variety of species from primates to guinea pigs. Each model had its unique advantages and disadvantages and offers different avenues for probing different aspects of the inflammatory process. For example, while primate models of allergic inflammation may closely mimic human disease the cost of primate research is prohibitive and most authors now prefer to work with small animal models, particularly rodents. Rodents are cheaper, easier to work with and there are many immunological probes available to support such an approach.

Hand in hand with this animal modeling there has been an explosion in basic immunology and genetic technology research, which has revolutionized the investigation of asthma and related disorders. For example, the identification of two distinct subsets of $CD4^+$ lymphocytes, namely Th-1 and Th-2 based on their cytokine expression was a turning point in the understanding of allergic inflammation (Mosmann 1986).

This single study spawned an infinite number of further studies and many of the current animals models induce airway eosinophilia by promoting a vigorous Th-2 response. Two of the most commonly employed strategies to provoke a Th-2 response in rodents are infection with gastrointestinal nematode Nippostrongylus brasiliensis (N.b.) or nebulisation of ovalbumin (OA) to the airways of OA-sensitised animals (Hook S et al 2000, Schramm CM et al 2000, Watkins AD et al 1996).

In view of the availability of the extensive battery of reagents capable of probing the murine immune response and its genetic variants, we developed a murine model for dissecting allergic disease mechanisms by exploiting the ability of parasite antigens to promote a vigorous Th-2 response. There is an overwhelming case to indicate that the cellular and molecular response to parasite antigens closely mimics the immune response seen in allergic

inflammation. This has led some authors to speculate whether they are causally related (Moqbel R et al 1990). Nippostrongylus is particularly relevant since *in vivo* N.b. infection is capable of overriding an established Th-1 response and effecting an isotype switch to a Th-2 response (Rocken M et al 1994). The important antigenic epitopes that drive the immune response towards a Th-2 response are probably located near the ventral secretory/excretory (S/E) apparatus of the adult worm. This view is based on the findings that the S/E apparatus are known to promote IgE and IgG_1 production, in contrast to the body wall antigens which initiate an IgG_{2a} response (Yamada M et al 1993).

It is widely known that OA models induce a relatively mild airway eosinophilia in the airways. So rather than use an OA-induced model of allergic inflammation we decided to investigate whether there were any advantages to employing a more powerful Th-2, stimulus namely N.b. infection. However, infection with the third stage N.b. larvae (L3) can still be overridden. For example, recent evidence has shown that specific monoclonal antibodies raised against the mouse eotaxin receptor, C-C chemokine receptor 3 (CCR3), anti-CCR3 mAbs, substantially depleted the number of eosinophils recruited to the blood, lung tissue and BAL fluid of mice infected with N.b. (Grimaldi JC et al 1999). Therefore N.b.-driven models could provide an alternative way to investigate more severe degrees of allergic inflammation of the airways. Initially we established a N.b. colony and then examined cellular recruitment to the BAL fluid and the histological response of the lungs to N.b. infection.

2.1.1 Life Cycle of Nippostrongylus Brasiliensis

The life cycle of N.b. has been well characterised (Ogilvie BM et al 1978). Rats and mice are the natural hosts and the adult bisexual worm, 4-6 mm long, lives predominantly in the small intestine. The female adult worm lays up to 1,000 eggs per day, which are passed in the rodent stool. Copulation never takes place until 120 hours after the initial infection and the female is infertile prior to insemination. In order to maintain an output of 1,000 eggs per day copulation needs to occur every other day. Eggs are passed in the stool from day 6 after the initial infection.

Four or five days after the eggs (L1 stage) are passed in the stool they hatch (L2) and develop into larvae (L3). These larvae are occasionally ingested or more commonly directly penetrate

the skin of mice and rats and migrate to the lungs. The route by which they reach the lungs is unclear but most believe they enter the blood stream or possibly the lymphatics or both. About 50% of the infective burden of L3 larvae reach the lungs. Within 15 hours of penetration L3 are being passively trapped in the capillary bed and distributed throughout the lungs. Respiratory movements aid this distribution. At this stage there is often significant alveolar haemorrhage due to enzymatic damage to the capillary bed.

About 70% of the L3 trapped in the lungs molt into L4 worms. This molting process is normally well under way 32 hours after the original skin penetration. The L4 worms then migrate from the lungs up the trachea and into the oesophagus and have started to reach the small intestine by day 3. The L4 larvae molt again over the next 48 hours and the adult worms (L5) take up residence in the small intestine, particularly the jejunum. It has been suggested that L3 larvae are less antigenic than adult worms and just one female adult can stimulate protective immunity. The antigenicity of the female worm is also known to be 10x greater than that of the male.

2.2 MATERIALS AND METHODS

2.2.1 Larval Culture and Infection

Five to six thousand third-stage N.b. larvae (L3) were injected subcutaneously (sc) in the cervical region of each adult male Spraque-Dawley (SD) rat (250-300 g). Stool was collected from the SD colony from day 6 to 10 post infection (pi) and washed vigorously to remove ammonia and debris. The clean stool was then left to soften in warm tap water for a minimum of 30 min. If the stool is left for too long this encourages fungal growth, which then stifles the culture of the N.b. larvae. Once softened, the stool was mixed (1:3) with activated granular charcoal (Aldrich Chemical Company Inc, Milwaukee, WI) and transferred to 1 litre freezer containers to incubate at 20^0C. A single day's rat stool harvest could generate up to fifteen 1-litre culture containers depending on the number of rats originally infected and the amount of stool harvested. The level of water and moisture in the culture containers was critical. Too much water flooded the culture, encouraged bacterial and fungal growth, and the surface tension prevented the larvae from moving up the culture bed. Too little water produced a desiccated culture bed and killed the larvae.

After 10 to 30 days of culture, the L3 migrated to the surface of the culture bed and were clearly visible (Photos P-1 to P-4).

Photo 1-4 N.b. larval culture two weeks after stool collection. Clusters of mobile stage three larvae (L3) can be clearly seen after migrating to the surface of the charcoal grains within culture bed.

Photo 2 of 4 N.b. larval culture two weeks after stool collection. Clusters of mobile stage three larvae (L3) can be clearly seen after migrating to the surface of the charcoal grains within culture bed.

Photo 3 of 4 N.b. larval culture two weeks after stool collection. Clusters of mobile stage three larvae (L3) can be clearly seen after migrating to the surface of the charcoal grains within culture bed.

Photo 4 of 4 N.b. larval culture two weeks after stool collection. Clusters of mobile stage three larvae (L3) can be clearly seen after migrating to the surface of the charcoal grains within culture bed.

Larvae were viable in this environment for up to eight weeks with no apparent loss in numbers or infectivity. Larvae were harvested using a Baerman apparatus (Figure 2-1).

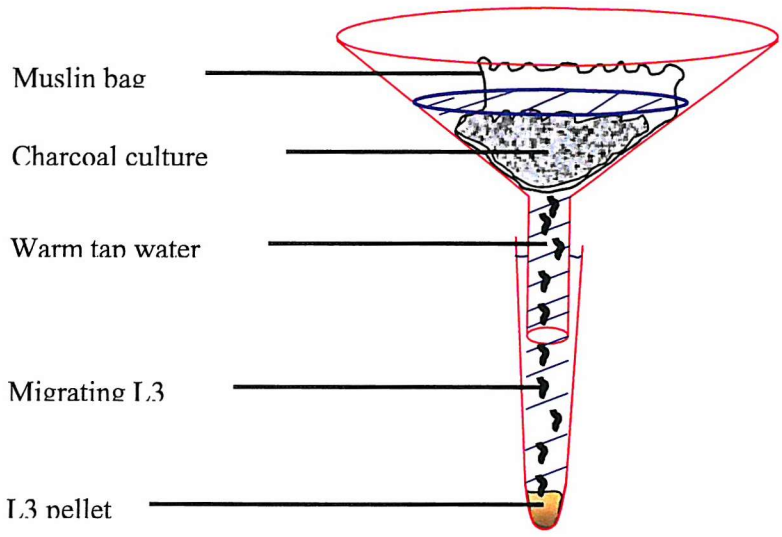

Figure 2-1. Baerman apparatus for recovery of N.b. L3 larvae from charcoal culture.

Basically a single 1-litre pot of charcoal culture was inverted onto a double folded muslin square. This was then placed in warm water in a Baerman apparatus and left for a minimum of 30 minutes and a maximum of four hours. Larvae migrated, under the force of gravity, into the collecting tube at the bottom of the Baerman apparatus.

The larval pellet obtained from the Baerman apparatus was washed in tap water 2-3 times and then 4 x 10 µl aliquots were transferred onto a slide for estimation of larval numbers. If the experimental protocol dictated that several groups of mice had to be infected over a number of days then one batch of larvae were harvested and kept in tap water in a 500 ml cell culture flask prior to injection. This ensured that all mice in the same experiment were infected with the same batch of larvae, to minimize variance in infectivity and virulence. Larvae could be kept in a 500-ml flask at room temperature for up to a week. Thereafter their infectivity was much reduced. Occasionally such precautions were not practical and therefore a number of cultures were harvested for inoculation into mice. Under such circumstances all attempts were made to ensure that harvested cultures were from the same initial rat stool samples taken on the same day.

Larvae were generally washed and counted 2-24 hours prior to injection, using a 26g needle, into the murine ventral abdominal wall to generate the murine model, or into the cervical region in rats to maintain the L3 colony. Although the effect of different infective doses of L3 was assessed (see chapter 9), the majority of experiments were conducted with a murine dose

of 750 L3 in 110 μl of water. Each infective dose was drawn up separately to avoid larval sedimentation in the 1ml syringe. Approximately 5-6,000 L3 were inoculated into each rat to maintain the L3 colony.

2.2.2 Animals

Female C57BL/6 mice (6 to 8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME), and housed under virus antibody-free conditions in the Animal Care Facility on a 12-hour light dark cycle. The access to this room was restricted to one investigator only. Mice were housed in wire-bottomed cages to prevent autoinfection and were also screened weekly to ensure they were free from ecto and endoparasites. Mice were maintained on standard mouse chow and acidified water *ad libitum*. Mice used in these studies were between 18 to 25 g and 10 to 16 wk of age. All procedures in this study were in compliance with the Animal Welfare Act Regulations (9CFR Parts 1,2, and 3), and with the Guide for the Care and Use of Laboratory Animals (DHEW Publication [NIH 85-23], 1985). The number of mice per experimental group generally varied between 8 to 20 animals.

2.2.3 Bronchoalveolar lavage and cell preparations

BAL fluid was acquired 3 to 35 days pi, as previously described (Kennedy et al 1995). Briefly, mice were anaesthetised with 30-60 μl of urethane ip (0.09-0.3 g/kg body weight) and a longitudinal incision was made in the neck to expose the trachea. This was then cannulated using a blunt-ended 23g needle, and secured with a silk tie. Two successive 500 μl aliquots of sterile ice-cold phosphate buffered saline (PBS 1x) were instilled into the airways. Even distribution was encouraged by gentle massage of the chest wall prior to withdrawal of the BAL fluid. Lavage fluid was transferred into 4 ml tubes containing 1 ml of sterile Ca^{++} and Mg^{++}-free Hanks balanced salt solution (HBSS) containing 5% fetal bovine serum (FBS), 10 mM HEPES, and antibiotics (penicillin-streptomycin) (HBSS-FBS) and kept on ice. The first aliquot usually yielded a 200-400 μl return, while the second yielded 500-600 μl.

The BAL fluid obtained was centrifuged at 300 X g for 15 minutes, and the cell pellet was resuspended in fresh HBSS-FBS. A 200 μl aliquot was then diluted in 10 ml of PBS and the red blood cells (RBC) were lysed using potassium cyanide (Lysing and Hemoglobin reagent, Baxter Diagnostics, Chicago, IL) for 2 minutes. The total number of white blood

cells was then enumerated for each sample using a Coulter counter (model ZM, Coulter Electronics, Luton, England.).

In addition, cytological examination was performed on slides prepared by diluting 50-100 µl of cells in an equal volume of HBSS and centrifuging in a cytospin centrifuge (Shandon) at 60 X g for 8 minutes. These cytospin slides were fixed and stained using Diff Quik™. Differential counts were made on 200 cells using standard morphological criteria to classify the cells either as neutrophils, eosinophils, lymphocytes, or other mononuclear leukocytes (primarily alveolar macrophages).

2.2.4 Lung histopathology

Following removal of the lungs from the chest cavity, they were gently inflated by infusing 0.5-1 ml of formalin down the tracheal cannulae. This was achieved using a lung inflation system (see Figure X). The lung inflation system generated a steady pressure, of approximately 10cm water. This system was previously tested to ensure that the inflation process did not, itself, cause alveolar damage. Inflated lungs were then preserved in a 50 ml Corning tube containing 20 ml of formalin and later imbedded in paraffin. Paraffin-embedded, middle and lower lobes from each mouse were sagitally sectioned through the mid-portion across the trachea and major bronchi. Six µm sections were cut, mounted on glass slides and stained with a standard hematoxylin, phloxine and eosin prior to histological assessment.

2.3 RESULTS

2.3.1 N.b. Larval Culture

An idea of larval health could be gauged from studying the activity of the larvae in the 10 µl aliquots that were taken for estimating the infective dose. The vast majority of larvae were highly motile, and therefore made difficult photographic subjects (Photo P-5 to P-7).

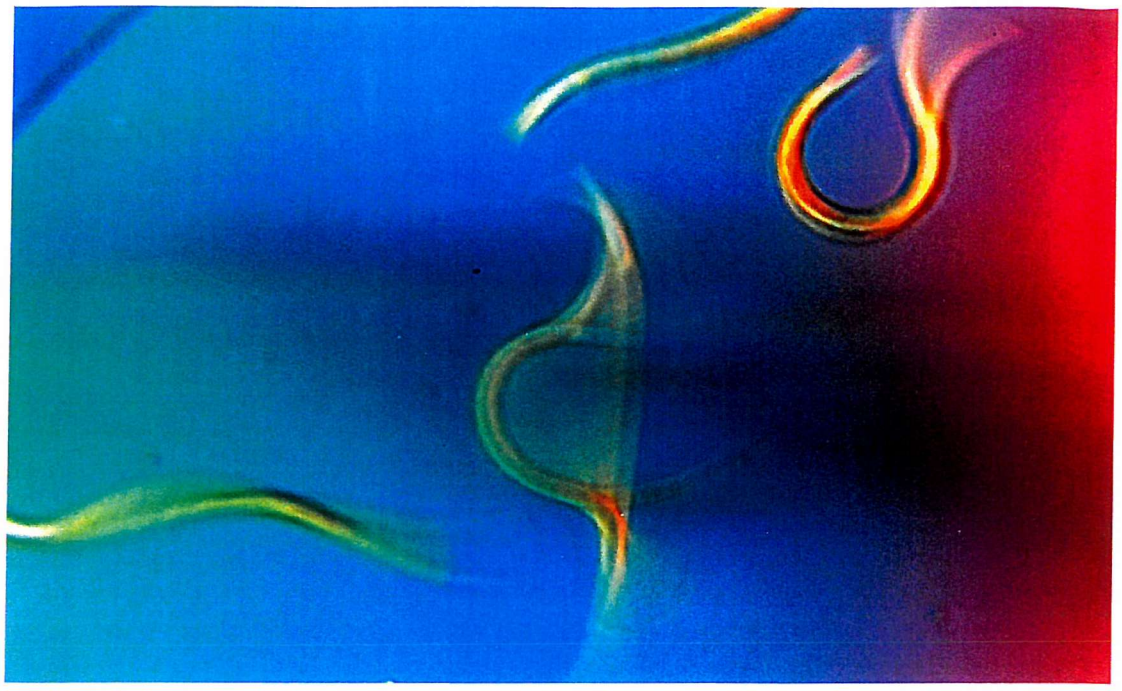

Photo 5 Highly motile N.b. L3 (Phase contrast). Motile L3 moving rapidly across the photographic field despite high velocity shutter speed.

Photo 6 Highly motile N.b. L3 (Dark field). Motile L3 moving rapidly across the photographic field despite high velocity shutter speed.

Photo 7 A single N.b. stage three larva (Phase contrast). Sinusoidal-shaped healthy L3.

If the larvae appeared too sessile it should not be assumed that they were dead. Normally centrifuging L3 at 1000rpm for 5-10 minutes stimulated them sufficiently to obtain a more accurate impression of mobility and likely infectivity. Non-viable larvae were identified normally by their shape and lack of motility following centrifugation. They lost their normal sinusoidal configuration and became "boat-shaped" (Photos P-8 to P-10).

Photo 8 N.b. stage three larva (Phase contrast). Healthy L3.

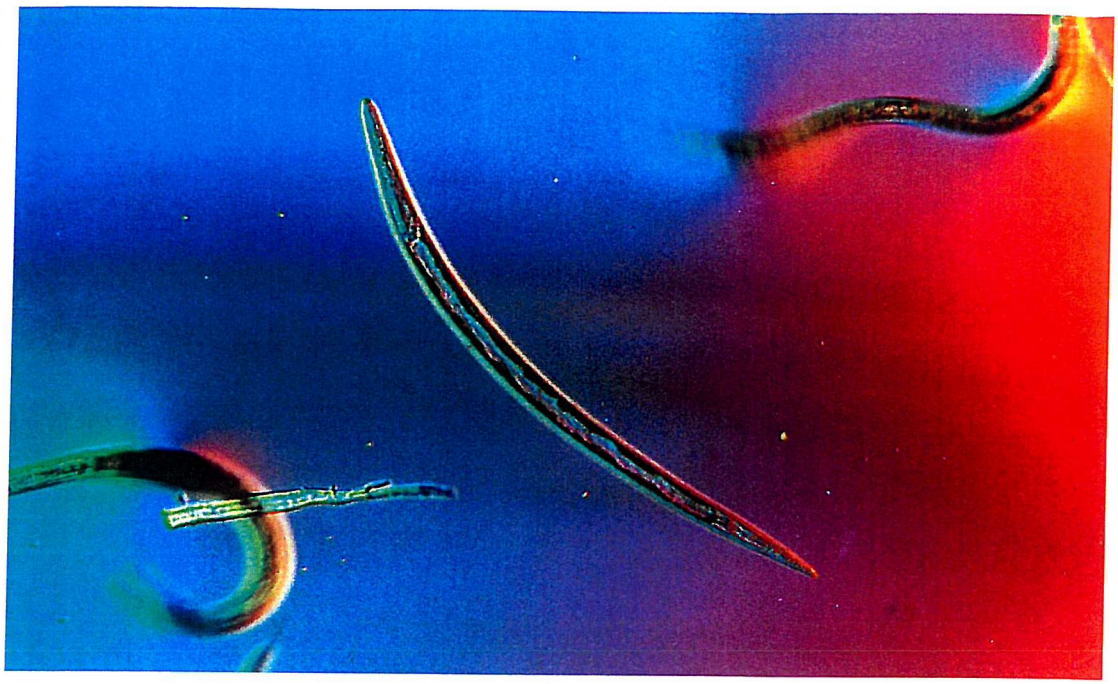

Photo 9 N.b. stage three larva (Phase contrast). Boat-shaped, sessile low infectivity or dead N.b. larvae.

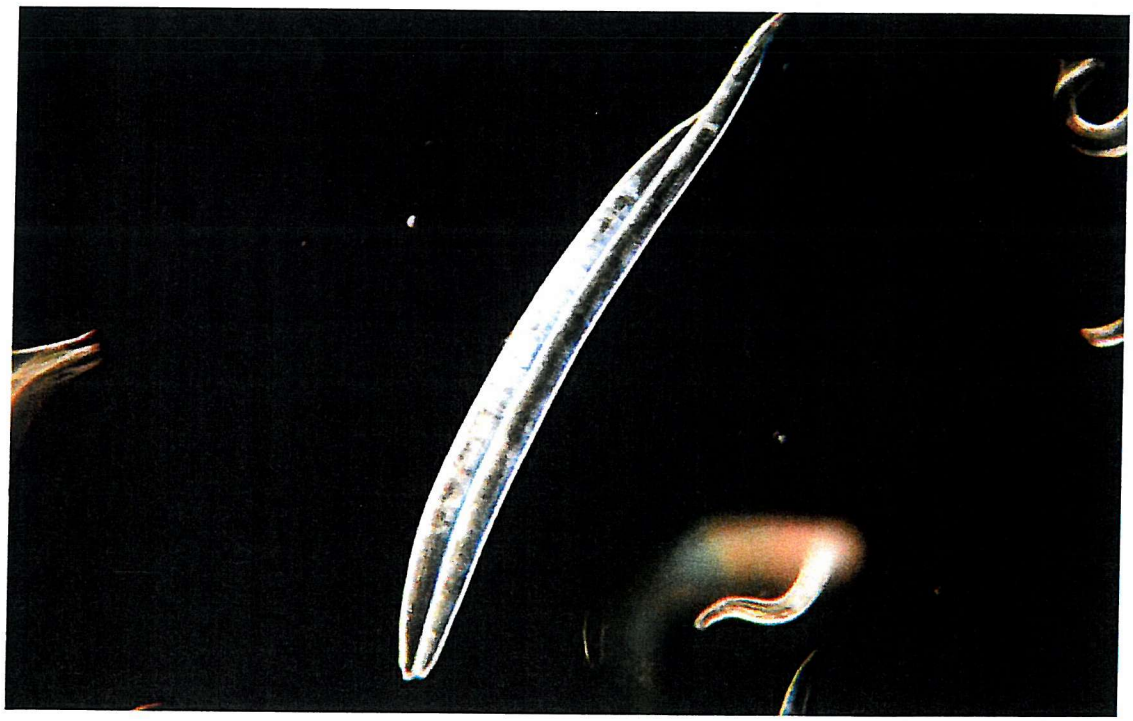

Photo 10 N.b. stage three larva (Dark field). Two boat-shaped, sessile low infectivity or dead N.b. larvae.

Viable larval numbers were determined by counting mobile L3 and sessile larvae with a least some deviation in their outline.

2.3.2 Histopathology of lung tissue

The lungs of the uninfected/naïve mice were normal in appearance and were without signs of peri-vascular or interstitial eosinophilic infiltration. In addition there were no signs of pulmonary haemorrhage or leukocyte infiltration of the alveolar bed in these mice (Photo P-11).

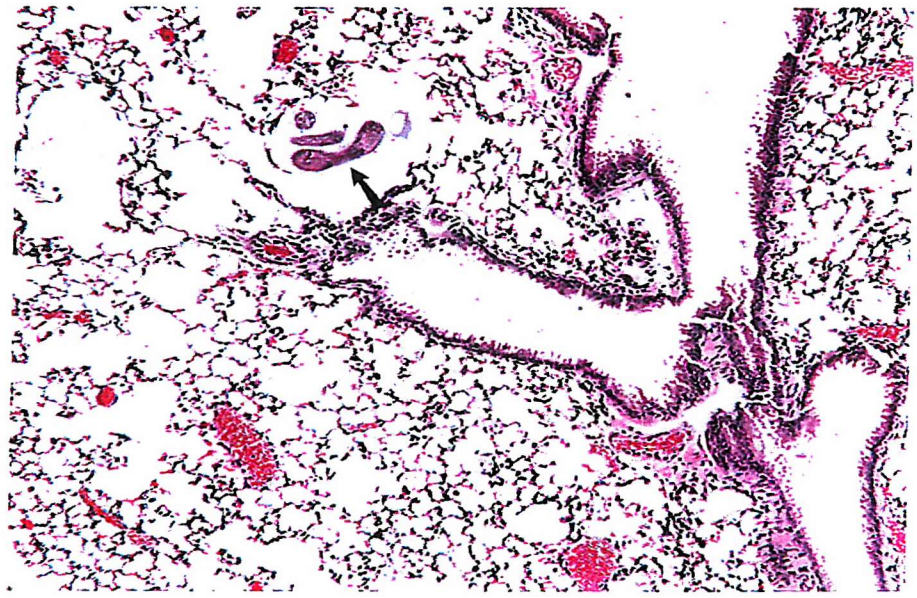

Photo 11 Normal airway architecture.
ALV = alveolus; BRCH = bronchiole; ART = arteriole

This was reflected in the BAL of uninfected mice, which contained approximately 96% monocytes and 4% lymphocytes. Following N.b. infection there were gross macroscopic changes visible from day 8 pi onwards, consisting of an irregular, nodular pleural surface, most marked on day 14 pi. At these time points the lungs were more resistant to the infusion of formalin than the control mice

Microscopic changes in the lungs of the N.b. infected mice were visible three days pi, with larvae clearly visualised in the alveolar bed (Photo P-12, arrow).

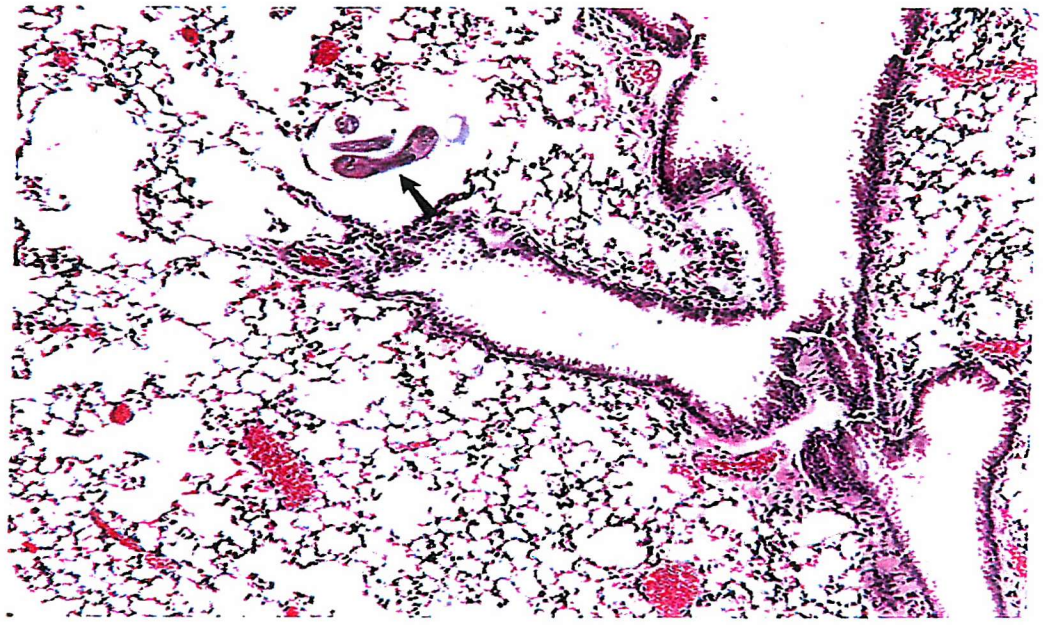

Photo-12 N.b. larvae migrating through alveolar bed on day 3 pi (Low Power). Arrowed =N.b. larvae in cross section

There was very little surrounding inflammatory response to the larvae, presumably because the larvae were still motile and migrating through the lung tissue. However, it was not uncommon to see alveolar haemorrhage at this stage (Photo P-13).

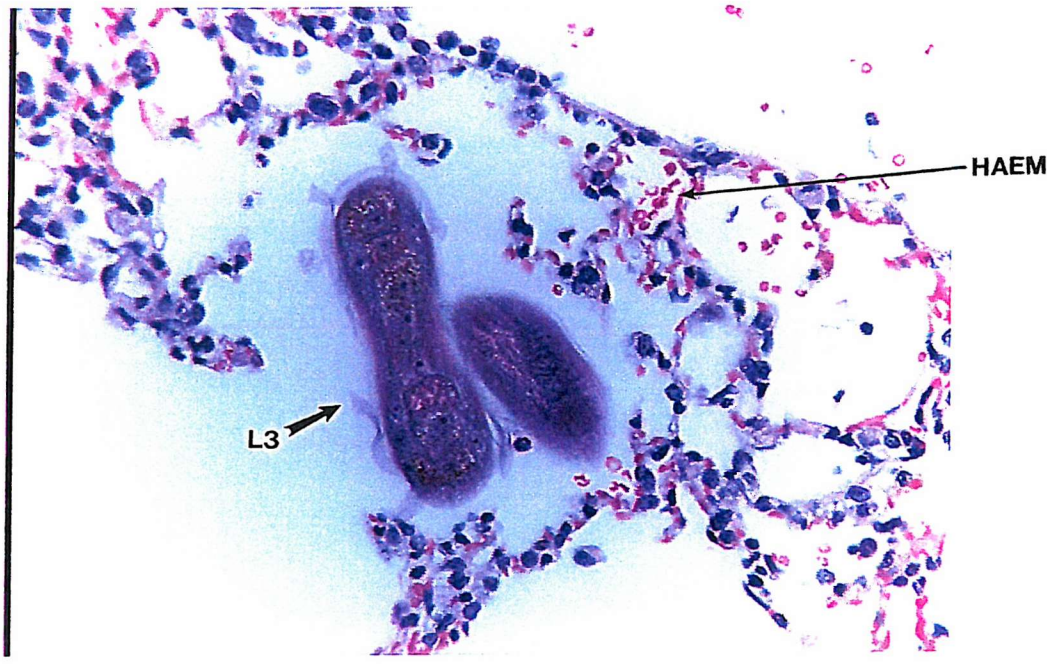

Photo-13 N.b. larvae migrating through alveolar bed on day 3 pi (High Power). HAEM=alveolar haemorrhage; L3=N.b. larvae in cross section

The BAL fluid was still predominantly monocytic with a few lymphocytes, occasional eosinophils and an increasing number of neutrophils seen (Photo P-14).

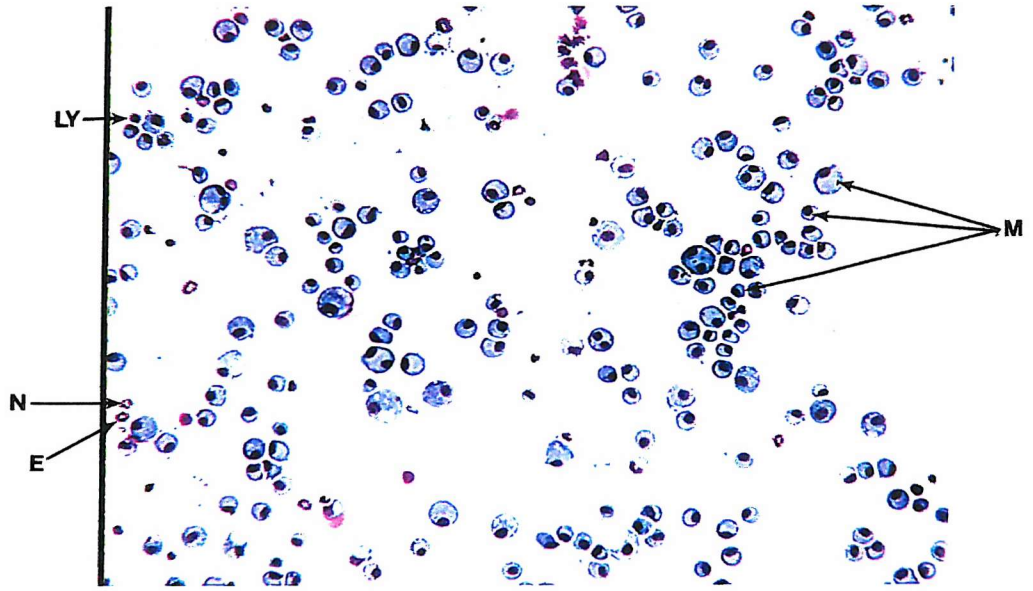

Photo 14 BAL fluid on day 3 pi.
M = monocyte; LY = lymphocyte; N = neutrophil; E = eosinophil

In some animals there was a marked alveolar haemorrhage with a heavily blood stained BAL. Such changes were often associated with the recovery of larvae and larval sheaths from the cytospin preparations of the BAL fluid (Photo P-15 & 16).

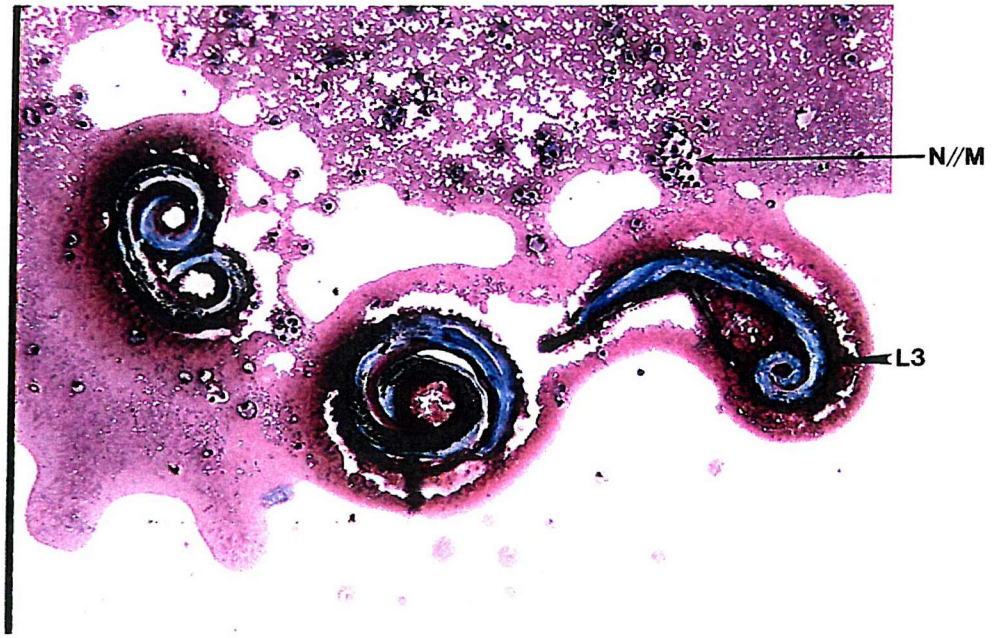

Photo 15 Haemorrhagic BAL fluid on day 3 pi (Low Power).
N//M = neutrophil/monocyte cluster L3 = N.b. larvae

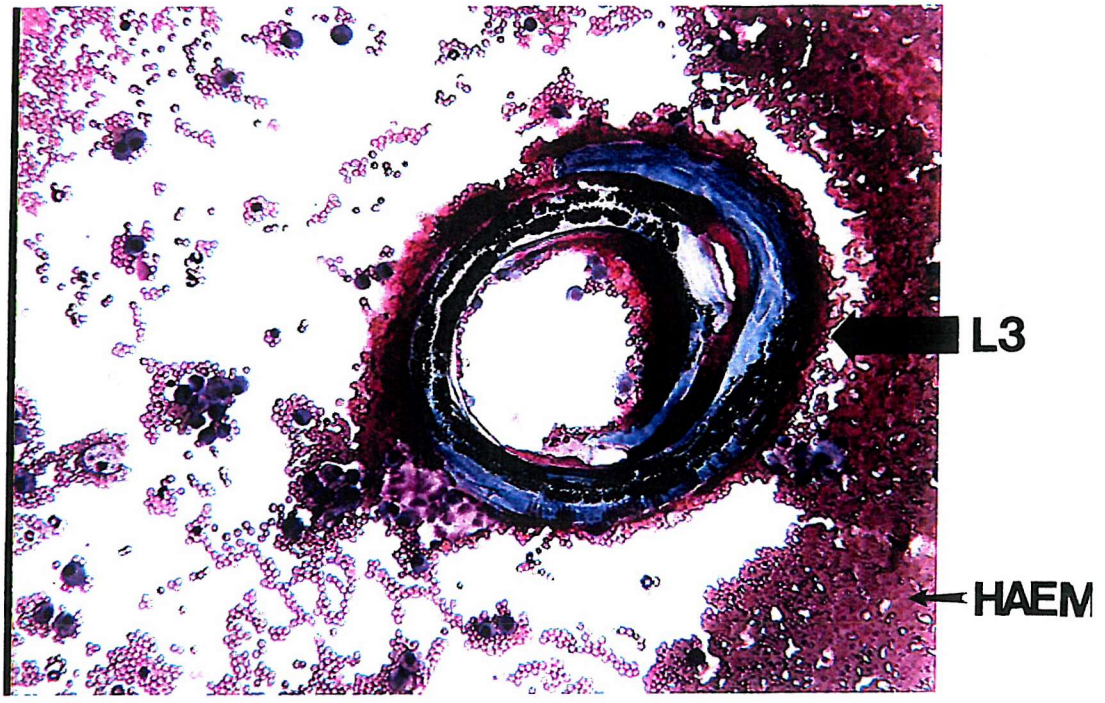

Photo 16 Haemorrhagic BAL fluid on day 3 pi (High Power).
L3 = N.b. larvae; HAEM = marked BAL erythrocytosis

Alveolar macrophages and lymphocytes were frequently seen adherent to the larval sheaths. In the presence of such haemorrhage leukocytes often clumped together (Photo P-15). The alveolar haemorrhage often persisted until day 8 (Photo P-17).

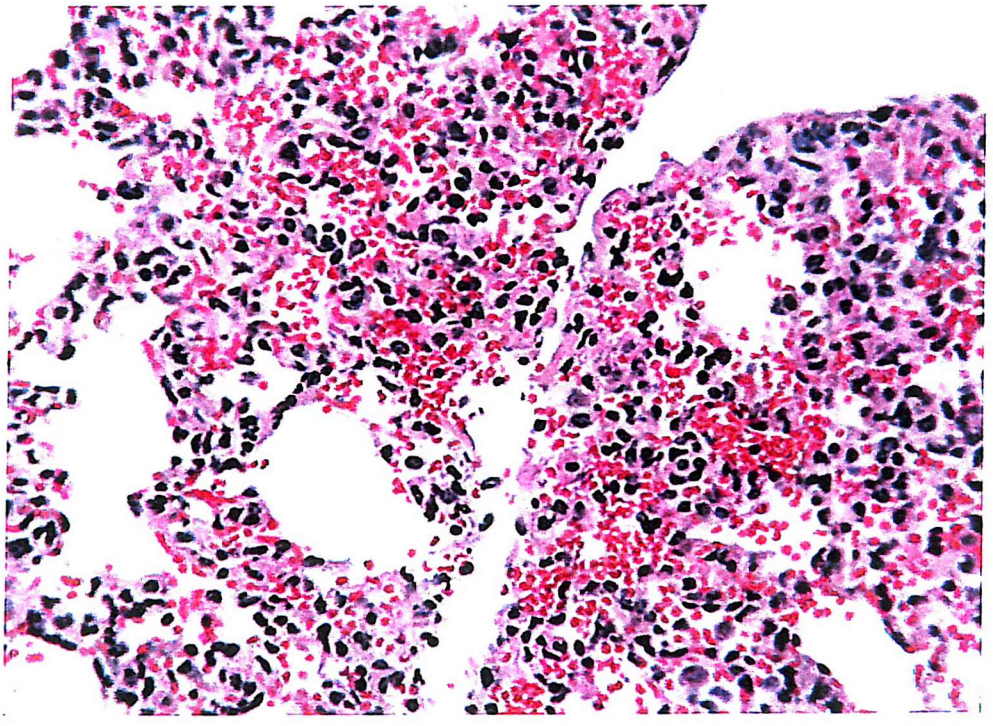

Photo 17 Alveolar Haemorrhage on day 8 pi.

By day 11 pi the L3 were cleared from the lung tissue and the alveolar haemorrhage subsided to be replaced by a significant eosinophilic infiltration. The histopathological changes were most pronounced on day 14 pi. In contrast to venular leukodiapedesis it appeared that the eosinophils were migrating across the arteriolar wall (Photo P-18), resulting in marked peri-arteriolar infiltration by day 14 pi (Photo P-19).

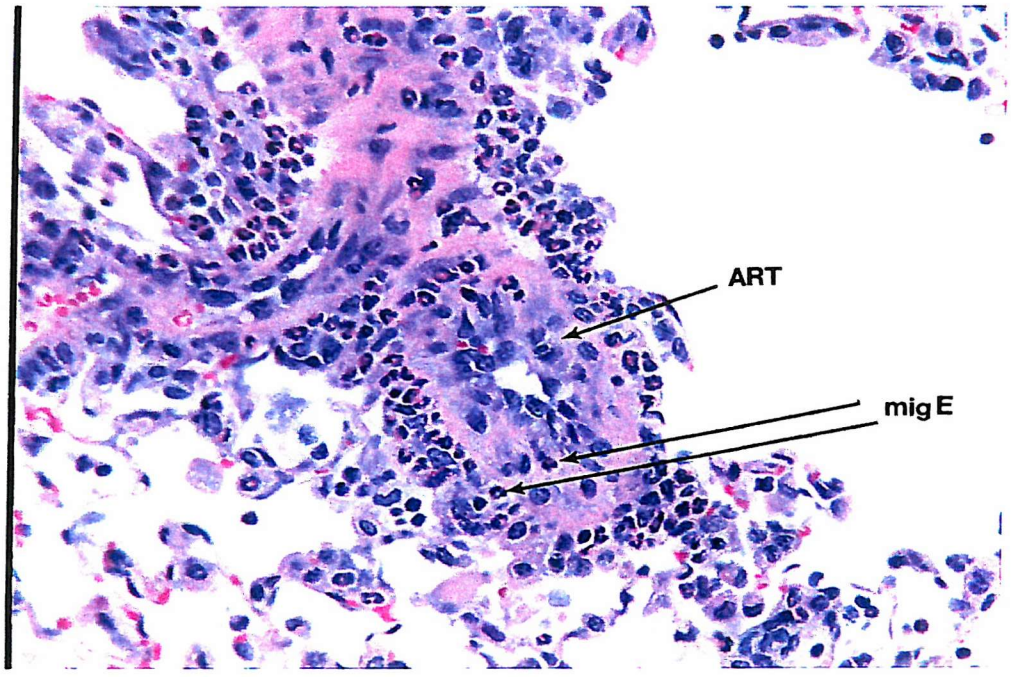

Photo 18 Eosinophilic migration across the arteriolar wall.
ART = arteriole; migE = migrating eosinophils.

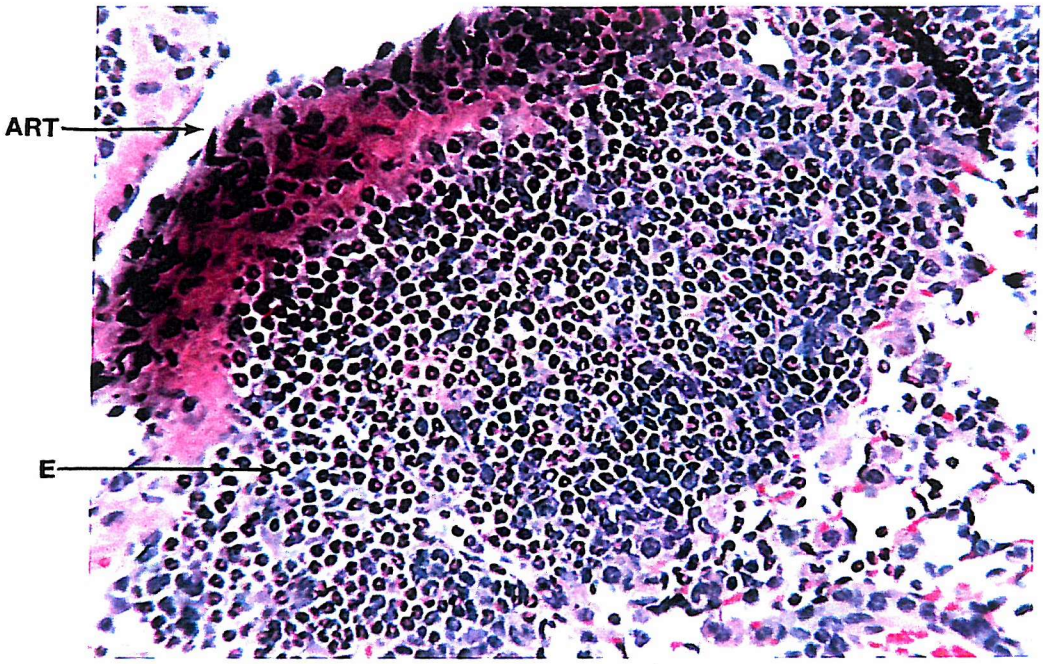

Photo 19 Peri-arteriolar eosinophilic inflammation.
ART = arteriole; E = eosinophils.

The significance of this finding is uncertain. On low power this peri-vascular infiltration seemed to promote the development of an eosinophilic granuloma (Photo P-20), with numerous eosinophils, lymphocytes, macrophages and giant cells present (Photo P 21).

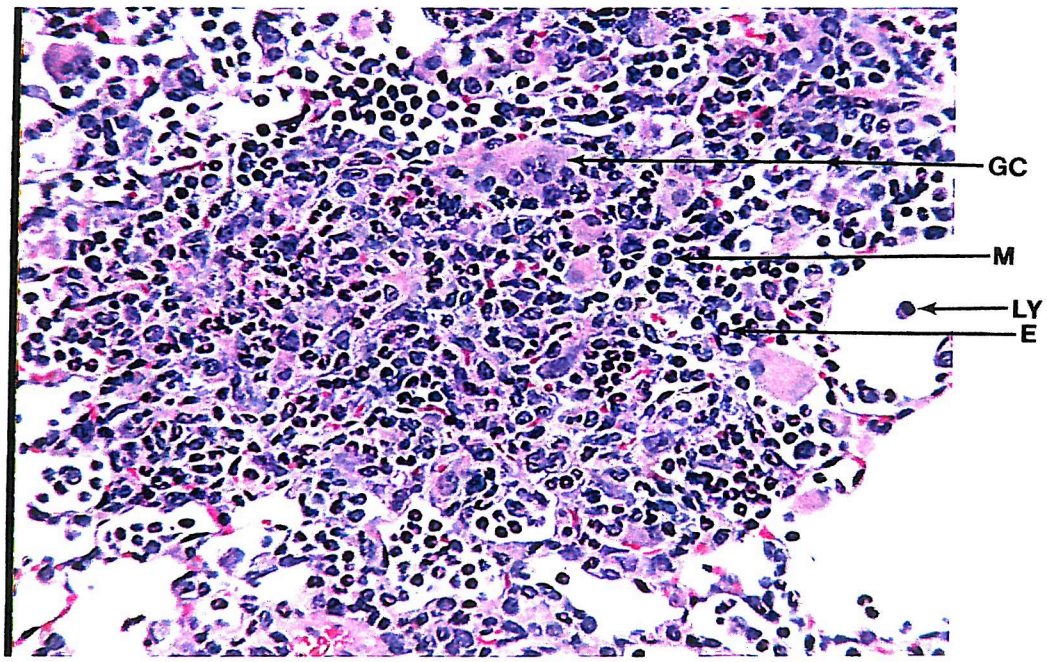

Photo 20 Eosinophilic granuloma (High Power).
EG = eosinophilic granuloma; B = bronchus

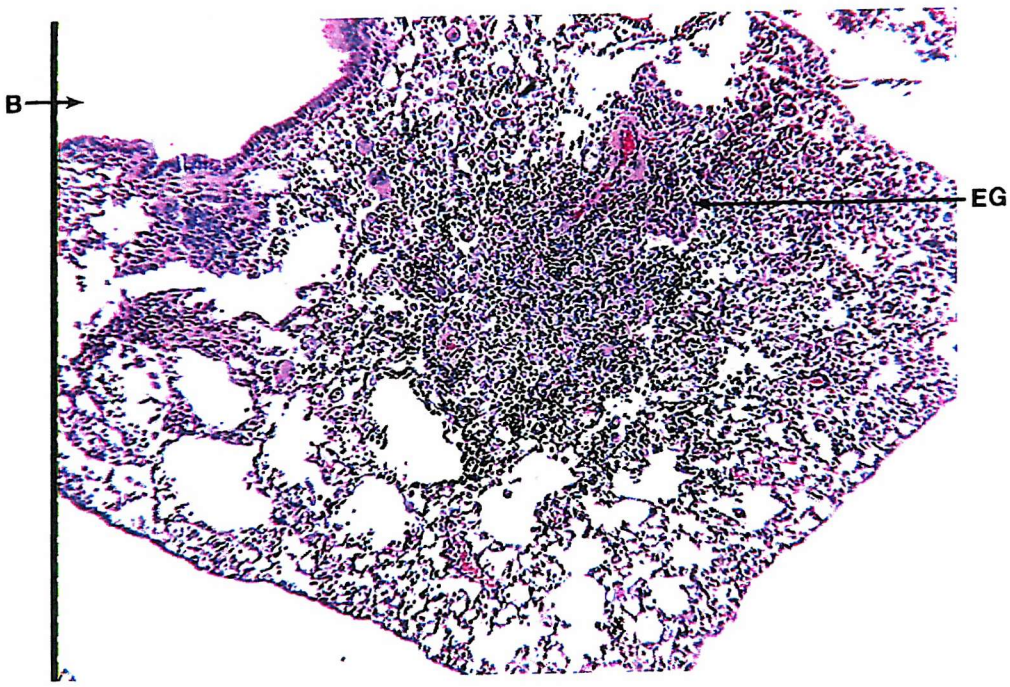

Photo 21 Eosinophilic granuloma (Low Power).
GC = giant cell; M = macrophage; LY = lymphocyte; E = eosinophil

The eosinophilic infiltration was reflected in a heavily eosinophil laden BAL with some monocyte/macrophages still present and only the occasional lymphocyte and neutrophil (Photo P-22).

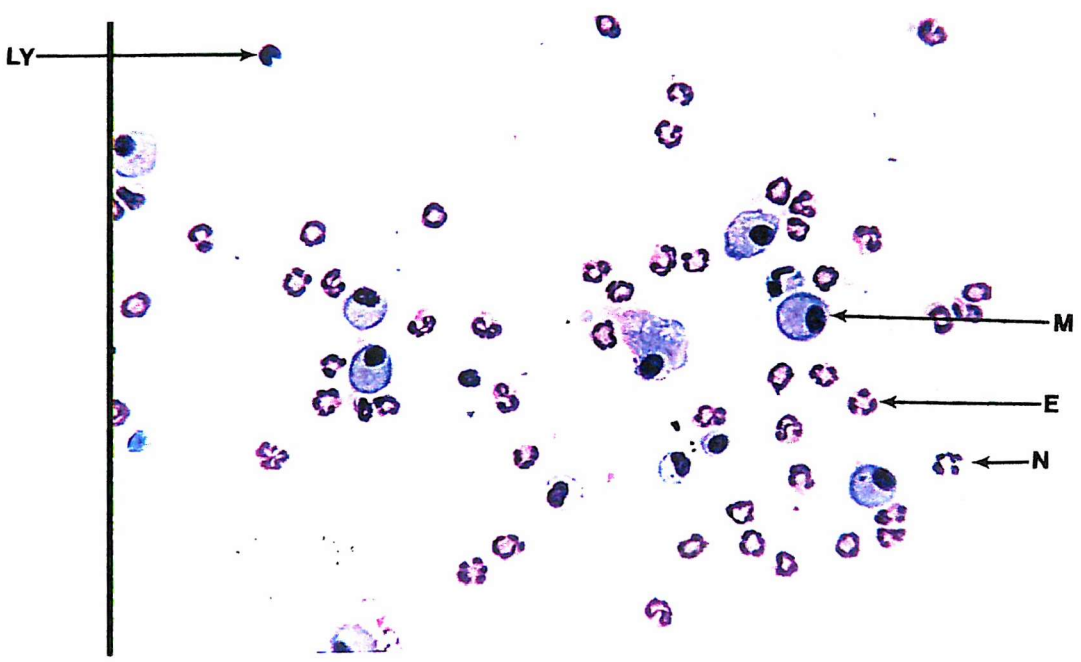

Photo 22 BAL fluid on day 14 pi.
M = monocyte/macrophage; E = eosinophil; N = neutrophil LY = lymphocyte;

The sub-epithelial cellular infiltration appeared to predominantly result from the massive peri-arteriolar migration (Photo P-23).

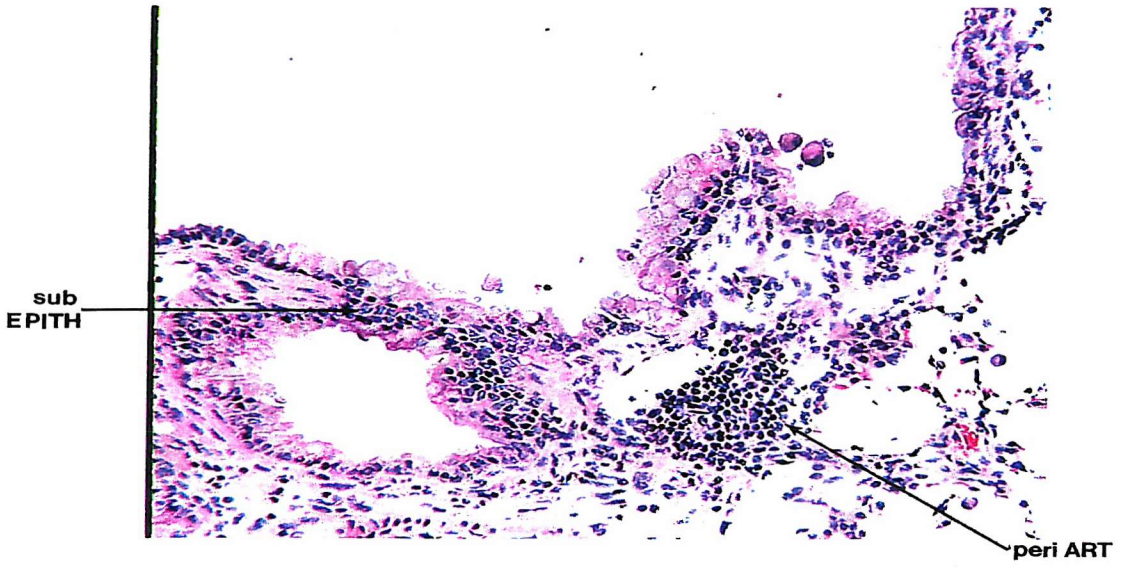

Photo-23 Peri-arteriolar and sub-epithelial infiltration of the airways. PeriART = peri-arteriolar infiltration; subEPITH = sub-epithelial infiltration.

Despite the influx of a substantial number of eosinophils, the airway epithelium remained remarkably intact. There were examples of epithelial loss and disruption (Photos P-24 to P-27), but these were rare and in view of the fact that they were not normally associated with areas of sub-epithelial inflammation they were thought to be predominantly the result of inflation trauma or histological processing.

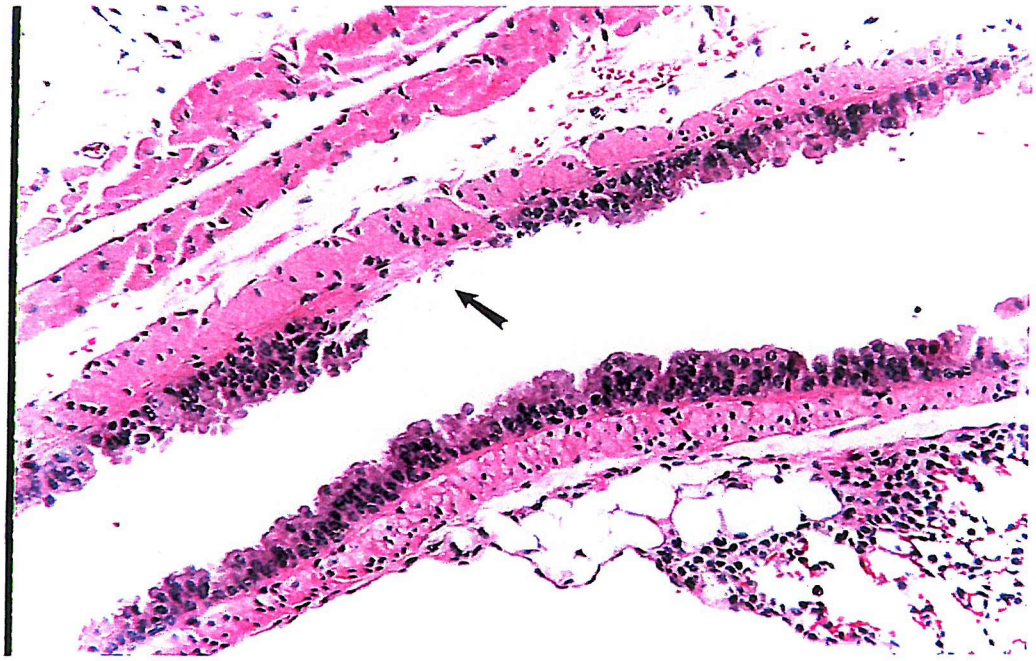

Photo 24 Epithelial loss
Loss of airway epithelium (arrowed) but no underlying inflammation.

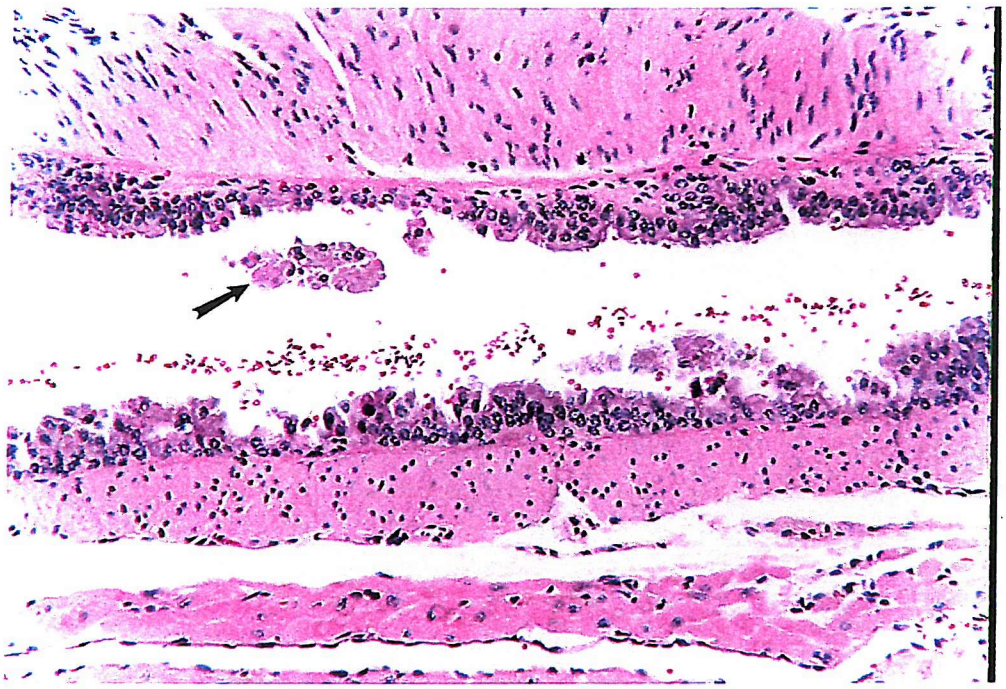

Photo 25 Epithelial detachment
Detached airway epithelium (arrowed) but no underlying inflammation.

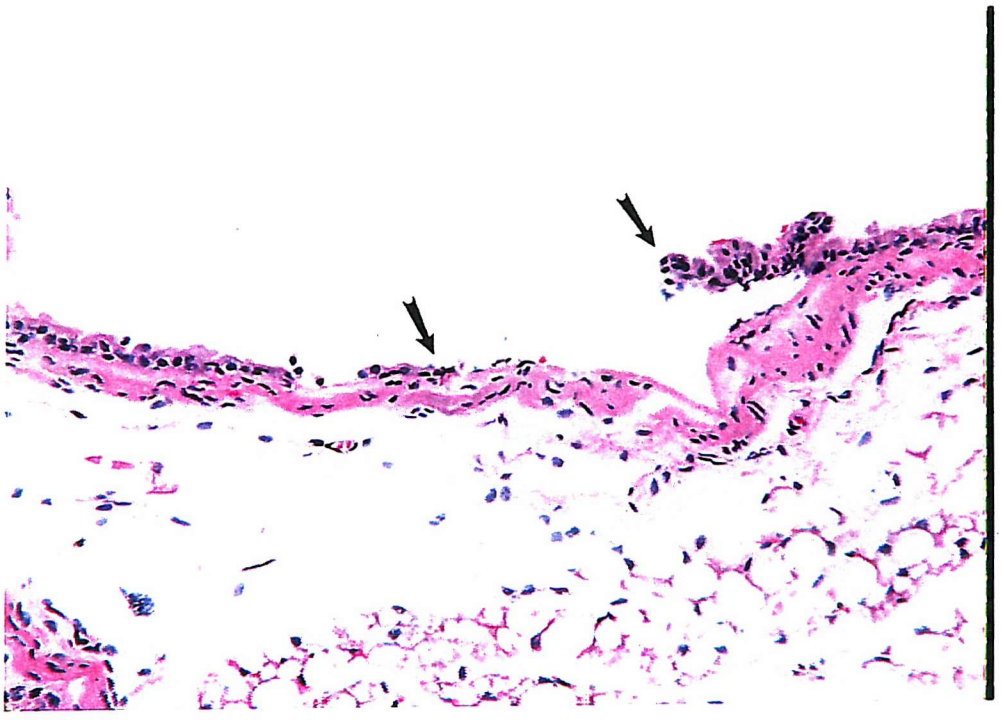

Photo 26 Epithelial detachment
Detached airway epithelium (arrowed) but no underlying inflammation.

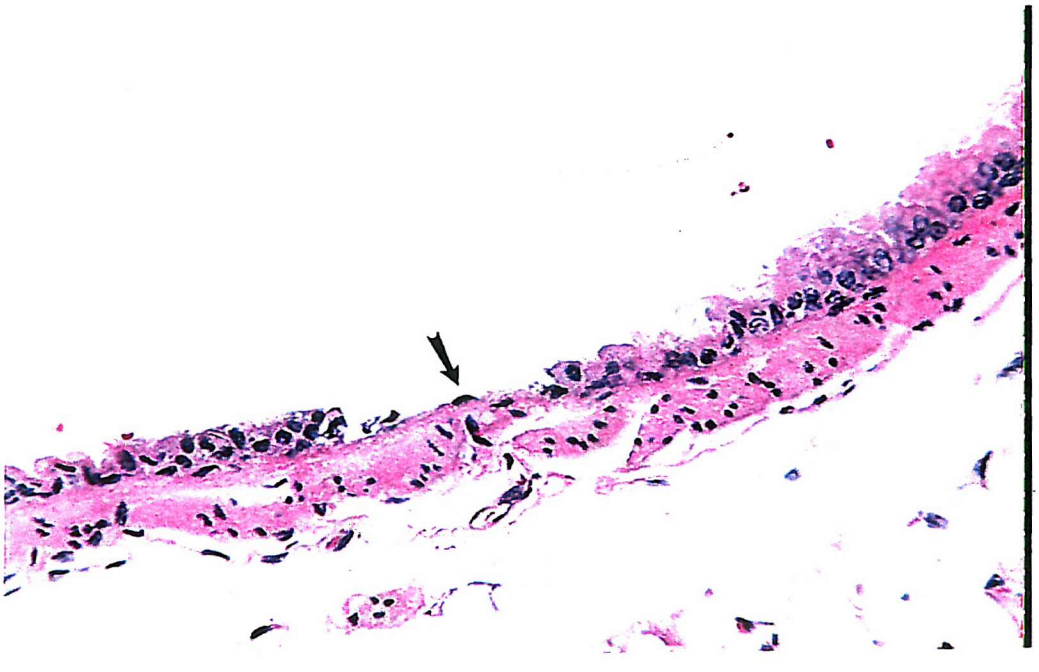

Photo 27 Epithelium loss
Loss of airway epithelium (arrowed) but no underlying inflammation.

However, N.b. infection did produce a minor degree of epithelial dysfunction and alveolar damage as evidenced by epithelial pyknosis (arrow Photo P-28) and pneumocyte type II hyperplasia visible in the airway wall (Photo P-29).

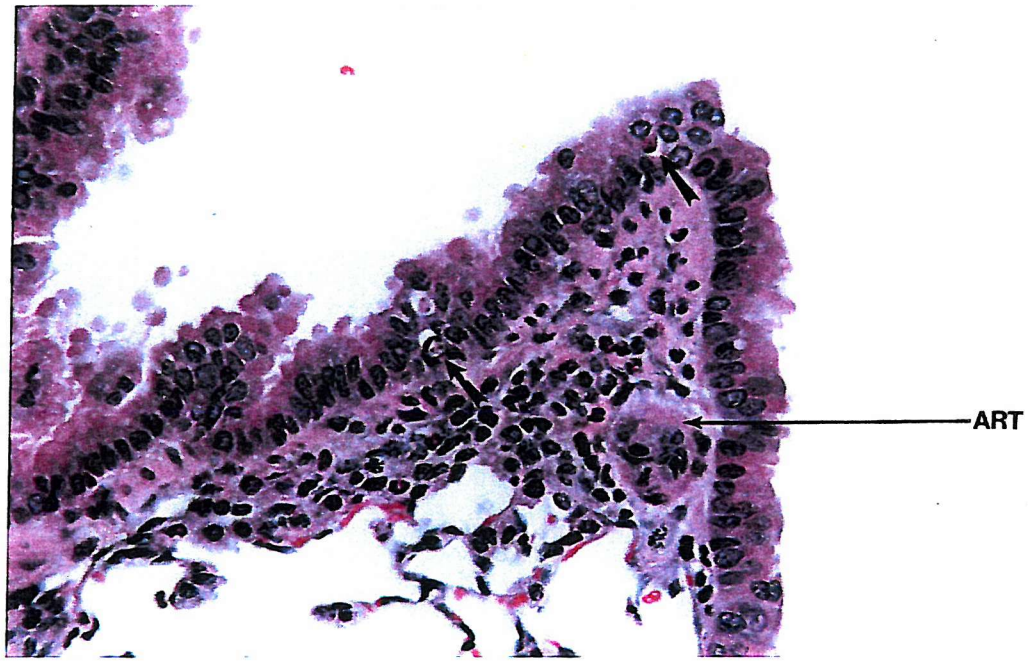

Photo 28 Epithelial dysfunction. Epithelial pyknosis (arrowed) with underlying inflammation and peri-vascular infiltration, ART = arteriole

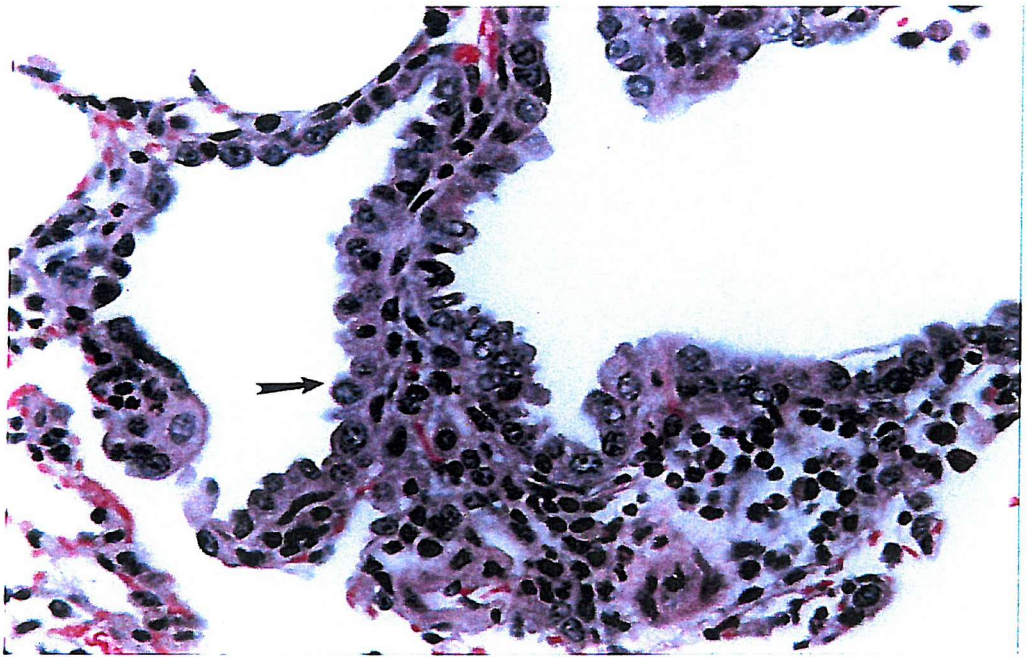

Photo 29 Epithelial dysfunction
Pneumocyte type II hyperplasia (arrowed) with underlying inflammation.

By day 26 the vast majority of these histological changes had subsided and the only consistent finding was some residual peri-vascular inflammation (Photo P-30).

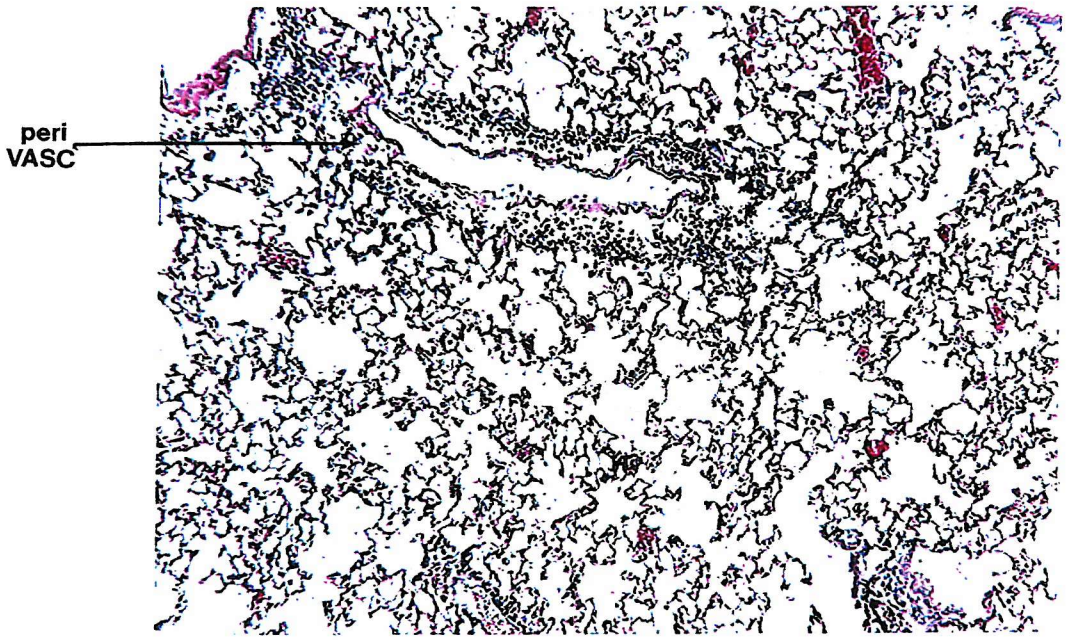

Photo 30 Resolution of inflammation on day 26 post infection. Peri-vascular infiltration less marked with a near normal pulmonary cytoarchitecture, periVASC = perivascular inflammation.

2.4 DISCUSSION

Several investigators have utilized N.b. to develop a helminth-based model of antigen-induced airway inflammation in rats (Ramaswamy et al 1993a & 1993b; Ramaswamy et al 1991 Egwang et al 1984). This nematode parasite changes its surface antigens during the course of maturation to the adult worm and its surface antigens are stage-specific. Thus lung larvae (L3), intestinal larvae (L4) and gut-living adults each possess a characteristic set of cuticular molecules. The adult surface appears to bear the greatest number of antigens, one of which is found only on the male worm. However it is probably the L3 antigens that drive a Th-2 response (see chapter 5). Interestingly when the L3 larvae reach the intestine they may initially provoke a local Th-1 response prior to initiating a strong Th-2 response (Ishikawa N et al 1998). This may be due to the changing nature of L3 antigens. Thus L3 larvae change their surface antigen expression substantially and very rapidly in contrast to the adult worm whose antigenic expression is very stable (Maizels RM et al 1983), and resistant to protease activity (Qureshi F et al 1987).

Having established a stable N.b. colony with consistent degree of infectivity and virulence we examined the pulmonary response to N.b infection in mice to determine how closely the histopathological changes mimicked allergic inflammation of the airways. While the response to N.b. infection in the lungs has been extensively studied in the rat, little is known about the histological response in murine lungs. Thus in rats helminth parasites provoke a significant destruction of the alveolar architecture concurrent with substantial alterations in broncho-alveolar lavage (BAL) cell numbers, differential counts, and in vitro helminthocidal activity. The histopathological changes in the rat are characterised by a type II pneumocyte hyperplasia, an eosinophil infiltration and a granulomatous reaction in the parenchyma (Ramaswamy K et al 1991). In the rat these granulomas have been shown to contain histiocytes/macrophages, multinucleate giant cells and occasionally larvae. These histological changes have been shown to be dependent on mast cell activation (Arizono N et al 1996) and had largely resolved by day 21 (Egwang 1984). In addition to these histological changes, 2-8 days post-infection in rats there is a significant increase in BAL cell numbers with a significant neutrophilia on day 2 and a significant increase in the absolute number of all cell types on day 8. By day 32 post-infection, BAL cell numbers return to control levels. The alveolar macrophages recovered from the BAL of infected rats adhered to and exhibited a complement-dependent killing of L3 (Egwang TG et al 1984b).

In contrast, the histopathological changes in the mice were more persistent, with signs of perivascular inflammation still apparent on day 26 pi. There was little objective evidence of inflammation during larval migration through the lungs but there was an early marked neutrophilia in the BAL fluid as seen in rats and also as occurs in infective exacerbations of allergic inflammation. During this early neutrophilia there were often signs of significant alveolar haemorrhage, with blood in the BAL and the alveolar spaces on light microscopy. Although severe exacerbations of asthma can result in bloody sputum it is not a common finding in allergic reactions, except in some anaphylactic deaths (Pumphrey RS 2000). However, the mechanism of alveolar haemorrhage seen in this murine model of allergic inflammation was almost certainly due to vascular leakage secondary to the enzymatic action of migrating larvae rather than severe inflammation per se.

By day 11 post-infection there was a different response in the lungs. This later response was slightly more typical of allergic inflammation with a significant eosinophilia in the lungs and

BAL fluid. In allergic inflammation of the airways there is a predominantly venular leukodiapedesis but in this model eosinophils appeared to be emigrating primarily from arterioles. The histological pattern seen here in mice was quite similar to that previously described in the rat with granulomas containing macrophages, eosinophils, lymphocytes and giant cells (Ramaswamy K et al 1991).

The epithelial damage characteristic of asthma was not a feature in this model with very little epithelial shedding. However, there was some evidence of alveolar damage with epithelial pyknosis and pneumocyte type II hyperplasia. But there was no evidence of collagen deposition in the epithelial subbasement membrane, which is a characteristic feature of the remodelling response in asthma. Some of these differences may have been due to the difference in the size and nature of the stimulating antigen in this model compared to asthma. In addition, it has been suggested that in asthma, there may be a primary defect in the epithelium such that it responds abnormally to various stimuli and cannot undergo the normal repair response. Thus upregulation of epidermal growth factor (EGF) by the 3v isoform of the adhesion molecule CD44 in addition to the increased expression of transforming growth factor (TGF)-β has been implicated in this epithelial dysfunction (Holgate ST et al 2000b). There was no primary defect in the airway epithelium of the mice used in this series of experiments.

In conclusion, the histopathological changes seen in this model were different from those seen in asthma. In this model there is an eosinophilic vasculitis with marked alveolar damage and haemorrhage on day 3, post infection. There is little evidence of inflammation in the proximal airway walls that is typical of asthma either during the early or later response to N.b. infection. Histopathologically, this model is probably more akin to Churg-Strauss syndrome than asthma. Although the histological mimickery of asthma in this model is not great it would still be accurate to describe this model as a Th-2 drive model of pulmonary inflammation. As such it may still help shed some light on the immunological mechanisms at play in allergic inflammation of the airways, particularly in the BAL as will be explored in subsequent chapters. This thesis proceeds to probe this murine model of pulmonary inflammation in greater detail in order to establish the crucial variables that determine the extent of the airway inflammation and attempts to optimise the usefulness of primary N.b. infection as a model for chronic inflammation of the airways in humans.

CHAPTER 3

The Systemic and Pulmonary Cellular Response to Nippostrongylus Brasiliensis

3.1 INTRODUCTION

There is now substantial evidence, in mice, to suggest that the Th-2 subpopulation of $CD4^+$ lymphocytes and two of their cytokines, interleukin (IL)-4 and IL-5 orchestrate the influx and activation of eosinophils into the airway epithelium. These are characteristic findings in allergic inflammation of the airways in humans (Coffman 1989, Finkelman et al 1988). In addition, Th-2 lymphocytes promote a B-cell isotype switch to IgE production (Kopf M et al 1993, Kuhn R et al 1991, Tepper RI 1990). Two of the most commonly employed strategies to provoke a Th-2 response in rodents are infection with gastrointestinal nematode Nippostrongylus brasiliensis (N.b.) or nebulisation of ovalbumin (OA) to the airways of OA-sensitised animals (Hook S et al 2000, Foster et al 2000, Schramm CM et al 2000, Watkins AD et al 1996).

One of the drawbacks of the OA models is that they do not mimic the chronic inflammation of the airways so typical of asthma in humans because the pulmonary inflammation in rodents subsides within a few days of the last nebulisation. In an attempt to overcome this problem some authors have used longer nebulisation protocols, involving up to 6 weeks of aerosolized OA, to try and provoke chronic inflammation of the rodent airways (Foster PS et al 2000). Others have started OA exposure in the neonatal period (Hamada K 2000). In addition, it is only recently that airway obstruction has actually been shown to occur in OA-induced airway inflammation (Neuhaus-Steinmetz U et al 2000), a fact that led many earlier researchers to choose guinea pigs in preference to mice, since mice were believed not to develop airway hyperresponsiveness unlike guinea pigs. However, the greater availability of immunological tools to probe the inflammatory response of mice shifted the balance away from the guinea pig models and has made murine models much more popular in recent years (Vargaftig BB et al 1999).

Despite the short-term nature of most OA models this does not prevent valuable information from being gathered about Th-2-driven inflammation of the airways. In fact over the last 15 years a great deal of the animal research into asthma has involved the provocation of a Th-2 driven model of allergic inflammation. In addition a great deal of basic immunology research has focused on the characterisation of the cytokine profiles of Th-1 and Th-2 cells, their cellular precursors, their cross regulation, their regulatory molecules and strategies that might shift the balance between them.

Some researchers have suggested that the polarisation of the immune response into a Th-1 predominant or Th-2 predominant response is an oversimplification. In fact, the existence of a distinct Th-2-like subpopulation was initially questioned, particularly in humans. However, numerous studies have now confirmed a pivotal role played by the IL-4 gene cluster encoded by Th-2-like T cells in human allergic disease. For example, T cell clones from atopic individuals can be stimulated to produce Th-2 cytokines in response to antigens (Parronchi P et al 1992), T cell mitogens (Field EH et al 1993), or allergens (Wierenga EA et al 1990, Parronchi P et al 1991), including house dust mite (O'Hehir RE et al 1993). In addition, late-phase allergic reactions are associated with increased gene transcription for IL-3, IL-4, IL-5, and GM-CSF (Robinson DS et al 1992, Del Prete GF et al 1993). These cytokines activate eosinophils and promote their recruitment into the bronchoalveolar lavage (BAL) fluid in asthmatics (Robinson DS et al 1993a).

However, the cytokine profiles traditionally associated with Th-1 or Th-2 populations may not always be clearly delineated *in vivo*. A Th-0 population, which may be common precursors to both Th-1 and Th-2 cells, may exist which secretes an intermediate cytokine profile. The factors that shift the balance between a Th-1 and a Th-2 driven response has been a source of considerable interest in the last ten years.

For example, a great deal of attention has been paid to the factors that induce the differentiation of naïve T cells into either a Th-1 or Th-2 subpopulation. Thus it has been suggested that the physicochemical nature of the antigens encountered *in utero* (Piccinni MP et al 1993, Warner JA et al 1994), or infancy (Holt PG et al 1994, Sporik R et al 1990), may lock the T cell response into a permanent Th-1-like or Th-2-like pattern. Experiments in mice have demonstrated that the generation of an interferon (IFN) or an IL-4-predominant response is dependent on the type and duration of antigenic stimulation. Thus, adult antigens from *Nippostrongylus brasiliensis* (N.b.) promote a Th-2 response, while BCG purified protein derivatives (PPD) or Brucella abortus antigens promote Th-1 responses, both *in vitro* (Saito S et al 1994), and *in vivo* (Street NE et al 1990). Similar findings have been reported in humans (Del Prete GF et al 1991).

The importance of this Th-1/Th-2 distinction has led some authors to develop novel therapeutic strategies designed to alter the balance between established populations of Th-1

and Th-2 lymphocytes. For example, recent research has led to the development of a series of Th-1 or Th-2-skewing *immunotherapy peptides* which, when given subcutaneously, are capable of enhancing or dampening airway eosinophilia and Th-2 cytokine production in a murine model of airway inflammation. These peptides were actually analogues of the dominant allergen epitopes (Janssen EM 2000). Alternatively, some authors have used genetic technology to skew the balance between Th-1 and Th-2 cells. For example, there is evidence to suggest that allergen-induced airway hyperresponsiveness (AHR) may respond to immunotherapy with Th1 cytokines. Thus, local administration of recombinant IL-12 or IFN-γ or intratracheal delivery of the genes for these cytokines has been shown to reduce the severity of AHR in rodent models. More recently some authors have successfully reduced allergen-induced AHR, airway eosinophilia, cytokine production and serum IgE concentrations by delivering cytokine genes to the lungs by intravenous injection of lipid-DNA complexes. In fact these authors demonstrated that systemic delivery of cytokines was more effective than either local intratracheal delivery of cytokine genes or the direct administration of recombinant IFN-γ (Dow SW et al 1999).

Other authors have identified complex immunomodulator molecules that promote a Th-1 response which are a mixture of antigen and oligodeoxynucleotides (Shirota H et al 2000). Such strategies raise the possibility that it may be possible to develop safe immunotherapy to desensitize asthmatics in the future. The ability to inhibit a Th-2 response has invigorated many pharmaceutical companies' drug discovery programmes. For example, a recent report indicated that an unnamed compound decreased the production of IL-4 and IL-5 by cultured splenocytes and also in bronchoalveolar lavage (BAL) fluid by 51 to 73%, while simultaneously increasing IFN-γ production 2- to 5-fold. In addition this compound was shown to suppress plasma IgE level by 55-85%, inhibit Ig-independent peritoneal eosinophilia by 54% and inhibit airway reactivity to acetylcholine (Kato Y et al 1999).

In an alternative approach other investigators have identified molecules, such as preproenkephalin (PPNK), that enhance Th-2 responses and have therefore suggested that the inhibition of these promoter molecules may also be a useful therapeutic strategy (Hook S et al 2000). Some researchers have demonstrated the importance of the Th-1/Th-2 balance by demonstrating how environmental triggers, such as pollution or endotoxins, can shift the Th-1/Th-2 balance and promote airway hyperresponsiveness (Hamada K et al 2000, Wan GH et al 2000).

However, strategies that are designed to shift the Th-1/Th-2 balance must be interpreted with caution, particularly in the light of recent evidence suggesting that, under some circumstances, Th-1 and Th-2 populations may not be mutually inhibitory. In fact in a recent series of adoptive transfer experiments it was suggested that both populations may co-operate in the pathogenesis of eosinophilic inflammation and recruitment of lymphocytes to the airways (Randolph DA et al 1999). Similarly, infection with powerful Th-2 stimulants such as N.b. has been shown to actually induce a Th-1 response in the mesenteric lymph nodes initially prior to inducing an isotype switch to a Th-2-type response, suggesting that the Th-1/Th-2 paradigm may not be an either/or phenomenon (Ishikawa N et al 1998).

One of the main reasons the Th-1/Th-2 paradigm has fired the imagination of so many scientists is because of the differing cytokine profiles typically produced by each population of lymphocytes. Of the numerous cytokines now described IL-4 and IL-5 have been the most widely studied. In fact, recent evidence employing cytokine knockout mice has demonstrated that IL-5 but not IL-4 plays a key role in chronic inflammation of the airways and the induction of airway hyperreactivity.

These studies demonstrated that IL-5 or IL-10 deficiency markedly decreased the recruitment of chronic inflammatory cells to the lamina propria and eosinophils to the epithelium. While the IL-5 or IL-10 deficient animals did not develop airway hyper-reactivity to inhaled methacholine they did develop epithelial hypertrophy and subepithelial fibrosis comparable with that observed in control wild type (WT) mice (Foster PS et al 2000). In contrast, IL-4-deficient mice exhibited exaggerated hyper-reactivity to methacholine as well as significantly greater epithelial hypertrophy and subepithelial fibrosis but no decrease in airway inflammation. This suggests that IL-5 and IL-10 play a greater role in airway inflammation while IL-4 plays a greater role in hyper-reactivity. Furthermore, the appearance of chronic epithelial and fibrotic changes in the absence of IL-5 and airway hyper-reactivity indicates that airway wall remodelling is independent of airway hyper-reactivity (Makela MJ 2000, Foster PS et al 2000).

These findings were confirmed by studies using Nippostrongylus brasiliensis. Thus IL-5 transgenic mice were highly resistant to primary infections with N.b. with few parasites found in the intestines of infected animals and minimal egg production. On closer

examination IL-5 transgenic mice generated a much more vigorous response, involving extensive eosinophil degranulation, at the site of N.b. inoculation. Thus 75-95% of the larvae were still in the subcutaneous tissues 24h after inoculation in the IL-5 transgenic mice compared to <20% in wild type (WT) control mice. These data strongly suggested that eosinophils restricted the movement of N.b. and therefore prevented most larvae from reaching the lungs (Daly CM et al 1999).

Having demonstrated some similarities between the histopathology of asthma and the histopathological response of N.b. infection (chapter 2), we examined systemic and pulmonary response to N.b. to determine how closely this mimicked allergic inflammation seen in humans. Specifically, we investigated the phenotype of the eosinophils and lymphocytes recruited to the airways and the activation and functionality of those eosinophils. Our results suggest that there are a number of similarities in the cellular response in the lungs and elsewhere between N.b. and the allergic inflammation seen in asthmatic airways. Specifically N.b. infection provoked an early and late inflammatory response, which culminated in the selective recruitment from the microvasculature of eosinophils and T cells expressing a restricted range of cell adhesion molecules. Therefore infection with N.b. may be a useful alternative to challenge with OA to induce a Th-2 response and may offer specific advantages over OA models.

3.2 MATERIALS AND METHODS

3.2.1 Larval culture and infection

Details of N.b. larval culture are given in chapter 2.

3.2.2 Animals

Details of animal husbandry are given inchapter 2.

3.2.3 Bronchoalveolar lavage and cell preparations

Details of brochoalveolar lavage and cell preparation are given in chapter 2. Data presented throughout this thesis are given per ml of BAL fluid drawn.

3.2.4 Lung tissue disaggregation

Following BAL fluid collection, lungs from three to seven mice/group were disaggregated as previously described (Kennedy et al 1995). Briefly lungs were removed, rinsed in PBS containing 5% FBS and then dissected from the bronchial tree, removing any peribronchial lymph nodes.

Then approximately 750 mg of lung tissue was minced into 2 mm squares and placed into four 2 ml, screw-cap microfuge tubes containing PBS-FBS. The lung was disaggregated by agitating the tissue for 1 hour at room temperature (RT) in 850 U/ml hyaluronidase Type I-S (Sigma, St. Louis, MO), 150 U/ml of type 3 collagenase (Worthington Biochemical Corp., Freehold, NJ), and 50 U/ml DNAse (Sigma). Undigested tissue was settled, and the supernatant was filtered through 55 μm nylon mesh. Erythrocytes were removed by lysis with TRIS-buffered ammonium chloride and the remaining cells were washed three times with HBSS-FBS.

3.2.5 Immunofluorescence staining and flow cytometry

The PE-anti-CD8 monoclonal antibody (mAb) used in these studies was purchased from Caltag (South San Francisco, CA) and all other mAb (biotinylated, PE-, or FITC-conjugated) were purchased from PharMingen, San Diego, CA. All mAb were titered for optimal staining with minimum signal overlap prior to use in multi-colour flow cytometry. In triple colour flow cytometry Thy1+ is stained red, CD3 is stained green and the other antibodies are stained yellow. The panel of mAbs used and the immunofluorescence staining technique have been previously described (Kennedy 1995). Briefly, BAL fluid or lung tissue cells were stained with mAb diluted in phenol red-free HBSS containing 2% FBS, 10 mM HEPES, and 0.02% Na Azide. Non-specific background staining was reduced by pre-treating samples with anti-CD32 ($Fc\gamma_{II}R$) mAb. Cells were then incubated with conjugated mAb. Biotinylated mAb was subsequently developed with Texas Red-streptavidin. Controls were generated by staining cells in the same manner with conjugated irrelevant myeloma Ig. Cells were fixed with 1% paraformaldehyde in PBS and 10 mM HEPES and run on a Coulter Epics 753 flow cytometer equipped with a 5 watt argon laser and a rhodamine-590 dye laser.

Forward angle and 90^{o} light scatter signals were used to determine the eosinophil and lymphocyte gates in the lung tissue and the BAL fluid. T cells were identified as $Thy1^{+}CD3^{+}$

cells, and B cells as $B220^+$ within the well-defined lymphocyte gate. The eosinophil gate was clearly defined in the lung tissue and BALF 14 days pi but less well demarcated in the lung tissue and BALF fluid of naïve animals and animals 3 days pi (Figure 3-9A and 3-9B). In the absence of a specific eosinophil surface marker, the gated eosinophil population may have contained other granulocytes. Contamination was thought to be minimal in the BAL but potentially confounding in the lung. Data acquired in list mode on 10,000-gated events were analysed using the Elite software (Coulter Electronics).

3.2.6 Blood: Leukocyte subsets

After BAL, the abdominal cavity was opened and ~200-600 µl of blood per animal was drawn from the inferior vena cava using a 23g needle and a 1 ml syringe. The blood was transferred into a microtube containing EDTA. The total number of leukocytes and the number of basophils, eosinophils, lymphocytes, monocytes and neutrophils were enumerated using a Technicon H1 instrument (Miles Diagnostics, Tarrytown, NY). Data presented throughout this thesis are given per ml of blood drawn.

3.2.7 Eosinophil Activation

BAL fluid cells from 20 mice, 14 days pi, were centrifuged at 1200 rpm for 5 minutes, resuspended in 2 ml HBSS without Ca^{++}, Mg^{++} and separated on a seven layer Percoll (1.123 g/ml) gradient. The densities of the gradient layers were as follows: 1.040 g/ml, 1.050 g/ml, 1.060 g/ml, 1.070 g/ml, 1.080 g/ml, 1.090 g/ml and 1.100 g/ml. This density gradient was centrifuged at 2500 rpm for 20 min. Eosinophil-enriched fractions were washed and resuspended in HBSS without Ca^{++}, Mg^{++}. Cell counts were performed and viability was determined. The fractions were resuspended at 1.0×10^6 cells/ml in HBSS containing 1.2 mM of $CaCl_2$ and 0.8 mM of $MgCl_2$, and treated with 5 µM of calcium ionophore A23187 for 30 min. The reaction was terminated with three volumes of methanol. Cell debris was removed by centrifugation at 1500 rpm for 10 min, and the supernatant was evaporated to dryness under vacuum in a centrifuge dryer (Savant Instruments, Hicksville, NY). The residue was dissolved in 500 µl of 1:3 HPLC grade methanol:dH_2O prior to injection into the high performance liquid chromatograph (HPLC).

3.2.8 High Performance Liquid Chromatography

Arachidonic acid metabolites were separated by a Varian 5000 HPLC using a solvent system

consisting of methanol: acetonitrile: 0.1% ammonium acetate (pH 5.7) with a flow rate of 1 ml/min. Separation was performed on a 5 μm Spherisorb 5 ODS-2 column (4.6 x 250 mm) (Phenomenex, Torrance, CA), protected by a Waters guard column (4.5 x 10 mm) packed with C18 microporosil. The octodecasilane (ODS) coats the silica and provides a very C18-rich (non-polar) medium, which mops up all the hydroxyl (OH) groups in the leukotrienes (LT), promoting retention. This is in contrast to the standard HPLC column, which is very polar and relies on electrostatic binding of the compounds injected. The Waters guard column protects the ODS column by acting as an in line filter

The samples were introduced through a Rheodyne 7125 valve with a 500 μl loop. Following sample injection a graded elution occurs starting with 100% solvent B (50% 0.1% pH 7.5 NH_4Ac (Ammonium Acetate) buffer; 20% Acetonitro, 30% methanol) and over the next 30 min switching to 75% solvent C (100% Methanol). From 30-45 minutes the elution moves from 75% C to 100% C, washing out the remaining debris. After injection the column effluent was collected in 1 ml fractions for 35 min, in silanized glass tubes. The tubes are silanised to prevent LT-OH groups sticking to the glass. Collection of these fractions commenced after 2 mins to allow the solvent to wash out the column and continued for the next 35 min. Comparing their HPLC retention times with retention times of authentic radiolabelled standards identified arachidonic acid metabolites.

The standardised retention times for the leukotrienes are as follows:

a.) LTB4 17.9 min

b.) LTC4 7.8 min

c.) LTD4 16.8 min

d.) LTE4 32.2 min

When leukotrienes are eluted from the column they absorb UV light @ 280nm, because of their three conjugated double bonds. In contrast HETE absorbs at 234 nm. This absorbance is automatically measured with an UV-100 ultraviolet detector set at 280 nm and a Radiometric HS Flo-One radioactivity flow counter.

Non-radiolabeled fractions were evaporated to dryness and redissolved in 0.25 ml EIA buffer (0.1 M potassium phosphate buffer pH 7.4, with 0.01% sodium azide, 0.4 M sodium chloride,

0.852 mM EDTA and 0.1% bovine serum albumin). Samples were stored at -30^{0}C prior to analysis by EIA.

In this experiment 3 samples were run with 35 fractions per sample: yielding 105 fractions. Each fraction acted as single 50µl sample in the ELISA immunoassay. Initially each sample was run neat in each of the 4 ELISAs; LTB4, LTC4 TxB2, PGE2 then once the main fraction was determined the sample was re-run as a 1 in 250 dilution.

3.2.9 Enzyme Immunoassay

The EIA reagents used in this study to quantify LTB_4, LTC_4, prostaglandin (PG)-E_2, and thromboxane (Tx)-B_2 were purchased from the Cayman Chemical Company (Ann Arbor, MI). Samples and standards were loaded at 50 µl/well into a 96 well immunoplates (MaxiSorbF96, Inter Med NUNC, Roskilde, Denmark) which had been previously coated with murine anti-rabbit IgG mAb (2 µg/well). The coated plates were washed 3X with 0.01 M potassium phosphate buffer containing 0.05% Tween 20 in a M96V Titertek microtiter plate washer (ICN Flow, McLean, VA). To each well, 50 µl of ACE (acetylcholine esterase) - linked eicosanoid conjugate and 50 µl of appropriate specific antibody were added and the plates were agitated and incubated overnight at room temperature. The following morning, the plates were washed as before. Two hundred microlitres of a freshly prepared mixture of 0.5 mM Ellman's reagent [5,5'-dithiobis(2-nitrobenzoate)] and 0.69 mM of ACE substrate [acetylthiocholine iodide] in 0.01 M potassium phosphate buffer pH 7.4 was added to each well and the plates were shaken in the dark for 1 to 2 h until adequate colour was developed. The absorbance at 412 nm were determined using a Molecular Devices Vmax 96-well plate reader (Menlo Park, CA) and the concentrations were calculated using the Beckman Immunofit EIA/RIA software package.

3.2.10 Total serum IgE

After removing an aliquot of whole blood for the total white blood cell count (WBC) and differential, the blood was centrifuged at 300 X g for 8 minutes and the plasma was removed for IgE estimation. Briefly, 96-well half-area plates (Costar, Cambridge, MA) were coated with 50 µl/well of sheep anti-mouse IgE (The Binding Site Ltd, Birmingham, England) diluted 1:250 in 0.02 M carbonate buffer, pH 9.6 and incubated overnight at 4^{0}C.

Plates were then washed on the morning of the assay with Dulbecco's Phosphate buffered saline (GIBCO BRL, Gaithersburg, MD) containing 0.05% Tween 20 (PBS/TW) and all non-specific binding was blocked with PBS/TW containing 1% BSA for 2 hours at RT.

Blocker was washed off using PBS/TW and serial dilutions of purified mouse IgE (SIGMA D-8406, Clone SPE-7) and unknown mouse sera (50 µl/well) were loaded onto the plate and incubated at RT for 2 hours. The unknown sera samples were diluted 1 in 10, 1 in 20 and 1 in 25 using PBS/Tween. Plates were then washed with PBS/TW and further incubated for 2 hours at RT with 50 µl/well of a 1:400 dilution of alkaline-phosphatase labelled anti-mouse IgE (Southern Biotechnology Associates, Inc., Birmingham, AL). Following a further wash with PBS/TW, 50 µl/well of p-nitrophenylphosphate (pNPP)(SIGMA 104 Phospahtase Substrate Tablets), at a concentration of 1 mg/ml of substrate buffer (Tris-sodium-barbital buffer) was added. After 1 hour at 37 ^{0}C the absorbance was read at 405 nm with an automated plate reader. Captured data was converted by Softmax software for analysis by Beckman Immunofit (Version 3.0) software.

3.2.11 Statistics

The data presented in this chapter on wild type C57BL/6 mice are pooled from several experiments conducted throughout this thesis. Thus the peripheral blood and BAL fluid data presented contains a variable number of mice per time point. There were an average number of 38 wild type C57BL/6 mice per time point pooling from several experiments. This enabled data to be analysed using a two-tailed paired t test assuming unequal variance in samples, but a normal distribution. When this overall test of treatment was found to be significant at the $p<0.05$, this was indicated by an asterisk (*). When the significance level reached $p<0.01$ this was indicated by two asterisks (**), and when the significance level reached $p<0.001$ this was indicated by three asterisks (***). If the result was borderline significant ($p=0.05$), then this was denoted by a # sign.

3.3 RESULTS

3.3.1 Peripheral blood changes

I. Total blood leukocyte count

Primary infection with third stage N.b. larvae produced a significant monophasic increase in

the total number of leukocytes in the blood, rising significantly from 5.31 x 10^6 in naïve mice to 7 x 10^6 by day 11 pi ($p<0.05$). The peripheral leukocytosis increased further to 8.93 x 10^6 by day14 pi ($p<0.001$) and peaked on day 19 at 9.54 x 10^6 ($p<0.001$). The leukocytosis persisted until day 26 pi, 8.94 x 10^6 ($p<0.01$) and subsided by day 35 pi to 6.31 x 10^6 (Figure 3-1 & Table 3-1).

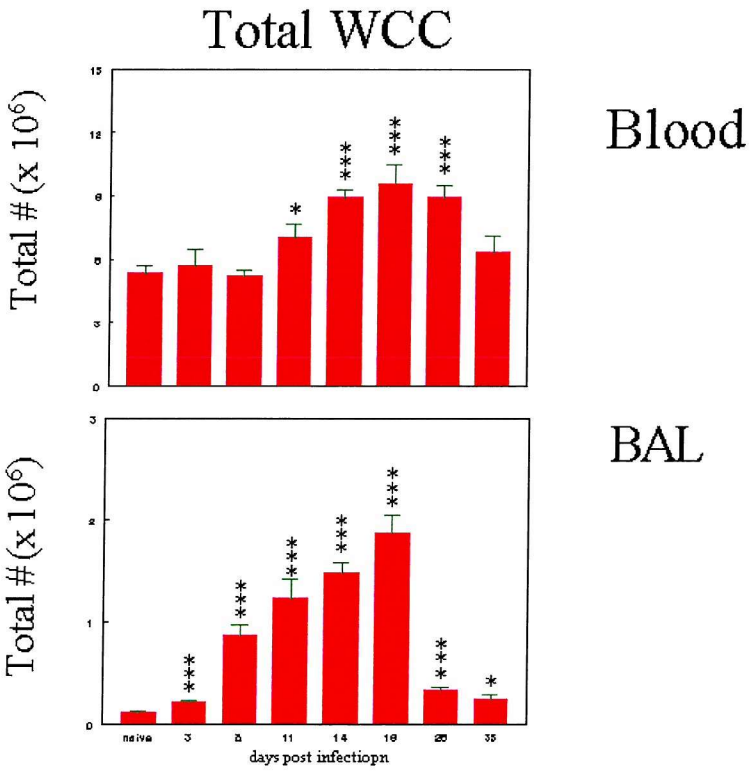

Figure 3-1. The total number of leukocytes recruited to the blood and airways of wild type mice following N.b. infection.

Cell subset	Naïve	Day 3	Day 8	Day 11	Day 14	Day 19	Day 26	Day 35
Total WCC x 10^6	5.31 ± 0.36	5.72 ± 0.73	5.18 ± 0.28	7.00 ± 0.70 *(p<0.05)*	8.93 ± 0.37 *(p<0.001)*	9.54 ± 0.98 *(p<0.001)*	8.94 ± 0.59 *(p<0.001)*	6.31 ± 0.82
Lymphocytes x 10^6	4.43 ± 0.32	4.49 ± 0.54	3.79 ± 0.23	5.58 ± 0.54	6.59 ± 0.27 *(p<0.001)*	7.99 ± 0.82 *(p<0.001)*	7.58 ± 0.52 *(p<0.01)*	4.39 ± 0.91
Neutrophils x 10^6	0.59 ± 0.03	0.75 ± 0.11	0.89 ± 0.07 (p<0.	0.60 ± 0.07	0.78 ± 0.04 *(p<0.05)*	0.62 ± 0.06	0.76 ± 0.05	0.46 ± 0.05
Eosinophils x 10^6	0.08 ± 0.01	0.21 ± 0.05	0.13 ± 0.02	0.46 ± 0.07	1.17 ± 0.09 *(p<0.001)*	0.60 ± 0.11 *(p<0.01)*	0.27 ± 0.04	0.14 ± 0.03
Monocytes x 10^6	0.12 ± 0.01	0.16 ± 0.04	0.21 ± 0.07	0.19 ± 0.03	0.20 ± 0.01	0.16 ± 0.02	0.17 ± 0.02	0.93 ± 0.79

Table 3-1. Peripheral blood total leukocyte and leukocyte subset counts at various time points after N.b. infection. Data are the means ± SEM from 20 to 30 mice/time point, from two to five experiments. Statistically significant differences compared to naïve or day 3 mice, as determined by a two-tailed t test assuming unequal variance, are indicated by * ($p<0.05$), ** ($p< 0.01$), and *** ($p< 0.001$).

Lymphocyte, eosinophil and neutrophil numbers and percentages changed significantly following N.b. infection, but there was no clear change in basophil and mononuclear cell numbers and percentages (see below).

II. Lymphocytes

The total number of lymphocytes in the peripheral dropped slightly on day 8 pi from 4.43 x 10^6 in naïve mice to 3.79 x 10^6. However this was not statistically significant. By day 14 pi the number of lymphocytes circulating had increased significantly to 6.59 x 10^6 ($p<0.001$). Lymphocyte numbers continued to increase and by day 19 pi they peaked at 7.99 x 10^6 ($p<0.001$). Lymphocyte numbers remained high on day 26 pi, 7.58 x 10^6 ($p<0.01$), before subsiding back to baseline levels by day 35 (Figure 3-2).

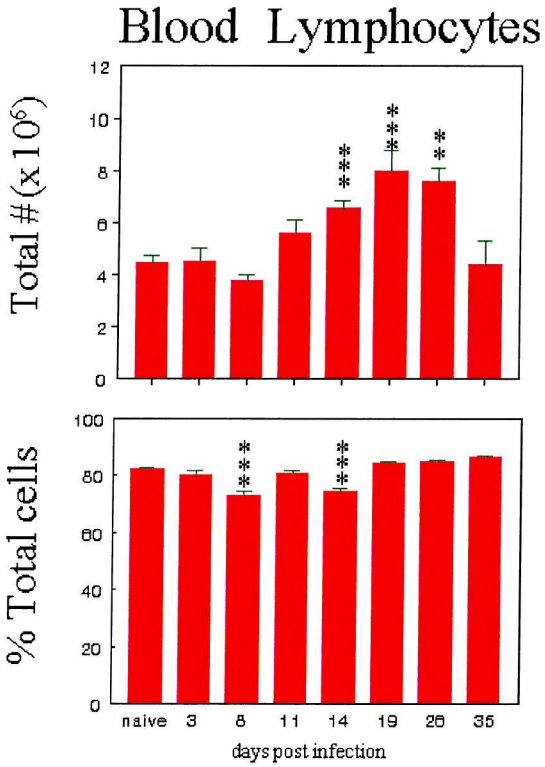

Figure 3-2. The total number and percentage of lymphocytes in the blood of wild type mice following N.b. infection.

In contrast, the percentage of lymphocytes in the peripheral blood remained relatively constant at approximately 80-82% throughout the infection. However, there was a small but significant drop in the percentage, to 73%, on day 8 pi ($p<0.001$) due, predominantly, to the neutrophilia. Similarly, the percentage of lymphocytes also dropped on day 14 pi to 75% ($p<0.001$), due to the eosinophilia (Figure 3-2).

III. Eosinophils

The total number of eosinophils in the peripheral blood increased significantly following N.b. infection rising from 80 x 10^3 in naïve mice to 1.17 x 10^6 by day 14 pi ($p<0.001$). Eosinophil numbers declined thereafter to 600 x 10^3 by day 19 pi ($p<0.01$). By day 26 pi eosinophil numbers had returned to normal levels (Figure 3-3).

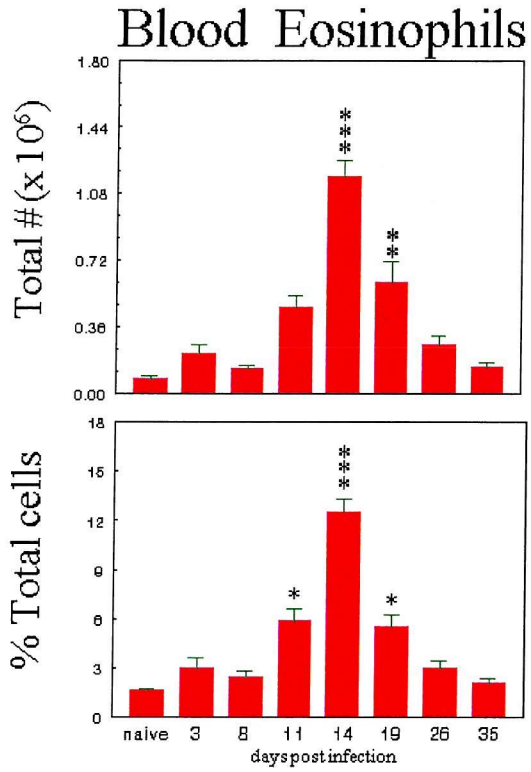

Figure 3-3. The total number and percentage of eosinophils in the blood of wild type mice following N.b. infection.

This peripheral eosinophilia was reflected in a significant increase in the percentage of eosinophils circulating. Thus the percentage of eosinophils increased significantly from <2% in naïve animals to 5.9% on day 11 pi ($p<0.05$), peaking at 12.5% on day 14 pi ($p<0.001$). Eosinophils levels remained high on day 19, at 5.5% ($p<0.05$) before subsiding to baseline levels by day 26 pi (Figure 3-3).

IV. Neutrophils

The total number of neutrophils in the peripheral blood increased from 0.59 x 10^6 in naïve mice to 0.89 x 10^6 on day 8 pi ($p<0.001$). The numbers dipped somewhat on day 11 pi but increased significantly again on day 14 pi to 0.78 x 10^6 ($p<0.05$). Thereafter numbers were not significantly different from naïve mice (Figure 3-4).

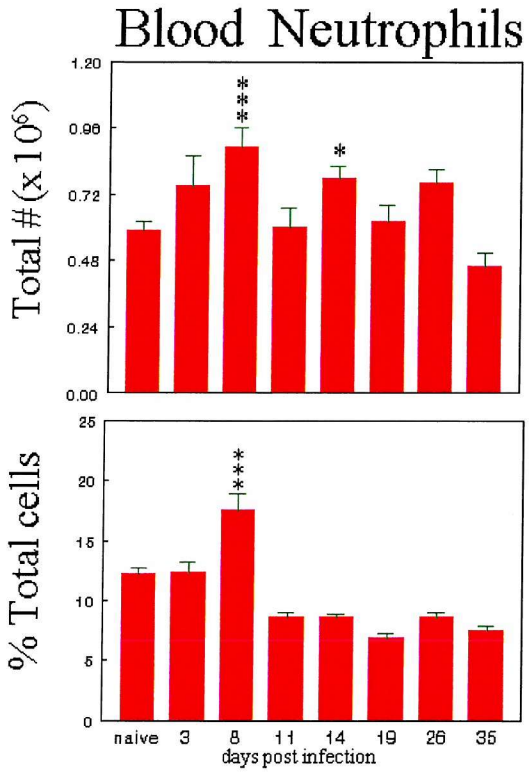

Figure 3-4. The total number and percentage of neutrophils in the blood of wild type mice following N.b. infection.

The peripheral neutrophilia was also reflected in a significant increase in the percentage of neutrophils in the blood. The percentage of neutrophils rose from 12.2% in naïve animals to a peak of 17.6% on day 8 pi ($p<0.001$), and subsided thereafter.

3.3.2 Leukocyte infiltration into the BAL fluid

I. Total leukocyte count

There was a significant cellular recruitment of leukocytes to the BAL fluid following N.b. infection (Figure 3-1). Thus leukocyte numbers doubled during the early phase response during larval migration through the lungs, rising from 0.11 x 10^6 in naïve mice to 0.21 x 10^6 on day 3 pi ($p<0.001$). However, there was an even more significant increase in leukocyte recruitment to the BAL fluid during the late phase response with leukocyte numbers increasing 17-fold (Figure 3-1). Thus leukocyte numbers peaked at 1.88 x 10^6 on day 19 pi ($p<0.001$), before subsiding on day 26 pi to 0.33 x 10^6 in ($p<0.001$). By day 35 pi leukocyte numbers had still not yet returned to normal ($p<0.05$)(Table 3-2).

Cell subset	Naïve	Day 3	Day 8	Day 11	Day 14	Day 19	Day 26	Day 35
Total WCC x 10^3	114 ± 9.1	212 ± 23.5 *(p<0.00*	877 ± 102.8 *(p<0.001)*	1235 ± 188 *(p<0.001)*	1485 ± 106.3 *(p<0.001)*	1879 ± 172 *(p<0.001)*	332 ± 25.2 *(p<0.001)*	250 ± 44 *(p<0.05)*
Neutrophils x 10^3	1.79 ± 0.76	76.8±20.5 *(p<0.00*	33.5 ± 4.10 *(p<0.05)*	30.0 ± 5.45	19.1 ± 2.99	1.0 ± 1.04	2.2 ± 0.85	0.3 ± 0.19
Lymphocytes x 10^3	12.8 ± 3.6	10.3 ± 1.6	159.1 ± 18.1 *(p<0.001)*	214.7 ± 34.8 *(p<0.001)*	241.6 ± 16.9 *(p<0.001)*	267.2 ± 25.5 *(p<0.001)*	46.7 ± 7.3	13.6 ± 4.3
Monocytes x 10^3	94.5 ± 4.7	122.1 ± 7.4	328 ± 31 *(p<0.001)*	164 ± 21	200 ± 11	286 ± 46 *(p<0.001)*	163 ± 9	229 ± 46 *(p<0.001)*
Eosinophils x 10^3	5.60 ± 3.5	3.02 ± 0.89	356.5 ± 63.4 *(P<0.05)*	826.7±14 7 *(p<0.001)*	1173 ± 94.2 *(p<0.001)*	1326 ± 418 *(p<0.001)*	24.2 ± 8.69	7.16 ± 2.14

Table 3-2. BAL fluid cellular profile at various time points after N.b. infection. Data are the means ± SEM from 20 to 30 mice/time point, from two to five experiments. Statistically significant differences compared to naïve or day 3 mice, as determined by a two-tailed t test assuming unequal variance, are indicated by * ($p<0.05$), ** ($p< 0.01$), and *** ($p< 0.001$).

II. Neutrophils

An examination of the leukocyte subset in the BAL fluid revealed a forty three fold increase in the total number of neutrophils, rising from 1.8 x 10^3 in naïve mice to 76.8 x 10^3 on day 3 pi ($p<0.001$). By day 8 pi neutrophil number had subsided, but were still significantly greater than in naïve animals at 33.5 x 10^3 ($p<0.05$). Thereafter neutrophil numbers returned to normal (Table 3-2). This BAL neutrophilia was reflected in a significant increase in the percentage of neutrophils, rising from < 1% in naïve animals to 26.9% three days pi ($p<0.001$) (Figure 3-5).

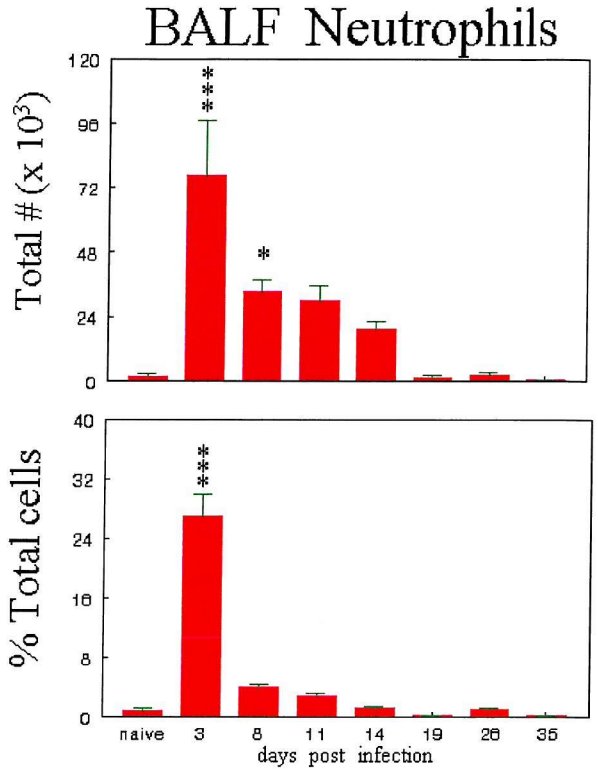

Figure 3-5. The total number and percentage of neutrophils in the BAL fluid of wild type mice following N.b. infection.

III. Eosinophils

The significant increase in cellular influx during the late phase response was largely due to a greater than two hundred-fold increase in eosinophil numbers when compared to naïve animals (Table 3-2). Thus eosinophil number rose from 5.6 x 10^3 in naïve animals to 356 x 10^3 by day 8 pi ($p<0.05$) and up to 826 x 10^3, 1173 x 10^3 and 1326 x 10^3 by days 11, 14 and 19 respectively ($p<0.001$). This significant recruitment of eosinophils to the airways of infected mice was reflected in an increase in the percentage of eosinophils recovered from the BAL fluid. Thus from a background level of 2% in naïve mice the percentage of eosinophils in the BAL fluid rose to 33% by day 8pi ($p<0.001$), and by days 11, 14 and 19 eosinophils accounted for 60%, 68% and 70% of BAL fluid cells recovered ($p<0.001$)(Figure 3-6).

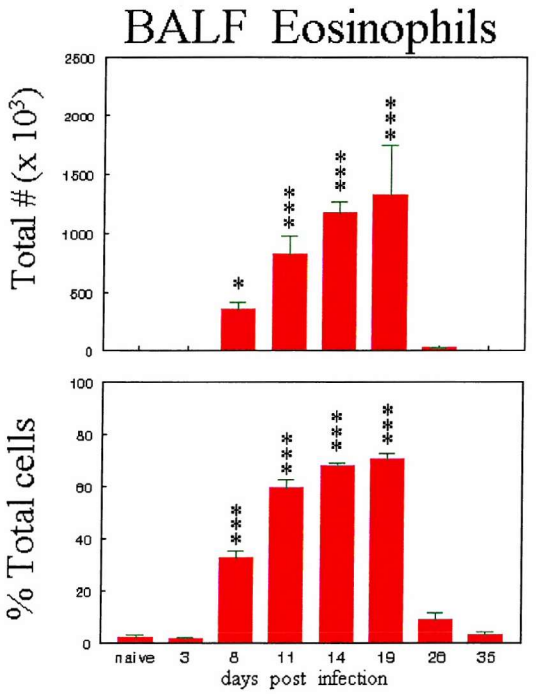

Figure 3-6. The total number and percentage of eosinophils in the BAL fluid of wild type mice following N.b. infection.

IV. Lymphocytes

The kinetics of lymphocyte migration into the lungs paralleled the eosinophil response with a 21-fold increase in numbers during the late response (Figure 3-7).

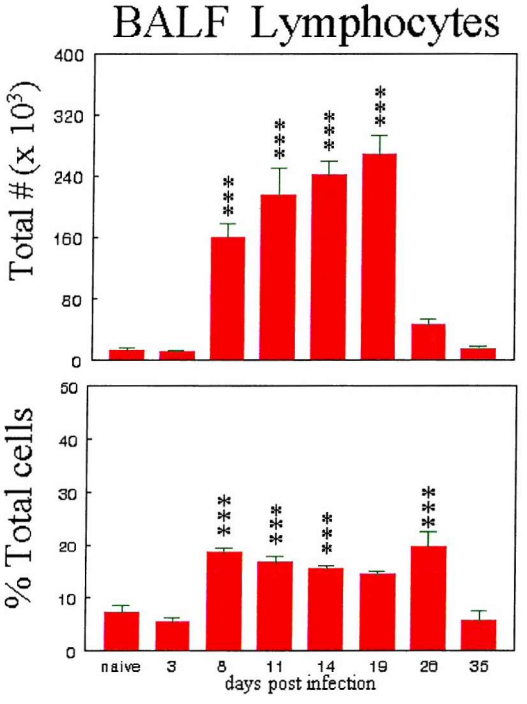

Figure 3-7. The total number and percentage of lymphocytes in the BAL fluid of wild type mice following N.b. infection.

Specifically lymphocyte numbers rose from 12.8 x 10^3 in naïve animals to 159 x 10^3 by day 8pi ($p<0.001$). Lymphocyte numbers increased further to 214 x 10^3 241 x 10^3 and 267 x 10^3 by days 11, 14 and 19 respectively ($p<0.001$)(Table 3-2). These changes were reflected in changes in the percentage of lymphocytes recovered from the BAL fluid. The percentage of lymphocytes in the BAL fluid rose from 7.2% in naïve mice to 18.6% by day 8 pi ($p<0.001$). This percentage remained high throughout the late response, varying between 14.4% and 19.6% ($p<0.001$) before subsiding back to 5.6% by day 35 pi (Figure 3-7).

V. Mononuclear cells

Overall there was a significant increase in the number of mononuclear cells in the BAL fluid during the late response, and this was most marked on days 8, 19 and 35 ($p<0.001$)(Table 3-2). However, this represented a significant drop when expressed as a percentage of total leukocytes from 90% in naïve animals to 19% on day 14 pi and 15% on day 19 pi ($p<0.001$)(Figure 3-8).

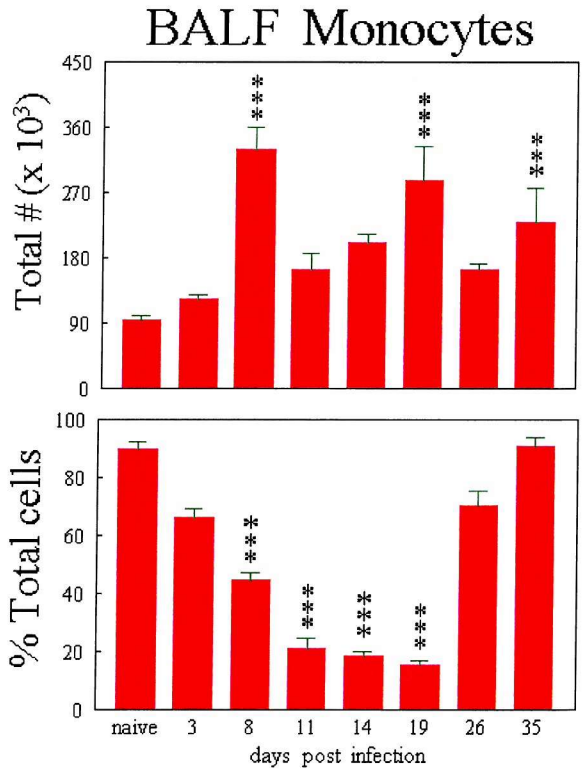

Figure 3-8. The total number and percentage of monocytes in the BAL fluid of wild type mice following N.b. infection.

3.3.3 Immunofluorescence staining and flow cytometry

The gates for lung and BAL fluid lymphocytes were well defined throughout. In contrast, the absence of a specific eosinophil surface marker made identification of an eosinophil gate difficult, particularly in the lung tissue and BAL fluid of naïve animals during the early phase response, 3 days pi (Figures 3-9A and 3-9B).

Consequently the gated eosinophil population may have been contaminated by other granulocytes, particularly in the BAL fluid, 3 days pi. In contrast, there was a clearly defined lung tissue and BAL fluid eosinophil gate 14 days pi (Figures 3-9C and 3-9D).

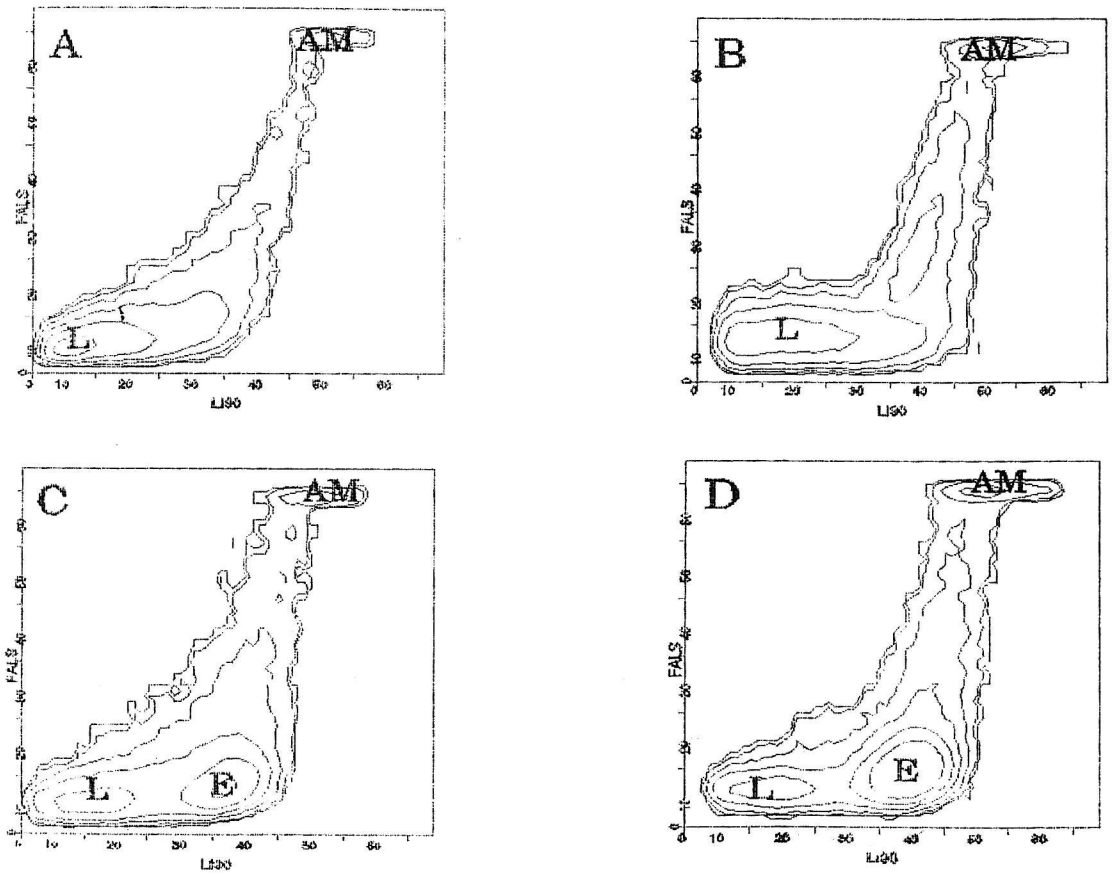

Figure 3-9. Forward angle light scatter (FALS) and 90 degree light scatter (Li 90) in lung tissue (panels A and C) and BAL fluid (panels B and D) in WT mice 3 days after N.b. infection (panels A & B) and 14 days pi.

3.3.4 Phenotypic analysis of lymphocytes in lung tissue and airway lumen

I. Lymphocyte subsets

- **Thy 1^+ and $B220^+$**

The number of $Thy1^+$ lymphocytes, a marker for T cells, in the BAL fluid and lung tissue increased significantly during the late phase response following N.b. infection. Specifically the number of T cells increased from 15.9 x 10^3 in naïve mice to 85.1 x 10^3 by day 8 pi ($p<0.05$) and increased still further to 123.8 x 10^3 by day 14 pi ($p<0.01$). By day 26 pi the number of T cells in the BAL fluid of infected mice was still substantially higher than naïve mice, although this was not statistically significant (Figure 3-10).

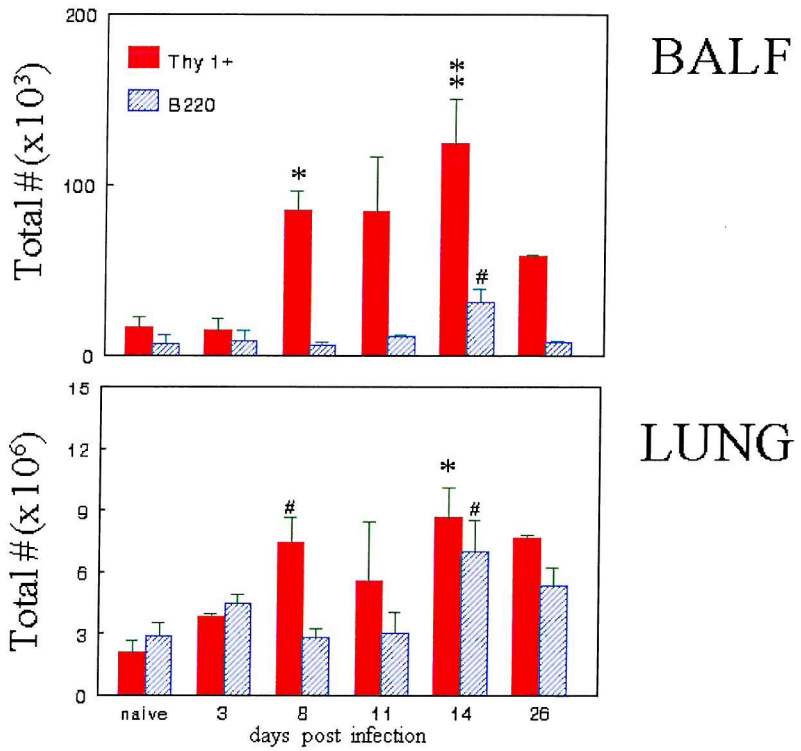

Figure 3-10. The total number of BAL fluid and lung tissue cells of wild type mice expressing the Thy1+ and B220 marker following N.b. infection.

In contrast, the number of lymphocytes expressing B220, the B cell marker, did not change significantly following N.b. infection. Although B cell numbers increased from 6.5 x 10^3 in naïve mice to 30.7 x 10^3 by day 14 pi this did not quite reach statistical significance ($p= 0.052$).

These changes in T and B cell recruitment to the BAL fluid following N.b. infection were reflected in changes in the number of T and B cells in the lung tissue (Figure 3-10). Thus the number of $Thy1^+$ lymphocytes in the lung tissue increased from 2.1 x 10^6 in naïve mice to 7.4 x 10^6 by day 8 pi ($p=0.05$), and increased still further to 8.7 x 10^6 on day 14 pi ($p<0.05$). In keeping with the changes in the BAL fluid the number of T cell in the lungs 26 days pi, 7.6 x 10^6, had still not returned to normal. The increase in the number of cells expressing B220 in the lungs increased substantially following N.b. infection from 2.9 x 10^6 in naïve mice to 7.0 x 10^6 on day 14 pi, but again this did not quite reach statistical significance ($p=0.05$).

- **T:B ratio**

Examination of the T:B cell ratio in the BAL fluid suggested a relative increase in the number of B cells during the early and late phase response. Thus the number of B cells recruited to the BAL fluid reduced the T:B cell ratio from 2.4:1 in naïve animals to 1.8:1 on day 3 pi. By day 8 pi the number of T cells in the BAL fluid had increased significantly increasing the T:B ratio to 14.1:1. However, by day 14 pi the number of B cells recruited to the BAL fluid had again reduced the T:B ratio to 4:1. Therefore relatively speaking there appeared to be a biphasic influx of B cells into the BAL fluid during both the early and late phase response.

In contrast, the T:B cell ratio in the lung showed a different pattern. Thus the T:B ratio increased steadily from 0.7:1 in naïve mice to 2.6:1 on day 8 pi before declining steadily to 1.4:1 on day 26 pi. This suggests an influx of T cells into the lung on day 8 pi, prior to the peak lymphocyte response in the BAL fluid. Roughly equal numbers of T and B cells were recruited to the lung during the late phase response, but in the BAL fluid there was a significantly greater number of T cells during the late response compared to B cells ($p<0.01$). This finding suggests a preferential migration of T cells into the BAL fluid.

II. T cell subsets

- **$CD4^+$ and $CD8^+$ T cells**

Phenotypic analysis of the lung tissue and BAL fluid lymphocytes revealed that the significant increase in lymphocyte numbers in the BAL fluid during the late response was predominantly due to an influx of $CD4^+$ T cells ($Thy1^+CD3^+$) lymphocytes. Thus the number of $CD4^+$ lymphocytes increased from 6.3 x 10^3 in naïve WT mice to 53.9 x 10^3 by day 8 pi ($p<0.05$),

increasing still further to 56.8 x 10^3 by day 11 pi ($p<0.05$). The recruitment of $CD4^+$ lymphocytes to the BAL fluid peaked on day 14 pi at 80.9 x 10^3 ($p<0.001$) before declining to 31.7 x 10^3 by day 26 pi. In contrast the number of $CD8^+$ lymphocytes in the BAL fluid remained largely unchanged following N.b. infection (Figure 3-11).

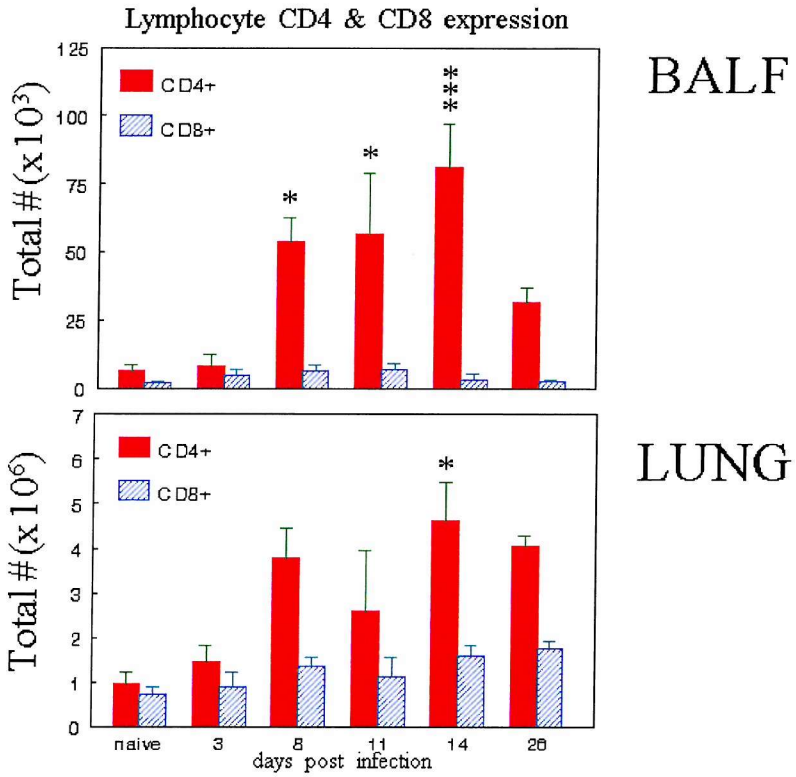

Figure 3-11. The total number of lymphocytes in the BAL fluid and lung tissue of wild type mice expressing the CD4 and CD8 phenotype following N.b. infection.

This change in $CD4^+$ lymphocyte numbers was reflected in a significant increase in the percentage of $Thy1^+CD3^+$ cells in the BAL fluid expressing CD4. Thus the percentage of $CD4^+$ lymphocytes increased from 50%, in naïve animals, to 73% on day 14 pi ($p<0.02$).

This significant increase in the number of $CD4^+$ lymphocytes in the BAL fluid was reflected in the lungs with a significant increase in $CD4^+$ lymphocytes in the lungs during the late phase response (Figure 3-11). Thus the number of $CD4^+$ T cells increased from 0.9 x 10^6 in naïve WT mice to 4.6 x 10^6 on day 14 pi ($p<0.05$). Again the number of $CD8^+$ T cells in the lung tissue did not change significantly following N.b. infection. The significant increase in the number of $CD4^+$ T cells in the lung tissue was reflected in a significant increase in the percentage of $Thy1^+CD3^+$ cells expressing CD4 in the lung tissue from 42%, during the early response, to 58% on day 14 pi ($p< 0.05$). In contrast to the changes in the percentage of $CD4^+$

T cells in the lung and BAL fluid there was no change in the percentage of $CD4^+$ cells in the peripheral blood (data not shown).

- **CD4:CD8 ratio**

The changes in recruitment of $CD4^+$ and $CD8^+$ cells were reflected in a change in the CD4:CD8 ratio in the BAL fluid and lung tissue. The CD4:CD8 ratio in the BAL fluid which decreased from 3.3:1 in naïve animals to 1.7:1 during larval migration through the lungs before increasing to 24.5:1 during the late phase response ($p< 0.05$). This suggests that there was a relative increase in $CD8^+$ T cells during the early phase response with the late phase response being dominated by $CD4^+$ T cells. In contrast, the change in the CD4:CD8 ratio in the lung during the late response was less dramatic, rising from 1.3:1 in naïve animals to 2.9:1 by day 14 pi, with no change during the early phase response.

- **$CD4^-CD8^-$**

The percentage of double negative T cells (expressing neither CD4 nor CD8) in the BAL fluid decreased from a background level of 34% in naïve animals to 15% during the early and late phase responses. No such trend in double negative T cells was apparent in the lung tissue.

- **α/β TCR, γ/δ TCR**

The number of T cells expressing γ/δ TCR in the BAL fluid following N.b. infection increased significantly during the late phase response, although due to the small number of FACS samples pooled from several animals across two experiments this only reached statistical significance on day 26 pi (Figure 3-12).

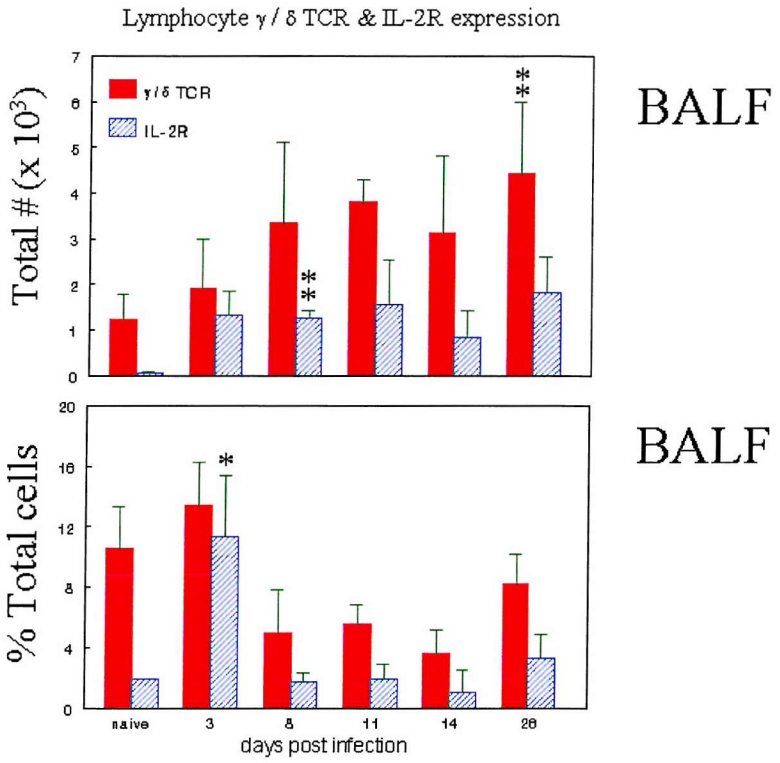

Figure 3-12. The total number and percentage of lymphocytes in the BAL fluid of wild type mice expressing γ/δ TCR and IL-2R following N.b. infection.

Thus numbers increased from 1.2 x 10^3 in naïve mice to 3.3 x10^3 on day 14 pi ($p<0.06$) and 4.4 x10^3 by day 26 pi ($p<0.01$).

The percentage of T cells expressing γ/δ TCR on day 3 pi was not significantly different to naïve mice due to the high level of γ/δ TCR expression in uninfected mice (Table 3-3).

Surface Marker	Naïve	Day 3	Day 8	Day 11	Day 14	Day 26
γδ-TCR	1,241 ± 544 (10.6)	1,921 ± 1,058 (13.4)	3,338 ± 1,786 (5)	3,797 ± 501 (5.6)	3,130 ± 1,697 (3.6)	4,433 ± 1568** (8.2)
CD62L (L-sel)	619 ± 257 (5.1)	5,191 ± 2,387 (38.1)**	4,768 ± 2,421 (7)	5,045 ± 2,671 (6.4)	2,677 ± 1,049 (4.1)	3,351 ± 1,179 (6.2)
CD25 (IL-2R)	57 ± 38 (1.9)	1,328 ± 514 (11.4)*	1,280 ± 159** (1.7)	1,561 ± 979 (1.9)	836 ± 410 (1.2)	1,803 ± 786 (3.3)
CD45R B†	317 ± 132 (7.1)	2,654 ± 1,238 (37.7)**	4,749 ± 3,356 (11.3)	1,529 ± 450 (14.1)	7,587 ± 2,758 (11.4)	2,580 ± 1,972 (7.3)
CD44† (Pgp-1)	5,161 ± 2,008 (69.2)	5,217 ± 3,557 (41.3)	34,333 ± 11,631 (72.5)	41,247 ± 9,198 (78.1)	44,782 ± 9,262 (82.8)	27,754 (75)
CD43	10,598 ± 4,058 (76.8)	9,727 ± 4,954 (72.8)	65,173 ± 10,639** (80.7)	59,629 ± 21,439 (80.9)	80,157 ± 20,868* (82.8)	31,711 ±5,295 (74.8)

Table 3-3. Phenotypic analysis of BAL fluid $Thy1^+CD3^+$ lymphocytes at various time points after N.b. infection († indicates $Thy1^+CD3^+CD4^+$ cells). Data are the means ± SEM from 20 to 30 mice/time point, from two to five experiments. Percentages are shown in brackets. Statistically significant differences compared to naïve or day 3 mice, as determined by a two-tailed t test assuming unequal variance, are indicated by * ($p<0.05$), ** ($p< 0.01$), and *** ($p< 0.001$). The number of cells analysed per experiment depends on the time point studied: 12-15 x 10^3 in naïve animals and mice 3 days pi, and 58-123 x 10^3 during the late response.

However, in comparison to the late phase response the percentage of T cells expressing γ/δ TCR during the early phase response was significantly greater ($p<0.02$). Thus 10.6% of naïve T cells recovered from the BAL fluid expressed γ/δ TCR compared to 13.4% on day 3 pi and 3.6% on day 14 pi (Figure 3-12). Similarly, there was a slight increase in the percentage of T cells expressing γ/δ TCR in the peripheral blood during larval migration, 2.4% in naïve animals to 5.8% 3 days pi (data not shown).

In keeping with the increased expression of γ/δ TCR on T cells recovered from the BAL fluid there was a significant increase in γ/δ TCR expression on T cells recovered from the lung tissue. Thus the number of T cells expressing this surface marker increased from 116 x 10^3 in naïve mice to 753 x 10^3 on day 8 pi, 508 x 10^3 on day 14, and 676 x 10^3 on day 26 pi ($p<0.01$). In keeping with this increase in the total number of T cells expressing γ/δ TCR in the lung tissue during the late phase response there was a significant increase in the percentage of T cells expressing this marker. Thus expression increased from 5.4% in naïve mice to 10% by day 8 pi ($p<0.001$), and remained high through to day 26 at 9.7% ($p<0.05$)(Table 3-4). This was in contrast to the changes seen in the percentage of BAL fluid cells expressing this marker, which appeared to increase during the early phase response and decline significantly during the late phase response.

In keeping with the significant increase in T cell expression of γ/δ TCR in the lungs and BAL fluid of WT mice following N.b. infection, the expression of α/β TCR on BAL fluid T cells also increased significantly during the late phase response. Thus the number of $Thy1^+CD3^+$ cells in the BAL fluid expressing α/β TCR increased from 8.7 x 10^3 in naïve mice to 55.7 x 10^3 on day 8 pi ($p<0.01$). Expression then remained high through the late phase response at 80.9 x 10^3 on day 14 pi ($p<0.05$), before declining somewhat to 34.1 x 10^3 on day 26 pi ($p<0.01$). This was reflected in a small but non-significant increase in the percentage of T cells expressing α/β TCR in the BAL fluid, rising from 66% in naïve mice to 70-75% during the late phase response (data not shown).

In contrast, the total number of lung T cells expressing α/β TCR during the late phase response increased but this did not reach statistical significance, rising from 1.6 x 10^6 in naïve mice to 5.9 x 10^6 on day 14 pi. Similarly the percentage of lung T cells expressing α/β TCR

did not really change following N.b. infection remaining around 75% throughout. No changes in α/β TCR expression were apparent in the blood.

III. Activation and memory markers

- **IL-2R (CD25)**

The percentage of BAL fluid cells expressing the T cell activation marker IL-2R peaked during larval migration, rising from a background level in naïve animals of < 1% to 11.4% on day 3 pi, and returning to background levels during the late response ($p<0.04$). The twenty-three-fold increase in the number of cells expressing IL-2R during the early response was masked by substantial influx of lymphocytes during the late phase. Thus, although the percentage of T cells expressing IL-2R did not exceed 3.5% during the late response the total number of cells expressing this activation marker increased significantly. Thus the number of IL-2R$^+$ T cells increased from 0.06 x 10^3 in naïve mice to 1.3 x 10^3 on day 8 pi ($p<0.01$), and remained at roughly that level throughout the late phase response (Figure 3-12).

In contrast to the upregulation of IL-2R on BAL fluid T cells during the early phase response there was little change in the percentage of lung T cells expressing this activation marker following N.b. infection, with expression remaining below 2.5% (Table 3-4). Consequently there was no change in the number of T cells expressing this marker in the lung tissue following N.b. infection.

- **L-selectin (CD62L)**

The percentage of T cells expressing L-Selectin recovered from the BAL fluid increased significantly during the early phase response from 5.1% in naïve mice to 38.1% on day 3 pi ($p<0.001$). By day 8 pi this level had dropped back to baseline levels (Figure 3-13). This was reflected by a substantial increase in the number of T cells expressing L-Selectin increasing from 0.6 x 10^3 in naïve mice to 5.2 x 10^3 on day 3 pi. However, due to the small number of samples across three experiments this increase did not reach statistical significance. The total number of T cells expressing L-Selectin remained high during the late phase response (Table 3 3).

The significant increase in the percentage of T cells in the BAL fluid expressing L-Selectin during the early phase response was not mirrored in the lung tissue. There was a small

increase from 33% in naïve mice to 34.4% on day 3 pi, with a subsequent reduction during the late phase response to 7-20% but this increase was not statistically significant (Figure 3-13).

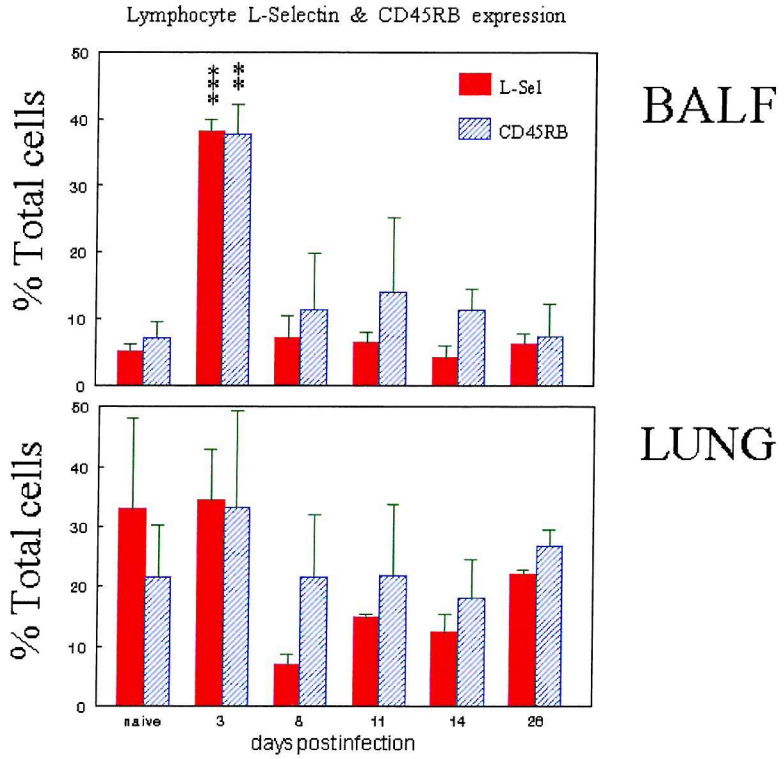

Figure 3-13. The percentage of lymphocytes in the BAL fluid and lung tissue of wild type mice expressing L-Selectin and CD45RB following N.b. infection.

The changes in the percentage of T cell expressing L-Selectin in the lung tissue were reflected in the changes in the number of T cells expressing L-Selectin. Thus there was a nearly two-fold increase in the number of T cells expressing L-Selectin in the lung tissue from 0.64 x 10^6 in naïve mice to 1.2 x 10^6 on day 3 pi, but this did not reach statistical significance. By the late phase response L-Selectin expression in the lung had returned to baseline levels of 0.7 x 10^6 by day 11 pi (Table 3-4).

Surface Marker	Naïve	Day 3	Day 8	Day 11	Day 14	Day 26
	x 10^{-6}/lung					
γδ-TCR	0.116 ± 0.019 (5.4)	0.203 ± 0.032 (6)	0.754 ± 0.325** (10)**	0.331 ± 0.184 (6.7)	0.509 ± 0.181** (6)	0.676 ± 0.034** (9.7)*
CD62L (L-sel)	0.635 ± 0.115 (33)	1.176 ± 0.357 (34.4)	0.513 ± 0.184 (7)	0.7 ± 0.336 (15)	1.04 ± 0.321 (12.5)	1.547 ± 0.052 (22.1)
CD25 (IL-2R)	0.000 ± 0.00 (0)	0.024 ± 0.024 (0.8)	0.167 ± 0.11 (2.1)	0.025 ± 0.025 (0.4)	0.067 ± 0.035 (0.7)	0.112 ± 0.001 (1.6)
CD45RB †	0.284 ± 0.182 (21.5)	0.417 ± 0.105 (33.2)	0.908 ± 0.495 (21.6)	0.73 ± 0.609 (21.8)	0.876 ± 0.334 (18)	1.081 ± 0.047 (26.8)
CD44† (Pgp-1)	0.506 ± 0.00 (62.9)	0.829 ± 0.422 (53.5)	2.048 ± 0.089 (63.5)	1.64 ± 0.657 (68.6)	2.559 ± 0.09 (69.5)	2.332 (60.8)
CD43	1.616 ± 0.308	2.705 ± 0.302	5.338 ± 1.112	4.12 ± 2.186 (85)	6.226* ± 1.18	5.434 ± 0.173

Table 3-4. Phenotypic analysis of lung tissue $Thy1^+CD3^+$ lymphocytes at various time points after N.b. infection († indicates $Thy1^+CD3^+CD4^+$ cells). Data are the means ± SEM from 20 to 30 mice/time point, from two to three experiments. Percentages are shown in brackets. Statistically significant differences compared to naïve or day 3 mice, as determined by a two-tailed t test assuming unequal variance, are indicated by * ($p<0.05$), ** ($p< 0.01$), and *** ($p< 0.001$).

- **CD45RB**

The percentage of BAL fluid T cells expressing CD45RB also increased significantly during larval migration through the lungs, rising from 7.1% in naïve animals to 38% 3 days pi ($p< 0.01$). By the late phase response the percentage of T cells expressing CD45RB had returned to baseline levels (Figure 3-13). Similarly, the percentage of T cell in the lung tissue expressing CD45RB increased during the early phase response rising from 21.5% in naïve mice to 34.4% on day 3 pi. However, this was not statistically significant. The percentage of lung tissue T cells expressing CD45RB during the late response dropped to baseline levels (Figure 3-13).

These changes in the percentage of BAL fluid and lung tissue T cells expressing CD45RB were not reflected in the total number of BAL fluid and lung tissue T cells expressing CD45RB. The total number of T cells expressing CD45RB generally tended to increase following N.b. infection in both BAL fluid and lung tissue (Tables 3-3 & 3-4).

- **CD43**

The percentage of T cells expressing the activation marker CD43 remained relatively constant in the BAL fluid and lung tissue following N.b. infection, generally around 75-80%. In contrast, the number of BAL fluid and lung tissue T cells expressing CD43 increased significantly during the late phase response (Tables 3-3 & 3-14). Thus the total number of BAL fluid T cells expressing CD43 increased from 10.6×10^3 in naïve mice to 65.1×10^3 on day 8 pi ($p<0.02$) and to 80.2×10^3 on day 14 pi ($p<0.05$) before declining day 26 pi (Table 3-3). Similarly, the total number of lung tissue T cells expressing CD43 increased during the late phase response, but these changes did not quite reach statistical significance. Thus numbers increased from 1.6×10^6 in naïve mice to 5.3×10^6 on day 8 pi ($p<0.08$) and to 6.2×10^6 on day 14 pi ($p<0.06$) before declining day 26 pi (Table 3-4).

- **CD44 (Pgp-1)**

In contrast to CD43 the percentage of T cells in the BAL fluid expressing CD44, the hyaluronate receptor Pgp-1, dropped substantially during the early phase response on day 3 pi (Figure 3-14).

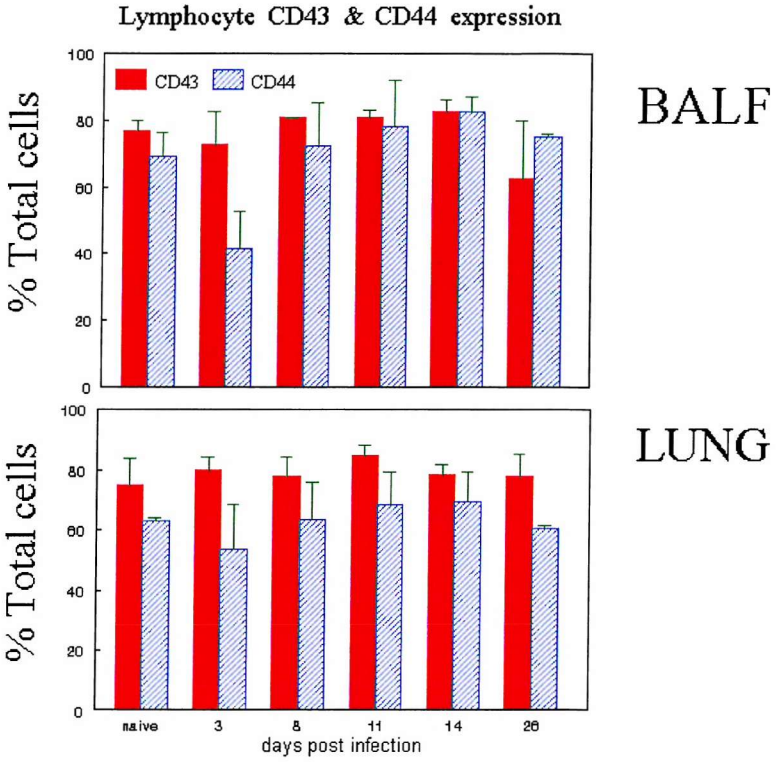

Figure 3-14. The percentage of lymphocytes in the BAL fluid and lung tissue of wild type mice expressing CD43 and CD44 (PgP-1) following N.b. infection.

Thus the percentage of T cells in the BAL fluid expressing CD44 decreased from 69.2% in naïve mice to 41.3% on day 3 pi before returning to normal levels during the late phase response (Table 3-3). However, these changes did not reach statistical significance due to the small number of samples from pooled data across five experiments. This reduction in the percentage of T cells in the BAL fluid expressing CD44 was mirrored by a reduction in the percentage of T cells from the lung tissue expressing CD44 during the early phase response. Thus the percentage of T cells from the lung tissue expressing CD44 dropped from 62.9% in naïve mice to 53.5% during larval migration through the lungs (Table 3-4). By the late phase response the percentage of T cells expressing CD44 in the lung tissue had returned to normal (Figure 3-14).

In keeping with the significant increase in the number of T cells expressing CD43 in the BAL fluid during the late phase response there was an nearly significant increase in the number of BAL fluid T cells expressing CD44. Thus the number of T cells expressing CD43 in the BAL fluid increased from 5.2 $x10^3$ in naïve mice to 44.8 $x10^3$ on day 14 pi ($p<0.06$). By day 26 pi the number of T cells expressing CD43 had not yet returned to normal (Table 3-3). The

number of T cells expressing CD43 in the lung tissue also increased substantially during the late phase response from 0.5 x10^6 in naïve mice to 2.6 x10^6 on day 14 pi. But this increase did not reach statistical significance owing to small sample sizes.

- **CD69**

The percentage of T cells in the BAL fluid expressing CD69 decreased gradually throughout the infection. CD69 was virtually undetectable on disaggregated lung tissue with expressed levels remaining below 2%. This low level of CD69 expression in the BAL fluid and lung tissue resulted in low numbers of T cells expressing this surface marker. In contrast to the changes in the expression of some of the above activation and memory surface markers there were no such in activation or memory markers on peripheral blood T cells (data not shown).

IV. Adhesion Markers

- **ICAM-1, VLA-4 and LFA-1**

The total number of T cells in the BAL fluid expressing ICAM-1 increased significantly during the late pulmonary response from 5.3 x 10^3 in naïve mice to 37.7 x 10^3 on day 8 pi ($p<0.03$). The number of ICAM-1^+ T cells remained high through day 14 pi at 33.9 x 10^3 ($p<0.02$) before subsiding on day 26 pi to 22.6 x 10^3 ($p<0.05$). These changes were not reflected in any changes in the percentage of T cells expressing ICAM-1 (Figure 3-15).

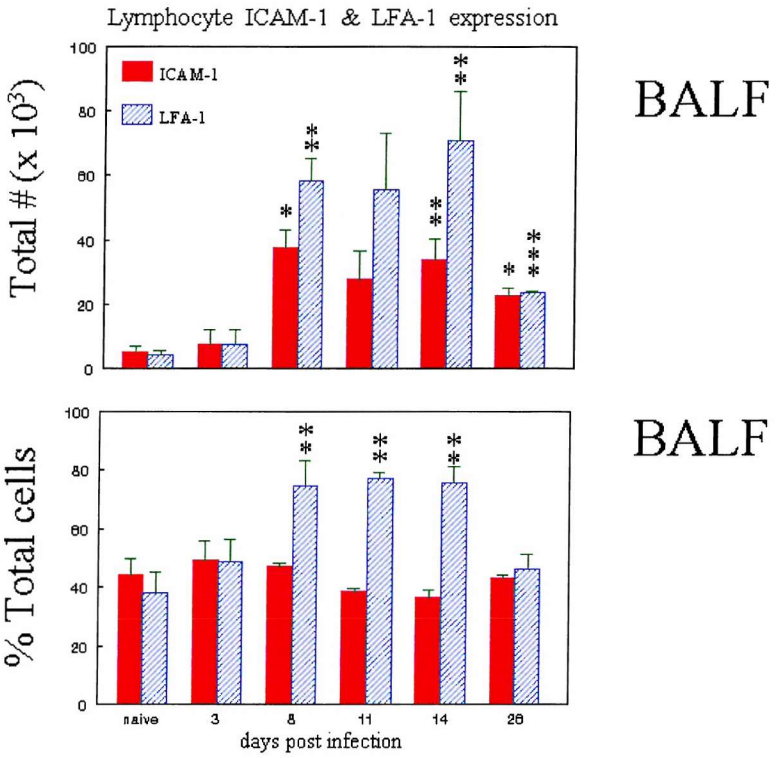

Figure 3-15. The total number and percentage of lymphocytes in the BAL fluid of wild type mice expressing ICAM-1 and LFA-1 following N.b. infection.

The changes in the number of BAL fluid T cells expressing ICAM-1 were mirrored by a near five-fold increase in the number of lung tissue T cells expressing ICAM-1 increasing from 0.6 x 10^6 in naïve mice to 2.97 x 10^6 on day 14 pi (Table 3-5). However, this increase was not statistically significant due to the small number of samples involved. In contrast to the lack of change in the percentage of BAL fluid T cells expressing ICAM-1 there was a non-significant increase in the percentage of lung tissue T cells expressing ICAM-1 rising from 26.3% in naïve mice to 40.3% during the late phase response (Table3-5). But again these changes were not statistically significant.

Surface Marker	Naïve	Day 3	**BAL fluid** Day 8	Day 11	Day 14	Day 26
CD54 (ICAM-1)	5,254 ± 1,695 (44.1)	7,727 ± 4,342 (49.3)	37,671 ± 5,249* (47)	27,934 ± 8,693 (38.6)	33,940 ± 6,442** (36.5)	22,636 ± 2,309* (43.2)
CD49d (VLA-4)	6,814 ± 2,650 (47.9)	5,759 ± 3,166 (37.7)	28,227 ± 1,113*** (36.4)	32,943 ± 8,177 (46.6)	42,063 ± 9,901* (43.4)	24,693 ± 1,547*** (47.7)
CD11a (LFA-1)	4,260 ± 1,247 (38.0)	7,517 ± 4,453 (48.5)	58,599 ± 6,726** (74.3)**	55,600 ± 17,361 (76.8)**	70,843 ± 15,150** (75.3)**	23,947 ± 292*** (46.2)
	Lung Tissue x 10^{-6}					
CD54 (ICAM-1)	0.622 ± 0.287 (26.3)	1.082 ± 0.076 (32.4)	2.956 ± 1.046 (40.3)	1.617 ± 1.064 (30.5)	2.966 ± 1.235 (33.9)	2.600 ± 0.500 (36.6)
CD49d (VLA-4)	0.538 ± 0.211 (23.3)	0.918 ± 0.080 (27.2)	2.108 ± 0.566 (29.7)	1.476 ± 0.819 (30.0)	2.706 ± 0.796 (32.6)	2.400± 0.200* (33.6)
CD11a (LFA-1)	1.451 ± 0.230 (67.9)	2.215 ± 0.457 (65.2)	5.528 ± 0.945* (81.5)	3.813 ± 1.792 (82.0)	6.159 ± 1.176 (77.9)	5.100 ±0.100*** (72.6)

Table 3-5. Adhesion molecule expression on BAL fluid and lung tissue lymphocytes at various time points after N.b. infection. Data are the means ± SEM from 20 to 30 mice/time point, from two to five experiments. Percentages are shown in brackets. Statistically significant differences compared to naïve or day 3 mice, as determined by a two-tailed t test assuming unequal variance, are indicated by * ($p<0.05$), ** ($p< 0.01$), and *** ($p< 0.001$).

In line with the changes in ICAM-1 we examined the expression of VLA-4 on BAL fluid T cells. Thus the number of BAL fluid T cells expressing VLA-4 increased significantly during the late pulmonary response from 6.8 x 10^3 in naïve mice to 28.2 x 10^3 on day 8 pi ($p<0.001$). Numbers peaked on day 14 pi at 42.1 x 10^3 ($p<0.04$), before declining on day 26 pi to 24.7 x 10^3 ($p<0.002$). These changes were not reflected in changes in the percentage of BAL fluid T cells expressing VLA-4 (Table3-5). Similarly, the number of lung tissue T cells expressing VLA-4 increased during the late phase response rising from 0.5 x 10^6 in naïve mice to 2.7 x 10^6 on day 14 pi and 2.4 x 10^6 on day 26 pi ($p<0.03$). These changes were reflected in a small but non-significant increase in the percentage of lung tissue T cells expressing VLA-4 from 23.3% in naïve mice to 32.6% on day 14 pi (Table 3-5).

In addition to the significant increase in the expression of the above two adhesion molecules on the surface of BAL fluid lymphocytes we also examined the expression of LFA-1. Both the percentage and the total number of T cells in the BAL fluid expressing LFA-1 increased significantly during the late phase response. Thus the total number of LFA-1^+ lymphocytes increased from 4.3 x 10^3 in naïve mice to 58.6 x 10^3 on day 8 pi ($p<0.01$), and to 70.8 x 10^3 on day 14 pi ($p<0.01$). By day 26 pi the number of T lymphocytes expressing LFA-1 had decreased to 23.9 x 10^3 but this was still significantly greater than in naïve mice ($p<0.001$). This significant increase in LFA-1 expression on BAL fluid T cells during the late phase response was matched by a significant increase in the percentage of T cells expressing this adhesion marker (Figure 3-15).

Thus LFA-1 expression increased from 38% in naïve mice to 74.3% on day 8 pi ($p<0.01$). This level of expression was sustained through the late phase response at 76.8% on day 11 ($p<0.01$), and 75.3 on day 14 pi ($p<0.01$), before declining on day 26 pi to background levels at 46.2%.

The expression of LFA-1 on lung tissue T cells also increased significantly during the late phase response (Table 3-5). Thus numbers increased from 1.5 x 10^6 in naïve mice to 5.5 x 10^6 on day 8 pi ($p<0.05$), and 6.2 x 10^6 on day 14 pi ($p<0.06$). By day 26 pi LFA-1 expression was still significantly greater than nice mice, 5.1 x 10^6 ($p<0.001$). In contrast, the percentage of LFA-1^+ lymphocytes during the late response did not quite reach significance rising from 68% in naïve animals to 78% on day 14 pi (Table 3-5). The percent of peripheral blood T cells expressing all three adhesion molecules was unchanged following N.b. infection.

3.3.5 Phenotypic analysis of eosinophils in lung tissue and airway lumen

I. Activation and memory markers

- **CD45RB**

There was a significant increase in the total number of eosinophils in the BAL fluid expressing CD45RB during the late phase pulmonary response (Figure 3-16).

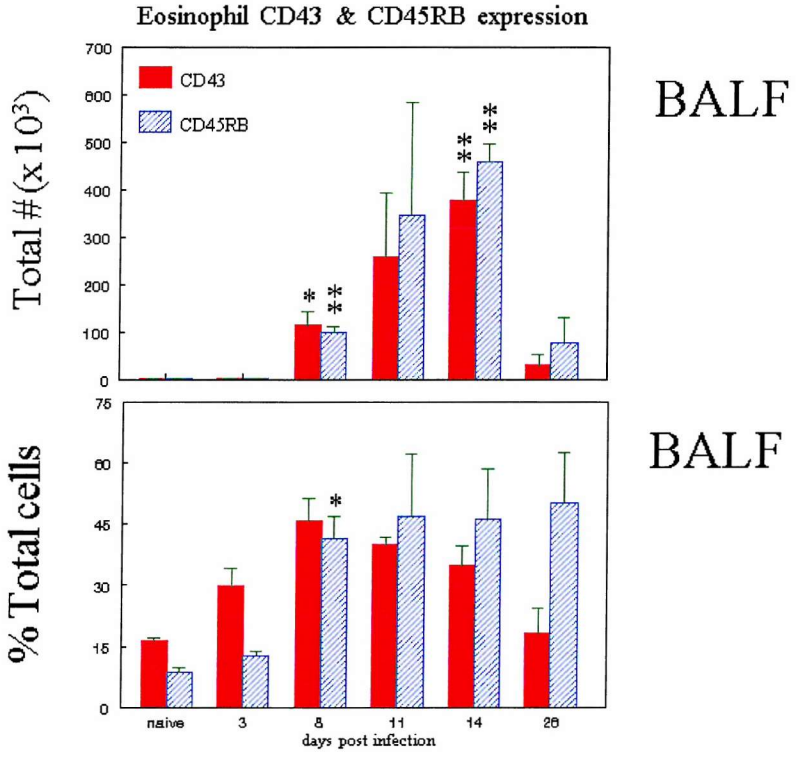

Figure 3-16. The total number and percentage of eosinophils in the BAL fluid of wild type mice expressing CD43 and CD45RB following N.b. infection.

Thus the number of $CD45RB^{+}$ eosinophil increased from 0.41 x 10^3 in naïve mice to 99 x 10^3 on day 8 pi ($p<0.02$). Numbers increased further on day 14 pi to 458.6 x 10^3 ($p<0.006$) before declining on day 26 pi to 75.5 x 10^3 (Table 3-6).

Surface Marker	Naïve	Day 3	BAL fluid Day 8	Day 11	Day 14	Day 26
CD43	770 ± (16.2)	1,849 ± 1,329 (29.9)	114,430 ± 27,072* (45.9)	259,856 ± 134,016 (39.8)	376,829 ± 58,831** (35.0)	28,497 ± 21,999 (18.1)
CD44 (PgP-1)	0 ± (0)	2,176 ± 1,040 (41.9)	198,817 ± 37,598 (80.6)	542,677 ± 248,828 (86)	657,558 ± 13,965** (82.1)	177,152 ± (84.9)
CD45RB	413 ± (8.7)	743 ± 508 (12.7)	98,983 ± 12,512** (41.3)*	344,926 ± 238,833 (46.9)	458,569 ± 38,413*** (46.2)	75,491 ± 55,130 (50.1)
	Lung Tissue x 10^{-6}					
CD43	0.596 ± 0.103 (34.4)	2.663 ± 1.549 (30.4)	7.825 ± 1.380* (43.0)	9.544 ± 6.997 (51.1)	12.399 ± 3.701 (39.0)	4.085 ± 1.481 (31.1)
CD44 (PgP-1)	1.347 ± (65.8)	6.349 ± 1.143 (70.9)	10.674 ± 0.105 (77.9)	14.432 ± 9.661 (83.4)	17.731 ± 0.380 (82.8)	11.391± (78.8)
CD45RB	0.136 ± 0.015 (8.0)	0.720 ± 0.463 (8.6)	4.609 ± 1.370 (25.2)	9.770 ± 9.166 (38.8)	11.567 ± 3.812 (36.4)	4.503 ± 3.130 (32.7)

Table 3-6. Activation marker expression on BAL fluid and lung tissue eosinophils at various time points after N.b. infection. Data are the means ± SEM from 20 to 30 mice/time point, from two to five experiments. Percentages are shown in brackets. Statistically significant differences compared to naïve or day 3 mice, as determined by a two-tailed t test assuming unequal variance, are indicated by * ($p<0.05$), ** ($p< 0.01$), and *** ($p< 0.001$)

This increase in the number of $CD45RB^+$ eosinophils was reflected in a more than five-fold increase in the percentage of eosinophils expressing this marker during the late phase response. Thus the percentage of $CD45RB^+$ eosinophils increased from 8.7% in naïve mice to 41.3% on day 8 pi ($p<0.04$), and 46.3% on day 14 pi. Levels remained high throughout the late phase response and by day 26 pi 50.1% of eosinophils were still $CD45RB^+$ (Figure 3-16).

Similarly there was a substantial increase in the percentage and total number of eosinophils in the lung tissue expressing CD45RB. Thus numbers increased 82-fold from 0.14 x 10^6 in naïve mice to 11.6 x 10^6 on day 14 pi. However, due to the small numbers of samples involved across three experiments this did not reach statistical significance (Table3-6). The percentage of lung tissue eosinophils expressing CD45RB also increased substantially from 8% in naïve mice to 38.8% during the late phase response. But again this increase did not reach statistical significance.

- **CD43**

The total number of eosinophils in the BAL fluid expressing CD43 also increased significantly following N.b. infection (Figure 3-16). The numbers rose from 0.7 x 10^3 in naïve mice to 168.3 x 10^3 on day 8 pi ($p<0.05$), and peaked on day 14 pi at 319.8 x 10^3 ($p<0.02$), before subsiding to 6.5 x 10^3 on day 26 pi. This increase in the number of eosinophils expressing CD43 was reflected in a substantial increase in the percentage of BAL fluid eosinophils expressing CD43. Thus expression increased from 16.2% in naïve animals to 45.9% on day 8 pi ($p<0.08$), falling back slightly by day 14 pi to 35% before declining by day 26 pi to 18.1% (Table 3-6).

A significant increase in the expression of CD43 on eosinophils was also seen in the lung tissue. Thus the total number of eosinophils expressing CD43 increased from 0.6 x 10^6 in naïve animals to 7.8 x 10^6 on day 8 pi ($p<0.03$), and 12.4 x 10^6 on day 14 pi ($p<0.08$), before waning by day 26 pi to 4.1 x 10^6 (Table 3-6). These changes were reflected in a small but non-significant increase in the percentage of lung tissue eosinophils expressing CD43 from 34.4% in naïve mice to 51.1% during the late phase response.

- **CD44 (Pgp-1)**

The expression of CD44 (Pgp-1) was undetectable in the BAL fluid of naïve mice. However, the total number of eosinophils in the BAL fluid expressing CD44 increased significantly during the late phase response compared to the early response (Table 3-6). Thus the number of eosinophils expressing CD44 increased from 2.2 x 10^3 on day 3 pi to 657.6 x 10^3 on day 14 pi ($p<0.01$). This nearly 300-fold increase in the number of eosinophils expressing CD44 was reflected in a doubling of the percentage of eosinophils expressing this marker from 41.9% on day 3 pi to 86% during the late phase response (Table 3-6).

Similarly there was a substantial increase in the number of lung tissue eosinophils expressing CD44. Thus the number of lung tissue eosinophils expressing CD44 increased from 1.3 x 10^6 in naïve mice to 17.7 x106 on day 14 pi ($p<0.08$). There was also a small but non-significant rise in the percentage of eosinophils in the lung tissue expressing CD44 from 65.8% in naïve mice to 82.8% on day 14 pi (Table 3-6).

II. Adhesion Markers

- **ICAM-1**

The total number of BAL fluid eosinophils expressing ICAM-1 increased substantially during the late pulmonary response (Table 3-7). Thus numbers increased from 1.3 x 10^3 mouse in naïve mice to 142 x 10^3 on day 8 pi ($p<0.06$). The number of eosinophils expressing ICAM peaked on day 14 pi at 761 x 10^3 but due to the small sample sizes and sample variance these changes did not reach statistical significance.

The percentage of BAL fluid eosinophils expressing ICAM-1 doubled from 27.2% in naïve mice to 63.9% during the late phase response, but this changes was not statistically significant. Similarly, although the number of eosinophils in the lung tissue expressing ICAM-1 increased from 0.6 x 10^6 in naïve mice to 17.7 x 10^6 during the late phase response this was statistically significant due to the small number of samples. The percentage of lung tissue eosinophils expressing ICAM-1 also showed a non-significant trend, increasing from 36% in naïve animals to 53% 14 days pi (Table 3-7).

Surface Marker	Naïve	Day 3	**BAL fluid**	Day 11	Day 14	Day 26
CD54 (ICAM-1)	1,292 ± (27.2)	3,055 ± 2,736 (30.1)	142,221 ± 34,985 (56.7)	358,935 ± 128,003 (60.4)	760,586 ± 258,385 (63.9)	74,881 ± 45,099 (56.3)
CD49d (VLA-4)	1,207 ± (25.4)	1,076 ± 760 (17.6)	131,807 ± 33,269 (52.5)*	334,080 ± 140,635 (54.2)	726,525 ± 237,241 (61.5)***	68,242 ± 39,218 (52.6)***
CD11a (LFA-1)	1,482 ± (31.2)	2,320 ± 1,550 (43.1)	191,603 ± 39,777* (77.8)	558,365 ± 249,121 (89.2)	1,011,668 ± 337,486 (85.4)	106,585 ± 70,359 (75.9)
	Lung Tissue x 10^{-6}					
CD54 (ICAM-1)	0.6 ± 0.1 (35.6)	2.035 ± 0.776 (33.3)	9.406 ± 3.086 (47.9)	8.762 ± 5.722 (51.6)	17.724 ± 6.796 (53.1)	5.856 ± 1.560 (45.2)
CD49d (VLA-4)	0.5 ± 0.1 (29.7)	1.398 ± 0.520 (20.8)	8.096 ± 2.150 (42.5)**	9.703 ± 6.810 (54.0)	18.540 ± 6.661 (56.4)***	5.292± 1.820 (40.4)
CD11a (LFA-1)	1.0 ± 0.30 (54.8)	4.045 ± 2.023 (50.4)	13.521 ± 3.585 (71.0)	13.962 ± 9.765 (77.9)	24.739 ± 7.794 (77.3)	7.232 ± 3.002 (54.7)

Table 3-7. Adhesion molecule expression on BAL fluid and lung tissue eosinophils at various time points after N.b. infection. Data are the means ± SEM from 20 to 30 mice/time point, from two to five experiments. Percentages are shown in brackets. Statistically significant differences compared to naïve or day 3 mice, as determined by a two-tailed t test assuming unequal variance, are indicated by * ($p<0.05$), ** ($p< 0.01$), and *** ($p< 0.001$).

- **VLA-4**

The total number and percent of eosinophils expressing VLA-4 in the BAL fluid increased during the late response (Figure 3-17).

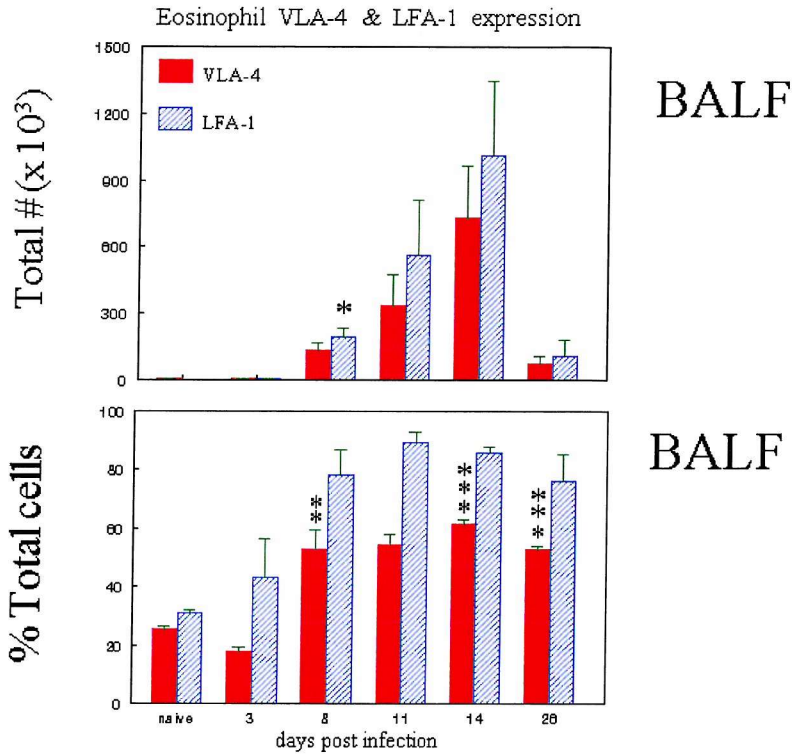

Figure 3-17. The total number and percentage of eosinophils in the BAL fluid of wild type mice expressing VLA-4 and LFA-1 following N.b. infection.

Thus the number of BAL fluid eosinophils expressing VLA-4 increased from 1.2 x 10^3 in naïve mice to 131.8 x 10^3 on day 8 pi ($p<0.06$), and peaked at 726.5 x 10^3 on day 14 pi before declining on day 26 pi. When expressed as a percentage of the total number of eosinophils recovered from the BAL fluid VLA-4 expression increased significantly during the late phase response compared to the early phase response. Thus the percentage of VLA-4^+ eosinophils increased from 25.4% in naïve mice, dropped during the early response to 17.6% before increasing significantly by day 8 pi to 52.5% ($p<0.04$). Percentage expression remained high throughout the late phase response with levels reaching 54.2% on day 11 pi ($p<0.07$), 61.5% on day 14 pi ($p<0.001$) and 52.6% on day 26 pi ($p<0.001$)(Figure 3-17).

This significant increase in the percentage of eosinophils in the BAL fluid expressing VLA-4 was mirrored by changes in the percentage of lung tissue eosinophils expressing VLA-4. Thus only 29.7% of eosinophils expressed VLA-4 in naïve mice and this dropped to 20.8%

during larval migration through the lungs. However, by the late phase this percentage had increased to 42.5% ($p<0.01$) and peaked on day 14 pi at 56.4% ($p<0.001$). Again due to the small number of samples pooled from three experiments the nearly forty-fold increase in eosinophil numbers expressing VLA-4 during the late phase response did not quite reach statistical significance.

- **LFA-1**

The total number of BAL fluid eosinophils expressing LFA-1 increased significantly during the late response (Figure 3-17). Thus the number of eosinophils expressing LFA-1 increased from 1.4×10^3 in naïve mice to 191.6×10^3 on day 8 pi ($p<0.04$). Numbers increased still further by day 14 pi to $1,011 \times 10^3$ but due to the large variation in expression in the samples this increase was not statistically significant. By day 26 pi number of eosinophils expressing LFA-1 in the BAL fluid had dropped substantially to 106.5×10^3 (Figure 3-17). These changes were reflected in a substantial increase in the percentage of eosinophils recovered from the BAL fluid that expressed LFA-1. Thus percentage expression increased from 31.2% in naïve mice to 77.8% on day 8 pi ($p<0.07$) and peaked at 89.2% on day 11 pi but still remained high on day 26 pi at 75.9% (Table 3 7).

A similar but non-significant trend in the total number and percentage of lung tissue eosinophils expressing LFA-1 was seen which increased from 55% (1.0×10^6/mouse) in naïve animals to 77% (24.7×10^6/mouse) 14 days pi ($p<0.07$).

3.3.6 Eosinophil eicosanoid production.

Percoll density gradient centrifugation of BAL fluid cells yielded a major eosinophil-enriched fraction at 1.080 g/ml and a minor fraction at 1.090 g/ml. A small number (5%) of lightweight eosinophils sedimented at 1.050 and 1.060 g/ml but these fractions were heavily contaminated with mononuclear cells. Morphological examination of the major eosinophil fractions, under light microscopy, showed the eosinophils to be small, compact and densely staining.
The eosinophil-enriched fractions obtained from the Percoll density gradient at 1.080 g/ml and 1.090 g/ml were activated with calcium ionophore and the arachidonic acid metabolites produced were qualitatively identified by reversed phase HPLC. By comparison with the retention times of eicosanoid standards (as determined with ultraviolet and radiometric detectors), the eosinophil enriched population generated significant peaks consistent with the

production of LTC_4, LTB_4, TxB_2 and PGE_2. Several additional peaks were produced in the regions of monohydroxyl and dihydroxyleicosanotetraenoic acids, but these were not analyzed further. Non-radiolabelled fractions eluted at times consistent with standard retention times for these four metabolites were then quantitatively evaluated using specific EIAs. The results revealed that murine BAL fluid eosinophils obtained, 14 days pi, were capable of producing 3.66 ± 1.14 ng of LTC_4, 4.35 ± 0.61 ng TxB_4, 1.33 ± 0.28 ng of LTB_4, and 0.42 ± 0.14 ng PGE_2 /10^6 cells (Table 3-8).

Fraction Density g/ml	Eosinophils (x 10^6/ml)	LTC_4	LTB_4	TxB_2	PGE_2
			ng/10^6 eosinophils		
1.080 (expt 1)	15	5.76	1.88	5.54	0.31
1.090	3.2	1.86	1.07	3.98	0.69
1.080 (expt 2)	3.6	3.35	1.03	3.53	0.25

Table 3-8. Eicosanoids production by BAL fluid eosinophils 14 days pi. Data are derived from two experiments containing 20 animals each.

3.4 DISCUSSION

These results demonstrate that infection of C57BL/6 mice with N.b. produces a distinctive early and late phase pulmonary response. The early response was characterised by a small but significant recruitment of neutrophils to the BAL fluid and a small increase in the percentage

of BAL fluid T lymphocytes of the $CD8^+$ phenotype. There was also an increased percentage of BAL fluid lymphocytes expressing naïve T cell markers (L-sel and CD45RB) and markers of T cell activation (IL-2R). In contrast, the late response was characterised by a substantial influx of eosinophils and activated $CD4^+$ T lymphocytes. During this late phase response there was also a significant increase in the percentage and total number of T lymphocytes and eosinophils expressing the adhesion molecules ICAM-1, VLA-4, and LFA-1. Further analysis of the eosinophils recruited to the airways suggested that they were immature, hypodense and highly activated. Functional analysis of these cells showed them to be capable of producing a range of eicosanoids that have been widely implicated in the pathophysiology of asthma.

Numerous reports have suggested that the Th-2 sub-population of $CD4^+$ lymphocytes is central to the pathogenesis of allergic inflammation in mice and humans (Coffman et al 1989; Finkelman et al 1988; Parronchi et al 1992). There are two commonly employed strategies for inducing a Th-2-driven model of allergic inflammation of the airways. The most popular is nebulisation of ovalbumin (OA) to the airways of OA-sensitised animals (Hook et al 2000). This approach tends to produce a short-term airway inflammation. Some authors have attempted to overcome this using a chronic exposure to OA (Foster et al 2000), but this may simply induce tolerance to the antigen (Yiamouyiannis CA et al 1997). An alternative strategy for provoking a Th-2-driven model is infection with the gut nematode Nippostrongylus brasiliensis – N.b. (Watkins AD et al 1996).

The assortment of antigens encountered during a N.b. infection are some of the most powerful inducers of the Th-2 response known (Coffman et al 1989), and investigation of parasite infections in rodents has been used to shed considerable light on the pathogenesis of allergic inflammation (Moqbel 1990). For example, the eosinophilia, mastocytosis, and increased serum IgE levels characteristic of allergic inflammation are also typical of helminth infections, and these changes have been shown to be T cell-dependent, since they do not occur in athymic, or T cell-depleted animals (Katona et al 1988). Specifically, the intestinal mastocytosis is IL-3 and IL-4-dependent (Madden 1991), the increase in serum IgE levels is IL-4-dependent (Daly CM et al 1999, Finkelman 1988; Kopf 1993), and the peripheral and pulmonary eosinophilia in addition to the inflammatory changes are IL-5 and IL-10-dependent but IL-3 and IL-4-independent (Makela MJ et al 2000, Foster et al 2000). So the *in vivo* differentiation of a Th-2 population from naïve T cells, appear to be critically dependent on

two factors namely an endogenous source of IL-4 (Gollob et al 1994) and the nature and duration of antigen stimulation (Saito et al 1994).

Employing N.b. rather than OA antigens offer another avenue for exploring the allergic inflammatory process. In fact, several investigators have utilized N.b. to develop a helminth-based model of antigen-induced airway inflammation in rats (Ramaswamy et al 1993a & 1993b; Ramaswamy et al 1991 Egwang et al 1984). In contrast, this chapter has described the pulmonary and peripheral blood responses to primary N.b. infection in mice.

In the rat model the peripheral leukocyte response to N.b. varies between different strains of rats, and is subject to wide daily and individual variation, especially if strict pathogen-free environments are not maintained (Ogilvie et al 1978). Some authors have reported a biphasic increase in all leukocyte subsets, excluding basophils following N.b. infection in rats (Ogilvie et al 1978), while others have found a biphasic increase with respect to eosinophils only, with neutrophil, lymphocyte, and mononuclear counts remaining largely unchanged (Roth et al 1980).

In contrast, the murine results presented here demonstrated a single peak in the total number of leukocytes, neutrophils, eosinophils, and lymphocytes in the blood commencing on day 8 and subsiding by day 35 pi. Although asthma is not associated with a marked lymphocytosis or eosinophilia, recent evidence has suggested that the peripheral blood eosinophil and lymphocyte counts may be correlated with airflow obstruction (Virchow et al 1994).

The analysis of BAL fluid is frequently used to investigate cellular trafficking in human and rodent lung, and the cells recovered are believed to reflect the underlying cellular profile of the lung tissue (Reynolds et al 1989; Curtis et al 1989). Previous reports in the rat, following primary infection with N.b. have demonstrated an increase in the total number of leukocytes, eosinophils, lymphocytes, macrophages, and neutrophils in the BAL fluid during the early and the late responses. If animals were then re-infected the biphasic nature of the cellular rises were even more marked, particularly for neutrophils and macrophages (Egwang 1984).

The response, seen here in the BAL fluid, to primary N.b. infection in mice was also biphasic. However, in contrast to the BAL changes in rats the early response in the BAL fluid in mice

was characterised by a small significant increase in the total number and percentage of neutrophils only, with no changes in eosinophil and lymphocyte numbers. In addition, a much larger increase in total number and percentage of eosinophils and lymphocytes in the BAL fluid dominated the late response. The magnitude of the changes seen, if not the cellular composition, was in keeping with those previously reported in the rat (Ramaswamy et al 1993a). The reason for these differences in the pattern of cellular recruitment to the BAL fluid between mice and rats are not clear.

Thus the early phase of the pulmonary response to N.b. infection was characterised by a significant increase in the number and percentage of neutrophils recruited to the BAL fluid. The role of the neutrophil in asthmatic airway inflammation has received little attention. However, a significant airway neutrophilia has been reported 6h after local allergen bronchoprovocation (Montefort et al 1994). In addition, an airway neutrophilia has been implicated in early (6 h) compared to late (24-48 h) asthmatic death following an acute asthmatic attack (Sur et al 1993). The functional significance of the early influx of neutrophils into the airways seen here is unclear. However, it has been suggested that the production of eosinophil chemotactic factors by neutrophils promote resolution of low level N.b. infections (Katy et al 1976; Czarnetzki et al 1978). In contrast, larger larval burdens may require a more sophisticated cellular response with the production of IL-4 by mast cells and lymphocytes in addition to an eosinophil recruitment in order to induce a sufficiently vigorous immune reaction to promote worm expulsion (Gounni et al 1994).

The early phase response was also characterised by a relative change in the B cell population with a reduction in the T:B cell ratio during larval migration through the lungs. The importance of the relative increase in the number of B cells during the early response is unclear, however they may form part of the immediate immune defence against N.b. infection. In fact recent evidence suggests that locally secreted IgE may be directly involved in airway inflammation by capturing antigen and forming antigen-specific immune complexes (Zuberi R et al 2000). In contrast, the relative recruitment of B cells during the late phase response may be associated with the rise in serum IgE. Contrary to previous reports, suggesting that C57BL/6 mice may be low IgE responders, we found that the total serum IgE in C57BL/6 mice was comparable to that previously reported in BALB/c mice and rats (Finkelman F et al 1994, Jarrett E et al 1977). Thus IgE levels reached a mean of 76 μg/ml on day 14 pi. During

the late response more T than B cells were recruited to the BAL fluid, while equal numbers of T and B cells were recruited to the lung. This preferential T cell recruitment may reflect the differing roles of T and B cells in response to N.b infection.

Flow cytometric analysis of BAL fluid and lung tissue lymphocytes and eosinophils revealed that changes in the percentage of cells expressing specific surface markers did not always reflect changes in the total number of cells expressing these markers. For example there was an increase in the percent, but not the total number of $CD8^+$ cells in the BAL fluid during larval migration. Whether $CD8^+$ T cells play a role in the early response to antigen is not clear but the significant reduction in the CD4:CD8 ratio suggests a role for this cell, although the inflammatory response to N.b. antigens may be independent of CD8 cells (Uchikawa R et al 1997).

The relative increase in $CD8^+$ T cells was succeeded by a substantial rise in the number and percentage of $CD4^+$ cells recruited to the BAL fluid and lung tissue. These findings differ from the cellular changes previously reported in the rat, where N.b. infection produced a sustained influx of $CD8^+$ cells and a much later recruitment of $CD4^+$ cells (Egwang et al 1984). The changes reported here are more in keeping with the changes seen in asthmatics following local antigen challenge (Montefort et al 1994, Gerblich et al 1991), and therefore the mouse may represent a more useful model of allergic inflammation than the rat.

So the early phase response was characterised predominantly by a neutrophilia in the BAL fluid with a relative increase in B cells and $CD8^+$ T cells. In addition there were specific changes in T cell surface marker expression. Thus in contrast to the α/β TCR expression, which remained unchanged, the percentage of BAL fluid T cell expressing γ/δ TCR during the early response was significantly greater than during the late phase response. The exact immunological role of γ/δ T cells is far from clear. However, it has been suggested that γ/δ T cells may be a first line of defence against allergens and antigens (Hiromatsu et al 1992). These cells are able to respond to a broad range of pathogens at sites of high antigen exposure (Janeway et al 1988), as a result of their less constrained, less MHC-restricted antigen recognition mechanism (Schild 1994). It has also been suggested that γ/δ T cells may be particularly important in initiating a Th-2 response by producing IL-4 (Ferrick et al

1995), and encouraging B cell isotype switching to IgE and IgG1 production (Wen et al 1994).

Recent evidence suggests that α/β TCR in addition to γ/δ TCR expression may be required for the induction of airway inflammation. Specifically data, from an OA-induced murine model of allergic inflammation, suggested that α/β TCR and γ/δ TCR expression may be required to provoke peribronchial inflammatory cell infiltration, eosinophilia, raised serum IgE levels, and increased in vivo airway responsiveness to methacholine. Furthermore, γ/δ TCR expression was specifically implicated in airway sensitization to antigen (Schramm CM et al 2000).

A significant proportion of these early responding T cells in the airways during larval migration may have been naïve. Naïve T cells express CD45RB and L-selectin, whereas the expression of Pgp-1 (CD44), CD43 and the loss of L-selectin are associated with cellular activation and the memory phenotype. In contrast to previous data in the rat (Egwang et al 1984), results in the murine model presented here suggest there was an increase in the percentage of naïve T cells in the BAL fluid during the early response. Support for this assertion is provided by the data indicating a reduction of CD44 expression in the lung and BAL fluid during the early phase response in addition to an increase in L-Selectin and CD45RB expression on T cells. It is tempting to speculate that this naïve population of $CD4^+$ cells may be Th-0 cells which develop into a more committed Th-2 population in response to N.b. antigens (Street et al 1990).

Not only were a significant proportion of these early responding T cells naïve but a substantial proportion of T cells recruited to the BAL fluid, during the early response, were activated, as indicated by the increase in the percent of cells expressing IL-2R and CD69. There is evidence for the selective recruitment of activated T cells to the airways in atopic asthma with increased expression of HLA-DR, IL-2R, and VLA-4 (Wilson et al 1992).

In contrast to the naïve cells recruited during the early response the cells recruited to the BAL fluid during the late response were predominantly of the memory phenotype, as indicated by the increased number of cells expressing CD43 and CD44. During the late response there was also a significant increase in the total number of T cells in the BAL fluid and lung tissue

expressing the β1 integrin VLA-4, and ICAM-1 and the β-2 integrin, LFA-1. These data support previous work implicating these vascular adhesion molecules in the recruitment of T cells to the airways (Kennedy et al 1995).

Blood and tissue eosinophilia are characteristic of helminth infections, and eosinophils are major effector cells of airway mucosal inflammation. Eosinophils, and their pro-inflammatory mediators, are found in increased numbers in the peripheral blood, sputum, bronchial biopsies and BAL fluid of patients with asthma (Beasley et al 1989). They are particularly prominent in the post-mortem airways in asthma deaths (Dunnill et al 1960 &1969). In this murine model up to 80% of all the leukocytes in the BAL fluid, during the late response, were eosinophils, strongly implicating them as effector cells of the severe pulmonary damage seen. Furthermore, the results presented here indicate that these eosinophils were functionally very active and capable of producing physiologically relevant amounts of the chemoattractant LTB_4 and the smooth muscle spasmogens LTC_4 and TxB_2.

Recruitment of eosinophils to the airways in allergic asthma involves the expression, by the endothelium, of a series of adhesion molecules including VCAM-1 (the endothelial ligand for VLA-4), and co-expression of the counter ligand on the surface of eosinophils, particularly ICAM-1 and LFA-3 (Mengelers et al 1994; Bentley et al 1993). Human eosinophils either constitutively express, or can be stimulated to express, LFA-1, L-selectin, HLA-DR, CD4, CD25 and CD69 (Hartnell et al 1993). A previous study was the first to report on the phenotypic characteristics and cell surface markers of murine eosinophils in inflammatory states and this thesis expands on that data (Watkins et al 1996b).

Specifically, very few of the eosinophils expressed CD69, CD62L (L-selectin), or CD25, and < 5% of eosinophils expressed CD4. In contrast, following N.b. infection, there was an increase in the number and percentage of eosinophils expressing CD43, CD44, and CD45RB both in lung tissue and the BAL fluid. The significance of these surface markers are not yet clear, however, previous reports suggest that activation markers, normally expressed by T lymphocytes, may also indicate eosinophil activation (Hartnell et al 1993). Based on the evidence presented here the data suggests that the eosinophils recruited to the airways during the late response were immature and activated. Further assessment of these eosinophils showed them to be small and hypodense, analogous to eosinophils in allergic asthma. The

substantial increase in the total number and percentage of lung tissue and BAL fluid eosinophils expressing VLA-4, ICAM-1, and LFA-1 seen here suggests a role for these molecules in the observed eosinophil influx. Similarly, VLA-4 and LFA-3 have also been implicated in eosinophil recruitment to human airways.

In conclusion, there are a number of similarities between asthmatic allergic inflammation and the pulmonary response to N.b. in mice. Asthmatic inflammation of the airways is characterised by an early and late phase response to allergen. The late phase response, is dominated by an influx of activated $CD4^+$ T cells and eosinophils, and usually commences approximately six hours after allergen exposure. However, it can be sustained and cause prolonged inflammatory changes and airway obstruction.

Similarly the pulmonary response to N.b. infection is also characterised by two distinct phases, albeit on a different time scale. There is an early pulmonary neutrophilia coincident with an increase in the proportion of naïve, activated T cells, and γδ T cells capable of synthesising IL-4 and promoting a Th-2 response (Ferrick et al 1995). This is followed by a later eosinophilic response involving the recruitment of a substantial number of activated $CD4^+$ cells. In line with previous reports using a model of ovalbumin-induced pulmonary inflammation (Kennedy et al 1995), these $CD4^+$ cells expressed increased levels of ICAM-1, VLA-4 and LFA-1. This lends support to the idea that these adhesion molecules may be important in T cell recruitment. The late recruitment of lymphocytes to the airways was accompanied by a large influx of hypodense eosinophils, coincident with significant pulmonary damage. Phenotypic analysis of these cells suggests that they may be immature and activated, and capable of producing spasmogens and pro-inflammatory chemoattractants. This airway lymphocytosis and eosinophilia was associated with a peripheral blood eosinophilia and raised serum IgE levels. Although there was no airway epithelial damage evident in this murine model, there are sufficient similarities in the phenotype and pattern of lymphocyte and eosinophil recruitment to make primary infection of C57BL/6 mice with N.b. a useful alternative to OA-induced animal models of human allergic inflammation.

This thesis proceeds to probe this murine model of allergic inflammation in greater detail in order to establish the crucial variables that determine the extent of the airway inflammation and attempts to optimise the usefulness of primary N.b. infection as a model for chronic inflammation of the airways in humans.

CHAPTER 4

L3 Dose Response Curve

4.1 INTRODUCTION

The dose of antigen is assumed to be one of the most important factors in determining the polarisation of T helper cell subsets into Th-1 or Th-2 populations. As previously discussed the nature of the stimulating antigen is also critical. Two of the most commonly employed antigens used to induce a Th-2 response are ovalbumin (OA)(Foster 2000) and infection with the gastrointestinal nematode Nippostrongylus brasiliensis – N.b. (Watkins AD et al 1996b).

A recent study using the OA-driven model of atopic asthma demonstrated that antigen dose was indeed critical. Thus, the authors investigated various sensitising doses of OA ranging from 0.1μg to 10, 100 and 1000μg plus $AlOH_3$ administered on days 0, 7 and 14. Mice were then challenged with a 50mg/ml OA aerosol for 20 minutes on days 15-20. There was a striking antigen dose-related difference in OA-specific antibodies. Specifically, high IgE and low IgG2a titres were found in mice sensitised at 10 μg, while low IgE and high IgG2a titres were seen in mice sensitised with 1000μg. In fact the sensitising dose was inversely correlated with the total cell count and the eosinophil count in bronchoalveolar lavage (BAL) fluid, as well as with the extent of histological changes such as goblet cell hyperplasia of the bronchial epithelium and cellular infiltration in the lungs. Furthermore, antigen-induced bronchial hyper-responsiveness (BHR) to methacholine was observed with sensitisation at 10μg but not at 1000μg.

Splenic mononuclear cells (SMNC) obtained from mice sensitised at either dose showed proliferation in response to OA. However, production of the Th-2 cytokines, interleukin (IL)-4 and IL-5, by OA-stimulated SMNC was inversely correlated with the dose of sensitising antigen. High-dose sensitisation resulted in general suppression of cytokine production by SMNC, including interferon (IFN)-γ. In addition, BALF levels of IL-4 and IL-5 production were increased by low-dose sensitisation, whereas IFN-γ and IL-12 levels were increased by high-dose sensitisation. These results suggest that the dose of sensitising antigen defines the phenotypic changes in murine models possibly by influencing the pattern

of cytokine production, with low dose promoting a Th-2 response and high dos promoting a Th-1 response (Sakai K et al 1999).

The ability of antigen dose to influence phenotype expression was confirmed recently but shown to be more complicated and dependent on the strain of mouse used. For example, low dose sensitisation (<8 μg of OA) elicited Th-2-type response in C57BL/6 mice, with high levels of serum IgE, IgG1 and low levels of IgG2a production. In contrast, the same dose elicited a Th-1 response in BALB/c mice with low levels of IgE, IgG1 and high levels of IgG2a. Conversely, high-dose sensitisation (>50 μg OA) elicited Th1-type response in C57BL/6mice, but a Th-2 type response in BALB/c mice.

Furthermore, the number of eosinophils infiltrating the lungs of low-dose OA-sensitised C57BL/6 mice was significantly greater than in BALB/c mice sensitised with the same amount of OA. Only a very high dose of OA (1000 μg) could induce greater eosinophilic infiltration into the lungs of BALB/c mice compared with C57BL/6 mice. Additionally, low-dose sensitisation generated Th-2 cytokines, including high levels of IL-4, IL-5 and a low level of IFN-γ in the lungs of C57BL/6 mice. But the same low dose induced a Th-1 pattern of cytokine production, including low levels of IL-4, IL-5 and a high level of IFN-γ, in the lungs of BALB/c mice

In contrast, high-dose sensitisation elicited Th-1 cytokine production in the lungs of C57BL/6 mice, while BALB/c mice generated Th-2 cytokines in their lungs. Interestingly, splenocyte cultures from C57BL/6 mice produced Th-1 cytokines, while cultures from BALB/c mice produced Th-2 cytokines regardless of OA sensitisation dose (100 ng-1 mg). These results indicate that C57BL/6 and BALB/c mice have different susceptibilities to OA-sensitisation and OA-induced pulmonary eosinophilia, regulated by Th-1 and Th-2 cytokines independent of splenic Th-1 and Th-2 cytokine production (Morokata T et al 2000).

In contrast to OA-driven models N.b.-driven models of pulmonary inflammation have been shown to produce a much greater pulmonary eosinophilia. Whether this is due to the nature of

the stimulating antigen or the dose of the antigen normally employed is unclear. Most studies in the literature have employed a dose of 750 L3/mouse to generate the airway eosinophilia.

Previous authors have demonstrated that as few as six infective N. brasiliensis larvae can elicit IL-4 production in the mesenteric lymph node, but only higher doses of larvae, such as 600 L3, provoke IL-4 secretion in the spleen (Lawrence RA et al 1996). So the purpose of this series of experiments was to try to determine if there was an incremental dose response relationship in the production of airway eosinophilia with varying infective burdens of L3. The generation of a milder airway eosinophilia, following N.b. infection could make the model more sensitive to pharmacological manipulation and might help in establishing a model of chronic airway inflammation.

The development of an animal model of chronic airway inflammation has been difficult for those working with either OA-driven or N.b. models (Foster PS et al 2000, Hamada K et al 2000). However, some authors have demonstrated that if primary N.b. infection is followed by trickle administration of further antigen then persistent airway eosinophilia can be generated (Ferens WA et al 1994). An alternative to trickle infection may be the subsequent administration of either nebulised N.b. antigen or administration of N.b. antigen by direct tracheal instillation.

Such an approach has worked in OA models. For example immunotherapy with OA323-339, the immunodominant epitope of ovalbumin, has been shown to produce a significant reduction in airway eosinophilia, OA-specific IL-4 and IL-5 production, OA-specific IgG1 production in addition to reduced airway hyperresponsiveness. In contrast a slight alteration in the antigenic epitope aggravated airway hyperresponsiveness and eosinophilia, suggesting that repeated challenge with antigen can dampen or enhance the Th-2 response (Janssen EM et al 1999).

This study confirmed an early report outlining the importance of antigen dose and sensitization regime. Thus, repeated challenge of OA-sensitized mice with intraperitoneal injections of OA prior to subsequent nebulisation significantly reduced the accumulation of $CD4^+$ lymphocytes

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and eosinophils recovered in the BAL fluid, in addition to attenuating the OA-induced proliferative response by blood lymphocytes (Ohnuma N et al 1998).

Therefore we decided to see if the N.b. infective burden could be substantially reduced yet still produce a significant peripheral eosinophilia and pulmonary inflammation. Such a reduced response would make the induced inflammatory response more amenable to therapeutic manipulation and provide a range in the severity of inflammation that could be contrasted to severity of disease. Therefore we compared the peripheral blood and pulmonary response to a challenge of 0, 50, 200 400 or 750 L3. All animals were harvested either on day 14, when the peak eosinophilia usually occurs or on day 19 when the peripheral blood eosinophilia is subsiding but the pulmonary response is still present.

4.2 MATERIALS AND METHODS

4.2.1 Larval Culture and Infection

Details of N.b. larval culture are given in chapter 2.

4.2.2 Animals

Details of animal husbandry are given in chapter 2.

4.2.3 Bronchoalveolar lavage and cell preparations

Details of brochoalveolar lavage and cell preparation are given in chapter 2. Data presented throughout this thesis are given per ml of BAL fluid drawn.

4.2.4 Blood: Leukocyte Subsets

Details of peripheral blood acquisition and cell analysis are given in chapter 3. Data presented throughout this thesis are given per ml of blood drawn.

4.2.5 Statistics

The data presented in this chapter on wild type C57BL/6 mice are pooled from three experiments. Thus the peripheral blood and BAL fluid data presented contains a variable number of mice per dose level. There was an average of 24 wild type C57BL/6 mice per dose level. This enabled data to be analysed using a two-tailed paired t test assuming unequal variance in samples, but a normal distribution. The data on the naïve C57BL/6 mice were taken from the general pool of naïve mice analysed in several experiments used in this thesis. When this overall test of treatment was found to be significant at the $p<0.05$, this was indicated by an asterisk (*). When the significance level reached $p<0.01$ this was indicated by two asterisks (**), and when the significance level reached $p<0.001$ this was indicated by three asterisks (***). If the result was borderline significant ($p=0.05$), then this was denoted by a # sign.

4.3 RESULTS

The N.b. driven model of allergic inflammation is characterized by a significant eosinophilia in the peripheral blood in addition to a biphasic recruitment of leukocytes to the airways. The late phase response in the airways consists of an influx of lymphocytes and eosinophils. The eosinophil response to N.b. infection in the peripheral blood peaks on day 14 pi and by day 19 the peripheral eosinophilia is subsiding. In contrast the airway eosinophilia is still present on day 19 pi. Therefore the peripheral blood response of mice to N.b. infection on day 14 and day 19 pi was compared to naïve, uninfected mice. In contrast the recruitment of leukocytes to the airways was compared on day 14 pi only, on the assumption that there would be very little difference between day 14 and 19 pi in the BAL fluid response.

A variety of infective doses were examined to determine whether there was a minimum dose of L3 that would provoke a peripheral or pulmonary eosinophilia. Unfortunately an error was made in the injection procedure of the mice due to be harvested on day 14 pi having been injected with 100L3 and therefore this group's data could not be presented.

4.3.1 Time course of peripheral blood changes following N.b. infection

I. Total Leukocyte Count

Primary infection with third stage N.b. larvae produced a significant increase in the total white cell count (WCC) in wild-type (WT) mice during the late phase response, which peaked on day 14-19 and had started to subside by day 26 pi (chapter 3). The same leukocytosis was documented in this experiment, however the peak occurred earlier with more leukocytes circulating on day 14 pi compared to day 19 pi (Figure 4.1).

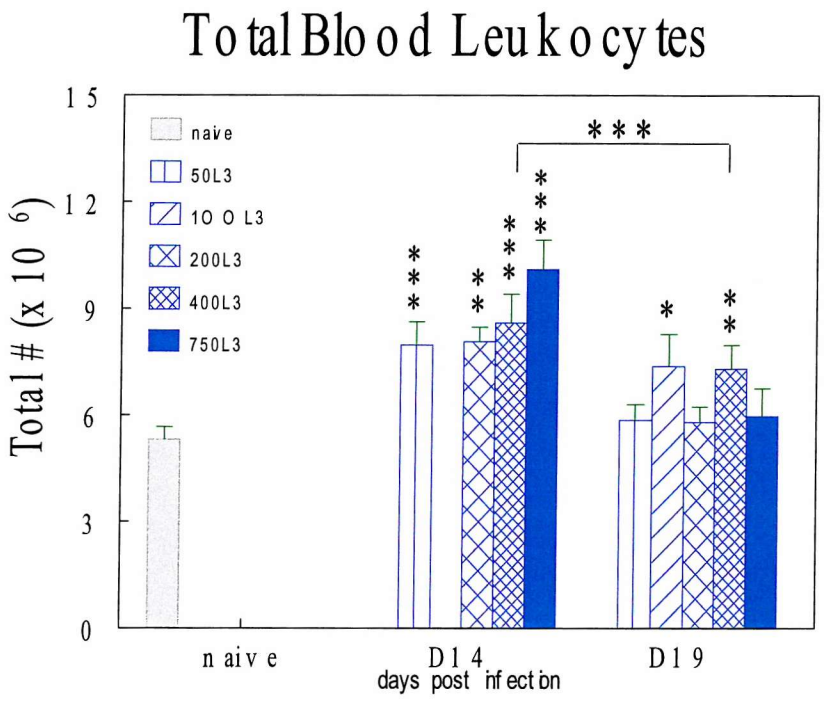

Figure 4-1. The total number of leukocytes in the peripheral blood of wild type mice in response to various doses of N.b. larvae.

Specifically, the mean number of leukocytes circulating increased significantly from 5.3 $x10^6$ in naïve mice to an average of 8.59 $x10^6$ on day 14 pi ($p<0.001$). By day 19 pi the leukocytosis had subsided significantly, compared to day 14 pi, to an average of 6.45 $x10^6$ ($p<0.002$).

All doses of L3 produced a significant increase in the peripheral leukocytosis. Thus, the number of circulating leukocytes increased significantly from 5.3 $x10^6$ in naïve mice to 7.97 $x10^6$ following infection with just 50 L3 on day 14 pi ($p<0.003$), and 8.06 $x10^6$ following

infection with 200 L3 ($p<0.001$). Similarly, numbers increased to 8.59 $x10^6$ following infection with 400 L3 on day 14 pi ($p<0.005$) and 8.93 $x10^6$ following infection with 750 L3 ($p<0.001$). In contrast, by day 19 pi the peripheral leukocytosis had subsided in three of the five groups infected with different doses of L3. Specifically, in mice infected with just 50 L3 the number of circulating L3 had dropped to 5.85 $x10^6$ compared to 5.3 $x10^6$ in naïve mice. Similarly, in the groups infected with 200 and 750 L3, numbers had also returned to near naïve levels, 5.8 and 5.96 $x10^6$ respectively. However, in groups infected with 200 and 400L3 leukocyte numbers still remained significantly higher than naïve levels, 7.4 and 7.29 $x10^6$ respectively ($p<0.05$ and $p<0.02$).

II. Eosinophils

The most significant change in the peripheral blood of either WT or genetically manipulated mice following N.b. infection was the increase in the number and percentage of circulating eosinophils, peaking on day 14 pi and subsiding by day 19 pi (chapters 3, 7-9).

This peripheral eosinophilia was again seen here with eosinophil numbers increasing to similar levels as seen in other chapters and decreasing by day 19 pi. Thus the mean number of circulating eosinophils increased significantly from 0.08 $x10^6$ in naïve mice to 0.74 $x10^6$ on day 14 pi ($p<0.001$) and this level decreased significantly to 0.22 $x10^6$ by day 19 pi ($p<0.03$). When comparing the different infective doses of L3 on day 14 pi, all doses generated a similar degree of eosinophilia except 750L3. The 750L3 dose stimulated a significantly greater peripheral eosinophilia, (1.28 $x10^6$), than either 50L3, (0.45 $x10^6$)($p<0.001$), or even 400L3, (0.52 $x10^6$) ($p<0.001$), and the number of circulating eosinophils in all these groups was significantly greater than in naïve mice ($p<0.001$)(Figure 4-2).

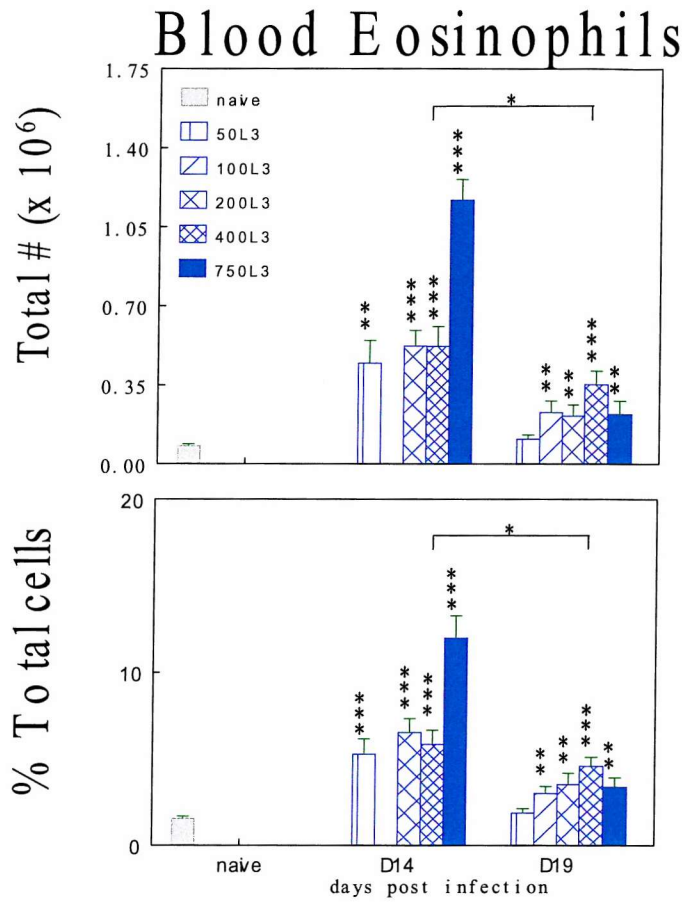

Figure 4-2. The total number and percentage of eosinophils in the blood of wild type mice in response to various doses of N.b. larvae.

By day 19 pi the eosinophilia had subsided to such an extent that the number of eosinophils in the blood of the mice infected with 750 L3 was not significantly different from other infective doses, but still greater than naïve mice. However, the exception was mice infected with 50L3 where eosinophil numbers had declined to normal by day 19 pi, at 0.11 $x10^6$. On day 19, the eosinophilia in all other groups was still significantly greater than naïve levels. Specifically, the numbers of eosinophils circulating in mice infected with 100L3, 200L3 400L3 and 750L3 was 0.23 $x10^6$ ($p<0.005$), 0.21 $x10^6$ ($p<0.01$), 0.35 $x10^6$ ($p<0.001$) and 0.22 $x10^6$ respectively ($p<0.009$). Nevertheless, on average there were still significantly fewer eosinophils in the peripheral blood of mice on day 19 pi compared to day 14 pi ($p<0.03$).

This significant increase in the number of eosinophils circulating in the peripheral blood was reflected in the significant increase in the percentage of eosinophils in the peripheral blood, 8.2%, on day 14 pi compared to 1.6% in naïve mice ($p<0.001$). Although by day 19 pi the percentage of eosinophils circulating, 3.3%, was significantly reduced compared to day 14 pi ($p<0.02$), it had not quite declined to naïve levels ($p<0.01$).

Again all four groups infected with different doses of L3 generated a significantly greater percentage of eosinophils in the blood on day 14 pi compared to naïve, uninfected mice. Thus 50L3 produced a 5.28% eosinophilia ($p<002$), while 200L3 generated a 6.53% ($p<0.001$), 400L3 generated a 5.84% eosinophilia ($p<0.001$) and 750L3 stimulated a even greater eosinophilia of 8.19% ($p<0.001$).

By day 19 pi the percentage of eosinophils in the blood of four of the five groups was still significantly greater than the percentage of eosinophils circulating in the blood of naïve mice. Thus, the percentage of eosinophils in mice infected with 100L3 had declined to 3.01% compared to 1.56% in naïve mice ($p<0.005$). Similarly, the percentage of eosinophils in mice infected with 200L3 had declined to 3.53% ($p<0.01$), the percentage in mice infected with 400L3 declined to 4.58% ($p<0.001$) and in mice infected with the standard dose of 750L3 the percentage of eosinophils had declined to 3.37% ($p<0.009$). Only in mice infected with 50L3 had the level of eosinophilia returned to baseline (1.87%, $p<0.29$)(Figure 4-2).

III. Neutrophils

Infection with N.b. larvae produces changes in the number and percentage of circulating neutrophils in the peripheral blood. Neutrophil numbers increase during the early phase response and peak somewhere between day 3 and day 8 pi (chapter 3, 7-9). By day 14 and 19 pi neutrophil numbers and percentages should be near naïve levels.

In this experiment the number of neutrophils circulating in the peripheral blood on day 14 pi was not significantly different compared to naïve mice for the three lower dosage groups, 50L3, 200L3 and 400L3. However, in the standard dosage group of 750L3, neutrophil numbers were still significantly greater on day 14 pi, 0.78 $x10^6$, compared to naïve levels, 0.59 $x10^6$

($p<0.001$). However, this greater number of neutrophils in the group infected with 750L3 was not significantly different from the groups infected with other doses of L3 on day 14 pi.

By day 19 pi neutrophil numbers had returned to normal naïve levels in all groups. In fact in two of the dosage groups numbers had dropped to significantly below naïve levels. Specifically, the group of mice infected with 200L3 had a neutrophil count of 0.45 $x10^6$ ($p<0.005$) and the mice infected with 750L3 had a neutrophil count of 0.43 $x10^6$ ($p<0.02$), compared to 0.59 $x10^6$ in naïve mice. Overall there was no significant difference between the mean number of neutrophils circulating on day 14 pi and day 19 pi (Figure 4-3).

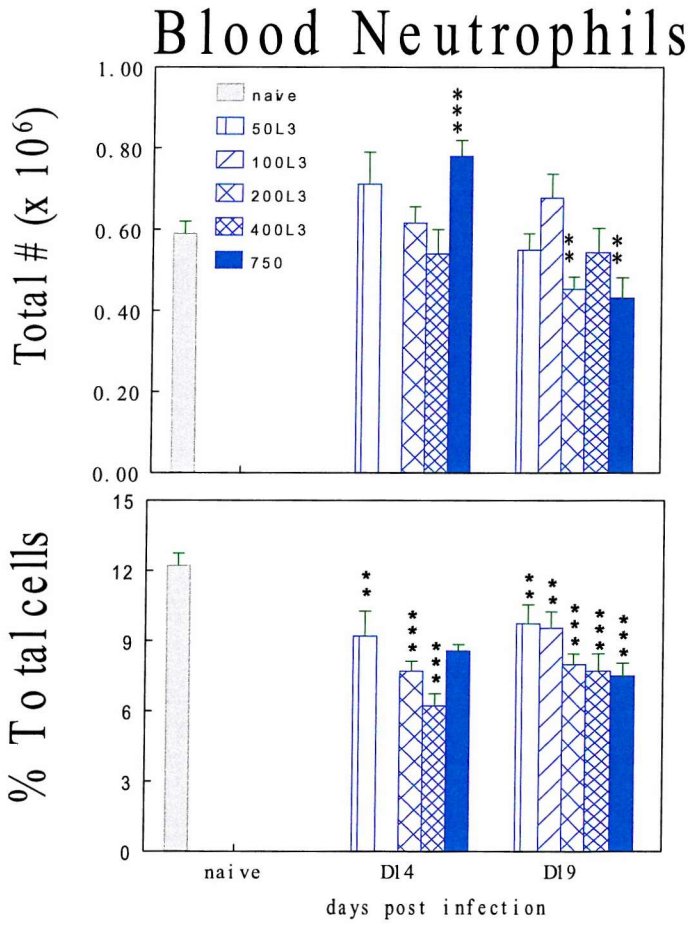

Figure 4-3. The total number and percentage of neutrophils in the blood of wild type mice in response to various doses of N.b. larvae.

On day 19 pi an infective dose of 200L3 did produce a small but significantly greater number of neutrophils in the blood compared to 400L3 ($p<0.008$), but such a difference was not seen at any other dose levels.

On day 14 and 19 pi there was a significant eosinophilia in the blood. Consequently the proportion of the other cell populations is often reduced. This was certainly true of neutrophils with a significantly lower proportion of neutrophils in the blood of infected mice on day 14 and 19 pi mice compared to naïve mice. Specifically, the percentage of neutrophils dropped from 12.2% in naïve mice to 9.2% in mice infected with 50L3 ($p<0.02$), to 7.7% in mice infected with 200L3 ($p<0.001$) and to 6.22% in mice infected with 400L3 ($p<0.001$). Similarly on day 19 pi the percentage of neutrophils in the blood was significantly lower than in naïve mice. In mice infected with just 50L3 the proportion of neutrophils dropped to 9.72% ($p<0.02$). Similarly it dropped to 9.54% in mice infected with 100L3 ($p<0.01$), to 8% in mice infected with 200L3 ($p<0.001$), to 7.69% in mice infected with 400L3 ($p<0.001$), and to 7.49% in mice infected with 750L3 ($p<0.001$). There was no difference in the percentage of neutrophils circulating on day 14 pi compared to day 19 pi ($p<0.71$)(Figure 4-3).

IV. Lymphocytes

The number of circulating lymphocytes in the peripheral blood of WT mice increased significantly following N.b. infection peaking on day 14-26 pi (chapter 3). Following N.b. infection in this experiment there was also a significant increase in lymphocyte numbers on day 14 pi compared to uninfected mice rising from 4.43 $x10^6$ in naïve mice to 6.71 $x10^6$ on day 14 pi ($p<0.001$). By day 19 pi lymphocyte numbers had returned to near normal, 5.46 $x10^6$, and therefore were significantly lower on average than on day 14 pi ($p<0.01$).

The significant lymphocytosis was independent of the infective dose with a similar number of lymphocytes circulating in mice infected with 50L3, 6.56 $x10^6$ ($p<0.01$), 200L3 - 6.64 $x10^6$ ($p<0.001$), 400L3 - 7.25 $x10^6$ ($p<0.004$), and 750L3, 6.59 $x10^6$ ($p<0.001$) respectively.

By day 19 pi only mice infected with 400L3 still had a significant lymphocytosis, 6.17 $x10^6$ ($p<0.02$), all other dosage groups had returned to naïve levels.

The percentage of lymphocytes circulating in the blood remained relatively constant over time. By day 14 pi there was no difference between the lymphocyte percentage in naïve mice, 82.19%, and the lymphocyte percentage in mice infected with three of the four doses, 50L3 (82.15%), 200L3 (82.19%), or 400L3 (84.92%)($p<0.06$). In contrast, mice infected with 750L3 had a significantly lower percentage of lymphocytes circulating, 74.6% ($p<0.001$), probably reflecting the greater percentage of eosinophils on day 14 pi in these mice (Figure 4 4).

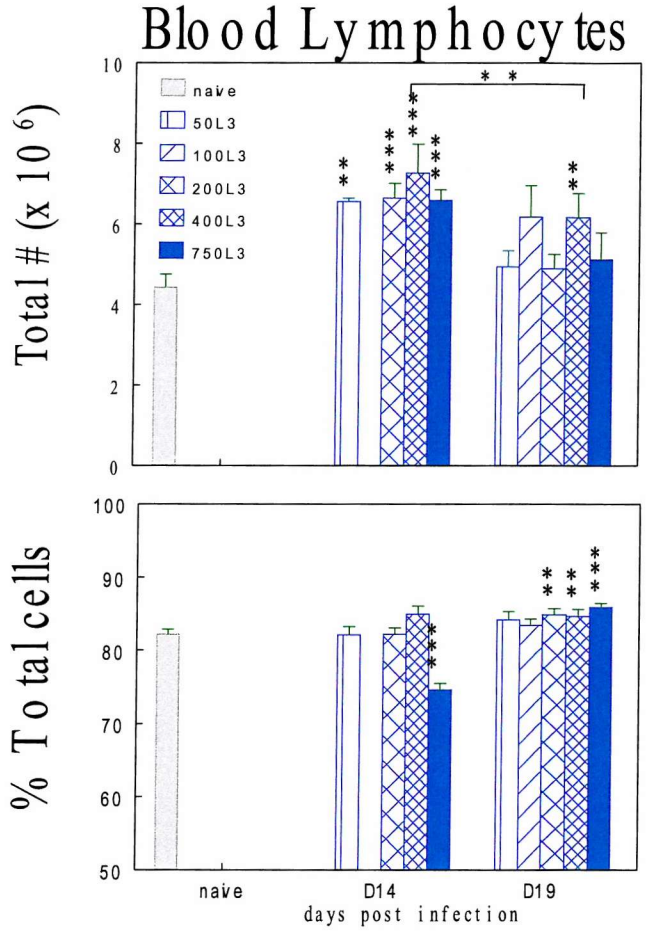

Figure 4-4. The total number and percentage of lymphocytes in the blood of wild type mice in response to various doses of N.b. larvae.

By day 19 pi the percentage of lymphocytes in the blood was equal to or slightly greater than naïve levels depending on the dosage of L3 used to infect mice. Thus, in the lower dosages, 50L3 and 100L3 the percentage of lymphocytes, 84.16% and 83.45%, were not statistically different from naïve mice. However, at the higher doses the percentage of lymphocytes in the

blood was significantly greater than that seen in naïve mice. Specifically, 84.89% in mice infected with 200L3 ($p<0.02$), 84.65% in mice infected with 400L3 ($p<0.05$), and 85.88% in mice infected with 750L3 ($p<0.0002$)(Figure 4-4).

V. Monocytes

The number of monocytes circulating in the peripheral blood is small and following N.b. infection this number remained largely unchanged on either day 14 or day 19 pi. However infecting mice with 750L3 stimulated such a substantial cellular response that even monocyte numbers increased significantly, from 0.12 $x10^6$ in naïve mice to 0.2 $x10^6$ on day 14 pi ($p<0.001$). Monocyte numbers on day 19 pi were not significantly different from naïve mice (Figure 4-5).

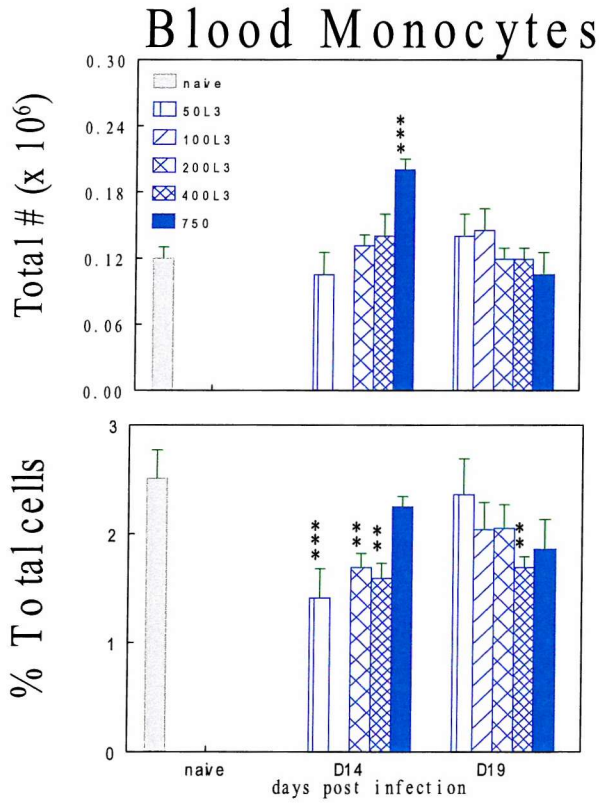

Figure 4-5. The total number and percentage of monocytes in the blood of wild type mice in response to various doses of N.b. larvae.

The percentage of monocytes in the peripheral blood is remarkably small whether in naïve or infected mice. Following infection with N.b. there was a small and statistically significant reduction in the percentage of monocytes in the peripheral blood. Specifically, the percentage

of monocytes decreased from 2.51% in naïve mice to 1.41% in mice infected with 50L3 on day 14 pi ($p<0.001$). Similarly, an infective dose of 200L3 produced a reduction in the percentage of monocytes to 1.69% ($p<0.01$), and 400L3 reduced the percentage to 1.59% ($p<0.003$), on day 14 pi. Apart from a dose of 400L3 which reduced the percentage of monocytes to 1.69% on day 19 pi ($p<0.005$), all other doses of L3 did not significantly impact the percentage of circulating monocytes of day 19 pi (Figure 4-5).

4.3.2 Time course of leukocyte infiltration into the lung following N.b. infection

I. Total Leukocyte Count

The main purpose of this experiment was to determine whether different infective doses of L3 would impact the cellular recruitment to the blood and more specifically the airways during the late phase response. From previous experiments (chapter 3) it was clear that the recruitment pattern of cells to the airways was not significantly different on day 19 pi compared to day 14 pi. Therefore, only the BALF of mice infected with different L3 doses and harvested on day 14 pi were compared to naïve mice.

The total leukocyte recruitment to the airways did appear to be incrementally dependent on the infective dose of L3 employed (Figure 4-6).

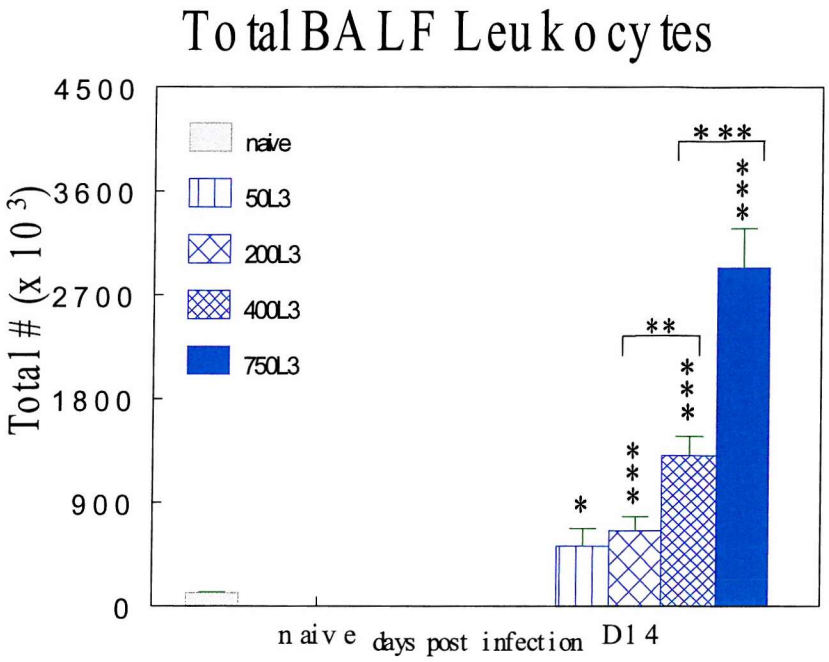

Figure 4-6. The total number of leukocytes in the BAL fluid of wild type mice in response to various doses of N.b. larvae.

Specifically, the total number of leukocytes in the airways rose significantly from 115 x10^3 in naïve mice to 520 x10^3 in mice infected with 50L3 ($p<0.03$). Leukocyte recruitment was greater following infection with 200L3, which increased the cell count significantly to 656 x10^3 ($p<0.002$), and to 1309 x10^3 following 400L3 ($p<0.001$). The greatest cellular influx occurred after infection with the standard dose of 750L3, with numbers rising to 2,939 x10^3 on day 14 pi ($p<0.001$)(Figure 4-6).

When comparing the impact of different infective doses we demonstrated that the cellular recruitment in response to 400L3 was significantly greater than the cellular influx following 200L3 ($p<0.006$). Similarly, the response to 750L3 was significantly greater than the response to 400L3 ($p<0.001$). In contrast there was no difference in the number of cells recruited by an infective dose of 50 or 200L3 (Figure 4-6).

II. Eosinophils

The cellular response in the lungs following N.b. infection was dominutesated by a significant increase in the number and percentage of eosinophils in the BAL fluid in WT mice (chapter 3). This series of experiments were designed to determine whether a much smaller infective dose of L3 would still induce a significant airway eosinophilia.

We demonstrated that even a small dose of 50 L3 produced a significant increase in the number of eosinophils recovered from the BAL fluid with numbers rising form 5.6 $x10^3$ in naïve mice to 273 $x10^3$ ($p<0.05$). A dose of 200L3 produced a greater rise in eosinophil numbers to 372 $x10^3$ ($p<0.003$), while 400L3 increased eosinophil numbers to 839 $x10^3$ ($p<0.001$) and 750L3 increased numbers to 2.2 $x10^6$ ($p<0.001$)(Figure 4-7).

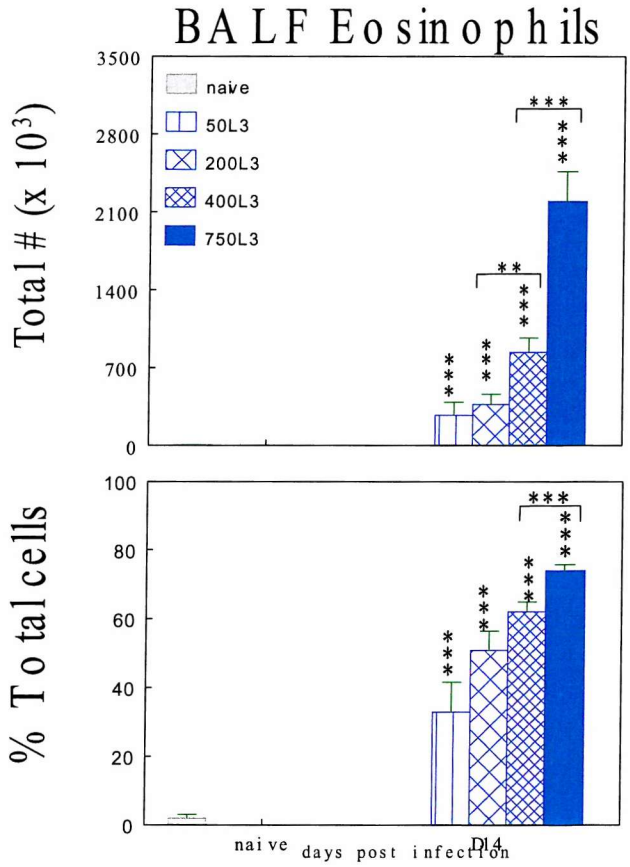

Figure 4-7. The total number and percentage of eosinophils in the BAL fluid of wild type mice in response to various doses of N.b. larvae.

While increasing the infective dose from 50L3 to 200L3 did increase the number of eosinophils recruited to the BAL fluid this increase was not statistically significant. However, an infective dose of 400L3 generated a significantly greater airway eosinophilia than 200L3 ($p<0.01$), and 750L3 stimulated a significantly greater airway eosinophilia than either 200L3 ($p<0.001$) or 400L3 ($p<0.001$).

These changes in eosinophil numbers were reflected in changes in the percentage of eosinophils recovered from BALF. Specifically, the smallest infective dose of 50L3 produced a significant increase in the percentage of eosinophils in the BALF, rising from 2% in naïve mice to 32.9% ($p<0.001$). A dose of 200L3 resulted in an increase in the percentage of eosinophils recovered from the BALF to 50.9% ($p<0.001$). The percentage of eosinophils recovered from the BAL fluid was greater with a dosage of 400L3 at 62% ($p<0.001$) and 74% in mice infected with the standard dose of 750L3 ($p<0.001$). Again, the response to 200L3 was not statistically greater than the response to 50L3. Similarly the response to 400L3 was not statistically different to 200L3 ($p<0.1$). However, the response to 400L3 was significantly greater than the response to 50L3 ($p<0.009$). The percentage of eosinophils recovered from the BALF in response to a dose of 750L3 was significantly greater than 400L3 ($p<0.003$), 200L3 ($p<0.0002$) and 50L3 ($p<0.001$)(Figure 4-7).

III. Neutrophils

In WT mice a biphasic cellular recruitment to the airways was seen. This was characterised by a significant influx of neutrophils to the airways during the "early phase" response peaking on day 3 pi, during larval migration through the lungs (chapter 3). By day 14 pi this early phase response had largely, but not always subsided.

In this experiment there was no day 3 time-point. Therefore in most infective doses of L3 the early airway neutrophilia had subsided by day 14 pi. Thus the total number of neutrophils recovered from the BAL fluid on day 14 pi following the smallest infective dose of 50L3 was no different from the number of neutrophils in the airways of naïve mice. Specifically, in naïve mice there were 1.8 $x10^3$ neutrophils in the BAL fluid compared to 2.1 $x10^3$ following an infection with 50L3.

However, the number of neutrophils recovered from mice infected with 200L3 had not yet returned to normal and was still significantly greater then naïve levels, namely 6.7 $x10^3$ ($p<0.04$). Following an infective dose of 400L3 although total numbers of neutrophils recovered from the BAL fluid had increased still further, to 7.5 $x10^3$, this was not statistically different from naïve mice due to the greater variability at this dosage level. At the standard infective dose of 750L3 neutrophil numbers on day 14pi, 42.3 $x10^3$, were still very significantly greater than in naïve mice ($p<0.006$), and mice infected with 400L3 ($p<0.02$), 200L3 ($p<0.01$) and 50L3 ($p<0.01$)(Figure 4-8).

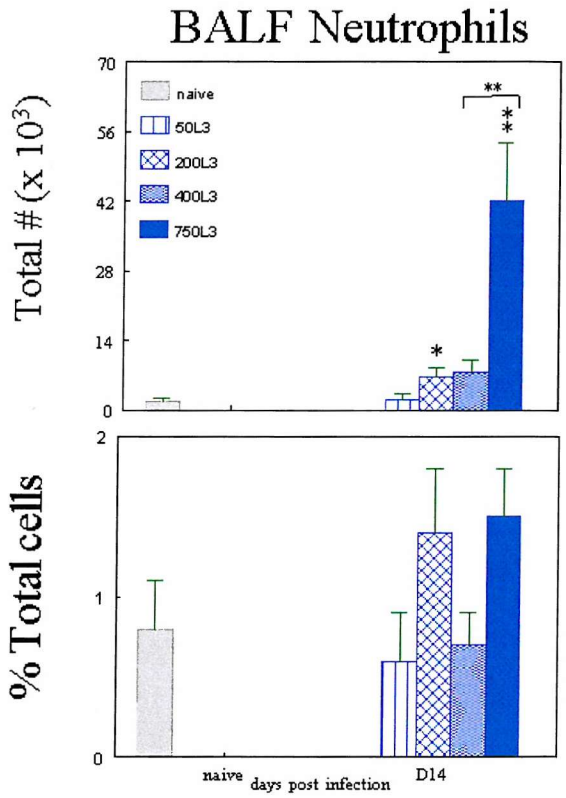

Figure 4-8. The total number and percentage of neutrophils in the BAL fluid of wild-type mice, in response to various doses of N.b. larvae.

This significant increase in neutrophil numbers in the airways of mice on day 14 pi probably reflected a generalized cellular recruitment at this time point since the percentage of neutrophils in naïve and mice on day 14 pi were not statistically different, both being <2%. In fact the percentage of neutrophils in the BAL fluid on day 14 pi did not differ at any dosage level (Figure 4-8).

IV. Lymphocytes

A significant increase in the number and percentage of lymphocytes, as well as eosinophils, dominated the cellular response in the lungs in WT mice following N.b. infection (chapter 3).

This airway lymphocytosis was seen in the airways of mice infected with just 50L3. Thus, 50 L3 produced a significant increase in the number of lymphocytes recovered from the BAL fluid with numbers rising form 12.7 $x10^3$ in naïve mice to 105 $x10^3$ following infection with 50L3 ($p<0.02$). A dose of 200L3 produced a greater rise in lymphocyte numbers to 132 $x10^3$ ($p<0.0003$), while 400L3 increased lymphocyte numbers significantly to 250 $x10^3$ ($p<0.0003$) and 750L3 increased numbers to 410 $x10^3$ ($p<0.0002$)(Figure 4-9).

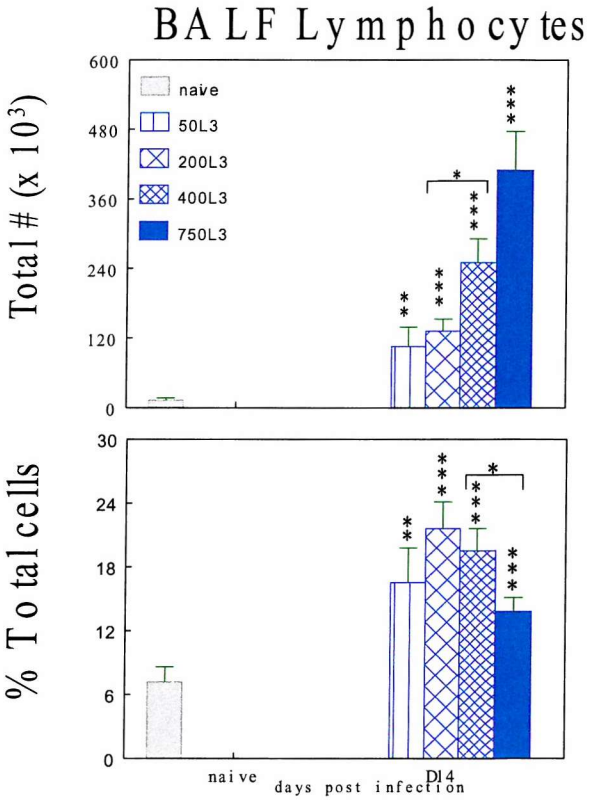

Figure 4-9. The total number and percentage of lymphocytes in the BAL fluid of wild type mice in response to various doses of N.b. larvae.

Increasing the infective dose from 50L3 to 200L3 did not significantly increase the number of lymphocytes recruited to the BAL fluid. However, an infective dose of 400L3 generated

a significantly greater airway lymphocytosis than either 50L3 or 200L3 ($p<0.01$). The airway lymphocytosis in response to an infective dose of 750L3 was not significantly greater than the lymphocytosis induced by 400L3, but was greater than that induced by 200L3 ($p<0.002$) and 50L3 ($p<0.001$)(Figure 4-9).

The proportion of lymphocytes in the BAL fluid did not change incrementally with increasing infective doses of L3. The smallest infective dose of 50L3 produced a significant increase in the percentage of lymphocytes in the BAL fluid, rising from 7.2% in naïve mice to 16.5% ($p<0.02$). Similarly a dose of 200L3 resulted in an increase in the percentage of lymphocytes recovered from the BAL fluid to 21.6% ($p<0.0002$). This percentage was much the same following infection with a dose of 400L3, 19.5% ($p<0.0001$) and was slightly lower following infection with the standard dose of 750L3, 13.8% ($p<0.002$).

The response to 200L3 was not statistically greater than the response to 50L3. Similarly the response to 400L3 was not statistically different to 200L3. The response to 400L3 was also not significantly greater than the response to 50L3 ($p<0.45$). However, the percentage of lymphocytes recovered from the BALF in response to a dose of 750L3 was significantly smaller than 400L3 ($p<0.04$), and 200L3 ($p<0.02$) but not 50L3 (Figure 4-9).

V. Monocytes

N.b. infection has been shown to produce an increase in the number of all cells types recruited to the airway including monocytes. This increase was apparent even with the smallest infective dose of 50L3 with numbers rising from 94.5 $x10^3$ in naïve mice to 140 $x10^3$ in mice infected with 50L3 ($p<0.01$). Similarly, an infective dose of 400L3 increased monocyte numbers in the airway to 210 $x10^3$ ($p<0.001$) and 750 L3 increased numbers to 279 $x10^3$ ($p<0.001$). Only 200L3 did not produce a statistically significant increase in monocyte numbers, probably due to the greater sample variability in this particular group of mice.

The numbers of monocytes in the airways following an infective dose of 50L3 did not differ statistically from the monocyte response to 200L3. An infective dose of 400L3 generated a

significantly greater airway lymphocytosis than 50L3 ($p<0.01$) but not 200L3. The airway monocyte recruitment in response to an infective dose of 750L3 was not significantly greater than the monocytosis induced by 400L3, but was greater than that induced by 200L3 ($p<0.02$) and 50L3 ($p<0.01$)(Figure 4-10).

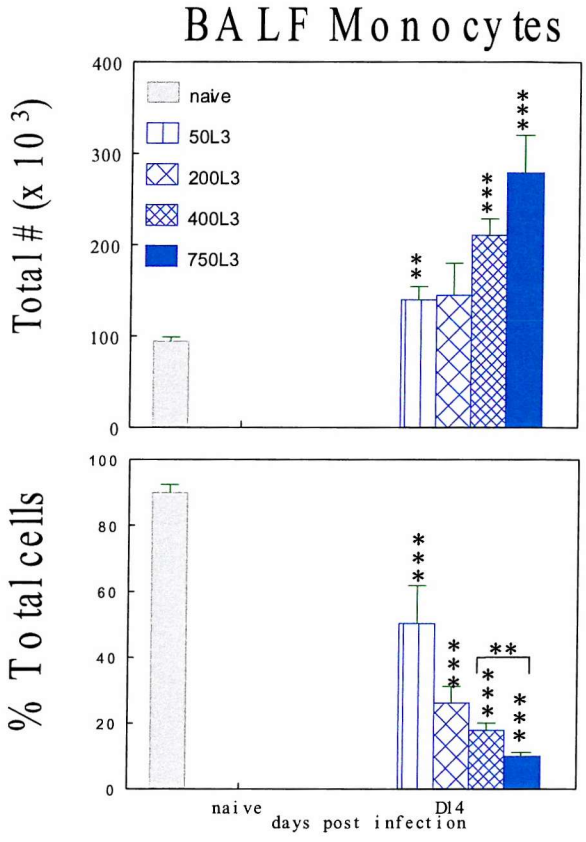

Figure 4-10. The total number and percentage of monocytes in the BAL fluid of wild type mice in response to various doses of N.b. larvae.

In view of the significant eosinophil and lymphocyte recruitment to the BAL fluid during the late-phase response the proportion of monocytes in the airways usually decreases significantly. This effect was seen clearly in this series of experiments. Thus the percentage of monocytes dropped from 90% in naïve mice to 50.3% in mice infected with 50L3 ($p<0.007$). Similarly, the percentage of monocytes recovered dropped to 26.2% in mice infected with 200L3 ($p<0.001$), to 17.8% in mice infected with 400L3 ($p<0.001$) and to 9.9% in mice infected with the standard dose of 750L3 ($p<0.001$). While the significant reduction in the proportion of monocytes in the airways induced by 200L3 was not statistically different from that induced by

50L3 ($p<0.07$), or 400L3, it was significantly less than the reduction seen following infection with 750L3 where the percentage reduction was at its greatest ($p<0.009$). Indeed, the recruitment of other cellular populations induced by the standard dose of 750L3 was so marked that the reduction of the proportion of monocytes induced by this dosage was significantly greater than that induced by 400L3 ($p<0.009$)(Figure 4-10).

4.4 DISCUSSION

The balance between irritation and inflammation is a fine line. Whether antigen exposure provokes a Th-1, Th-2 response or induces immunological tolerance is dependent on many factors. For example, the nature and dose of the stimulating antigen, the interval between antigen exposure, the route of administration of the antigen, the type of sensitisation regime, the nature of the sensitising agent and its adjuvant and possibly repeated exposure to the same antigen. Such complexity has been shown to be true of OA models of allergic inflammation (Sakai K et al 1999), and attempts to develop chronic models of inflammation of the airways have often led to a dampening of airway inflammation and the development of tolerance rather than chronic inflammation (Yiamouyiannis CA et al 1999). Similar difficulties have been seen in N.b.-driven models of airway inflammation (Murray M et al 1979).

In our attempt to optimise the N.b.-driven model of airway inflammation we explored the effects of varying doses of N.b. antigen exposure on the recruitment of leukocytes to the blood and lungs of wild-type mice. Previous authors have shown that as little as six L3 are capable of inducing a Th-2 cytokine response in regional lymph nodes (Lawrence RA et al 1996). Similarly, others have reported that different doses of N.b. larvae can induce different IgE responses. Specifically, rats infected with 2,500 N.b. larvae (L3) generated a specific IgE response 4 weeks post infection. In contrast, rats infected with 10 or 100 L3 produced a sustained rise in IgE levels, which started at 4 weeks post infection and continued to rise until at least 8 weeks after infection. The IgE level at 8 weeks was significantly higher in rats infected with 10 or 100 larvae than in rats infected with 2,500

larvae. A similar picture was seen in total IgE responses. These results suggest that the low-level infections induce a much more sustained specific and non-specific IgE response than that induced after heavy infection (Yamada M et al 1992).

However, the minimum dose of L3 required to induce a peripheral and pulmonary eosinophilia has yet to be established. In this series of experiments we demonstrated that 50L3 were more than sufficient to induce a significant peripheral leukocytosis. Unfortunately, we did not explore doses less than 50L3 but we suspect that this dose was probably close to the minimum required to produce a peripheral eosinophilia.

There was a flat dose response curve in the peripheral blood with no significant difference in the number or percentage of eosinophils recruited to the blood. This may have been due to increased levels of IL-5 induced by N.b. infection limiting eosinophil egress from the bone marrow. Although, the peripheral eosinophilia generated by 50L3 was no different in magnitude than the eosinophilia generated by 400L3, by day 19 pi all other doses still generated an eosinophilia in the blood except 50L3. Supporting the assertion that the 50L3 dose may have been close to the minimum dose required to induce a peripheral eosinophilia. A similar pattern was seen in the lymphocyte response to 50L3 in the peripheral blood.

In the BAL fluid there was an incremental dose response relationship between number of infective larvae administered and the total number of leukocytes, lymphocytes and eosinophils recruited to the airways. Again we were unable to establish the minimum dose required to induce a significant inflammatory cell recruitment to the airways. However, a dose of 50 L3 produced an increase in the number of eosinophils analogous to the level produced in OA-induced models of allergic inflammation (Kennedy et al 1995).

In conclusion, reducing the standard infective dose to 6% of the normal larval burden of 750 L3 still produced a significant eosinophilia and lymphocytosis in the blood and airways of infected mice. However, the number of these cells recruited was substantially lower than that seen with the standard infective burden. These results may be worth pursuing if the N.b. model is to be developed either for therapeutic manipulation or in order to develop a chronic model of

pulmonary inflammation. Therefore N.b. models still offer an antigenic alternative to OA models for explore the allergic immune response and the different impact of these two Th-2 stimulants may yet shed further light on the subtlety of immune regulation that is characteristic of asthmatic inflammation of the airways.

CHAPTER 5

Treatment with an Antihelminthic Agent

5.1 INTRODUCTION

This thesis has attempted to develop a model that can be used to investigate the complexity of interacting factors involved in inflammation of the airways. As previously discussed mice are now the animals of choice in most models of airway inflammation. This is partly because they are cheap, their husbandry is easy and there is an extensive range of immunological tools capable of probing the complexity of immunological responses. In addition, the most popular method of inducing a Th-2 driven model of pulmonary inflammation is to aerosolise ovalbumin to the airway (Hook S et al 2000, Foster et al 2000, Schramm CM et al 2000). We have attempted to develop an alternative strategy using Nippostrongylus brasiliensis (N.b.) antigens to drive a Th-2 response.

Since the induction of Th-2 response has been shown to be central to subsequent airway inflammation many authors have attempted to develop therapeutic strategies that will either inhibit or turn off the Th-2 response or promote a Th-1 response. For example, some authors have developed Th-1 or Th-2-skewing immunotherapy peptides that work directly (Janssen EM et al 2000), or that act as promoters of a Th-1 response (Hook S et al 2000). Other authors have attempted to administer, intravenously or locally, cytokines or cytokine genes to the lungs (Dow SW et al 1999). Yet other authors have delivered antigen/peptide complexes to promote a Th-1 response (Shirota H et al 2000).

An alternative approach has been to work downstream of the evolution of the two T helper cell subpopulations and modulate some of the effector molecules involved in the Th-2 response such as IgE (Fick RB Jr et al 1999, Fahy JV et al 2000). But despite all these attempts we are still some way short of effectively preventing the cascade of inflammatory processes that occur in asthma and allergic inflammation. This chapter attempts to explore in more detail the nature of the stimulating antigens expressed by N.b. that may be responsible for inducing the isotype switch to a Th-2 response.

Earlier chapters have demonstrated that there is a biphasic response to infection with N.b. larvae. The early phase response is predominantly characterised by an airway neutrophilia peaking on day

3-8 pi. In contrast the late phase response is characterised by a lymphocyte and eosinophil influx into the airways, peaking on day 14 pi. By this stage the N.b. larvae have migrated through the pulmonary tissue to the gastrointestinal tract where they mature into adult worms. This series of experiments was designed to determine whether it was the larval or adult antigens that were responsible for the pulmonary eosinophilia and lymphocytosis, or both.

Recent evidence has implicated the excretory-secretory proteins released by adult parasites as the dominant antigens not only inducing a Th-2 response to N.b. but also to bystander antigens that may be introduced at the same time as N.b. infection. Thus immunization of a number of murine strains with N.b. excretory-secretory proteins (NES) has been shown to increase the production of IL-4, total serum IgE and specific IgG1 antibodies. This occurs whether NES is administered with or without Freund's adjuvant, which normally favors a Th1 response. Thus, NES possesses intrinsic adjuvanticity. Furthermore, co-administration of hen egg lysozyme (HEL) with NES in the absence of other adjuvants results in generation of HEL-specific Th-2 response indicating that NES can act as an adjuvant for third-party antigens (Holland MJ et al 2000).

This series of experiments was designed to test the hypothesis that adult antigens are more important than larval antigens in the generation of the pulmonary inflammatory response. The identification of the key N.b. antigens could help identify what antigens could be isolated from N.b. to drive future developments of the N.b. model. For example, N.b. antigens could be nebulised directly into the airways in a manner similar to OA-driven models. In order to determine which are the key antigens ivermectin was administered orally to naïve and infected mice. Ivermectin is a potent macrolytic lactone that causes paralysis in many nematodes by inducing an influx of chloride ions across cell membranes. Ivermectin was administered after larvae had migrated through the lungs and before adult worms became established in the gut. Therefore effectively removing adult antigen exposure. Therefore if ivermectin abolished the pulmonary eosinophilia it could be concluded that the pulmonary changes were due to the adult worm antigens, which had been ablated. If, however, ivermectin did not abolish the pulmonary eosinophilia then it could be concluded that the airway changes were due to the larval antigens. If the pulmonary eosinophilia was only partially abolished this would suggest that both sets of antigens may be involved.

5.2 MATERIALS AND METHODS

5.2.1 Larval culture and infection

Details of N.b. larval culture are given in chapter 2.

5.2.2 Animals

Details of animal husbandry are given in chapter 2.

5.2.3 Ivermectin dosage schedule assessment

In order to determine the optimum dose of ivermectin that would inhibit the development of the adult worm but not cause toxicity to the mice three different dosage regimes were assessed, namely 2.5mg/kg, 5mg/kg and 7.5mg/kg. Doses of ivermectin were made up fresh each day and administered orally in a volume of 100µl, assuming an average weight of 21gms/mouse. There were three mice per dosage group and the intestines of all three mice were pooled to determine adult worm counts.

In addition, four different dosage days were assessed in order to determine the optimum day on which to administer the ivermectin. Thus ivermectin was given on day 1, 2, 3 and 4 pi. All mice were killed on day 8 pi, which is when adult worm population in the gut should be at its peak. There were three mice per time point and the intestines of all three mice were pooled to determine adult worm counts.

Table 2. Ivermectin Dosage Schedule

Group	**Ivermectin dosage day**				**ORAL DOSE**	Nos. killed on day 8
A	D1				2.5mcg/kg	3
B	D1				5.0mg/kg	3
C	D1				7.5mg/kg	3
D		D2			2.5mcg/kg	3
E		D2			5.0mg/kg	3
F		D2			7.5mg/kg	3
G			D3		2.5mcg/kg	3
H			D3		5.0mg/kg	3
I			D3		7.5mg/kg	3
J				D4	2.5mcg/kg	3
K				D4	5.0mg/kg	3
L				D4	7.5mg/kg	3

On day 8 pi all mice were killed and the small intestines were removed, from the duodenal bulb to the caecum. The intestines from each group A-L were pooled. Longitudinal incisions were made in the small intestines and the everted gut was placed on top of three layers of muslin in a plastic champagne glass containing RPMI. These glasses were then incubated at 37 ^{0}C for three hour. Adult N.b. worms migrated, under the force of gravity, into the base of the stem from where they were subsequently recovered. They were then washed thoroughly in RPMI containing 10% pen/strep, prior to resuspending in RPMI + pen/strep diluted w/v 1:5. Worm numbers were then estimated by microscopy.

5.2.4 Bronchoalveolar lavage and cell preparations

Details of brochoalveolar lavage and cell preparation are given in chapter 2. Data presented throughout this thesis are given per ml of BAL fluid drawn.

5.2.5 Blood: Leukocyte subsets

Details of blood acquisition and cell analysis are given in chapter 3. Data presented throughout this thesis are given per ml of blood drawn.

5.2.6 Spleen mononuclear preparations

Three to five spleens/group across three separate experiments were removed aseptically into RPMI 1640 (GIBCO) with 5% FBS. Cells were dissociated, centrifuged at 1500 rpm for 10-12 mins, and RBCs were lysed using tris buffered 0.84% NH_3Cl. Cells were then washed, centrifuged and resuspended in 10 mls of RPMI with FBS, 50μM 2-ME, 100U/ml pen, 100 μg/ml strep 2mM L-Glutamine, 1mM sodium pyruvate (GIBCO) ready for counting using a haemocytometer. Splenocytes were loaded into cell culture plates (Costar #3690) previously coated with a range of concentrations of N.b. adult antigen, concavalin A and anti-CD3 at a concentration of 2×10^6 cells/well for the measurement of cytokine production and 0.5×10^6 cells/well for cell proliferation studies. Cell culture plates were then incubated for approximately 64 Hrs (37 ^{0}C with 5% CO_2) prior to pulsing with 1μCu/well of tritiated thymidine. Cell proliferation plates were harvested 8 hours after pulsing onto glass fibre and radioactivity was determined using the Betaplate system (Pharmacia/LKB, Pictaway N.J.).

5.2.7 Cytokine ELISAs

Costar 96 well half area EIA plates (Costar #3690) were coated overnight at 4 ^{0}C with 50μl/well of anti-IL-5 (1μg/ml) and anti-IFN-γ (1μg/ml) (PharMingen, San Diego, CA). Plates were washed using Dulbecco's PBS plus 0.05% Tween 20, pH 7.2 (PBS/TW), and blocked for 1hr at room temperature (RT) with PBS/TW plus 3% Bovine Serum Albumin (BSA). Standard curves were constructed for each cytokine and unknown samples were loaded at 50μl/well and plates incubated at RT for 3-4hr. Biotin-labeled anti-cytokine antibodies (PharMingen, San Diego, CA) were diluted in blocker to a concentration of 2μg/ml and loaded into washed plates at 50μl/well prior to incubation at RT for 1hr. Streptavidin-horseradish peroxidase (Southern Biotechnology Associates) was diluted in blocker 1:2000 and loaded into washed plates at 50μl/well prior to incubation at RT for 30 min. ABTS solution (Kirkegaard & Perry Labs) was mixed and added to washed plates at 50μl/well prior to incubation at RT for 60min (30min for IFN-γ). Plates were then read on a microtiter plate reader at 405 nm.

5.2.8 Generation of N.b. specific antigen

Adult SD rats were killed 8 days pi and the small intestines were removed. Longitudinal incisions were made in the small intestines and the everted gut was placed on top of three layers of muslin in a plastic champagne glass containing RPMI. These glasses were then incubated at 37 ^{0}C for three hour. Adult N.b. worms migrated, under the force of gravity, into the base of the stem from where they were subsequently recovered. They were then washed thoroughly in RPMI containing 10% pen/strep, prior to resuspending in RPMI + pen/strep diluted w/v 1:5. Worms were homogenized by hand, and settled prior to high-speed centrifugation and protein filtration using a 0.45μ filter. The concentration of N.b. adult antigen was calculated using a standardized Bradford Biorad protein assay.

5.2.9 Statistics

The data presented in this chapter on wild type C57BL/6 mice that were not treated with ivermectin are pooled from several experiments conducted throughout this thesis. Thus the peripheral blood and BAL fluid data presented contains, on average, 38 mice per time point. The ivermectin treated mice were pooled from three experiments and contained, on average, 15 mice per time point. The data was analysed using a two-tailed paired t test assuming unequal variance in samples, but a normal distribution. When this overall test of treatment was found to be significant at the $p<0.05$, this was indicated by an asterisk (*). When the significance level reached $p<0.01$ this was indicated by two asterisks (**), and when the significance level reached $p<0.001$ this was indicated by three asterisks (***). If the result was borderline significant ($p=0.05$), then this was denoted by a # sign.

5.3 RESULTS

5.3.1 Ivermectin dosage schedule

Ivermectin given on day 1,2 or 3 all stopped the development of adult worms in the intestines. Ivermectin administered on day 4 pi largely stopped adult worm development, although a few adult worms were recovered from the pooled intestinal preparations. In addition, all three dosage regimes were sufficient to inhibit worm development without any obvious signs of distress or

toxicity to the mice. Therefore the smallest dose, namely 2.5mg/kg, was used in all subsequent experiments and ivermectin was administered on day 3 pi once the larval migration had become established in the lungs. The rationale for choosing day 3 was that the mice would receive maximum exposure to larval but not adult antigens, whereas day 1 administration might inhibit larval antigen exposure to some extent.

5.3.2 Time course of peripheral blood changes following N.b. infection

I. Total leucocyte count

Primary infection with third stage N.b. larvae produced a significant increase in the total white cell count (WCC) in WT mice during the late phase response, which peaked on day 14 (chapter 3). Similarly, in this series of experiments the total WCC increased significantly from 7.43 $x10^6$ to 12.14 $x10^6$ following N.b. infection in untreated C57BL/6 mice ($p<0.001$). In contrast, treating the mice with ivermectin on day 3 pi completely abolished this increase in the WCC. In fact the WCC was slightly lower in infected ivermectin treated mice compared to naïve ivermectin treated mice, 8.48 $x10^6$ and 8.0 $x10^6$ respectively ($p<0.58$). Consequently, the WCC on day 14 pi in untreated mice was significantly greater than that on day 14 pi in ivermectin treated mice ($p<0.003$). Ivermectin had no effect on the WCC in naïve mice ($p<0.24$) (Figure 5-1).

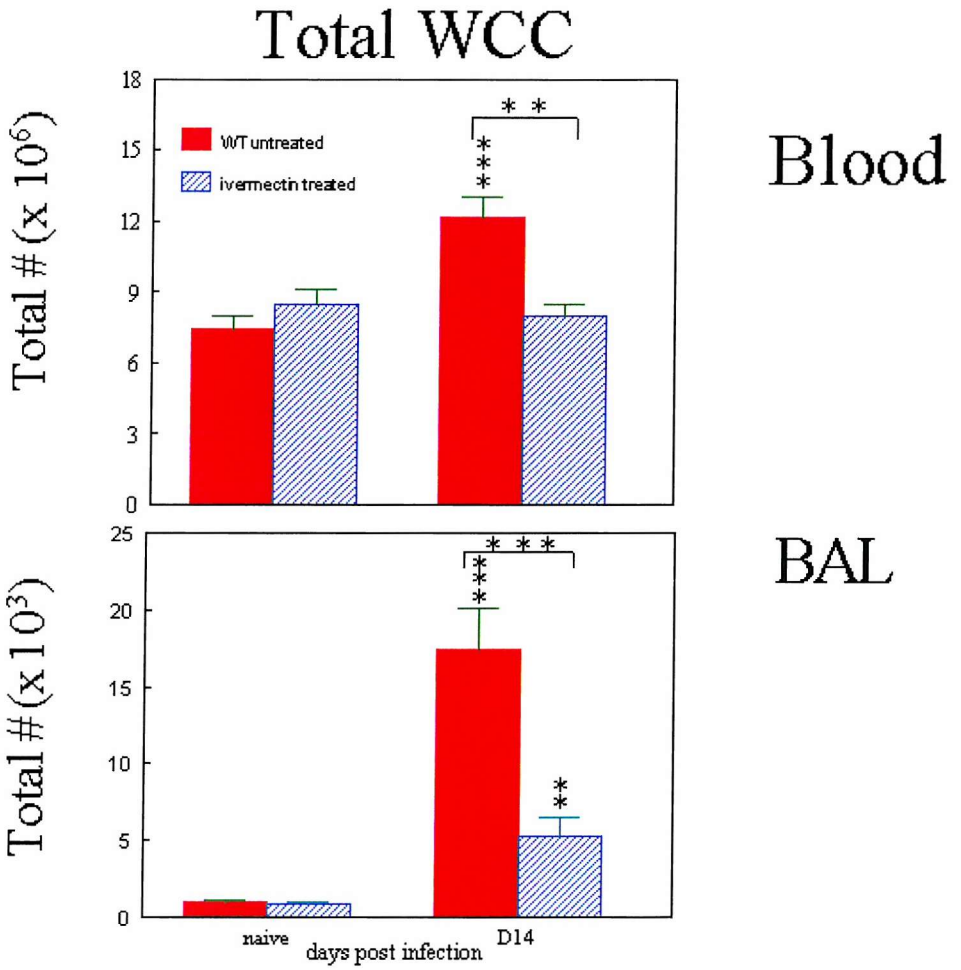

Figure 5-1. The total number and percentage of leukocytes recruited to the blood and airways in ivermectin treated and untreated wild-type control mice following N.b. infection.

II. Eosinophils

The most significant change in the peripheral blood of WT, or genetically manipulated mice following N.b. infection was the increase in the number and percentage of circulating eosinophils (chapters 3-6). This peripheral eosinophilia was again seen here with eosinophil numbers increasing significantly from 0.14 x10^6 in naïve untreated mice to 1.71 x10^6 in untreated mice on day 14 pi ($p<0.001$). In contrast, the increase in circulating eosinophils following N.b. infection was virtually abolished in mice treated with ivermectin. The eosinophil count in ivermectin treated mice increased from 0.12 x10^6 in naïve treated mice to 0.32 x10^6 in infected mice treated with ivermectin ($p<0.003$)(Figure 5-2).

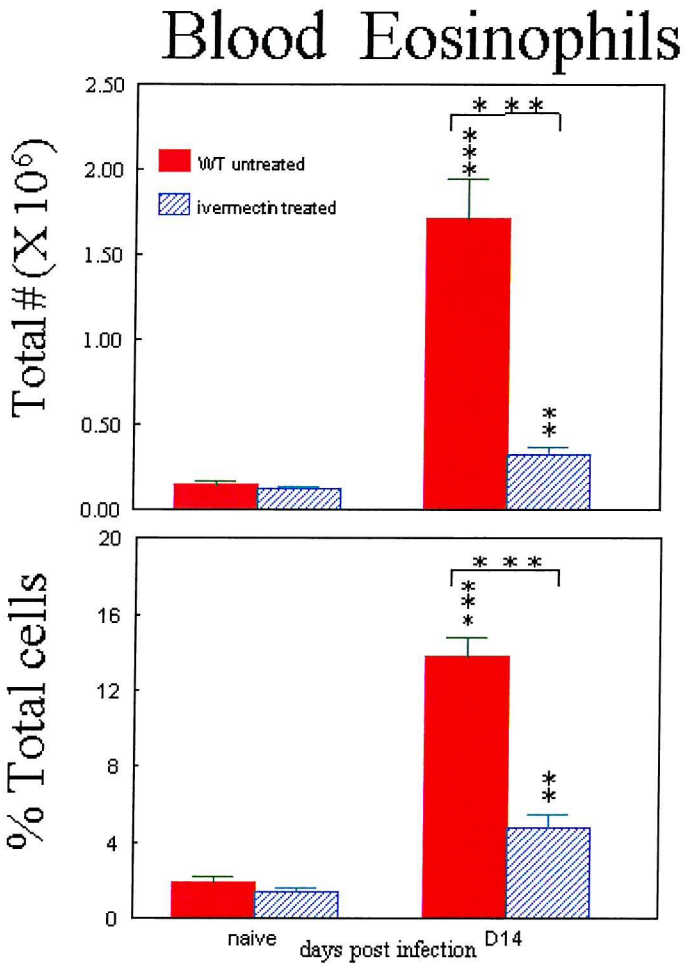

Figure 5-2. The total number and percentage of eosinophils in the blood in ivermectin treated and untreated wild-type control mice following N.b. infection.

This substantial reduction in the peripheral eosinophil count following ivermectin treatment resulted in a significant difference in the eosinophil numbers in treated compared to untreated mice on day 14 pi ($p<0.001$). Ivermectin treatment had no discernable effect on the eosinophil count in uninfected mice ($p<0.37$)(Figure 5-2).

The reduction in the peripheral eosinophilia count following ivermectin treatment in infected mice was reflected by changes in the percentage of eosinophils in the peripheral blood. Specifically, the percentage of eosinophils increased significantly in untreated mice from 1.91% in naïve mice to 13.76% by day 14 pi ($p<0.001$). Again treatment with ivermectin significantly reduced this increase in the percentage of eosinophils in the peripheral blood. Thus in ivermectin treated mice

the percentage of eosinophils increased from 1.43% in naïve ivermectin treated mice to only 4.73% by day 14 pi ($p<0.003$). Consequently there was a significantly greater percentage of eosinophils in the peripheral blood in untreated mice compared to ivermectin treated mice on day 14 pi ($p<0.001$). Ivermectin made no difference to eosinophil levels in uninfected mice (Figure 5.2).

III. Neutrophils

Infection with N.b. larvae produces changes in the number and percentage of circulating neutrophils in the peripheral blood. Neutrophil numbers increase during the early phase response and peak somewhere between day 3 and day 8 pi (chapter 3-6). By day 11 pi the neutrophilia has largely, though not always completely, subsided. In this experiment the number of neutrophils in the peripheral blood of uninfected mice was still elevated on day 14 pi. Specifically numbers increased from to 0.58×10^6 in naïve untreated mice to 1.29×10^6 by day 14 pi ($p<0.01$). However, in ivermectin treated mice there was an almost identical number of neutrophils in naïve and infected ivermectin treated mice, 0.62×10^6 in and 0.63×10^6 respectively.

Since there was no increase in neutrophil numbers following N.b. infection in ivermectin treated mice, the number of neutrophils in the peripheral blood of untreated mice was significantly greater on day 14 pi than neutrophil numbers in ivermectin treated mice ($p<0.01$). Ivermectin made no difference to neutrophil numbers in uninfected mice (Figure 5-3).

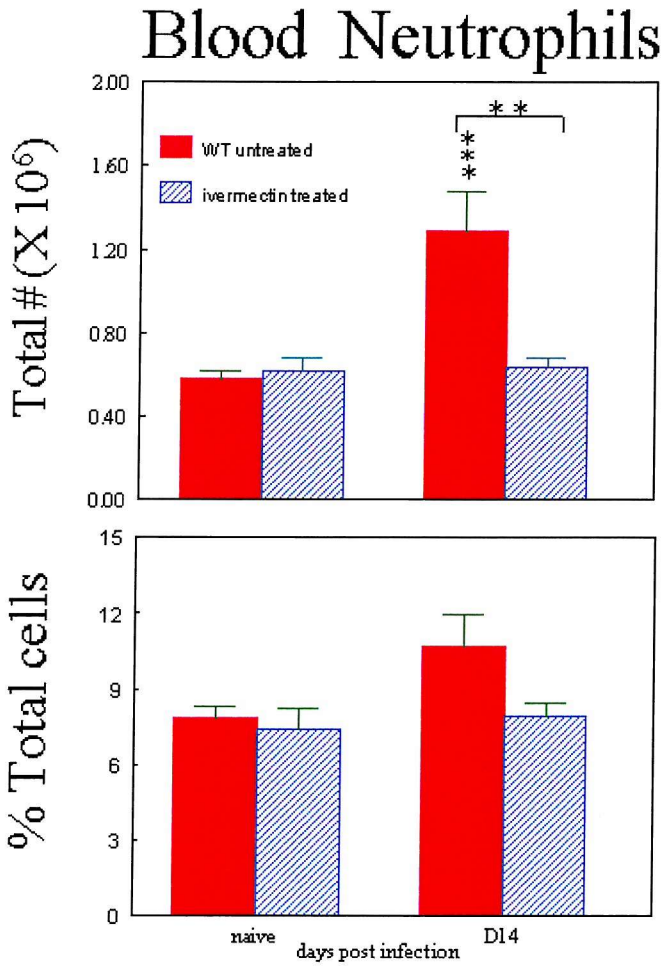

Figure 5-3. The total number and percentage of neutrophils in the blood in ivermectin treated and untreated wild-type control mice following N.b. infection.

In contrast to the changes in neutrophil numbers there was no difference in the percentage of neutrophils in the peripheral blood of untreated or ivermectin treated mice following N.b. infection. Specifically, the percentage of neutrophils in untreated mice rose from 7.86% in naïve untreated mice to 10.66% on day 14 pi, but this was not statistically significant ($p<0.08$). In addition, the percentage of neutrophils in the blood of ivermectin treated mice rose from 7.41% in naïve ivermectin treated mice to 7.94% on day 14 pi. Therefore there was no significant difference in the percentage of neutrophils in the blood of naïve treated and naïve untreated mice, or infected treated and infected untreated mice ($p<0.09$) (Figure 5-3).

IV. Lymphocytes

The number of circulating lymphocytes in the peripheral blood of WT mice increased significantly following N.b. infection peaking on day 14-19 pi (chapter 3). Following N.b. infection the untreated mice in this experiment also developed a peripheral lymphocytosis. Specifically, lymphocyte numbers increased significantly from 6.5 $x10^6$ in naïve untreated mice to 8.6 $x10^6$ in infected untreated mice ($p<0.02$). Ivermectin treatment abolished this lymphocytosis. Therefore lymphocyte numbers actually decreased following N.b. infection in treated mice from 7.48 $x10^6$ in naïve ivermectin treated mice to 6.7 $x10^6$ in infected ivermectin treated mice. This reduction in circulating lymphocyte numbers in ivermectin treated mice compared to the increase in lymphocyte numbers in untreated mice resulted in there being significantly more lymphocytes in the blood of untreated mice compared to treated mice on day 14 pi ($p<0.02$)(Figure 5-4).

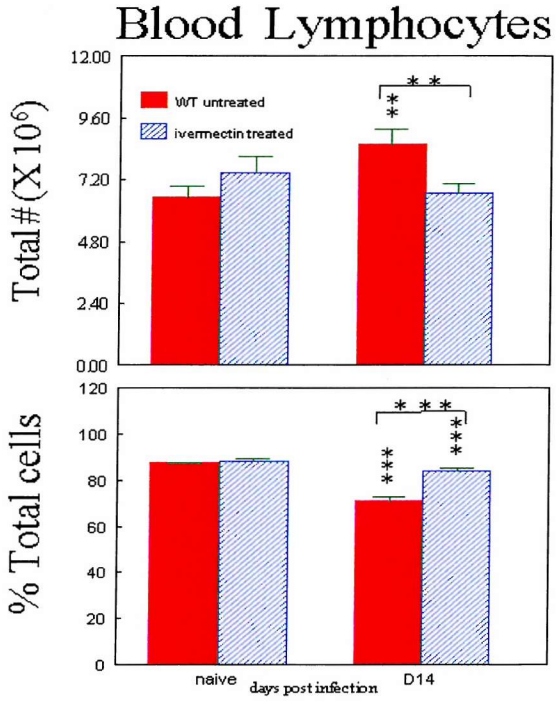

Figure 5-4. The total number and percentage of lymphocytes in the blood in ivermectin treated and untreated wild-type control mice following N.b. infection.

There was no difference in the number of lymphocytes in the blood of naïve treated and naïve untreated mice.

The significant increase in the proportion of eosinophils in the peripheral blood of infected mice following N.b. infection results in a significant decrease in the proportion of lymphocytes in the blood particularly on day 14 pi (chapter 3). This was also seen in this experiment with untreated mice showing a significant reduction in the percentage of lymphocytes in the blood on day 14 pi. Specifically the percentage of lymphocytes dropped from 87.37% in naïve untreated mice to 71.19% in infected untreated mice ($p<0.001$). In contrast, because ivermectin treatment largely eradicated the peripheral blood eosinophilia following N.b. infection there was less of a reduction in the proportion of lymphocytes in the peripheral blood of these mice. Specifically, the percentage of lymphocytes in the peripheral blood of ivermectin treated mice only dropped from 87.97% in naïve mice to 83.9 ($p<0.02$). Consequently there was a significantly greater percentage of lymphocytes in the blood of ivermectin treated mice on day 14 pi compared to the proportion of lymphocytes in the blood of untreated mice on day 14 pi ($p<0.001$). The proportion of lymphocytes in the blood of naïve treated, and untreated mice, was virtually identical (Figure 5-4).

V. Monocytes

The increase in eosinophil numbers during the late phase response is mirrored by the increase in monocyte numbers in WT mice. There was also a significant increase in monocyte numbers in this experiment following N.b. infection. Specifically, monocyte numbers increased from 0.13 $x10^6$ in naïve untreated mice to 0.23 $x10^6$ in infected untreated mice ($p<0.02$).

However, ivermectin treatment abolished the increase in monocyte numbers following N.b. infection. There was, therefore, very little difference in monocyte numbers between naïve ivermectin treated mice and infected ivermectin treated mice, 0.14 $x10^6$ in naïve and 0.16 $x10^6$ in infected mice respectively ($p<0.69$). Ivermectin treatment also had no impact on monocyte numbers in naïve treated compared to naïve untreated mice (Figure 5-5). But despite abolishing the increase in monocyte numbers in the ivermectin treated group this did not produce a statistically significant difference between day 14 mice treated with ivermectin (Figure 5-5).

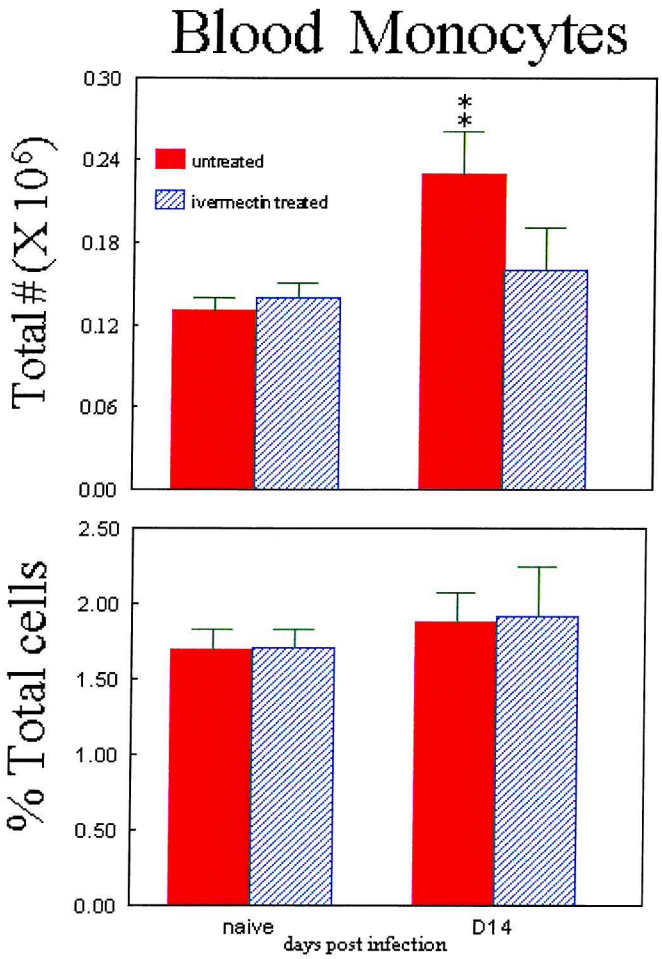

Figure 5-5. The total number and percentage of monocytes in the blood in ivermectin treated and untreated wild-type control mice following N.b. infection.

There was very little difference in the percentage of monocytes in the peripheral blood of naïve untreated and naïve ivermectin treated mice, 1.69% and 1.7% respectively. This low proportion of monocytes in the blood did not change much after N.b. infection, rising in untreated mice to 1.87% and in ivermectin treated mice to 1.91%. Therefore there was no difference in the proportion of monocytes in the blood of treated and untreated mice following N.b. infection (Figure 5-5).

5.3.3 Time course of leukocyte infiltration into the lung following N.b. infection

I. Total Leukocyte Count

In WT mice there was a biphasic cellular response in the BAL fluid following N.b. infection (chapter 3). This consisted of an early cellular influx into the airways during larval migration through the lungs, primarily on day 3 pi, followed by a late phase response peaking on day 14 pi. This cellular recruitment to the airways was seen in both untreated and ivermectin treated mice.

Specifically the total number of leukocytes in the airways of untreated mice rose from 0.893 x 10^3 in naïve mice to 17.45 x 10^3 in infected untreated mice ($p<0.001$)(Figure 5-1). This cellular recruitment was substantially reduced by ivermectin treatment. Thus, leukocyte numbers increased from 0.817 x 10^3 in naïve ivermectin treated mice to only 5.2 x 10^3 in infected ivermectin treated mice ($p<0.02$). This represented a significant reduction in leukocyte recruitment to the airways ($p<0.003$). The level of leukocytes recovered from the BALF of naïve untreated and naïve ivermectin treated mice was not statistically different (Figure 5-1).

II. Eosinophils

The cellular response in the lungs following N.b. infection was dominated by a significant increase in the number and percentage of eosinophils in the BAL fluid in WT mice (chapter 3). This airway eosinophilia was again seen in this experiment. Specifically, the number of eosinophils in the airways of untreated mice increased very significantly from 0.27 x 10^3 in naïve mice to 1.32 x10^6 on day 14 pi ($p<0.001$). However, this eosinophil recruitment was significantly reduced by ivermectin treatment. Thus, eosinophil numbers increased from 0.13 x 10^3 in naïve ivermectin treated mice to 0.30 x10^6 on day 14 pi ($p<0.03$). Therefore the number of eosinophils in the BALF of infected but untreated mice was significantly greater than the number of eosinophils in the BAL fluid of infected but ivermectin treated mice ($p<0.001$)(Figure 5-6).

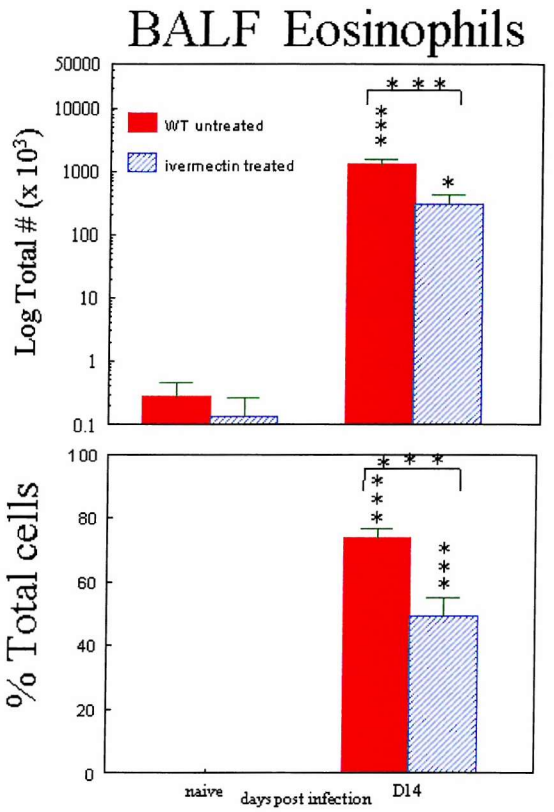

Figure 5-6. The total number and percentage of eosinophils in the BAL fluid in ivermectin treated and untreated wild-type control mice following N.b. infection. Eosinophil counts are given on a log scale for visual representation purposes only.

Ivermectin treatment had no effect on the number of eosinophils recovered from the BAL fluid of naïve mice compared to untreated mice.

The changes in airway eosinophil numbers following N.b. infection were reflected in changes in the percentages of eosinophils recovered from the BAL fluid. Specifically, the percentage of eosinophils in the airways of untreated mice increased significantly from 0.2% in naïve untreated mice to 74% in infected untreated mice ($p<0.001$). However, the increase in the percentage of eosinophils in the airways of ivermectin treated mice was less dramatic. The proportion increased from 0.1% in naïve treated mice to 49.2% in infected ivermectin treated mice ($p<0.001$). Although the percentage of eosinophils in the BAL fluid did not differ in naïve untreated and naïve ivermectin treated mice, by day 14 pi there was a significantly greater percentage of eosinophils in the BAL fluid of untreated mice compared to ivermectin treated mice ($p<0.001$)(Figure 5-6).

III. Neutrophils

In WT mice a biphasic cellular recruitment to the airways was seen. This was characterised by a significant influx of neutrophils to the airways during the "early phase" response peaking on day 3 pi, during larval migration through the lungs (chapter 3). By day 14 pi this early phase response had largely, but not always subsided. In this experiment there was no day 3 time-point. Nevertheless, the neutrophil influx had not quite subsided by day 14 pi. Specifically the number of neutrophils in the BAL fluid of untreated mice on day 14 pi was still significantly greater than the number seen in naïve untreated mice, 33.4 x 10^3 and 0.53 x 10^3 respectively ($p<0.01$).

Similarly, the number of neutrophils in the BAL fluid of ivermectin treated mice increased significantly from an undetectable level in naïve ivermectin treated mice to 7.8 x 10^3 in infected ivermectin treated mice ($p<0.04$). However, this neutrophilia was significantly less in the ivermectin treated group compared to the untreated controls ($p<0.03$). Neutrophil numbers in naïve treated and untreated mice were comparable (Figure 5-7).

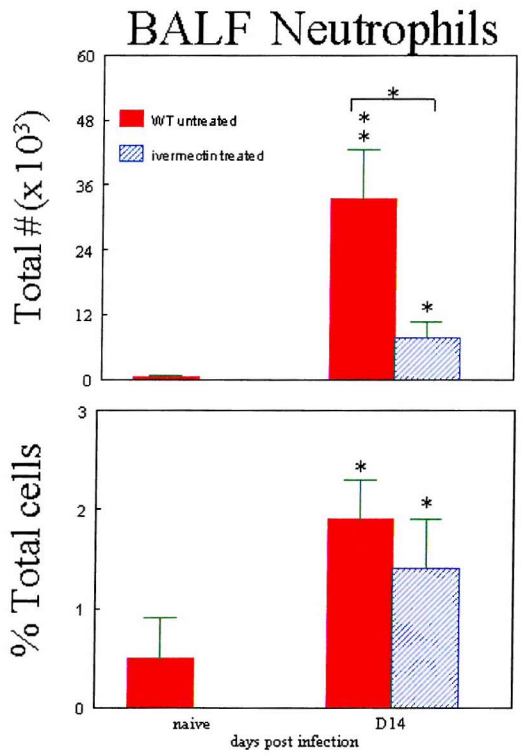

Figure 5-7. The total number and percentage of neutrophils in the BAL fluid in ivermectin treated and untreated wild-type control mice following N.b. infection.

The percentage of neutrophils recovered from the BAL fluid of treated and untreated mice never rose above 2% throughout the experiment. However when comparing naïve and infected mice there was a small yet statistically significant increase in the proportion of neutrophils in the BAL fluid following N.b. infection in both treated and untreated mice. Specifically, the percentage rose from 0.5% to 1.9% in untreated mice ($p<0.04$) and from 0% to 1.4% in ivermectin treated mice ($p<0.03$). The percentage of neutrophils in naïve treated or untreated mice did not differ, neither did the percentage of neutrophils in infected treated or untreated mice (Figure 5-7).

IV. Lymphocytes

During the late phase response there was a significant increase in lymphocyte numbers in WT mice (chapter 3). This was also seen in both untreated and ivermectin treated mice. Specifically the number of lymphocytes in the BAL fluid of untreated mice increased significantly following N.b. infection from 4.08 x 10^3 in naïve untreated mice to 234 x 10^3 on day 14 pi ($p<0.001$). In contrast, the lymphocyte recruitment to the BAL fluid in ivermectin treated mice was significantly smaller with lymphocyte numbers increasing from 1.57 x 10^3 in naïve treated mice to 103 x 10^3 on day 14 pi ($p<0.006$). Thus there were significantly more lymphocytes in the BAL fluid of untreated mice compared to treated mice on day 14 pi ($p<0.02$)(Figure 5-8).

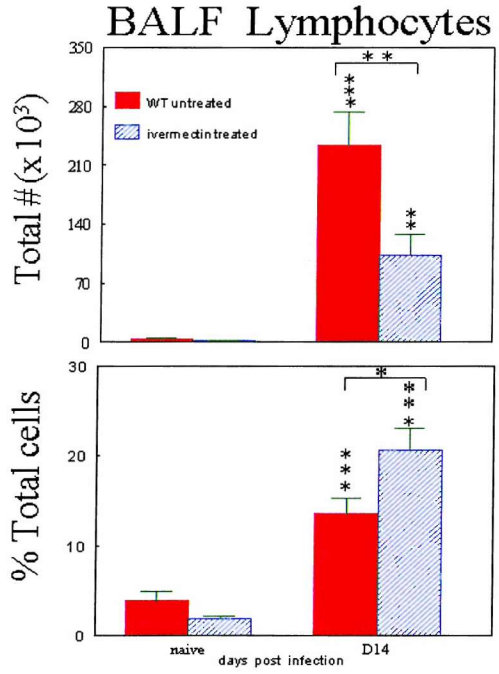

Figure 5-8. The total number and percentage of lymphocytes in the BAL fluid in ivermectin treated and untreated wild-type control mice following N.b. infection.

This increase in the lymphocyte recruitment during the late phase response in both treated and untreated mice, was reflected in changes in the percentages of lymphocytes recovered from the BAL fluid. However, since the recruitment of eosinophils to the BAL fluid was very significantly reduced by ivermectin treatment, the proportion of lymphocytes in the BAL fluid following N.b. infection was relatively greater in ivermectin treated mice compared to untreated controls ($p<0.03$). Specifically, the percentage of lymphocytes recovered from the BAL fluid of untreated mice increased from 3.9% in naïve mice to 13.5% on day 14 pi ($p<0.001$). This increase was much greater in ivermectin treated mice rising from 1.8% in naïve mice to 20.6% on day 14 pi ($p<0.001$). Ivermectin treatment had no effect on the proportion of lymphocytes recovered from naïve mice (Figure 5-8)

V. Monocytes

Following N.b. infection there was an increase in monocyte numbers in ivermectin treated and untreated mice. Specifically the number of monocytes increased from 86.2×10^3 in naïve untreated mice to 192×10^3 on day 14 pi, although this did not quite reach statistical significance ($p<0.07$). The number of monocytes in the BAL fluid of ivermectin treated mice also increased from 81.7×10^3 in naïve treated mice to 116×10^3 on day 14 pi ($p<0.03$). Treatment with ivermectin again appeared to reduce the monocyte recruitment to the BAL fluid, although there was no statistical difference between the number of monocytes in naïve or infected mice comparing the treated and the untreated groups (Figure 5-9).

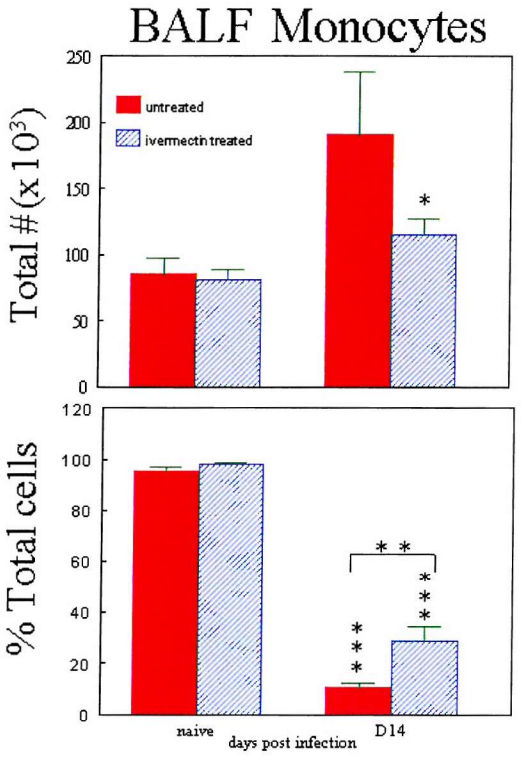

Figure 5-9. The total number and percentage of monocytes in the BAL fluid in ivermectin treated and untreated wild-type control mice following N.b. infection.

In contrast, there was a significant reduction in the percentage of monocytes in the BAL fluid of both ivermectin treated and untreated mice, reflecting the recruitment of other cellular populations to the BAL fluid following N.b. infection. Specifically, the percentage of monocytes in the BAL fluid of untreated mice decreased from 95.4% in naïve mice to 10.6% on day 14 pi ($p<0.001$). Similarly, the percentage of monocytes in the BAL fluid of ivermectin treated mice decreased from 98.1% in naïve mice to 28.6% on day 14 pi ($p<0.001$). Since there were fewer eosinophils and lymphocytes recruited to the airways of ivermectin treated mice the reduction in the proportion of monocytes on day 14 pi was less dramatic than in untreated controls. This resulted in a significantly greater proportion of lymphocytes in the BAL fluid of ivermectin treated mice on day 14 pi compared to untreated controls ($p<0.02$). Ivermectin had no effect on the proportion of monocytes in the airways of naïve mice (Figure 5-9).

5.3.4 Splenocyte proliferation

I. To N.b.-specific antigen

Spleen cells were stimulated with a variety of specific and non-specific mitogens. Ranges of specific and non-specific antigen doses were used since the optimum conditions for splenocyte proliferation in response to antigen were unknown. The response of splenocytes to N.b. specific antigen following N.b. infection was compared in ivermectin treated and untreated mice (Figure 5-10).

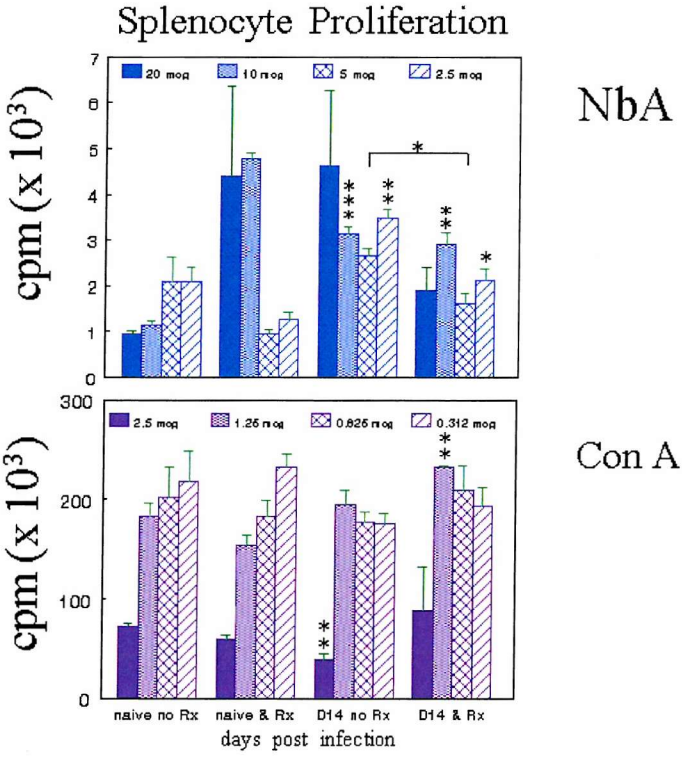

Figure 5-10. Splenocyte proliferative response to stimulation with N.b. specific antigen (NbA) and Con A in ivermectin treated (Rx) and untreated wild-type control mice following N.b. infection.

This showed that there was a significant response to N.b. specific antigen in untreated splenocytes after they had been sensitized by N.b. infection, while the response of ivermectin treated splenocytes was variable.

Specifically there was an increase in the proliferative response to N.b. antigen in untreated mice at 10mcg/well ($p<0.001$) and 2.5mcg/well ($p<0.03$). While there was a greater splenocyte proliferative response at the other two dosages, 20mcg/well and 5mcg/well, following N.b.

infection this did not reach statistical significance. Overall there was a significant increase in the mean proliferative response in infected untreated mice compared to naïve untreated mice ($p<0.01$)(Figure 5-10).

Ivermectin abolished this increase in the proliferative response in infected splenocytes. Thus the mean proliferative response in ivermectin treated splenocytes following N.b. infection was no greater than in naïve treated splenocytes ($p<0.54$). Looking at the specific doses of N.b. antigen, there was a decrease in the proliferative response following N.b. infection in splenocytes stimulated with 20mcg/well and 10mcg/well, although only the latter reached statistical significance ($p<0.008$). In contrast, there was an increase in the proliferative response of splenocytes following N.b. infection at the smaller doses of 5mcg/well and 2.5mcg/well. But again only the smaller dose reached statistical significance ($p<0.03$) (Figure 5-10).

Comparing the mean proliferative response in untreated and ivermectin treated mice, following N.b. infection, there was a significantly greater proliferative response in the untreated mice suggesting that ivermectin impaired splenocyte proliferation ($p<0.04$)(Figure 9-10). In detail this greater proliferative response in untreated murine splenocytes, following N.b. infection occurred at 10mcg/well ($p<0.001$), 5mcg/well ($p<0.02$), and 2.5mcg/well ($p<0.01$) but not at 20mcg/well.

The proliferative response of naïve untreated and naïve ivermectin treated splenocytes did not differ except at 10mcg/well ($p<0.001$). This result was not in keeping with the other responses at different doses and did not affect the mean proliferative response in naïve ivermectin treated mice which did not differ from untreated mice (Figure 5-10).

II. To non-specific Antigen:

Spleen cells were also stimulated with two different non-specific mitogens, ConA and anti-CD3. Different doses of ConA, but not anti-CD3, were used since the optimum conditions for splenocyte proliferation in response to this mitogen were unknown. The response of splenocytes to ConA was compared in ivermectin treated and untreated mice (Figure 5-10). This showed that, unlike the response to specific antigen there was no real increase in the mean proliferative response in infected ivermectin treated and untreated mice compared to naïve ivermectin treated and untreated mice.

This lack of an effect with non-specific antigen was borne out by the splenocyte proliferative response to specific doses of ConA. Specifically, there were very few differences in the proliferative response of ivermectin treated and untreated murine splenocytes in infected mice compared to naïve mice. There were two examples where this finding was not true. Thus at 2.5mcg/well ($p<0.01$) in the untreated group the proliferative response was reduced. And at 1.25mcg/well in the ivermectin treated group where the proliferative response was significantly increased. In the absence of changes at the other dosages and no change in the mean proliferative response the overall importance of these results is likely to be minimal (Figure 5-10).

The splenocyte proliferative response to stimulation with a-CD3 in ivermectin treated mice was unchanged following N.b. infection. In addition, there was no difference in the proliferative response of naïve mice, comparing ivermectin treated and untreated mice. However, there was a significant reduction in the proliferative response of untreated splenocytes on day 14 pi compared to naïve untreated splenocytes ($p<0.02$). This resulted in a significant difference in the proliferative response in treated and untreated mice on day 14 pi ($p<0.02$), but the importance of this result is unclear (Figure 5-11).

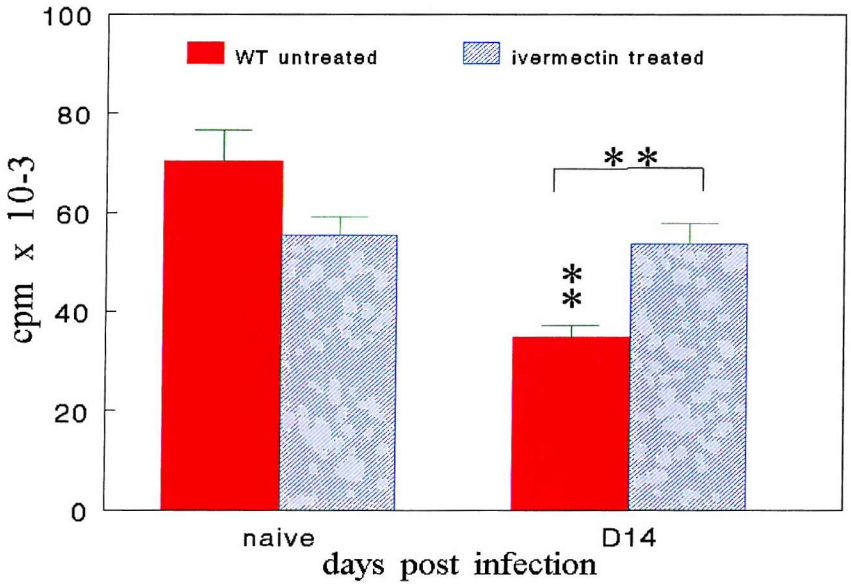

Figure 5-11. Splenocyte proliferative response to stimulation with anti-CD3 in ivermectin treated and untreated wild-type control mice following N.b. infection.

5.3.5 Splenocyte cytokine production in response to N.b.-specific antigen

The cytokine production, in response to stimulation with N.b. specific antigen, of ivermectin treated and untreated murine splenocytes on day 14 pi was compared with the cytokine production of naïve ivermectin treated and untreated murine splenocytes.

Spleen cells were stimulated with N.b. specific antigen and the production of the Th-1 cytokine IFN-γ and the Th-2 cytokine IL-5 were measured in naïve and infected mice.

There was an increase in splenocyte IFN-γ production in both ivermectin treated and untreated groups following N.b. infection. Specifically IFN-γ production increased from an undetectable level in naïve untreated mice to 1,983pg/ml on day 14 pi ($p<0.02$). There was an even greater increase in IFN-γ production in ivermectin treated mice rising from 441pg/ml in naïve mice to 1,581pg/ml on day 14 pi, but due to the small number of data points in this group this result did not reach statistical significance (Figure 5-12).

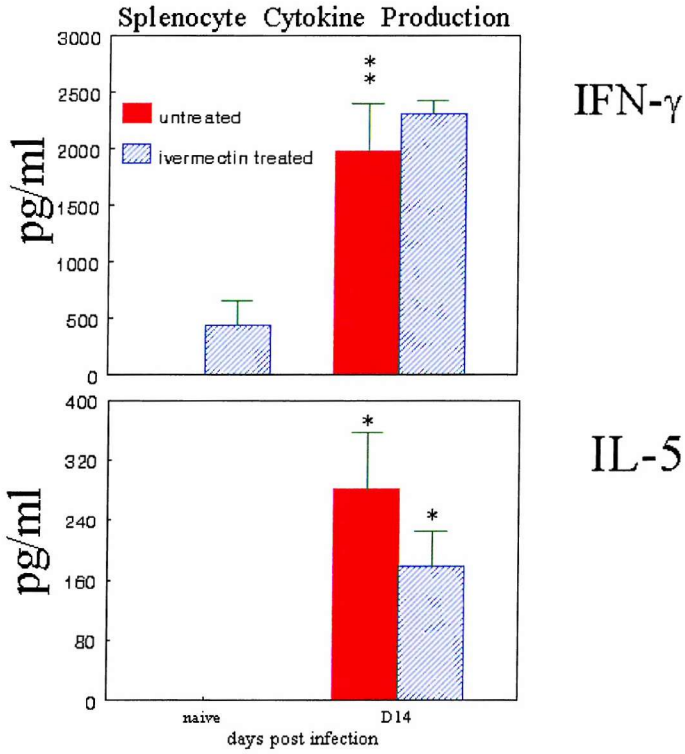

Figure 5-12. Splenocyte IFN-γ and IL-5 production in response to N.b specific antigen in ivermectin treated and untreated wild-type control mice following N.b. infection.

In contrast, the increase in IL-5 production following N.b. infection was much less in ivermectin treated mice compared to untreated controls. Specifically, IL-5 production increased in untreated mice from undetectable levels in naïve mice to 282pg/ml on day 14 pi in untreated mice ($p<0.03$), and to 178pg/ml in ivermectin treated mice ($p<0.03$). However, this difference in IL-5 production following N.b. infection in ivermectin treated and untreated controls did not reach statistical significance (Figure 5-12).

5.4 DISCUSSION

In order to investigate whether adult or larval antigens are the main drivers of the switch to a Th-2 response following N.b. infection we administered the anthelminthic ivermectin to naïve and infected mice. We only examined naïve and infected on day 14 pi, at the peak of the inflammatory response, either with or without ivermectin treatment. Previous research has suggested that drugs such as ivermectin are most effective when they are administered on the same day as nematode infection and their effect diminish progressively thereafter (el-Azzouni MZ et al 1997). However our preliminary results suggested that it made no difference whether the ivermectin was administered on day 1, 2 or 3. Therefore mice were treated with the ivermectin on day 3 pi prior to the maturation of adult worms in the intestine.

Treated mice received 2.5mg/kg of ivermectin as a one-off oral dose on day 3 pi. A single bolus of ivermectin has previously been shown to be effective in maintaining a steady state with consistent plasma and faecal levels (Alvinerie M et al 1999). Although the dosage used here was slightly below the normally recommended rodent dosage our preliminary studies had indicated that this small dose was as effective in abolishing the development of the adult stage as larger dosage regimes and was free from obvious side effects or toxicity.

Three animals from the infected groups were killed on day 8 pi to confirm the efficacy of the ivermectin treatment by examining the number of adult worms in the gastrointestinal tract at this time point. Previous authors have demonstrated that there is a wide dose range that is effective and the exact oral formulation of ivermectin also does not appear to be critical (Daurio CP et al 1992).

In fact ivermectin is extremely well tolerated with little toxicity at normal doses and little impact on the behaviour, body weight, motor coordination, or spatial learning in most murine strains (Davis JA 1999). Nevertheless we decided to use the minimum effective dose in light of the evidence that ivermectin itself can produce a significant decrease in the number of circulating eosinophils, in addition to reducing polyclonal IgG, IgE, and parasite-specific IgG antibody production (Steel C et al 1991). Other authors have shown that ivermectin may reduce T cell responsiveness to a range of antigens and mitogens, at least in sheep (Stankiewicz M 1995). Conversely, some authors have suggested that ivermectin may enhance antibody production and T helper cell and macrophage responsiveness (Uhlir J et al 1992, Uhlir J et al 1991, Blakley BR et al 1991).

We demonstrated that obliteration of N.b. adult antigens by administering a small dose of ivermectin on day 3 pi completely inhibited the normal peripheral leukocytosis. When examining the leukocyte subsets, ivermectin treatment also inhibited the peripheral neutrophilia, lymphocytosis and monocytosis. However, ivermectin failed to completely inhibit the peripheral eosinophilia. These results are in keeping with recent data demonstrating that ivermectin inhibited peripheral eosinophilia but did not inhibit the local recruitment of eosinophils to the skin in patients with onchocerciasis or the release of eotaxin or RANTES (Cooper PJ et al 2000, Cooper PJ et al 1999). In contrast, some authors have demonstrated the opposite effect with ivermectin treatment enhancing local eosinophilia (Wildenburg G et al 1994). So the effects of ivermectin treatment on eosinophil recruitment may be dependent on the model and clinical situation examined.

The results generated in our model suggest that the recruitment of neutrophils, lymphocytes and monocytes to the blood may be dependent on N.b. adult antigen but independent of larval antigens. In contrast, eosinophil recruitment to the blood may not be entirely dependent on N.b. adult antigens since there was still a significant peripheral eosinophilia on day 14 pi in mice treated with ivermectin. Thus eosinophil recruitment to the blood can be partially induced by N.b. larval antigens.

In contrast to the systemic response , the recruitment of leukocytes to the airways appeared to be less dependent on adult larval antigen since there was still a significant leukocytosis in the airways of ivermectin treated mice following N.b. infection.

The significant difference between airway leukocytosis in untreated and treated mice following N.b. infection suggested that adult antigens may be more important than larval antigens in inducing the late phase pulmonary response. This suggestion was borne out by the recruitment of all other cellular subsets to the airways. Thus N.b. infection still resulted in a significant recruitment of eosinophils, neutrophils, lymphocytes and monocytes to the airways of ivermectin treated mice. However, the number of each cell type recruited was significantly greater in untreated mice compared to treated mice. This suggests that recruitment of each cell type is only partially dependent on adult antigen and can also be stimulated by larval antigens. However, adult antigens may be more powerful stimulants to airway recruitment.

The suggestion that adult antigens may be a more powerful stimulus of a Th-2 response than larval antigens was borne out by the splenocyte proliferative response. Thus ivermectin treatment and ablation of adult antigens reduced the proliferative response to specific but not non-specific antigen. Previous research had suggested that ivermectin itself increases T cell proliferative responses to specific antigen, at least in humans, but we found no evidence of this (Steel C et al 1991).

The greater ability of adult antigens to induce a Th-2 response may have been due to the greater production of Th-2 cytokines. This suggestion is borne out by our data demonstrating that the ablation of adult antigens resulted in a lower level of IL-5 production but a higher level of IFN-γ production in ivermectin treated mice. Although this interpretation requires further investigation since the differences in the production of both cytokines was not statistically significant when comparing treated and untreated mice. In addition, there has been some evidence to suggest that ivermectin may actually increase IL-5 levels (Cooper PJ et al 1999), and may not affect IFN-g production (Soboslay PT et al 1992). However, such discrepancies may simply reflect differences in antigen load (Steel C et al 1994).

In conclusion, this series of experiments suggest that N.b. larval antigens are capable of inducing a systemic and pulmonary Th-2 response in this mouse model. However, adult N.b. antigens appear to be amore powerful Th-2 stimulus. The investigation of the factors involved in inducing the Th-2 switching in allergic inflammation of the airways is complex. These data may help identify N.b. antigen that could be refined and used as alternative antigenic stimulants for inducing a Th-2 rsponse. Therefore future developments of this model could use aerosolised N.b. antigens as an alternative to aerosolised OA sensitisation models. Such alternative N.b. sensitisation regimes may open up another front against allergic inflammation.

CHAPTER 6

Balb/c Mice

6.1 INTRODUCTION

Over the last twenty years it has become abundantly clear that allergic inflammation of the airways involves a diverse range of cells and a multitude of chemical mediators (Pearlman DS et al 1999). Experiments in animal models and in vitro, as well as an increasing number of studies in atopic human subjects, have revealed that allergic inflammation of the airways is orchestrated by T lymphocytes. Specifically, the Th-2 lymphocyte subset, and their cytokines, interleukin (IL)-4, IL-5, IL-6, IL-9, IL-10, and IL-14, have been widely implicated as the key orchestrator of airway inflammation. Therefore various animals models have been developed which provoke a Th-2 response in the lungs, and elsewhere, enabling a deeper exploration of the allergic inflammatory response.

The two most commonly employed strategies for inducing such a Th-2 response are nebulisation of ovalbumin (OA) to animals sensitised by intraperitoneal injection of OA (Hook et al 2000), or infection with the gastrointestinal nematode Nippostrongylus brasiliensis - N.b. (Watkins AD et al 1996b).

In view of the wide array of immunological probes available to explore the murine immune response the humble mouse has become the rodent of choice in these animal models of allergic inflammation. The mouse has, therefore, replaced the sensitive guinea pig, the rat and larger species in many laboratories around the world.

Since there are wide differences among inbred rat or mouse strains in their susceptibility to develop asthma-like pulmonary changes the choice of strain may be critical to the optimisation of each model (Uchikawa R et al 1996). For example, airway sensitivity to challenge with intravenous methacholine can vary three-fold depending on the mouse strain involved. In addition, in OA-sensitized mice, the percentage of eosinophils recovered from the bronchoalveolar lavage (BAL) fluid can vary from 3% to 91% and pulmonary inflammation can vary from being undetectable to being widespread and severe. Furthermore OA-specific IgE concentrations have been shown to vary from less than 3 ng/ml to 455 ng/ml in different strains. Shifts in responsiveness correlated significantly with pulmonary eosinophilia among strains but not with antigen-specific IgE levels (Brewer JP et al 1999).

Of the numerous studies reported a significant proportion has been conducted in C57BL/6 mice and their genetic variants. However, it has been suggested that C57BL/6 mice are more IL-2 dependent and therefore may to respond a parasite infection in a more Th-1 predominant fashion. As a consequence a number of the existing murine models of asthma may have been less than optimum. This may go some way to explain the criticism that numerous murine models lacking many of the inflammatory and epithelial changes that are typical of the human disease. Moreover, these models are frequently complicated by allergic alveolitis.

In contrast Balb/c mice are believed to be more IL-4 dependent and therefore respond to parasite infections or OA challenge with a more Th-2-driven response and higher IgE production. In fact a series of recent studies demonstrated that BALB/c mice, systemically sensitised to OA and chronically challenged for up to eight weeks with low particle mass concentrations of aerosolised OA, developed histopathological changes typical of human allergic inflammation.

Specifically these Balb/c mice developed a progressive inflammatory response in the airways characterised by the presence of intraepithelial eosinophils, goblet cell hyperplasia, epithelial thickening and subepithelial fibrosis. In addition, the lamina propria was infiltrated with lymphoid/mononuclear cells and there was no associated alveolitis. In parallel, these mice developed increased airway sensitivity and bronchospasm, as well as increased maximal reactivity and high titres of antigen- specific-IgE (Temelkovski J et al 1998).

But generating an animal model with airway hyperreactivity to OA has been a particularly difficult problem and has led some authors to develop genetic strains, such as BP2 'Biozzi' mice, which develop airway hyperresponsiveness after a single antigenic provocation (Vargaftig BB et al 1999). Other authors have persisted with the OA model and have recently been successful in inducing non-specific airway hyperresponsiveness (AHR)(Neuhaus-Steinmetz U et al 2000).

The difficulties experienced by many researchers in inducing airway hyperreactivity may have been strain specific. Thus in an OA-model of airway inflammation, increased pulmonary resistance in response to intravenously delivered methacholine occurred in BALB/c, but not C57BL/6 or B6D2F1 mice.

Furthermore, this AHR was observed despite the fact that OA-challenged BALB/c mice had less airway eosinophilia and smaller increases in total IgE than either C57BL/6 or B6D2F1 mice. Balb/c mice also had less pulmonary inflammation and OA-specific IgE than B6D2F1 mice. Thus the airway sensitivity exhibited by Balb/c mice may closely mimic that seen in humans and may not require significant degrees of airway eosinophilia, pulmonary inflammation, or high serum levels of total or antigen-specific IgE (Wilder JA et al 1999). This dissociation between AHR and inflammatory cell infiltration has been shown in numerous strains. In fact, under certain circumstances inflammatory changes in the airways can be induced in the airways of Balb/c mice with little or no airway hyperresponsiveness (Haile S et al 1999).

The airways of Balb/c mice may be more reactive due, in part, to lower levels of endogenous IL-5 and higher levels of endogenous IL-4 production compared to other murine strains. For example, recent evidence suggests that Balb/c variants with IL-5-deficiency lost the ability to develop airway hyperreactivity to inhaled methacholine. These mice also had reduced number of inflammatory cells in the lamina propria and fewer eosinophil in the airway epithelium, although epithelial hypertrophy and subepithelial fibrosis were still present (Foster PS et al 2000). In contrast, Balb/c mice deficient in IL-4 actually had exaggerated airway responsiveness to methacholine, in addition to a much greater degree of epithelial hypertrophy and subepithelial fibrosis.

A reduced endogenous production of IL-5 and a more vigorous Th-2 response in Balb/c mice may simply be due to a poorer IL-5 response to specific antigens rather than a deficiency in IL-5 production per se (Dehlawi MS et al 1987). This suggests that the control of the immune response may be quite subtle. Thus, small differences in the nature and quality of the antigenic

epitope encountered by an animal or slight differences in the gene expressed by that animal may drive quite significant differences in the shape or size of the immune response observed (Lee TD et al 1982).

Confirmation of such subtlety and the extent to which differences in IL-5 expression contribute to genetic variability in parasite immunity was investigated in a recent series of studies by comparing eosinophilia, IgE production, mastocytosis and IL-5 mRNA expression following N.b. infection. In uninfected C57BL/6 and Balb/c mice IL-5 mRNA$^+$ cells, detected by in situ hybridization, were distributed throughout the lamina propria and crypt regions of the small intestine. However, expression of IL-5 mRNA was twice as high in BALB/c mice than in C57BL/6 mice.

In fact IL-5 expression in BALB/c mice increased until day 11 post-infection (pi), when there were 4 times the number of IL-5$^+$ cells than seen in uninfected control mice. In contrast, in C57BL/6 mice, IL-5 expression peaked on day 7 pi and the number of cells expressing IL-5 was only 1.5 times higher than uninfected C57BL/6 controls. At all time points the numbers of IL-5$^+$ cells in the gut of C57BL/6 mice were significantly lower than BALB/c mice.

The difference in the number of IL-5$^+$ cells correlated with changes in blood and intestinal eosinophilia, mastocytosis and IgE production and was reflected in differences in worm expulsion and egg counts. This suggests that not only is IL-5 an important regulatory factor determining host immunity to parasite infection but also that differential regulation of IL-5 expression explains in part the observed strain differences with respect to parasite resistance (Zhou Y et al 1996).

Taken together all these studies suggest that Balb/c mice may respond to OA or N.b. antigens with a more Th-2 predominant response. However, some authors have demonstrated that the response of Balb/c mice to N.b. infection is no different to many other common strains, including C57BL/6 (Stadnyk AW et al 1990), and some authors have even suggested that Balb/c mice may actually produce a poorer Th-2 response to OA or nematode parasites (Morokata T et al 1999).

For example, in an OA model of allergic inflammation it has been shown that serum IgE and IgG1 levels were higher in C57BL/6 mice than in BALB/c mice. In contrast, IgG2a levels in C57BL/6 mice were lower than in BALB/c mice. Furthermore, the number of eosinophils infiltrating the lungs in C57BL/6 mice was significantly higher than in BALB/c mice after OA challenge. The levels of the Th2 cytokines, IL-4 and IL-5, generated in challenged C57BL/6 lung tissue, were also higher than in BALB/c lung tissue. This higher IL-4 and IL-5 production in C57BL/6 mice was implicated in the greater eosinophilia seen in these animals since the eosinophilia was reduced in both strains of mice by injection of anti-IL-4 and anti-IL-5 monoclonal antibodies (mAbs)(Morokata T et al 1999).

However, the pulmonary response to Th-2 antigens may not reflect the systemic response since cultured splenocyte from C57BL/6 mice produced less IL-4 and IL-5 in vitro than splenocytes from BALB/c mice. These results suggest that C57BL/6 mice induce a Th2-type response in the lungs, while BALB/c mice induce a Th1-type response in the lungs, despite considerable production of IL-4 and IL-5 from splenocytes. Therefore, it may be that local immune responses are more important in the induction of allergic inflammation in the lungs and are different from systemic immune responses, which are may depend on genetic background (Morokata T et al 1999).

Given the contradiction in the literature we compared the local and systemic response, not to OA antigens but to nematode antigens in C57BL/6 and Balb/c mice in order to determine which strain generated a more Th-2 response. This would help determine which murine strain would be more appropriate in a nematode driven model of allergic inflammation of the airways. Since the peak splenocyte proliferative response was believed to occur on day 11 pi, when a significant late phase response is also occurring (chapter 3), the cytokine and cellular response in both strains of mice was compared at this time point in addition to comparing it to naïve, uninfected mice.

6.2 MATERIALS AND METHODS

6.2.1 Larval Culture and Infection

Details of N.b. larval culture are given in chapter 2.

6.2.2 Animals

Details of animal husbandry are given in chapter 2.

6.2.3 Bronchoalveolar lavage and cell preparations

Details of brochoalveolar lavage and cell preparation are given in chapter 2. Data presented throughout this thesis are given per ml of BAL fluid drawn.

6.2.4 Blood: Leukocyte Subsets

Details of blood acquisition and cell analysis are given in chapter 3. Data presented throughout this thesis are given per ml of blood drawn.

6.2.5 Spleen mononuclear preparations:

Details of spleen cell preparation are given in chapter 5.

6.2.6 Cytokine ELISAs

Costar 96 well half area EIA plates (Costar #3690) were coated overnight at 4 ^{0}C with 50µl/well of anti-IL-2 (2µg/ml), anti-IL-4 (1µg/ml), anti-IL-5 (1µg/ml), anti-IL-10 (4µg/ml), and anti-IFN-γ (1µg/ml) (PharMingen, San Diego, CA). Plates were washed using Dulbecco's PBS plus 0.05% Tween 20, pH 7.2 (PBS/TW), and blocked for 1hr at room temperature (RT) with PBS/TW plus 3% Bovine Serum Albumin (BSA). Standard curves were constructed for each cytokine and unknown samples were loaded at 50µl/well and plates incubated at RT for 3-4hr. Biotin-labeled anti-cytokine antibodies (PharMingen, San Diego, CA) were diluted in blocker to a concentration of 2µg/ml and loaded into washed plates at 50µl/well prior to incubation at RT for 1hr. Streptavidin-horseradish peroxidase (Southern Biotechnology Associates) was diluted in blocker 1:2000 and loaded into washed plates at 50µl/well prior to incubation at RT for 30 min. ABTS

solution (Kirkegaard & Perry Labs) was mixed and added to washed plates at 50µl/well prior to incubation at RT for 60min (30min for IFN-γ). Plates were then read on a microtiter plate reader at 405 nm.

6.2.7 Generation of N.b. specific antigen

Details of N.b. specific antigen generation are given in chapter 5.

6.2.8 Statistics

The data presented in this chapter on wild type C57BL/6 mice are pooled from several experiments conducted throughout this thesis. Thus the peripheral blood and BAL fluid data presented contains, on average, 38 mice per time point. The Balb/c mice data were pooled from two experiments and contained, on average, 10 mice per time point. Total and differential cell counts in the BAL fluid, blood splenocyte proliferation and cytokine production were analysed using a two-tailed paired t test assuming unequal variance in samples and a normal distribution. When this overall test of treatment was found to be significant at the $p<0.05$, this was indicated by an asterisk (*). When the significance level reached $p<0.01$ this was indicated by two asterisks (**), and when the significance level reached $p<0.001$ this was indicated by three asterisks (***). If the result was borderline significant ($p=0.05$), then this was denoted by a # sign.

6.3 RESULTS

6.3.1 Time course of peripheral blood changes following N.b. infection

I. Total Leukocyte Count

Primary infection with third stage N.b. larvae produced a significant increase in the total white cell count (WCC) in WT mice during the late phase response starting on day 11 pi (chapter 3). In contrast in this series of experiments N.b. infection did not produce a peripheral leukocytosis in either C57BL/6 or Balb/c mice. This may have been to the smaller number of animals examined. In addition there was no significant difference in the peripheral WCC in naïve or infected Balb/c and C57BL/6 mice. Specifically, the total WCC in naïve Balb/c mice was 7.5×10^6 compared to

8.43 x10^6 in naïve C57BL/6 mice and 7.55 x10^6 in infected Balb/c mice compared to 8.86 x10^6 in infected C57BL/6 mice (Figure 6-1).

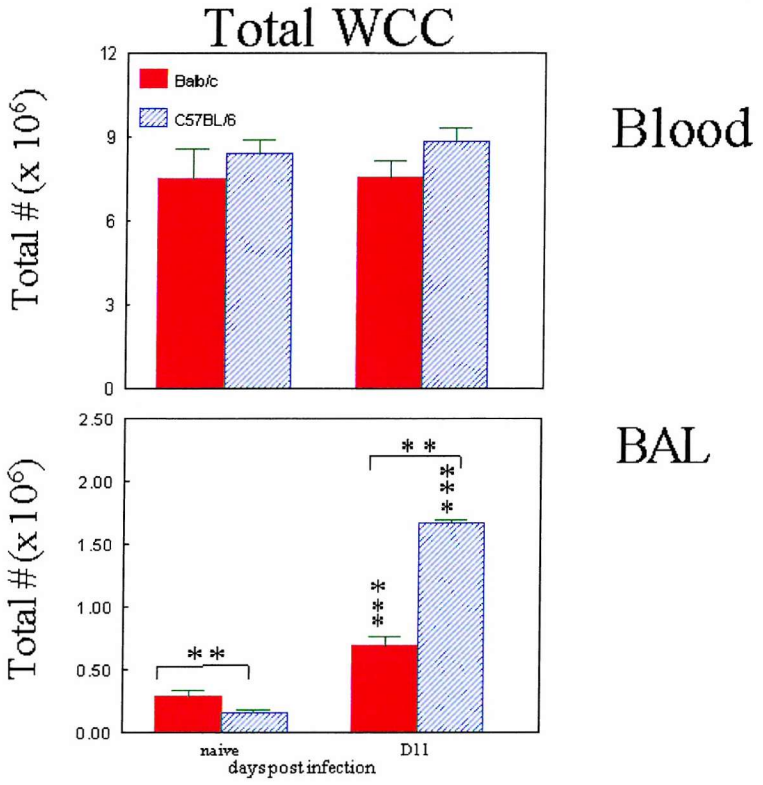

Figure 6-1. The total number and percentage of leukocytes recruited to the blood and airways in Balb/c and wild-type control mice following N.b. infection.

II. Eosinophils

The most significant change in the peripheral blood of WT and genetically manipulated mice following N.b. infection was the increase in the number and percentage of circulating eosinophils (chapters 7-9). Similarly, the number of circulating eosinophils increased significantly in both C57BL/6 and Balb/c mice by day 11 pi. Specifically eosinophil numbers increased from 0.10 x10^6 in naïve Balb/c mice to 0.70 x10^6 by day 11 pi ($p<0.001$) and from 0.15 x10^6 in naïve C57BL/6 mice to 0.64 x10^6 by day 11 pi ($p<0.001$). However, the eosinophil count in naïve Balb/c or C57BL/6 mice did not differ from each other. Similarly there was no difference in the eosinophil response in infected Balb/c or C57BL/6 mice (Figure 6-2).

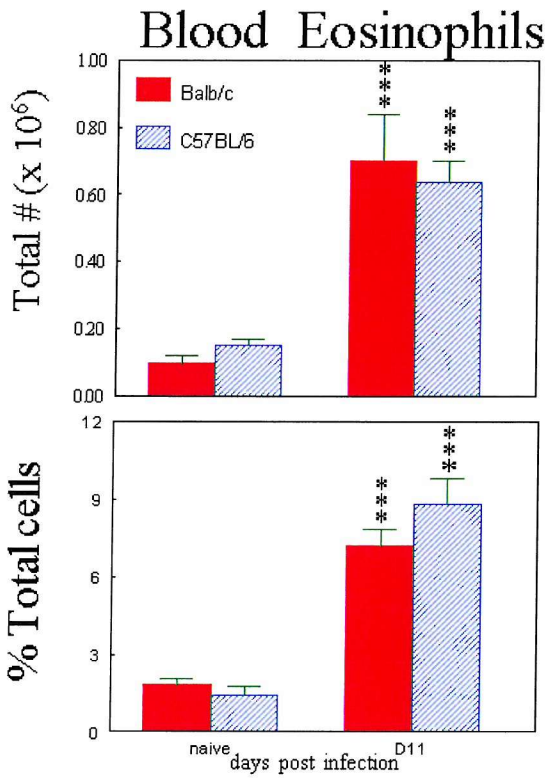

Figure 6-2. The total number and percentage of eosinophils in the blood in Balb/c and wild-type control mice following N.b. infection.

This significant increase in eosinophil numbers was reflected in significant changes in the percentage of eosinophils in the peripheral blood in both Balb/c and C57BL/6 mice. Specifically the percentage of eosinophils increased significantly in Balb/c mice from 1.44% in naïve mice to 8.78% by day 11 pi ($p<0.001$). Similarly, the percentage of eosinophils in C57BL/6 mice increased significantly from 1.81% in naïve mice to 7.19% by day 11 pi ($p<0.001$). However, there was again no difference between the percentage of eosinophils in the blood of naïve or infected Balb/c or C57BL/6 mice (Figure 6-2).

III. Neutrophils

Infection with N.b. larvae produces changes in the number and percentage of circulating neutrophils in the peripheral blood. Neutrophil numbers increase during the early phase response and peak somewhere between day 3 and day 8 pi (chapter 3). By day 11 pi the neutrophilia has

largely, though not always completely, subsided. In this series of experiments there was also very little change in the neutrophil numbers between naïve and infected mice in either Balb/c or C57BL/6 strains. Specifically, neutrophil numbers rose from 1.37 x10^6 in naïve Balb/c mice to 1.40 x10^6 by day 11 pi and from 0.73 x10^6 in naïve C57BL/6 mice to 0.79 x10^6 by day 11 pi. However, there were significantly more neutrophils in the peripheral blood of naïve Balb/c compared to naïve C57BL/6 mice ($p<0.05$) and this significant difference remained following N.b. infection ($p<0.001$)(Figure 6-3).

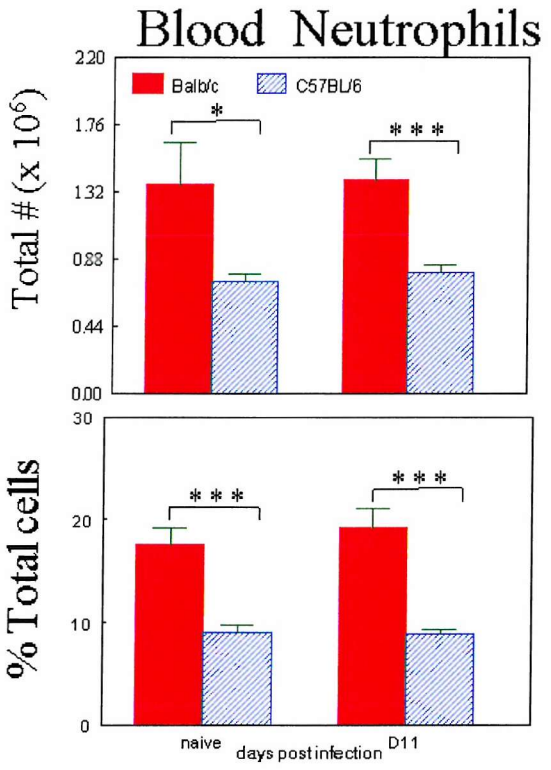

Figure 6-3. The total number and percentage of neutrophils in the blood in Balb/c and wild-type control mice following N.b. infection.

The differences in the total number of neutrophils in the peripheral blood of Balb/c compared to C57BL/6 mice was reflected in a difference in the percentage of neutrophils in the peripheral blood of naïve and infected Balb/c and C57BL/6 mice. Specifically, the percentage of neutrophils in the peripheral blood of Balb/c mice was unchanged by day 11 post infection, 17.63% and 19.245% in naïve and infected mice respectively. Similarly, the percentage of neutrophils in the blood of C57BL/6 mice was unchanged by day 11 post infection, 8.98% and

8.94% respectively. However, there was a significantly greater percentage of neutrophils in the blood of naïve Balb/c mice compared to naïve C57BL/6 mice ($p<0.001$) and also in infected Balb/c mice compared to infected C57BL/6 mice ($p<0.001$)(Figure 6-3).

IV. Lymphocytes

The number of circulating lymphocytes in the peripheral blood of WT mice increased significantly following N.b. infection (chapter 3). However this did not occur until day 14 pi. On day 11 pi lymphocyte numbers were not significantly different from naïve mice. Similarly, in these experiments there was no increase in lymphocyte numbers by day 11 post N.b. infection in either Balb/c or C57BL/6 mice compared to naïve mice. In fact there was a small, but non-significant reduction from 5.78 $x10^6$ to 4.95 $x10^6$ in Balb/c mice and 7.2 $x10^6$ to 6.93 $x10^6$ in C57BL/6 mice. The reduction in circulating lymphocyte numbers was greater in Balb/c mice than in C57BL/6 mice resulting in significantly less lymphocytes in the blood of infected Balb/c mice compared to infected C57BL/6 mice ($p<0.04$)(Figure 6-4).

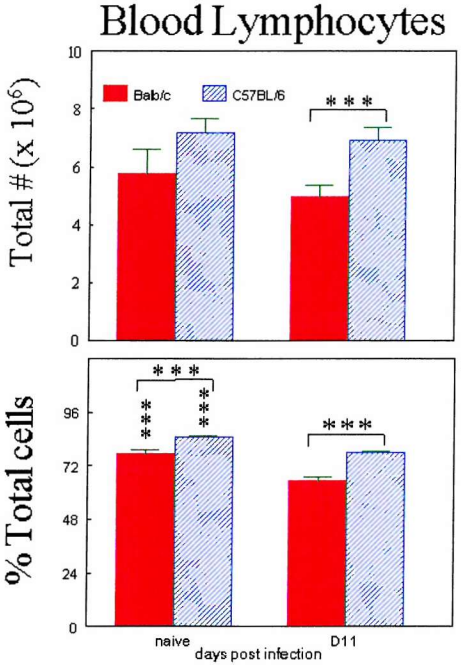

Figure 6-4. The total number and percentage of lymphocytes in the blood in Balb/c and wild-type control mice following N.b. infection.

This lower number of circulating lymphocytes in the peripheral blood of infected Balb/c mice was reflected in the percentage of lymphocytes in the blood. Specifically, the percentage of

lymphocytes in the blood of Balb/c mice dropped from 77.53% in naïve mice to 65.34% in infected mice ($p<0.001$). There was a similar and significant reduction in the percentage of lymphocytes in the blood of C57BL/6 mice from 85.08% in naïve mice to 78.01% in infected mice ($p<0.001$). The percentage of lymphocytes in the blood of Balb/c mice was significantly less than in C57BL/6 mice in both naïve mice ($p<0.001$) and infected mice ($p<0.001$) (Figure 6-4).

V. Monocytes

The increase in eosinophil numbers during the late phase response was mirrored by the increase in monocyte numbers in both Balb/c and C57BL/6 mice. Specifically, monocyte numbers increased from 0.19 $x10^6$ in naïve Balb/c mice to 0.29 $x10^6$ in infected Balb/c mice ($p<0.05$). Similarly, monocyte numbers increased from 0.17 $x10^6$ in naïve C57BL/6 mice to 0.26 $x10^6$ in infected C57BL/6 mice ($p<0.001$). However, there was no difference between the number of monocytes in the blood of naïve or infected C57BL/6 mice compared to Balb/c mice (Figure 6-5).

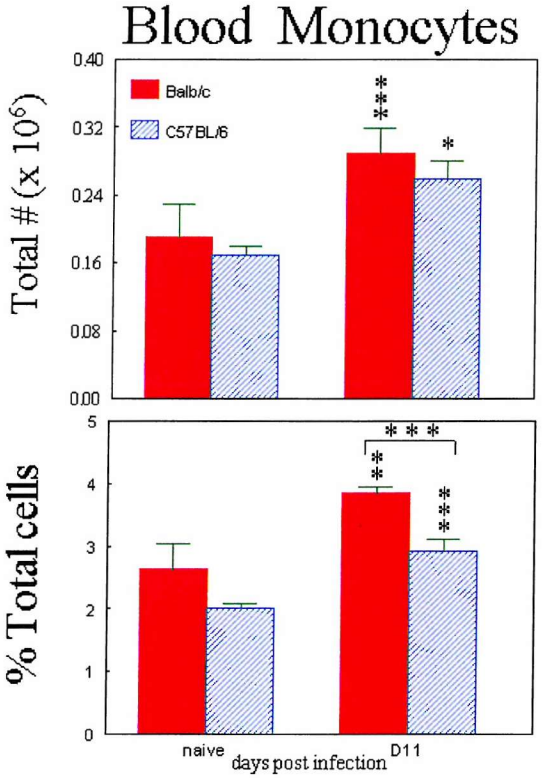

Figure 6-5. The total number and percentage of monocytes in the blood in Balb/c and wild-type control mice following N.b. infection.

In line with the increased number of monocytes in the blood of both Balb/c and C57BL/6 mice there was a significant increase in the percentage of monocytes in the peripheral blood following N.b. infection. Specifically, monocyte percentages increased from 2.64% in naïve Balb/c mice to 3.86% in infected mice ($p<0.02$), and from 2.01% in naïve C57BL/6 mice to 2.92% in infected mice ($p<0.001$). In addition, the increase in the percentage of monocytes in the peripheral blood was greater in Balb/c mice, with a significantly greater percentage of monocytes in the blood of Balb/c mice following N.b. infection compared to C57BL/6 mice ($p<0.001$)(Figure 6-5).

6.3.2 Time course of leukocyte infiltration into the lung following N.b. infection

I. Total Leukocyte Count

In WT mice there was a biphasic cellular response in the BAL fluid following N.b. infection (chapter 3). This consisted of an early cellular influx into the airways during larval migration through the lungs, primarily on day 3 pi, followed by a late phase response peaking on day 14 pi. This late phase cellular recruitment to the airways was seen in both Balb/c and C57BL/6 mice.

Specifically the total number of leukocytes in the airways of Balb/c mice rose from 2.8×10^3 in naïve Balb/c mice to 6.9×10^3 in infected mice ($p<0.01$). Similarly, the total number of leukocytes in the airways of C57BL/6 mice rose from 1.5×10^3 in naïve C57BL/6 mice to 16.6×10^3 in infected mice ($p<0.01$)(Figure 6-1). The cellular recruitment to the airways following N.b. infection was significantly more vigorous in C57BL/6 mice compared to Balb/c mice. Thus although there were significantly more leukocytes in the airways of naïve Balb/c mice compared to C57BL/6 mice ($p<0.04$), after N.b. infection the number of leukocytes in the airways of C57BL/6 mice was significantly greater than that seen in Balb/c mice ($p<0.01$) (Figure 6-1).

II. Eosinophils

The cellular response in the lungs following N.b. infection was dominated by a significant increase in the number and percentage of eosinophils in the BAL fluid in both Balb/c and C57BL/6 mice. In Balb/c mice the number of eosinophils in the airways of increased very

significantly from 0.24 x 10^3 in naïve mice to 194 x10^3 on day 11 pi ($p<0.001$). Similarly, in C57BL/6 mice the number of eosinophils in the airways increased very significantly from 17.98 x 10^3 in naïve mice to 1.18 x10^6 on day 11 pi ($p<0.001$). Although the number of eosinophils in the BALF of naïve C57BL/6 and naïve Balb/c mice were not significantly different by day 11 pi there was a considerably greater influx of eosinophils into C57BL/6 airways compared to Balb/c airways ($p<0.003$)(Figure 6-6).

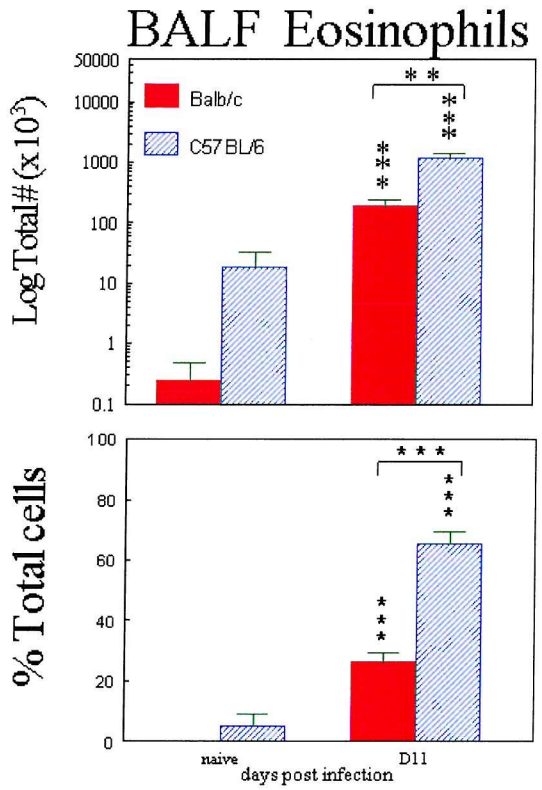

Figure 6-6. The total number and percentage of eosinophils in the BAL fluid in Balb/c and wild-type control mice following N.b. infection. Eosinophil counts are given on a log scale for visual representation purposes only.

The changes in airway eosinophil numbers following N.b. infection were reflected in changes in the percentages of eosinophils recovered from the BALF. Specifically, the percentage of eosinophils in the airways increased significantly from 0.1% in naïve Balb/c mice to 26.2% in infected Balb/c mice ($p<0.001$). In contrast, the percentage of eosinophils in the airways of C57BL/6 mice increased by a much greater amount from 5.2% in naïve to 65.6% in infected

C57BL/6 mice ($p<0.001$). Although the percentage of eosinophils in the BALF did not differ in naïve Balb/c and C57BL/6 mice, by day 11 pi there was a significantly greater percentage of eosinophils in the BALF of C57BL/6 mice compared to Balb/c mice ($p<0.001$)(Figure 6-6).

III. Neutrophils

In WT mice a biphasic cellular recruitment to the airways was seen. This was characterised by a significant influx of neutrophils to the airways during the "early phase" response peaking on day 3 pi, during larval migration through the lungs (chapter 3). By day 11 pi this early phase response had subsided. In this series of experiments there was no day 3 time-point. Nevertheless, the neutrophil influx had not quite subsided by day 11 pi (Figure 6-7).

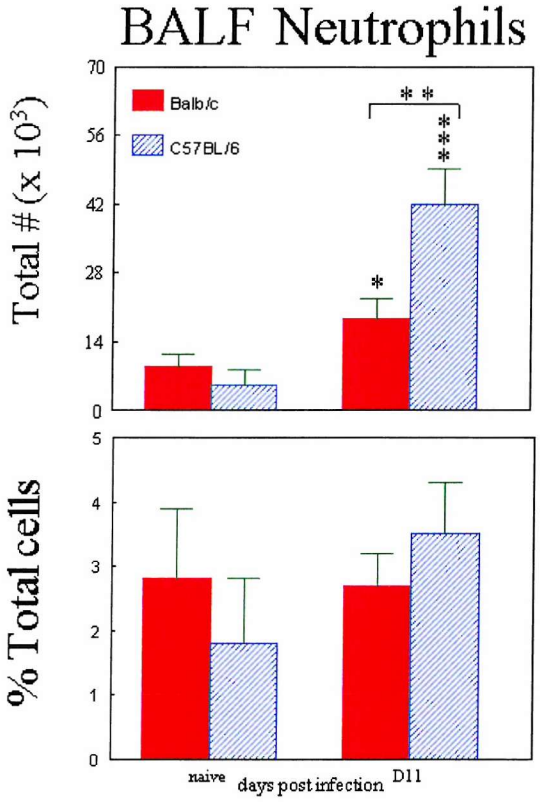

Figure 6-7. The total number, and percentage, of neutrophils in the BAL fluid in Balb/c and wild-type control mice following N.b. infection.

Specifically the number of neutrophils in the BALF of Balb/c mice on day 11 pi was significantly greater than the number seen in naïve Balb/c mice, 18.8 x 10^3 and 8.9 x 10^3 respectively ($p<0.05$).

Similarly, the number of neutrophils in the BALF of C57BL/6 mice on day 11 pi was significantly greater than the number seen in naïve C57BL/6 mice, 41.8 x 10^3 and 5.13 x 10^3 respectively ($p<0.001$). There was also a significantly greater airway neutrophilia in C57BL/6 mice following N.b. infection ($p<0.02$) while neutrophil numbers in naïve C57BL/6 and Balb/c mice were comparable (Figure 6-7).

In contrast, the percentage of neutrophils recovered from the BALF of infected Balb/c mice was not statistically different from the percentage of neutrophils in the BALF of naïve mice, 2.7% and 2.8% respectively. Similarly, there was no significant difference between the percentage of neutrophils in the BALF of naïve and infected C57BL/6 mice, 3.5% and 1.8% respectively. Therefore there was no difference between the percentage of neutrophils recovered from the BALF of Balb/c or C57BL/6 mice in naïve or infected mice (Figure 6-7).

IV. Lymphocytes

During the late phase response there was a significant increase in lymphocyte numbers in WT mice starting on day 8 pi and persisting through to day 19 pi (chapter 3). This was also seen in both Balb/c and C57BL/6 mice. Specifically the number of lymphocytes increased significantly following N.b. infection from 5.5 x 10^3 in naïve Balb/c mice to 148 x 10^3 on day 11 pi ($p<0.001$). This lymphocyte recruitment to the BALF was also seen in C57BL/6 mice with lymphocyte numbers increasing significantly from 29.36 x 10^3 in naïve to 318 x 10^3 on day 11 pi ($p<0.001$) (Figure 6-8).

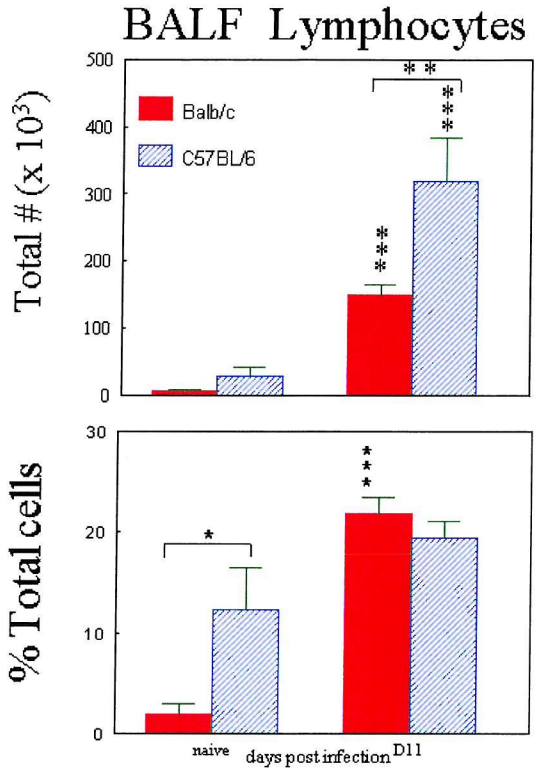

Figure 6-8. The total number and percentage of lymphocytes in the BAL fluid in Balb/c and wild-type control mice following N.b. infection.

The airway lymphocytosis was much greater in infected C57BL/6 mice compared to infected Balb/c mice ($p<0.03$), despite there being no difference in lymphocyte numbers in naïve C57BL/6 and Balb/c mice ($p<0.12$)(Figure 6-8).

This increase in the lymphocyte recruitment during the late phase response in Balb/c and C57BL/6 mice was reflected in changes in the percentages of lymphocytes recovered from the BALF. The increase in the percentage of lymphocytes recovered from the BAL fluid of infected Balb/c mice was significantly greater than the percentage of lymphocytes in naïve Balb/c airways, 21.9% and 2.0% respectively ($p<0.001$). In contrast, the increase in the percentage of lymphocytes recruited to the BALF of infected C57BL/6 mice compared to naïve C57BL/6 mice did not quite reach statistical significance, 19.4% and 12.4% ($p<0.14$).

This finding was probably due to the fact that there were a significantly smaller percentage of lymphocytes in the airways of naïve Balb/c mice compared to naïve C57BL/6 mice ($p<0.03$). Thus the significant increase in the percentage of lymphocytes in the airways of Balb/c mice resulted in the percentage of lymphocytes in the airways of C57BL/6 and Balb/c mice being roughly equivalent (Figure 6-8).

V. Monocytes

Following N.b. infection there was an increase in monocyte numbers in Balb/c and C57BL/6 mice. Specifically the number of monocytes in the BALF of Balb/c mice increased from 273 x 10^3 in naïve Balb/c mice to 338 x 10^3 on day 11 pi, but this was not statistically significant. The number of monocytes in the BALF of C57BL/6 mice also increased from 104 x 10^3 in naïve C57BL/6 mice to 151 x 10^3 on day 11 pi, although this did not quite reach statistical significance ($p<0.07$). However, the number of monocytes in the BALF of Balb/c mice was significantly greater than in C57BL/6 mice in both naïve ($p<0.01$) and infected mice ($p<0.001$)(Figure 6-9).

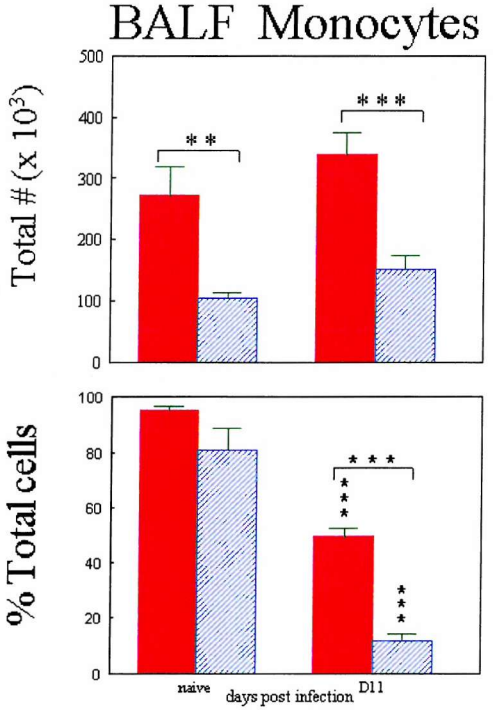

Figure 6-9. The total number and percentage of monocytes in the BAL fluid in Balb/c and wild-type control mice following N.b. infection.

In contrast, there was a significant reduction in the percentage of monocytes in the BALF of both Balb/c and C57BL/6 mice, reflecting the recruitment of other cellular populations to the BALF following N.b. infection. Specifically, the percentage of monocytes in the BALF of Balb/c mice decreased from 95.2% in naïve mice to 49.3% on day 11 pi ($p<0.001$). Similarly, the percentage of monocytes in the BALF of C57BL/6 mice decreased from 99.5% in naïve mice to 11.8% on day 11 pi ($p<0.001$). Although the percentage of monocytes in the BALF of both naïve C57BL/6 mice and naïve Balb/c did not differ, by day 11 pi there were significantly less monocytes in the BALF of C57BL/6 mice ($p<0.001$)(Figure 6-9).

6.3.3 Splenocyte proliferation

I. Splenocyte response to N.b.-specific antigen

Spleen cells were stimulated with a variety of specific and non-specific mitogens. Ranges of specific and non-specific antigen doses were used since the optimum conditions for splenocyte proliferation in response to antigen were unknown. The response of splenocytes to N.b. specific antigen (NbA) following N.b. infection was compared in Balb/c and C57BL/6 mice (Figure 6-10).

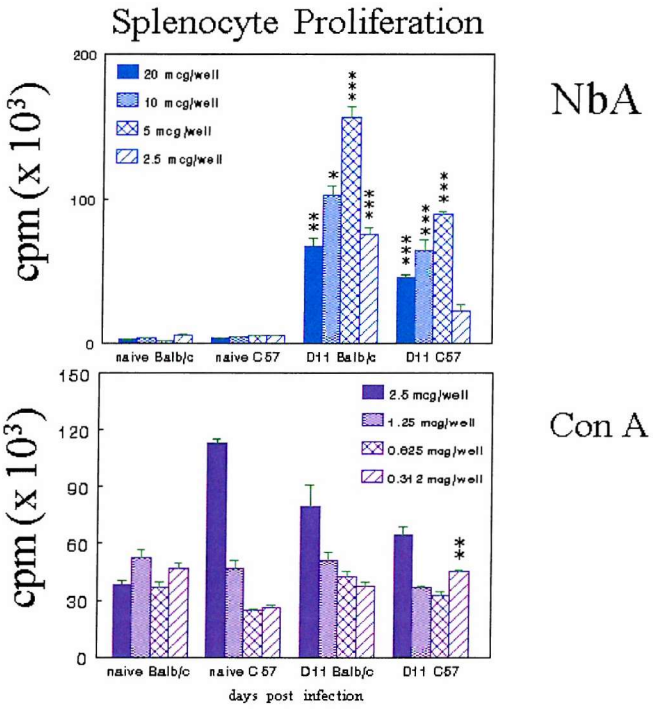

Figure 6-10. Splenocyte proliferation in response to N.b specific antigen, and ConA, in Balb/c and wild-type control mice following N.b. infection.

This showed that there was a significant response to N.b. specific antigen in both Balb/c and C57BL/6 splenocytes after they had been sensitized by a N.b. infection.

Specifically there was a significant increase in the proliferative response to NbA in Balb/c murine splenocytes right across the NbA dose range of 20mcg/well ($p<0.007$), 10mcg/well ($p<0.04$), 5mcg/well ($p<0.002$) and 2.5mcg/well ($p<0.004$). The mean increase in proliferative response, comparing naïve and infected Balb/c mice, was also statistically significant ($p<0.02$).

Similarly, there was a significant increase in the proliferative response of C57BL/6 murine splenocytes following N.b. infection at 20mcg/well ($p<0.001$), 10mcg/well ($p<0.01$), 5mcg/well ($p<0.001$) but the increase did not quite reach statistical significance at 2.5mcg/well of NbA (Figure 6-10). The mean increase in proliferative response, comparing naïve and infected C57BL/6 mice, was also statistically significant ($p<0.04$).

When looking at the detail of the proliferative response to various N.b. antigen doses naïve C57BL/6 splenocytes responded significantly more vigorously at the 5mcg/well dose compared to Balb/c mice ($p<0.001$). However such a difference in response was not seen at either 20mcg/well, 10mcg/well or at 2.5mcg/well. Furthermore there was no difference in the mean proliferative response between naïve Balb/c and naïve C57BL/6 mice. In contrast, in infected mice, the proliferative response of Balb/c splenocytes was significantly greater at the 20mcg/well ($p<0.05$), 10mcg/well ($p<0.02$), 5mcg/well ($p<0.01$) and at the 2.5mcg/well dose ($p<0.0.001$). Although the mean proliferative response of infected Balb/c and C57BL/6 was not significantly different due to the greater variability.

II. Splenocyte response to non-specific antigen.

Spleen cells were also stimulated with two different non-specific mitogens, ConA and anti-CD3. Different doses of ConA, but not anti-CD3, were used since the optimum conditions for splenocyte proliferation in response to this mitogen were unknown. The response of splenocytes to ConA was compared in Balb/c and C57BL/6 mice (Figure 6-10). This showed that, unlike the

response to N.b. specific antigen there was no real increase in the mean proliferative response in infected Balb/c or C57BL/6 mice compared to naïve Balb/c and C57BL/6 mice.

However when looking at the detail of the splenocyte proliferative response to specific doses of ConA certain significant differences emerged. There seemed to be an unusually high proliferative response in naïve C57BL/6 mice to 2.5mcg/well of ConA. This resulted in a significantly greater proliferative response in naïve compared to infected C57BL/6 mice at this dose of ConA ($p<0.001$). However, no such difference was apparent when comparing the proliferative response of naïve and infected C57BL/6 splenocytes at the 1.25mcg/well dose of ConA.

In contrast, the greater proliferative response of C57BL/6 splenocytes to specific antigen following N.b. infection was seen in the two smaller non-specific antigen doses. Thus by day 11 pi C57BL/6 splenocytes proliferated more vigourously in response to 0.625mcg/well of ($p<0.08$) and 312mcg/well ($p<0.002$) of ConA compared to naïve C57BL/6 splenocytes (Figure 6-10).

This trend towards increased proliferative responsiveness to ConA following N.b. infection was not as clear-cut in Balb/c splenocytes where there was an increased proliferation at 2.5mcg/ well but this was not statistically significant ($p<0.06$). Furthermore, there was reduced proliferative responsiveness at 0.312mcg/well, but again this was not statistically significant ($p<0.08$). The two middle doses of 1.25mcg/well and 0.625mcg/well of ConA also did not stimulate a greater proliferative response form day 11 splenocytes compared to uninfected murine splenocytes (Figure 6-10).

The splenocyte proliferative response of naïve and infected Balb/c and C57BL/6 mice to α-CD3 was also compared. The trend toward increased splenocyte proliferation following N.b. infection was borne out in Balb/c mice with day 11 splenocytes proliferating more vigourously than naïve Balb/c splenocytes ($p<0.02$). However, no such difference was seen in C57BL/6 mice. This was despite the fact that naïve Balb/c splenocytes proliferated less in response to a-CD3 than naïve C57BL/6 mice ($p<0.008$). This greater responsivity of C57BL/6 splenocytes disappeared

following N.b. infection when Balb/c splenocytes responded just as vigourously as C57BL/6 splenocytes ($p<0.06$)(Figure 6-11).

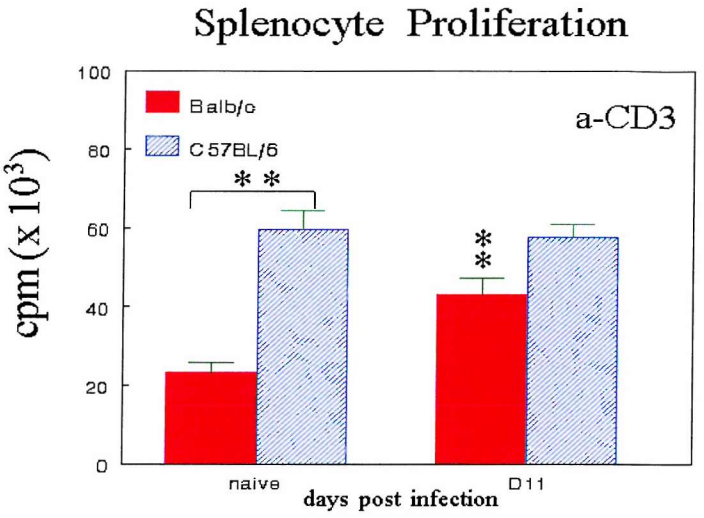

Figure 6-11. Splenocyte proliferation in response to anti-CD3 in Balb/c and wild-type control mice, following N.b. infection.

6.3.4 Splenocyte cytokine production in response to N.b.-specific antigen

The cytokine production of naïve and infected Balb/c and C57BL/6 splenocytes in response to stimulation with specific and non-specific antigen was compared.

I. Splenocyte cytokine production in response to N.b.-specific antigen.

Spleen cells were stimulated with N.b. specific antigen and the production of the Th-1 cytokines, IL-2 and IFN-γ and the Th-2 cytokines IL-4, IL-5 and IL-10 was measured in naïve and infected mice.

There was an increase in splenocyte IFN-γ production in both Balb/c and C57BL/6 mice following N.b. infection. Specifically IFN-γ production increased from 410pg/ml naïve Balb/c mice to 1909pg/ml on day 11 pi, and from 498pg/ml in naïve C57BL/6 mice to 3,033pg/ml on day

11 pi ($p<0.06$). These increases were not statistically significant and the production of IFN-γ did not differ in naïve or infected Balb/c mice compared to naïve or infected C57BL/6 mice (Figure 6-12).

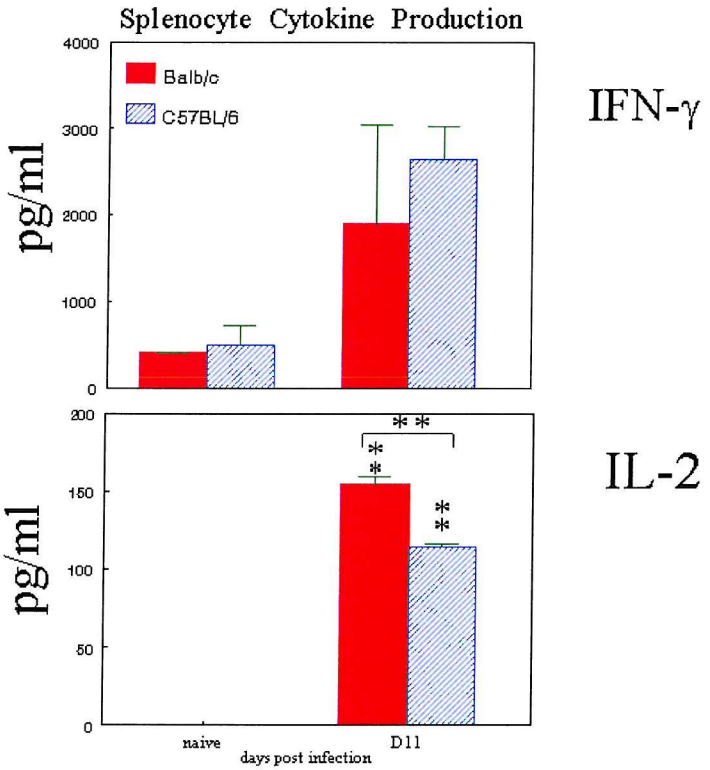

Figure 6-12. Splenocyte IFN-γ and IL-2 production, in response to N.b specific antigen, in Balb/c and wild-type control mice following N.b. infection.

In contrast, the production of splenocyte IL-2 increased significantly in both Balb/c and C57BL/6 mice following N.b. infection. Specifically IL-2 production increased from an undetectable level to 155pg/ml in Balb/c mice ($p<0.02$) and to 114pg/ml in C57BL/6 mice ($p<0.01$). The IL-2 production was significantly greater in Balb/c mice compared to C57BL/6 mice ($p<0.03$) (Figure 6-12).

The production of IL-4 by murine splenocytes also increased significantly in both Balb/c and C57BL/6 mice following N.b. infection. Thus IL-4 production increased from an undetectable level to 160pg/ml in Balb/c mice ($p<0.001$) and to 30pg/ml in C57BL/6 mice ($p<0.001$). The IL-4

production was also significantly greater in Balb/c mice compared to C57BL/6 mice ($p<0.001$)(Figure 6-13).

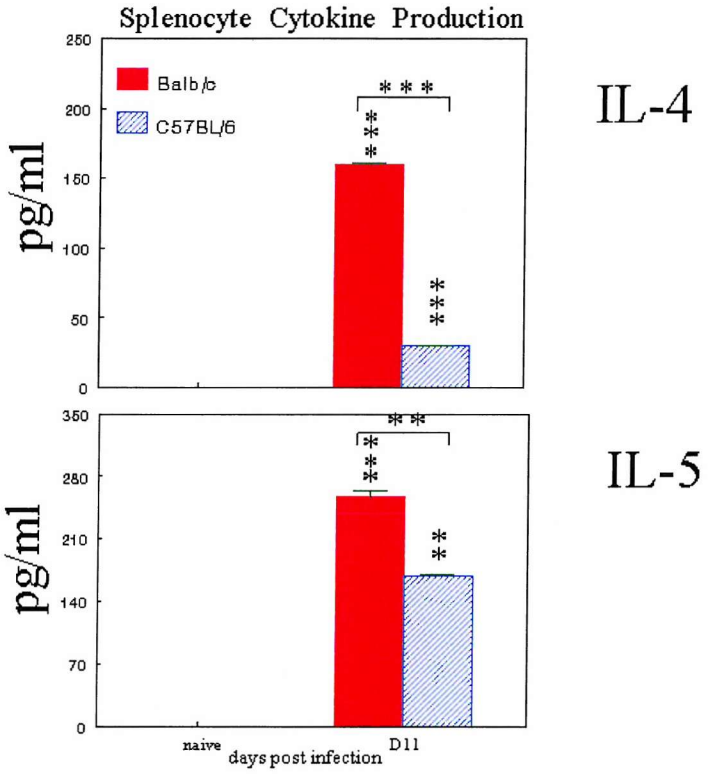

Figure 6-13. Splenocyte IL-4 and IL-5 production, in response to N.b specific antigen, in Balb/c and wild-type control mice following N.b. infection.

Similarly the production of IL-5 by murine splenocytes increased significantly in both Balb/c and C57BL/6 mice following N.b. infection. Thus IL-5 production increased from an undetectable level to 258pg/ml in Balb/c mice ($p<0.001$) and to 171pg/ml in C57BL/6 mice ($p<0.01$). The IL-5 production was again significantly greater in Balb/c mice compared to C57BL/6 mice ($p<0.02$)(Figure 6-13).

The production of IL-10 by murine splenocytes followed an identical pattern and increased significantly in both Balb/c and C57BL/6 mice following N.b. infection. Thus IL-10 production increased from undetectable level in naïve Balb/c mice to 3043pg/ml on day 11 pi in Balb/c mice ($p<0.06$) and from 40.2pg/ml to 1014pg/ml on day 11 pi in C57BL/6 mice ($p<0.002$). The IL-10

production was greater in Balb/c mice compared to C57BL/6 mice although this did not reach statistical significance ($p<0.08$)(Figure 6-14).

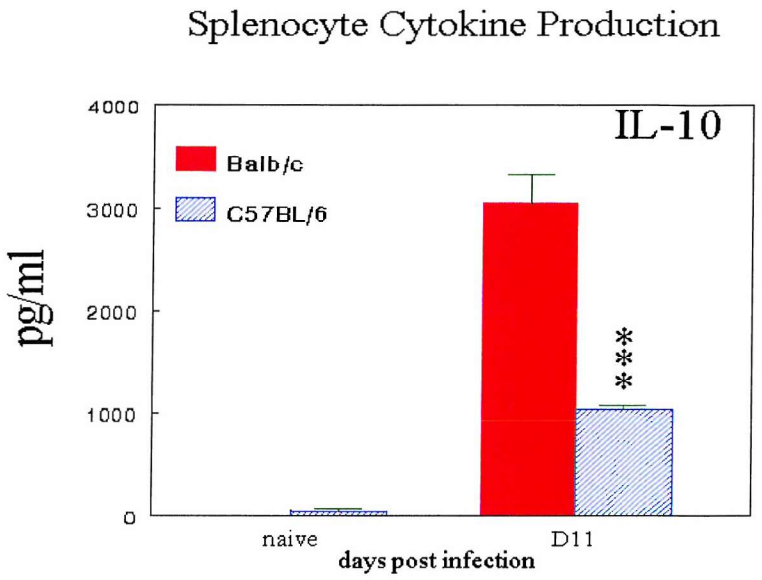

Figure 6-14. Splenocyte IL-10 production in response to N.b specific antigen in Balb/c and wild-type control mice following N.b. infection.

II. Splenocyte response to non-specific antigen

Spleen cells were stimulated with the non-specific antigens ConA and a-CD3 and the production of IFN-γ, IL-4, IL-5 and IL-10 was measured in naïve and infected mice.

There was a reduction in splenocyte IFN-γ production in both Balb/c and C57BL/6 mice following N.b. infection in response to stimulation with ConA. Specifically IFN-γ production decreased from 12.3ng/ml in naïve Balb/c mice to 4.3ng/ml on day 11 pi ($p<0.01$) and decreased from 53.2ng/ml in naïve C57BL/6 mice to 1.7ng/ml on day 11 pi (Figure 6-15).

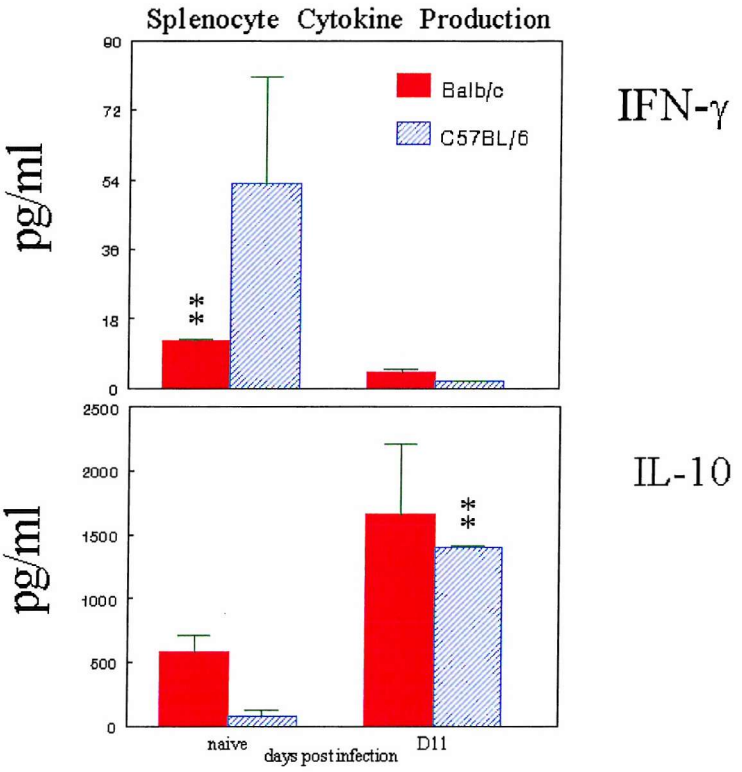

Figure 6-15. Splenocyte IFN-γ and IL-10 production in response to ConA in Balb/c and wild-type control mice following N.b. infection.

The level of IFN-γ production was greater in naïve C57BL/6 mice compared to naïve Balb/c mice but this was not statistically significant. Similarly, IFN-γ production was greater in infected C57BL/6 mice compared to infected Balb/c mice but again this was not quite statistically significant ($p<0.07$)(Figure 6-15).

The production of IL-10 by murine splenocytes in response to ConA followed an opposite pattern with IL-10 increasing in both Balb/c and C57BL/6 mice following N.b. infection. Thus IL-10 production increased from 582pg/ml to in naïve Balb/c mice to 1655pg/ml on day 11 pi in Balb/c mice but this did not reach statistical significance. In contrast, there was a significant increase in IL-10 production by C57BL/6 splenocytes in response to Con A from 75pg/ml to 1405pg/ml on day 11 pi ($p<0.02$). There was no significant difference in the IL-10 production when comparing naïve or infected Balb/c mice to naïve or infected C57Bl/6 mice (Figure 6-15).

The production of IL-4 by murine splenocytes in response to ConA also increased in both Balb/c and C57BL/6 mice following N.b. infection. Thus IL-4 production increased from 34.6pg/ml in naïve Balb/c mice to 354pg/ml in Balb/c mice on day 11 pi ($p<0.09$), and from an undetectable level to 14pg/ml in C57BL/6 mice ($p<0.03$)(Figure 6-16).

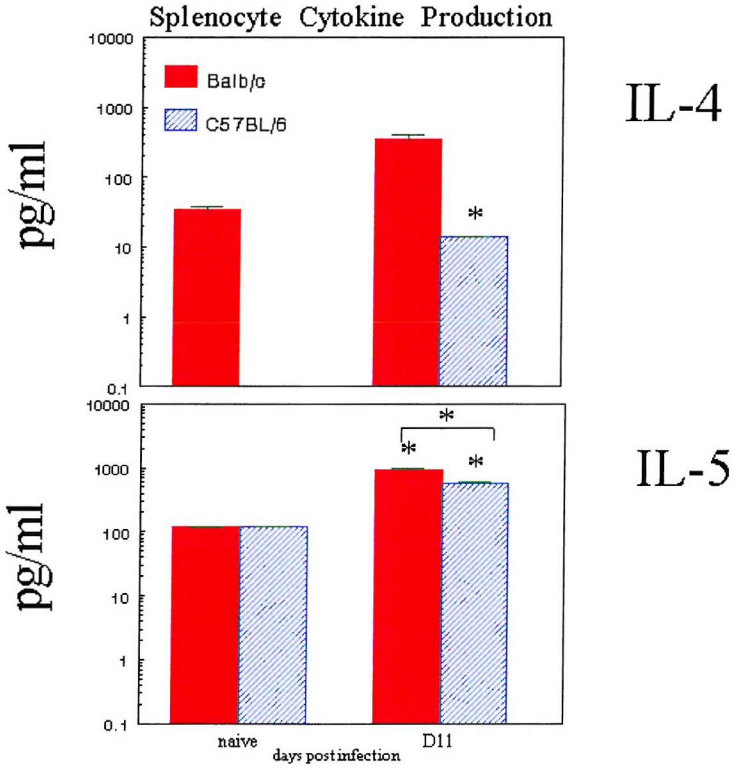

Figure 6-16. Splenocyte IL-4 and IL-5 production in response to ConA in Balb/c and wild-type control mice following N.b. infection.

There was no significant difference in the IL-4 production of naïve or infected Balb/c mice compared to naïve or infected C57BL/6 mice in response to ConA.

The production of IL-5 by murine splenocytes also increased significantly in both Balb/c and C57BL/6 mice following N.b. infection. Thus IL-5 production increased from 121pg/ml to 924pg/ml in Balb/c mice ($p<0.04$) and from 118pg/ml to 560pg/ml on day 11 pi in C57BL/6 mice ($p<0.05$). The IL-5 production was significantly greater in Balb/c mice compared to C57BL/6 mice ($p<0.04$), but not in naïve mice ($p<0.41$)(Figure 6-16).

The cytokine production in response to splenocyte stimulation with a-CD3 was more variable. In Balb/c mice splenocyte IFN-γ production decreased significantly following N.b. infection ($p<0.001$). In contrast, the decrease in IFN-γ production by C57BL/6 murine splenocytes was not statistically significant. Furthermore, the production of IFN-γ by Balb/c splenocytes was significantly greater than that generated by infected C57BL/6 murine splenocytes following N.b. infection ($p<0.02$). In contrast, there was no difference in the production of this cytokine between naïve Balb/c and naïve C57BL/6 mice (Figure 6-17).

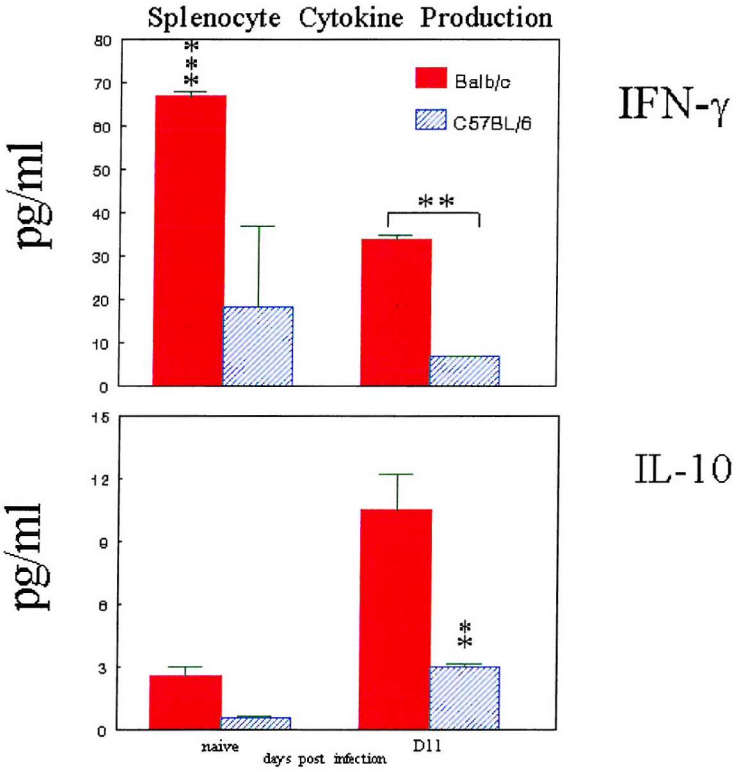

Figure 6-17. Splenocyte IFN-γ and IL-10 production in response to anti-CD3, in Balb/c and wild-type control mice following N.b. infection.

The production of IL-10 by murine splenocytes, in response to a-CD3 stimulation, followed an opposite pattern with IL-10 increasing in both Balb/c and C57BL/6 mice following N.b. infection. Thus IL-10 production increased from 2.6ng/ml in naïve Balb/c mice to 10.5ng/ml on day 11 pi in Balb/c mice. This increase did not reach statistical significance. In contrast, the increase in IL-10 production by C57BL/6 mice following N.b. infection from 541pg/ml to 2958pg/ml on day 11 pi

in was significant ($p<0.02$). However, there was no significant difference in the IL-10 production in naïve or infected Balb/c mice compared to naïve or infected C57BL/6 mice (Figure 6-17).

The production of IL-4 by murine splenocytes in response to a-CD3 stimulation increased significantly in Balb/c mice following N.b. infection but not in C57BL/6 mice. Thus IL-4 production increased from 85pg/ml in naïve Balb/c mice to 631pg/ml on day 11 pi ($p<0.04$). In contrast, the production of IL-4 in C57BL/6 mice did not increase significantly following N.b. infection ($p<0.07$)(Figure 6-18). The production of IL-5 by murine splenocytes also increased in both Balb/c and C57BL/6 mice following N.b. infection. However neither increase was statistically significant (Figure 6-18).

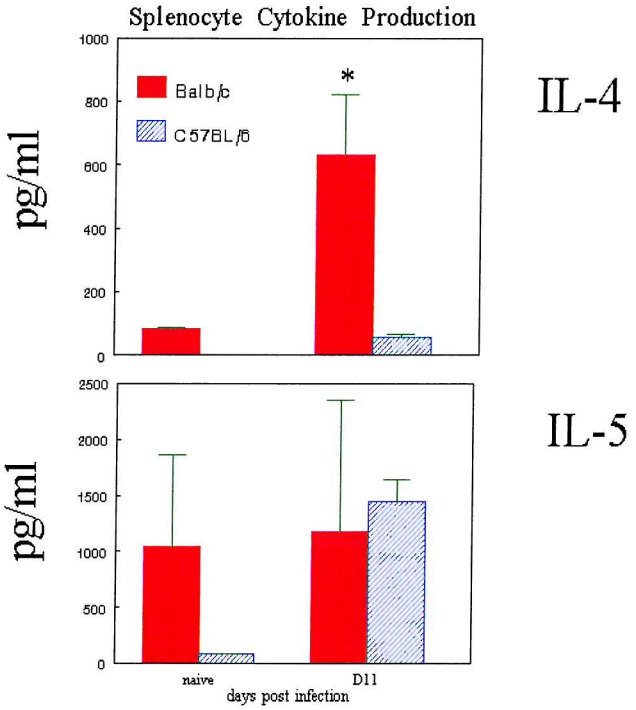

Figure 6-18. Splenocyte IL-4 and IL-5 production in response to anti-CD3 in Balb/c and wild-type control mice following N.b. infection.

6.4 DISCUSSION

Laboratory models of allergic inflammation of the airways using intestinal nematode infections have played an important role in developing our understanding of the immune mechanisms that operate to maintain airway function. The type of helper T cell response that develops following infection with intestinal nematode parasites is critical to the inflammatory process. The early events that mediate polarisation of the helper T cell subsets towards either Th-1 or Th-2 response during intestinal nematode infection are not well understood. However, it is likely that multiple factors influence the induction of a Th 1 or Th-2 type response, and multiple effector mechanisms are involved in the response to nematode antigens.

Costimulatory molecules have been shown to be important in driving T helper cell development down a specific pathway, as has the immediate cytokine environment during T cell activation (Huang Y et al 1998, Else KJ et al 1998). In addition, the genotype of laboratory strains may determine whether Th1- or Th2-type immune responses are elicited. However, the literature is contradictory on whether some strains tend towards Th-1 or Th-2 responses.

For example, some authors have demonstrated that C57BL/6 mice develop a much greater pulmonary eosinophilia, IgE and local cytokine response than Balb/c (Morokata et al 1999), or DBA/2 mice (Morokata T et al 1999b). In contrast, others have reported data suggesting that eosinophil recruitment into the airways of BALB/c mice was more than 20 times greater than that seen in C57BL/6 mice despite equivalent systemic levels of IL-4, IL-5 and antigen-specific IgE titres in both strains (Nakajima H et al 2000).

Given the disagreement in the literature (Nakajima H et el 2000, Temelkovski 1998, Morokata T et al 1999, 1999b), and in order to optimise the model of pulmonary inflammation developed here we decided to explore the airway and systemic response to N.b. infection in two different murine strains. We examined the response of Balb/c mice, which have traditionally been considered to respond in a Th-2 predominant fashion and C57BL/6 mice which have traditional been thought to respond in a Th-1 predominant manner (Brenner GJ et al 1994).

Specifically we explored the kinetics of the cellular response in the blood and airways in addition to the splenic cytokine response to N.b. infection. We demonstrated that C57BL/6 mice developed a much greater airway leukocytosis than Balb/c mice despite equivalent white cell counts in the peripheral blood. In direct contradiction to the recent findings of others (Nakajima H et al 2000, Herz U et al 1998) we demonstrated that there were significantly more eosinophils recruited to the airways of C57BL/6 mice compared to BALB/c mice despite a slightly greater peripheral blood eosinophilia in Balb/c mice.

This suggests that the pulmonary response of C57BL/6 mice was more Th-2 driven than the pulmonary response in Balb/c mice, who developed a more Th-1-type response. The greater Th-2 skewed response in C57BL/6 mice may have been due to a greater local production of IL-5 in C57BL/6 mice since this cytokine has been heavily implicated in eosinophil recruitment (Urban JF Jr et al 1993).

However a number of recent studies have suggested that the balance between Th-1 and Th-2 responses may be less clear-cut (Randolph DA et al 1999, Ishikawa N et al 1998). For example, the response of BALB/c and C57BL/6 to certain infections may not follow a clear polarized Th-1 or Th-2-type cytokine response (Ulett GC et al 2000). In addition, local cytokine production may be more important than the systemic response to antigen since systemic IL-5 production may be insufficient to cause antigen-induced eosinophil recruitment into the airways (Nakajima H et al 2000).

We specifically investigated the local and the systemic response to nematode antigens by assessing the systemic production of Th-1 and Th-2 cytokines and the proliferative response of murine splenocytes in addition to the cellular provenance of the BAL fluid in Balb/c and C57BL/6 mice. Previous authors have found that spleen cells from N.b.-infected mice exhibit a dramatically increased proliferation, in response to Con A and anti-CD3, and this proliferative response was IL-6 dependent (Liwiski RS 1999). However, in our hands only C57BL/6 mice exhibited a small but significantly increased proliferative response to Con A and conversely only

Balb/c mice showed a significantly increased proliferative response to anti-CD3. Both strains generated a dramatically increased response to N.b. specific antigens.

In contrast to their lower airway eosinophilia, the splenocytes from Balb/c mice produced more Th-2 cytokines compared to C57BL/6 mice. The greater production of IL-4 by Balb/c splenocytes compared to C57BL/6 splenocytes in response to a variety of antigenic stimulants has been repeatedly reported (Nakajima H et al 2000, Helmby H et al 2000). However, in the above series of experiments Balb/c splenocytes also produced more Th-1 cytokines than C57BL/6 mice. This supports earlier data suggesting that the development of the immune response may not be polarized into a Th-1 or Th-2-type cytokine response in vivo (Kendall LV et al 2000, Ulett GC et al 2000).

This lower pulmonary eosinophilia despite a higher systemic IL-5 production confirms the importance of compartmentalisation of the immune response as suggested by others (Morokata T et al 1999). The compartmentalisation of the immune response has led some authors to measure cytokine production in the BAL fluid (Tang C et al 1997, Krug N et al 1996), although this may not directly reflect what is happening in the airway epithelium either.

The importance of local or systemically produced increases in IL-5 production in the induction of bronchial hyperreactivity, and therefore airway symptomatology, has been the focus of much debate recently (Wilder JA et al 1999, Coyle AJ et al 1998). However, the weight of evidence still supports a substantial role for IL-5 and eosinophils in bronchial hyperreactivity (Hamelmann E et a l 1999, Hogan SP et al 1997). Data suggesting that airway hyperreactivity may be separately regulated to airway inflammation may simply be reflecting the significant redundancy and overlap in inflammatory mechanisms.

In addition to the lower number of eosinophils recruited to the airways of Balb/c compared to C57BL/6 mice we also demonstrated a significantly lower lymphocyte and neutrophil recruitment to their airways. However, there was a much greater monocyte population in the airways of Balb/c mice. This suggests that the pulmonary response to N.b. infection in Balb/c

mice may be more slightly more Th-1 driven. These data contradicts some earlier reports (Herz U et al 1998), but confirms others (Morokata et al 1999, 1999b).

In conclusion we demonstrated an ambivalent systemic cytokine response in the spleen of Balb/c mice, with neither Th-1 nor Th-2 cytokines predominating. However, both were produced in greater quantities than in C57BL/6 mice. In contrast, to this more vigorous systemic response in the spleen of Balb/c mice there was more vigorous leukocyte recruitment to the airways of C57BL/6 mice. This serves to emphasise the importance of selecting the appropriate strain depending on the nature of the research question being asked and the immunological compartment being studied. Subtle variations in response to antigenic stimulation exist and these must be considered when designing experimental protocols. Overall, the use of Balb/c mice did not seem to confer any advantage over C57BL/6 mice in the development of the N.b. model of airway inflammation.

CHAPTER 7

ICAM-deficient Mice

7.1 INTRODUCTION

Reversible or partially reversible airway obstruction, inflammation, and bronchial hyper-responsiveness to various stimuli are the defining characteristics of asthma. Airway obstruction in asthma is a complex event, due to bronchospasm, inflammation, and mucus formation. Recently inflammation has assumed centre stage in the pathogenesis of the disease, since it contributes not only to airflow obstruction, but also to bronchial hyper-responsiveness, which is typical of asthma. The provoking trigger or inhaled allergen induces activation of mast cells and macrophages with the subsequent release of several pro-inflammatory mediators, including leukotrienes, chemotactic factors, and cytokines.

Antigen processed by macrophages is presented to undifferentiated T helper cells, inducing differentiation to the Th-2 phenotype, with the subsequent release of interleukin (IL)-4 and IL-5, causing IgE synthesis and eosinophil infiltration, respectively. Macrophage-derived cytokines, such as IL-1, tumor necrosis factor (TNF)-alpha, and interferon (IFN)-gamma, activate endothelial cells, upregulating the expression of adhesion molecules such as intercellular adhesion molecule (ICAM)-1 (CD54), and vascular cell adhesion molecule (VCAM)-1, which permit egression of leukocytes from the vasculature to the airway mucosa. Several inflammatory cells, such as eosinophils, mast cells, and macrophages, not only cause airway damage, but also synthesise cytokines that perpetuate the inflammatory process. This complex interplay of inflammatory cells and mediators causes the classic histopathophysiological features seen in the airways of both symptomatic and asymptomatic individuals with asthma (Bjornsdottir US et al 1999).

The cellular recruitment to the airways has been the focus of particular attention and is known to be a highly sophisticated process with much built in redundancy. Recruitment of leukocytes to the airways is brought about by the sequential interaction of pro-inflammatory cytokines, such as IL-4 and TNF-α, and cell adhesion molecules (Striz I et al 1999, Pilewski JM et al 1995). These cytokines may be derived from a number of cell types, including mast cells (see chapter 9) and the Th-2 subpopulation of $CD4^+$ lymphocytes. Th-2 cells have been implicated as the major orchestrator of the inflammatory process. Specifically, Th-2 cytokines have been shown to upregulate the expression of adhesion molecules, encouraging the influx of a variety

of inflammatory cells, in addition to promoting activation and survival of eosinophils. A potential therapeutic approach to the treatment of asthma, therefore, would be to specifically target the recruitment of eosinophils and lymphocytes by disrupting the adhesion molecules responsible for the cellular influx.

Numerous adhesion molecules have been identified as important in the recruitment of inflammatory cells to the airways, including ICAM-1, VCAM-1 and their leukocyte counter ligands, Leukocyte Function Antigen-1 (LFA-1, CD11a/CD18), Mac-1 (CD11b/CD18) in addition to Very Late Activation Antigen-4 (VLA-4), and E-selectin (Gosset P et al 1995, Bentley AM et al 1993).

This series of experiments focuses on one of these ICAM. ICAM was first characterised in 1986 and shown to be a member of the immunoglobulin gene family, containing five immunoglobulin-like domains. As well as binding LFA-1 and Mac-1 ICAM also binds *Plasmodium falciparum* and 90% of the rhinoviruses. To date the majority of evidence implicating ICAM in pulmonary inflammation comes from studies of human asthmatic biopsies or bronchoalveolar lavage (BAL) fluid and animal models of allergic lung inflammation (Grunstein MM et al 2000, Matsuse H et al 2000).

The first study to suggest a potential role for ICAM-1 in allergic inflammation of the airways was performed in non-human primates using chronic antigen challenge in animals naturally sensitised to *Ascaris suum*. In this study antigen challenge induced increased expression of ICAM-1 on the bronchial vasculature and bronchial epithelium and promoted an airway eosinophilia and hyperresponsiveness to inhaled methacholine (Wegner CD et al 1990). Subsequent studies demonstrated that local allergen challenge in humans produced an increased level of soluble (s)-ICAM in the bronchoalveolar lavage (BAL) fluid and blood of asthmatics (Takahashi N et al 1994, Sedgwick JB 1992) in addition to an increase in the expression of ICAM-1 in bronchial biopsies. ICAM upregulation has also been reported on the sputum eosinophils of asthmatics (Montefort S et a 1994a, Gundel RH et al 1992, Hansel TT et al 1991), the nasal secretions and skin of allergic individuals (Ciprandi G et al 1994, Kyan Aung J et al 1991) and on cultured human airway smooth muscle from asthmatics. In fact airway smooth muscle released s-ICAM in response to stimulation with interleukin

(IL)-5, an effect that was blocked with an IL-5-receptor-alpha blocking antibody (Grunstein MM et al 2000).

The expression of ICAM-1, in asthmatics, is upregulated during periods of airflow limitation as a result of *de novo* synthesis and in concert with increased expression of the leukocyte integrins, LFA-1 and Mac-1 (Ohkawara Y et al 1995). However, not all studies have found an upregulation of ICAM-1 in bronchial biopsies (Montefort S et al 1992) or increased serum levels of s-ICAM-1 in acute asthma when compared to stable asthmatics and normal subjects (Montefort S et al 1994b). In fact there is evidence to suggest that the level of soluble (s)-ICAM-1, s-L-selectin, s-VCAM-1, s-E-selectin, and s-P-selectin vary according to the severity and nature of the underlying inflammatory process, with different patterns being seen in acute asthma, stable asthma and acute bronchiolitis, for example (Oymar K et al 1998, Bagnato G et al 1998).

This variability in ICAM expression may also be secondary to asthmatic treatment since a number of therapeutic interventions are known to alter ICAM expression. For example, a recent study demonstrated that both budesonide and formoterol significantly inhibited the cytokine-induced ICAM-1 and VCAM-1 expression on human lung fibroblasts (Spoelstra FM 2000). Similarly, a study in children with only moderate atopic asthma treated for 2 months with inhaled budesonide demonstrated a significant reduction in the circulating (c)-ICAM concurrent with improved pulmonary function test (Cengizlier R et al 2000).

Alternatively, changes in s-ICAM may be due to local changes in the inflammatory microenvironment. For example changes in local cytokine production may induce local changes in levels of s-ICAM expression. This suggestion is supported by recent data indicating that nebulised human IL-5 increased the concentrations of sICAM-1 in the sputum 2 hours after nebulisation, peaking 48 hours later and lasting no less than 72 hours in allergic asthmatics but not in non-allergic matched controls (Shi HZ et al 1999). Furthermore, many of the Th-2 cytokines are known to specifically upregulate ICAM expression in epithelial cells. Thus, IL-4, IL-5, IL-10 and IL-13 have been shown to produce a 2 to 5-fold enhancement of ICAM-1 expression on epithelial cells uninfected with rhinovirus and a further twofold increase in infected cells. In contrast, IFN-γ only slightly reduced, but did not override, the Th-2-induced level of ICAM-1 expression on both

uninfected and virus-infected epithelial cells. Since the airway mucosa in atopic asthma is predominantly infiltrated by Th-2 lymphocytes, these results could explain the increased susceptibility to human rhinovirus infection in asthmatic patients (Bianco A et al 1998). Other authors have suggested that TNF-α may be critical in upregulating ICAM expression (Krunkosky TM et al 1996).

However, studies examining the levels of c-ICAM must be interpreted with caution since the results may be misleading (Bagnato G et al 1998). The level of c-ICAM may reflect the level of peripheral lymphocyte surface marker shedding rather than lymphocyte recruitment. This idea is supported by the finding of increased serum levels of s-ICAM in association with reduced peripheral T lymphocyte expression following local allergen challenge (De Rose V et al 1994).

Nevertheless ICAM is still considered a critical molecule in the inflammatory process since the patterns of ICAM-1 expression along the cell surface in addition to an epithelial gradient for RANTES have been shown to be critical in facilitating T cell traffic through epithelium (Taguchi M et al 1998). In addition, recent data suggest that ICAM not only facilitates cellular recruitment to the airways but also plays a critical role in mediating T-cell/T-cell, T-cell/target cell, T-cell/B-cell T-cell eosinophil interaction (Douglas IS et al 2000, Stanciu LA et al 1998). Thus, eosinophils have been shown to adhere to activated T cells via ICAM-3, but not ICAM –1, and this interaction augmented the secretion of leukotriene C4 (LTC4) from eosinophils (Douglas IS et al 2000).

Furthermore interaction of inflammatory cells with these adhesion molecules has been shown to alter cell function. Thus incubation of peripheral blood eosinophils from asthmatics with VCAM-1 induced spontaneous eosinophil adhesion whereas eosinophil adhesion to ICAM-1 required a second signal, such as granulocyte macrophage colony-stimulating factor (GM-CSF). In addition, ICAM-1 and VCAM-1 promoted superoxide anion (O2-) generation by eosinophils, an effect enhanced by GMCSF, as well as eosinophil-derived neurotoxin (EDN). ICAM-1, without GM-CSF, also promoted calcium ionophore-induced leukotriene C4 (LTC4) release from eosinophils and the release of all these pro-inflammatory mediators by eosinophils was inhibited by anti-β_2-integrin antibody. These results suggest that ICAM-1 and VCAM-1 are important in determining the eventual

function of airway eosinophils (Nagata M et al 1998). Similarly, ICAM-1 on the surface of T-cells is thought to participate in signal transduction and may thus modulate several functions including activation, proliferation, cytotoxicity and cytokine production.

Recently ICAM has been implicated in bronchospasm accompanying the common cold (Stanciu LA et al 1998). Thus rhinovirus infections, which are associated with the majority of asthma exacerbations, have been shown to upregulate ICAM-1 expression on airway epithelial cells *in vitro*. This rhinovirus-induced upregulation of ICAM-1 is critically dependent upon nuclear factor (NF)-kappaB proteins binding to the ICAM-1 promoter region (Papi A et al 1999). This effect has been shown to be inhibited by topical and systemic corticosteroids in a dose dependent manner by some (Papi A et al 2000), but not all authors (Grunberg K et al 2000). Furthermore, p50 subunit of NF- κB may be the critical factor since mice deficient in the p50 subunit are incapable of mounting eosinophilic airway inflammation compared with wild-type (WT) mice. This lack of an eosinophil response, *in vivo*, was not due to a problem with T cell activation or proliferation in the WT mice, nor was it due to a defect in the expression of the cell adhesion molecules VCAM-1 and ICAM-1. Rather it appeared to be due to the lack of IL-5 and eotaxin production, which are crucial for the proliferation, differentiation and recruitment of eosinophils into the asthmatic airway (Yang L et al 1998).

Similar results have been seen following respiratory syncytial virus (RSV) infection. Thus RSV has also been shown to upregulate ICAM expression, IgE secretion and to promote airway hyperresponsivenes (AHR) in a murine model of allergic inflammation (Matsuse H et al 2000). These findings have increased the interest in therapeutic strategies to attack ICAM to prevent the airway inflammation that accompanies upper respiratory tract infection (Stanciu LA et al 1998).

Some authors have employed blocking monoclonal antibodies (mAbs) to investigate the role of ICAM in lymphocyte and eosinophil recruitment to the airways in animal or human models of allergic inflammation. Some (Tohda Y et al 1999, Bentley AM et al 1993, Nagase T et al 1995), but not all of these studies (Gundel RH et al 1991, Nakajima H et al 1994), have shown that airway eosinophilia, epithelial shedding and bronchial hyper-responsiveness can be blocked using monoclonal antibodies directed against ICAM-1. These effects were predominantly seen in the late asthmatic response (LAR)(Tohda Y et al 1999). The reason

for the discrepancy in these results may be due to the activation status of the epithelial cells or the eosinophils. Thus recent data using anti-ICAM mAbs suggests that eosinophil adhesion to activated epithelial cells is CD18/ICAM-dependent but adhesion to resting epithelial cells is CD18/ICAM-independent (Burke-Gaffney A et al 1998).

Anti-ICAM has also been shown to reduce airway hyper-responsiveness without altering airway inflammatory cell numbers (Sun J et al 1994). This separation of the anti-inflammatory and physiological effects of anti-ICAM confirms the complex role adhesion molecules play in airway inflammation (Pilewski JM et al 1995), and such complexity is not specific to studies involving blocking antibodies to ICAM-1 (Milne AAY et al 1994).

In order to help clarify the reasons for some of the discrepant findings on the role of ICAM-1 in cellular recruitment to the airways we carried out a rigorous assessment of the role of this adhesion molecule in antigen-induced lung inflammation in ICAM knockout (ICAM-ko) mice. The aim was to test the hypothesis that knocking out ICAM-1 would significantly reduce the pulmonary inflammatory response to N.b. antigens.

The use of transgenic ICAM-ko mice confers a number of specific advantages over blocking antibody studies. For example, it circumvents the problems of antibody delivery, construction of dose response curves, the determination of adequate plasma levels, problems of toxicity and ICAM binding avidity to endothelial sites. Our results indicate that helminth antigens induced a greater pulmonary and peripheral eosinophilia in ICAM-ko mice than in wild type controls. These findings are in direct contrast to earlier results that demonstrated that the anti-ICAM monoclonal antibody, 1A29, completely inhibited the pulmonary eosinophilia in an ovalbumin-induced model of pulmonary inflammation (Richards IM et al 1996). Taken together these data suggest that the nature and strength of the stimulating antigen may play a key role in determining the level of cellular recruitment to the airways, and therefore the importance of anti-ICAM strategic.

7.2 MATERIALS AND METHODS

7.2.1 Larval Culture and Infection

Details of N.b. larval culture are given in chapter 2.

7.2.2 Animals

Details of animal husbandry are given in chapter 2.

7.2.3 Bronchoalveolar lavage and cell preparations

Details of brochoalveolar lavage and cell preparation are given in chapter 2. Data presented throughout this thesis are given per ml of BAL fluid drawn.

7.2.4 Immunofluorescence staining and flow cytometry

Details of cell preparation and the reagents used in flow cytometry are given in chapter 3.

7.2.5 Blood: Leukocyte Subsets

Details of blood acquisition and cell analysis are given in chapter 3. Data presented throughout this thesis are given per ml of blood drawn.

7.2.6 Spleen mononuclear preparations:

Details of spleen cell preparation are given in chapter 5.

7.2.7 Cytokine ELISAs

Details of specific cytokine ELISAs are given in chapter 6.

7.2.8 Generation of N.b. specific antigen

Details of N.b. specific antigen generation are given in chapter 5.

7.2.9 Statistics

The data presented in this chapter on wild type C57BL/6 mice are pooled from several experiments conducted throughout this thesis. Thus the peripheral blood and BAL fluid data presented contains, on average, 38 mice per time point. The ICAM-1 data presented were drawn from three separate experiments and therefore the number of mice pooled in each time point ranged from 8 to 39 although the average was 16 mice per time point. Although for day 3, 8 and 14 the average was 33 mice. Total and differential cell counts in the BAL fluid, blood splenocyte proliferation and cytokine production were analysed using a two-tailed paired t test assuming unequal variance in samples and a normal distribution. When this overall test of treatment was found to be significant at the $p<0.05$, this was indicated by an asterisk (*).

When the significance level reached $p<0.01$ this was indicated by two asterisks (**), and when the significance level reached $p<0.001$ this was indicated by three asterisks (***). If the result was borderline significant ($p=0.05$), then this was denoted by a # sign. The key findings were tested using Mann Whitney U-test to check that the assumption of normal distribution was not generating spuriously significant results.

7.3 RESULTS

7.3.1 Time course of peripheral blood changes following N.b. infection

I. Total Leukocyte Count

Primary infection with third stage N.b. larvae produced a significant increase in the total number of blood leukocytes in ICAM-ko and wild type (WT) mice, peaking on day 14 pi in both ($p <0.001$) and subsiding significantly in ICAM-ko but not WT controls ($p<0.001$)(Figure 1). In addition, there was a significantly greater number of circulating leukocytes in naïve ICAM-ko mice compared to naïve controls ($p<0.001$) as well as on day 8 pi ($p<0.005$) and day 14 pi ($p<0.003$). In contrast, the greater number of leukocytes in ICAM-ko mice on day 3 pi did not quite reach statistical significance ($p<0.06$) and the number of leukocytes on day 26 pi was significantly less in ICAM-ko mice than wild type controls on day 26 pi ($p<0.02$)(see Figure 7-1).

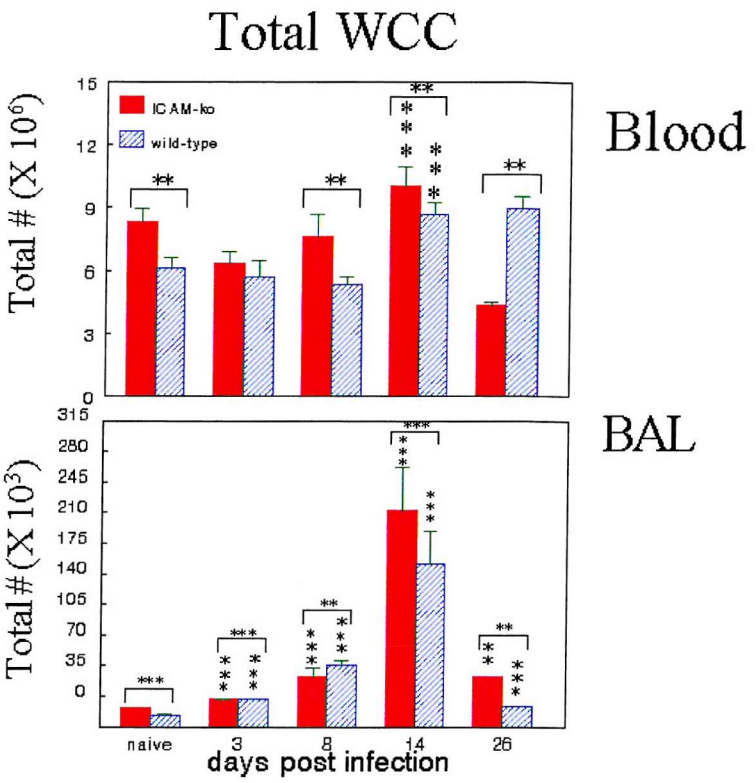

Figure 7-1. The total number of leukocytes recruited to the blood and airways in ICAM-knockout mice and wild-type control mice following N.b. infection.

Compared to naïve mice there was a significant leukocyte recruitment to the airways of both ICAM-ko and wild type controls on day 3 pi ($p<0.0002$ and $p<0.0003$ respectively), as well as on day 8 and day 14 ($p<0.0001$), and day 26 pi ($p<0.003$ and $p<0.0001$ respectively). The greater number of circulating leukocytes in ICAM-ko mice compared to controls was reflected in a greater number of leukocytes recovered from the BAL fluid in naïve ICAM-ko mice compared to controls ($p<0.0001$). However, this finding was reversed on day 3 and 8 pi with more leukocytes recovered from the BAL fluid of control mice ($p<0.0006$ and $p<0.02$ respectively). By day 14 pi there was a significantly greater number of leukocytes recovered from the airways of ICAM-ko mice ($p<0.0001$) and this effect was sustained on day 26 pi ($p<0.02$)(Figure 7-1).

II. Lymphocytes

The significant increase in the total leukocyte count following N.b. infection was reflected in the significant increase in the total number of lymphocytes in the peripheral blood in WT and ICAM-ko mice on day 14 pi. Thus the total lymphocyte count increased from 4.8 $x10^6$ in WT naïve mice to 6.7 $x10^6$ 14 days pi ($p<0.001$), and from 5.9 $x10^6$ in naïve ICAM-ko mice to 7.3

x10^6 14 days pi ($p<0.03$). This lymphocytosis had not subsided by day 26 pi in WT but had returned to naïve levels in ICAM-ko mice ($p<0.002$). In line with the greater number of circulating leukocytes in ICAM-ko mice there were significantly more circulating lymphocytes in the blood of naïve ICAM-ko mice compared to controls, 5.9 x10^6 and 4.8 x10^6 respectively ($p<0.02$). In addition, following N.b. infection there was a significantly greater lymphocytosis in ICAM–ko mice on day 3 pi 5.6 x10^6 and 4.5 x10^6 respectively ($p<0.02$), on day 8 pi 4.8 x10^6 and 3.8 x10^6 respectively ($p< 0.006$). However, there was no such difference on day 14 pi ($p<0.16$) and by day 21 pi the difference had reversed to 3.6 x10^6 in ICAM-ko compared to 7.6 x10^6 in WT controls ($p<0.002$)(Figure 7-2).

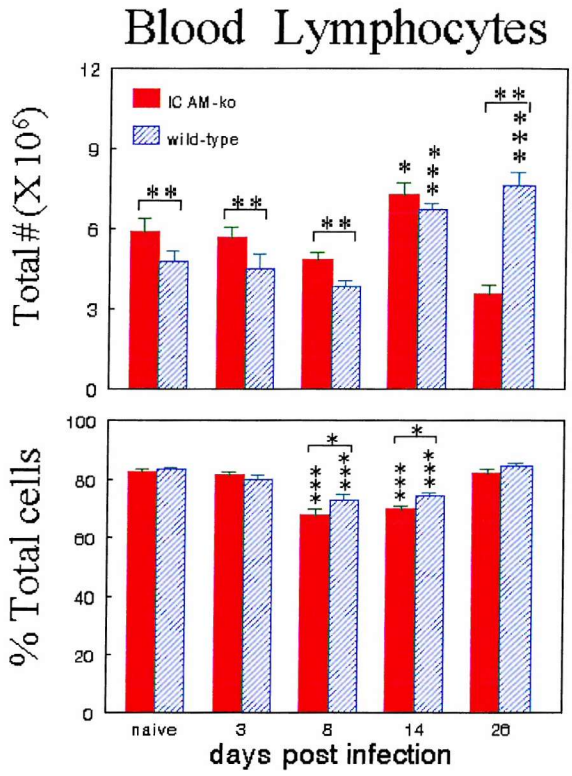

Figure 7-2. The total number and percentage of lymphocytes in the blood in ICAM-knockout mice and wild-type control mice following N.b. infection.

In contrast, the percentage of lymphocytes circulating in the blood of naïve WT and ICAM-ko mice was remarkably similar (83.2% and 83.5% respectively). There was a significant decrease in the percentage of lymphocytes circulating in the blood of both ICAM-ko and WT controls on day 8 pi ($p<0.001$) and day 14 pi ($p<0.001$). Furthermore this significant reduction in the percentage of lymphocytes circulating was more marked in ICAM-ko mice on day 8 pi ($p<0.04$) and on day 14 pi ($p<0.03$). This was almost certainly due to the significant blood eosinophilia during the late phase response reducing the overall percentage of lymphocytes in the infected animals (Figure 7-2).

III. Neutrophils

There was a significant increase in the number of circulating neutrophils following N.b. infection in WT control mice on 8 day pi ($p<0.0003$), day 14 ($p<0.0005$) and day 26 pi ($p<0.01$). Similarly, there was also a significant increase in the number of circulating neutrophils in ICAM-ko 8 days after N.b. infection, from 0.92 $x10^6$ in naïve ICAM-ko mice to 1.85 $x10^6$ on day 8 pi ($p<0.01$). In keeping with the greater number of circulating leukocytes in ICAM-ko blood compared to WT control mice there was also a significantly greater number of neutrophils circulating in ICAM-ko compared to controls, 0.92 $x10^6$ and 0.6 $x10^6$ respectively ($p<0.001$). This discrepancy was also apparent on day 3 pi, 0.96 $x10^6$ and 0.75 $x10^6$ respectively but this was not statistically different. The difference was most marked on day 8 pi with significantly more neutrophils circulating in ICAM-ko mice compared to controls, 1.85 $x10^6$ and 0.89 $x10^6$ respectively ($p<0.0001$). This difference remained at day 14 pi ($p<0.0007$) and day 26 pi ($p<0.01$)(Figure 7-3).

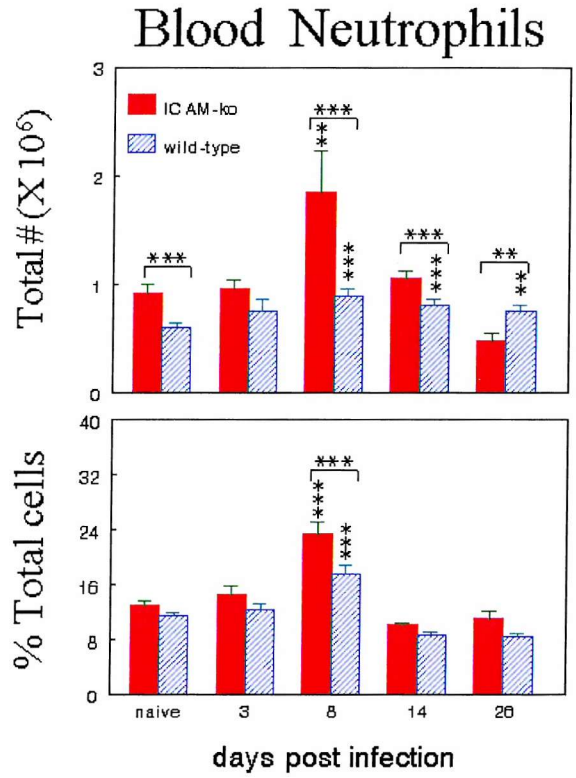

Figure 7-3. The total number and percentage of neutrophils in the blood in ICAM-knockout mice and wild-type control mice following N.b. infection.

The change in the percentage of neutrophils in the peripheral blood mirrored the change in the number of neutrophils circulating. Thus there was a significant increase from 13% in naïve ICAM-ko mice to 23.5% on day 8 pi ($p<0.001$). Similarly, the percentage of neutrophils increased from 11.5% in naïve control mice to 17.6% on day 8 pi ($p<0.0002$). The increase in

neutrophil recruitment to the vascular compartment on day 8 pi was significantly greater in ICAM-ko mice compared to control mice ($p<0.0001$)(Figure 7-3).

IV. Eosinophils

Of all the changes in the peripheral blood the eosinophilia was the most marked. In contrast to the total leukocyte, lymphocyte and neutrophil numbers there were no differences between naïve ICAM-ko mice and naïve WT mice in the eosinophil count. There was a significant increase in eosinophil numbers in wild type mice following N.b. infection. Thus the number of eosinophils in the blood increased from 0.08 $x10^6$ in naïve WT mice to 0.21 $x10^6$ on day 3 pi ($p<0.04$), 0.13 $x10^6$ on day 8 pi ($p<0.05$), 1.15 $x10^6$ on day 14 pi ($p<0.001$) and 0.27 $x10^6$ day 26 pi ($p<0.001$). In contrast, the number of eosinophils in the blood of ICAM –ko mice only increased significantly during the late phase response rising from 0.11 $x10^6$ in naïve mice to 1.79 $x10^6$ on day 14 pi ($p<0.001$).

While the eosinophilia in WT mice was significantly greater on day 3 pi ($p<0.02$) and day 26 pi ($p<0.003$) during the late phase response there were significantly more eosinophils in the blood of ICAM-ko mice than in WT controls. This suggests that a lack of ICAM did not hinder the recruitment of eosinophils to the vascular compartment (Figure 7-4).

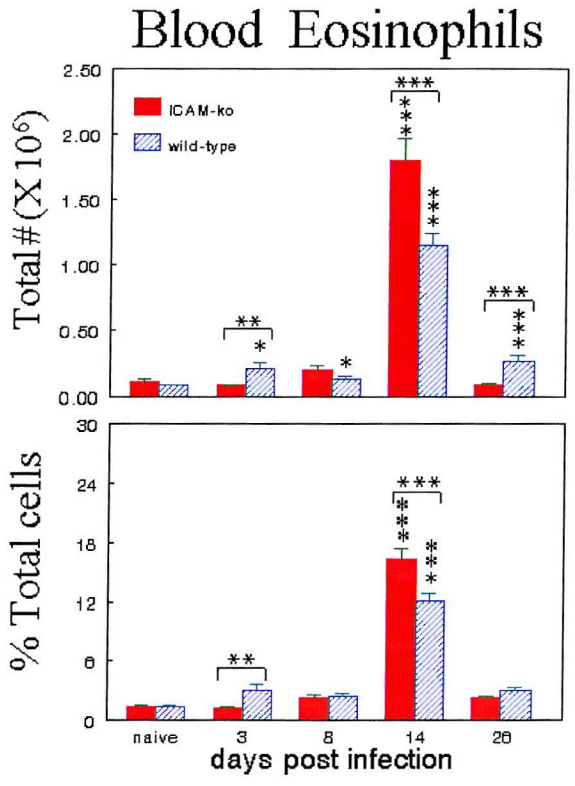

Figure 7-4. The total number and percentage of eosinophils in the blood in ICAM-knockout mice and wild-type control mice following N.b. infection.

This significant rise in eosinophil numbers in both ICAM-ko and WT controls was reflected in a significant rise in the percentage of eosinophils in the peripheral blood in both ICAM-ko and WT mice peaking at 16.3% and 12.1% on day 14 pi respectively ($p<0.001$). The increase in the percentage of eosinophils in the blood followed the same pattern as the total number of eosinophils. Thus there were significantly more eosinophils in the blood of WT mice during the early phase response ($p<0.01$), but during the late phase response there was significantly more eosinophils in the blood of ICAM-ko mice ($p<0.001$)(Figure 7-4).

7.3.2 Time course of leukocyte infiltration into the lung following N.b. infection

I. Lymphocytes

Lymphocyte recruitment to the BAL fluid increased significantly in ICAM-ko and wild type control mice. Specifically, there was an increase in lymphocyte numbers in ICAM-ko mice, from 12.9×10^3 in naïve animals to 119×10^3 on day 8 pi ($p<0.001$). Numbers increased further to 245×10^3 on day 14 pi ($p<0.0003$), before falling to 136×10^3 on day 26 pi ($p<0.001$). This represented a 14-fold increase in lymphocyte numbers during the late phase response. Similarly there was an increase in lymphocyte numbers in WT mice, rising from 12.8×10^3 in naïve animals to 159×10^3 on day 8 pi ($p<0.001$). Numbers increased further to 242×10^3 on day 14 pi ($p<0.001$), before falling to 47×10^3 on day 26 pi ($p<0.001$). This represented a 19-fold increase in lymphocyte numbers during the late phase response. The recruitment of lymphocytes to the BAL fluid in ICAM-ko and WT controls was not significantly different, apart from on day 26 pi when there were still significantly more lymphocytes remaining in the BAL fluid of ICAM-ko mice ($p<0.01$)(Figure 7-5).

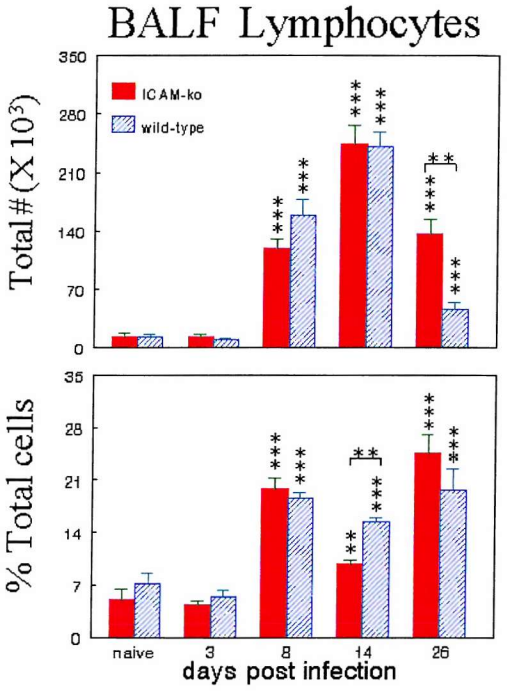

Figure 7-5. The total number and percentage of lymphocytes in the BAL fluid in ICAM-knockout mice and wild-type control mice following N.b. infection.

This airway lymphocytosis was reflected in the percentages of lymphocytes recovered from the BAL fluid which increased significantly during the late phase response from 5% in naïve ICAM-ko mice to 19.8% on day 8 pi ($p<0.001$). By day 14 pi this had decreased somewhat due to the proportionate increase in airways eosinophils but was still significantly greater than naïve mice at 9.8% ($p<0.01$). The percentage of lymphocytes in the airways remained high on day 26 pi at 24.6% ($p<0.001$). Similarly, the percentage of lymphocytes recruited to the airways of WT control mice increased significantly from 7.2% in naïve WT mice to 18.6% on day 8 pi ($p<0.001$). By day 14 pi this had also decreased somewhat due to airways eosinophilia but was still significantly greater than naïve mice at 15.4% ($p<0.01$). This reduction on day 14 pi was significantly less in WT control mice than in ICAM-ko mice ($p<0.01$). By day 26 pi the percentage of lymphocytes in the airways remained higher than naïve WT controls at 19.6% ($p<0.001$)(Figure 7-5).

II. Neutrophils

Neutrophils dominated the early cellular response in both ICAM-ko and WT control mice. Thus the total number of neutrophils increased 43-fold in wild type mice from 1.7×10^3 in naïve animals to 76.8×10^3 on day 3 pi ($p<0.001$). Similarly, the total number of neutrophils increased in ICAM-ko mice from zero in naïve animals to 50.3×10^3 on day 3 pi ($p<0.001$).

By the late phase response this neutrophilia had substantially subsided in ICAM-ko mice and WT controls. Although numbers on day 8 pi in WT controls were still significantly greater than in naïve WT mice ($p<0.05$).

Similarly, the percentage of neutrophils increased 34-fold from 0.8% to 26.9% in WT mice ($p<0.001$) and from zero to 15.4% in ICAM-ko mice ($p<0.001$). The recruitment of neutrophils to the airways in WT controls was significantly greater than in ICAM-ko mice during the early phase response ($p<0.001$). By day 8 pi this early neutrophilia had all but disappeared in both ICAM-ko and WT control mice (Figure 7-6).

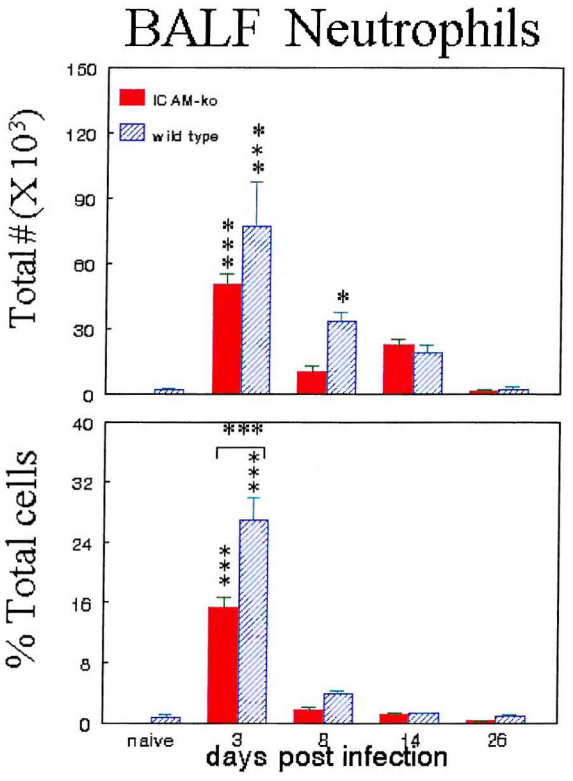

Figure 7-6. The total number and percentage of neutrophils in the BAL fluid in ICAM-knockout mice and wild-type control mice following N.b. infection.

III. Eosinophils

The late phase pulmonary response was dominated by a significant increase in the number and percentage of eosinophils in the BAL fluid in WT and ICAM-ko mice. In ICAM-ko mice the number of eosinophils in the airways increased from zero in naïve mice to 3.39 $x10^3$ 3 days pi ($p<0.001$), to139 $x10^3$ on day 8 pi ($p<0.001$) and increased still further by day 14 pi to 2.1 $x10^6$ ($p<0.001$). Similarly, the number of eosinophils in the airways of WT mice increased from 5.6 $x10^3$ in naïve WT mice to 356 $x10^3$ on day 8 pi ($p<0.001$) and increased still further by day 14

pi to 1.2 x10^6 ($p<0.001$). While the increase in eosinophil numbers was significantly greater on day 8 pi in WT mice ($p<0.002$) by day 14 pi there were significantly more eosinophils in the airways of ICAM-ko mice than in WT controls ($p<0.001$)(Figure 7-7).

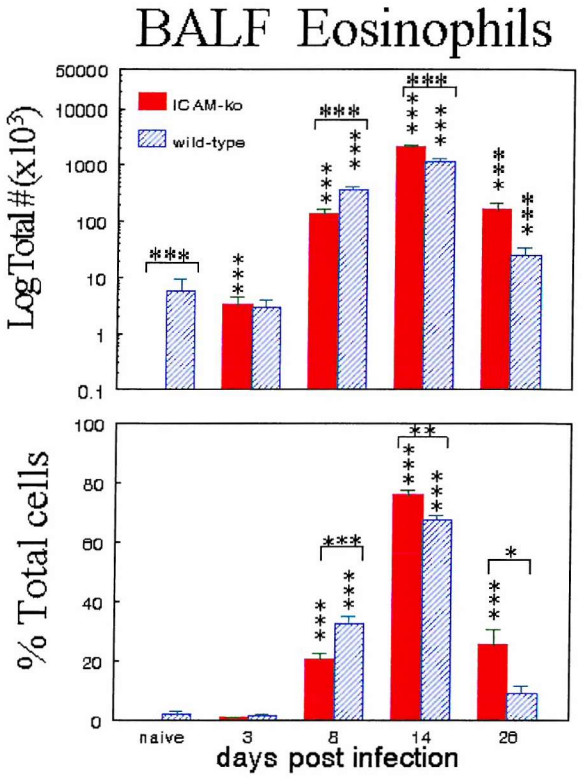

Figure 7-7. The total number and percentage of eosinophils in the BAL fluid in ICAM-knockout mice and wild-type control mice following N.b. infection. Eosinophil counts are given on a log scale for visual representation purposes only.

The changes in airway eosinophil numbers following N.b. infection were reflected in changes in the percentages of eosinophils recovered from the BAL fluid. Thus the percentage of eosinophils in the airways of ICAM-ko mice rose from zero in naïve mice to 20.4% on day 8 pi ($p<0.001$), rising still further to 76% by day 14 pi ($p<0.001$) before falling to 25.4% on day 26 pi ($p<0.001$). Similarly, the percentage of eosinophils recruited to the airways in WT controls also increased significantly during the late phase response. Thus the eosinophils made up 2% of the airways leukocytes in naïve WT mice but by day 8 pi this had risen to 32.6% ($p<0.001$) and 67.8% by day 14 pi ($p<0.001$), before falling back to 9.1% on day 26 pi. The percentage of eosinophils recruited from the airways of ICAM-ko mice was significantly greater than WT mice on day 14 pi ($p<0.01$) and day 26 pi ($p<0.05$), but was significantly less on day 8 pi ($p<0.001$). This suggested that the eosinophil recruitment to the airways in ICAM-ko mice may have occurred a little later than in WT controls (Figure 7-7).

In addition to the cellular changes outline above there was a significant increase in the number of mononuclear cells in the BAL fluid 14 days pi. However, this represented a significant decrease when expressed as a percentage of total leukocytes from 90 and 95% in naïve WT and ICAM-ko mice to 16% and 13% 14 days pi in naïve WT and ICAM-ko mice respectively ($p<0.001$) (data not shown).

7.3.3 Splenocyte proliferation and cytokine production to specific antigen

The splenocyte proliferative response to specific antigen was a significantly greater on day 14 pi compared to naïve spleens and spleens harvested 26 days pi ($p<0.001$)(data not shown). In contrast, there was no significant difference in the proliferative response to the non-specific mitogens concavalin A or anti-CD3 between naïve and infected animals (data not shown). In two experiments the day 14 splenocyte proliferative response in WT mice was compared to ICAM-ko mice. In both cases WT mice had a significantly greater proliferative response to specific antigens than the ICAM-ko mice ($p<0.001$), although no such difference in the proliferative response to specific antigen was found between WT and ICAM-ko mice on day 8 pi (data not shown).

Cultured splenocytes were stimulated with varying dose of heterogenous N.b. adult antigens *in vitro* and the production of the Th-2 cytokines, IL-5 and IL-10, were assayed. Both ICAM-ko and WT mice produced detectable amounts of IL-5 and IL-10 during the late phase pulmonary response, peaking on day 14 pi and decreasing somewhat by day 26 pi. Generally speaking splenocyte Th-2 cytokine production appeared to be optimally stimulated by 40 μg/well of N.b. antigen (Figure 7-8). There was no statistically significant difference between the amount of either IL-5 or IL-10 produced by WT or ICAM-ko at any of the stimulating doses of N.b. antigen (data not shown).

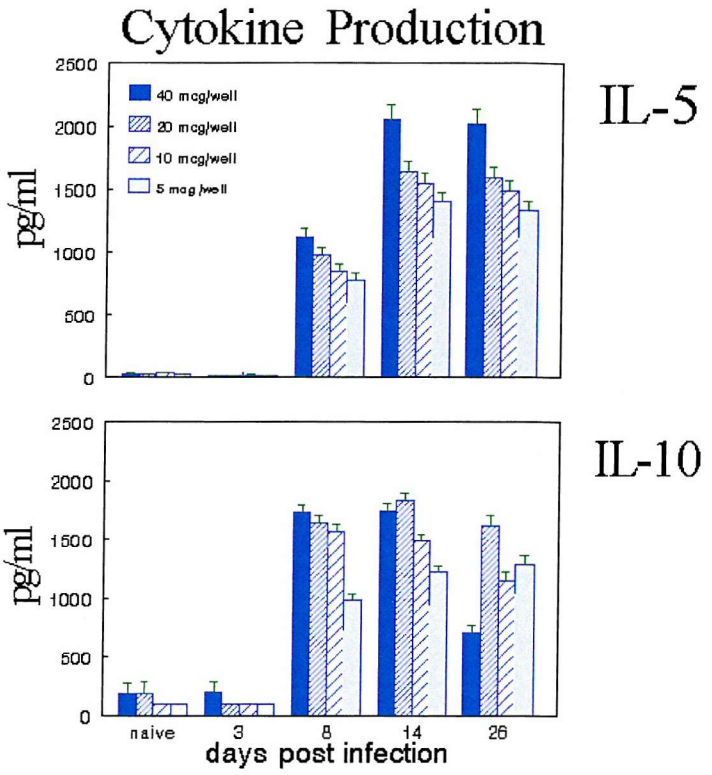

Figure 7-8. Splenocyte cytokine production, in response to varying doses of N.b. specific antigen, in ICAM-knockout mice following N.b. infection.

Based since there seemed to be a trend in favour of 40 μg/well of N.b. antigen this dose was used in subsequent experiments to stimulate Th-2 cytokine production in ICAM-ko mice and compared this to WT controls.

The production of IL-5 and IL-10 in both ICAM-ko and WT control mice was greatest during the late phase pulmonary response on day 14 pi. Thus in ICAM-ko mice, IL-5 production increased from 32 pg/ml in naïve mice to 1,121 pg/ml on day 8 pi ($p<0.01$). IL-5 levels increased still further on day 14 pi to 2051 pg/ml ($p<0.001$) before starting to decline on day 26 pi to 2023 pg/ml ($p<0.001$). Similarly, IL-5 levels in WT control mice increased significantly during the late phase response. IL-5 was undetectable in naïve WT mice or by 3 days pi but by day 8 levels had increased to 1517 pg/ml. By day 14 pi IL-5 levels had increased significantly, compared to day 8 pi, to 2319 pg/ml ($p<0.001$). No data was recorded for WT mice on day 26 pi (Figure 7-9).

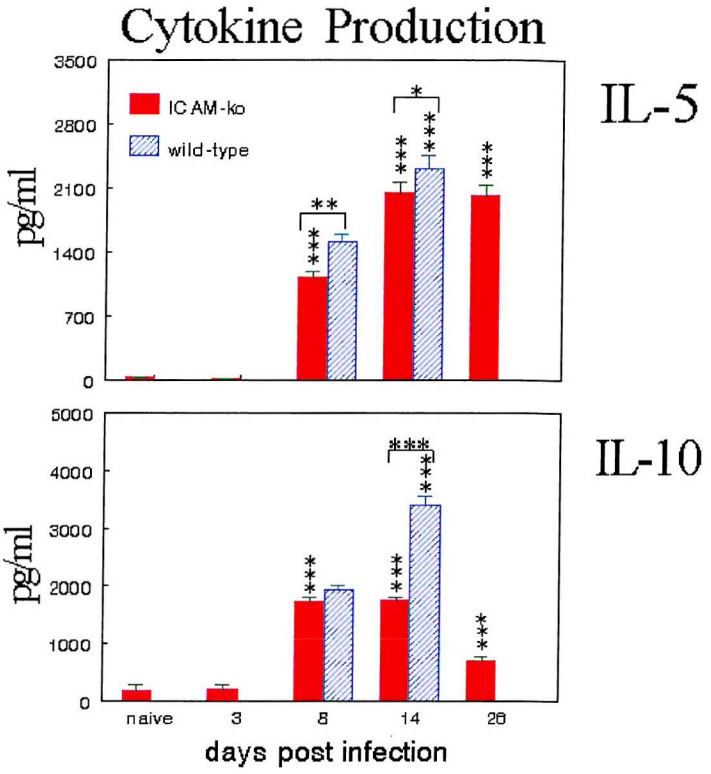

Figure 7-9. Splenocyte cytokine production in response to stimulation with N.b. specific antigen in ICAM-knockout mice and wild-type control mice following N.b. infection.

The production of IL-5 in WT mice was significantly greater on day 8 and day 14 pi compared to ICAM-ko mice ($p<0.01$ and $p<0.05$ respectively).

Similarly, the production of IL-10 increased significantly during the late phase pulmonary response in both ICAM-ko and WT control mice. Thus in ICAM-ko mice, IL-10 production increased from 184 pg/ml in naïve mice to 1,735 pg/ml on day 8 pi ($p<0.001$). IL-5 levels increased slightly on day 14 pi to 1746 pg/ml ($p<0.001$) before declining on day 26 pi to 704 pg/ml ($p<0.001$). IL-10 was undetectable in naïve WT mice or just 3 days pi but by day 8 levels had increased to 1939 pg/ml. By day 14 pi IL-10 levels had increased significantly, compared to day 8 pi, to 3414 pg/ml ($p<0.001$). No data was recorded for WT mice on day 26 pi. The production of IL-10 in WT mice was significantly greater on day 14 pi compared to ICAM-ko mice ($p<0.001$)(Figure 7-9).

7.3.4 Immunofluorescence staining and flow cytometry

The gates for lung and BAL fluid lymphocytes were well defined throughout, whereas the absence of a specific eosinophil surface marker made identification of an eosinophil gate

difficult, particularly in the lung tissue and BAL fluid of naïve animals and animals 3 days pi. The gated eosinophil population may have been contaminated by other granulocytes, particularly in the BAL fluid, 3 days pi. In contrast, the lung tissue and BAL fluid eosinophil gate was a clearly defined 14 days pi.

7.3.5 Phenotypic analysis of lymphocytes in airway lumen following N.b. infection

I. Thy 1^+.

Phenotypic analysis of the BAL fluid lymphocytes revealed a significant increase in the total number of $Thy1^+$ lymphocytes in the BAL fluid, in both the ICAM-ko and wild type mice but only during the late phase pulmonary response. Specifically, in ICAM-ko mice $Thy1^+$ lymphocyte numbers increased slightly from 15.2 x 10^3 in naïve mice to 16.7 x 10^3 on day 3 pi. But by day 8 pi lymphocyte numbers had increased significantly to 98 x 10^3 ($p<0.05$) and increased still further by day 14 pi to 155 x 10^3 ($p<0.001$), before subsiding somewhat to 37 x 10^3 on day 26 pi. These changes very closely mirrored the changes seen in the recruitment of $Thy1^+$ lymphocytes to the BAL fluid of WT control mice (Figure 7-10).

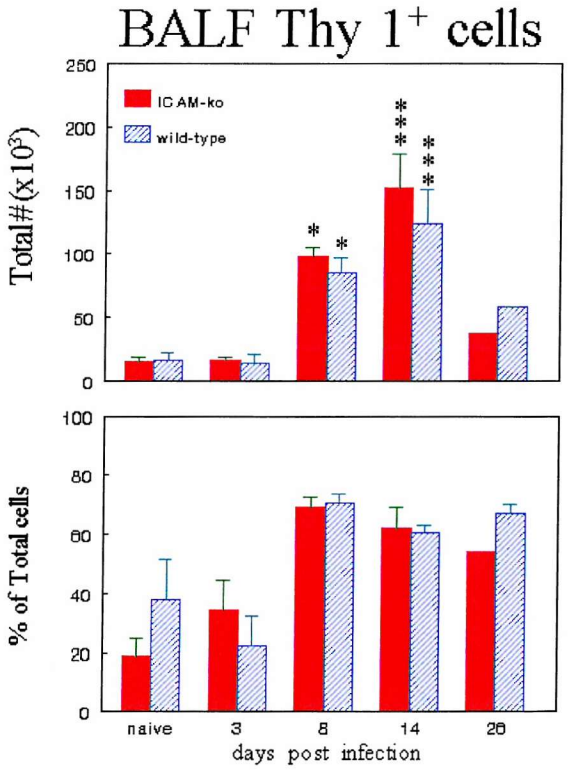

Figure 7-10. The total number, and percentage, of Thy1+ T cells in the BAL fluid in ICAM knockout and wild-type control mice following N.b. infection.

There was also a non-significant increase in the percentage of $Thy1^+$ lymphocytes in the BAL fluid of ICAM-ko and WT control mice, during the late phase pulmonary response. The total number and percentage of $Thy1^+$ lymphocytes recruited to the BAL fluid of ICAM-ko mice did not differ significantly from WT control mice (Figure 7-10).

II. $CD4^+$

The total number of $Thy1^+CD3^+CD4^+$ lymphocytes recruited to the BALF fluid increased significantly during the late phase pulmonary response. Thus, in ICAM-ko mice the number of $CD4^+$ lymphocytes increased from 8.3×10^3 in naïve mice to 44×10^3 on day 8 pi ($p<0.01$). By day 14 pi there was an 8-fold increase in the number of $CD4^+$ lymphocytes in ICAM-ko mice, to 68.6×10^3 when compared to naïve mice ($p<0.001$). Similarly, the number of $CD4^+$ lymphocytes in WT mice increased from 6.3×10^3 in naïve mice to 54×10^3 on day 8 pi ($p<0.01$) and by day 14 pi there was a 13-fold increase in the number of $CD4^+$ T cells in WT mice, to 81×10^3($p<0.001$)(Figure 11). By day 26 pi the number of $CD4^+$ T cells in ICAM-ko and WT mice had subsided to 17×10^3 and 32×10^3 respectively. The total number of $CD4^+$ cells recruited to the airways of ICAM-ko and WT was not significantly different.

There was a substantial increase in the percentage of BAL fluid T cells expressing $CD4^+$ in WT mice from 50% in naïve animals to 73% during the late response, although these changes did not reach statistical significance. In contrast, the percentage BAL fluid T cells expressing $CD4^+$ in ICAM-ko mice did not change significantly following N.b. infection (Figure 7-11).

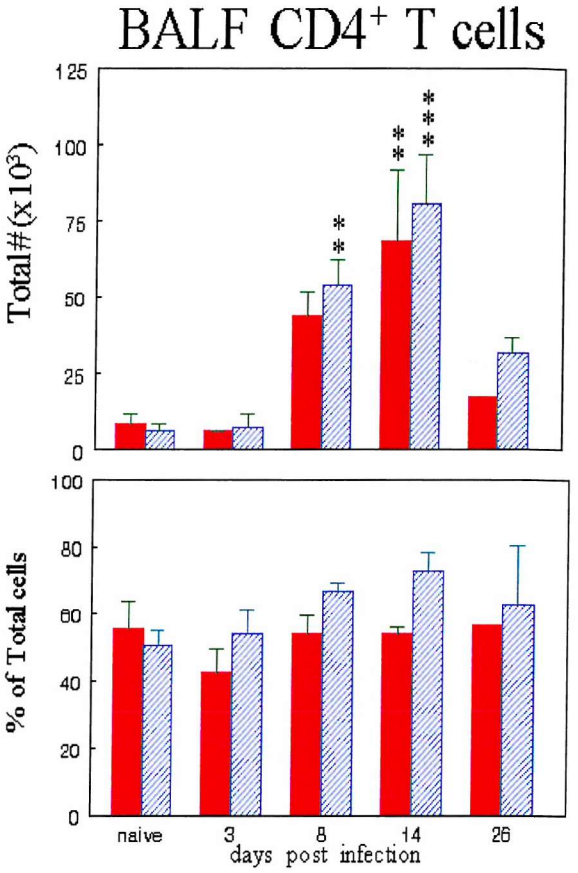

Figure 7-11 The total number and percentage of CD4+ T cells in the BAL fluid in ICAM knockout and wild-type control mice following N.b. infection.

III. IL-2R (CD25).

The percentage of BAL fluid cells expressing the T cell activation marker IL-2R peaked during larval migration in WT mice, rising from a background level, in naïve animals, of < 1% to 11.4% on day 3 pi ($p<0.03$), and returned to background levels during the late phase response (Table 1).

Surface Marker	Naïve	Day 3	Day 8	Day 14	Day 26
CD25 (IL-2R)	57 ± 38 (0.5)	1,328 ± 514 (11.4)***	1,280 ± 159 (1.7)	851 ± 580 (1.1)	1,803 ± 786 (3.3)
CD69	802 ± 230 (8.2)	1,112 ± 536 (6.6)	1,435 ± 763 (2.1)	629 ± 325 (0.7)	1478 (3.2)
CD43	10,598 ± 4,058 (76.8)	9,727 ± 4,954 (72.8)	65,173 ± 10,639 (80.7)	91,682 ± 24,603 (79.7)	31,711 ±5,295 (62.5)
CD45RB	317 ± 132 (7.1)	2,654 ± 1,238 (37.7)**	4,749 ± 3,356 (11.3)	8,788 ± 3,511** (10.9)	2,580 ± 1,972 (7.3)
ICAM-1 (CD54)	5,254 ± 1,695 (44.1)	7,727 ± 4,342 (49.3)	37,671 ± 5,249 (47)	28,807 ± 10,772 (35.3)	22,636 ±2,309 (43.2)

Table 1: Phenotypic analysis of BAL fluid T lymphocytes at various time points after N.b. infection in WT mice. Data are the means ± SEM from 20 to 40 mice/time point, from two to more experiments. Percentages are shown in brackets. Statistically significant differences compared to naïve or day 3 mice, as determined by ANOVA, are indicated by asterix, (* = $p<0.05$, ** = $p<0.01$,*** = $p<0.001$).

There was no detectable expression of IL-2R during the early response in ICAM-ko mice and no significant increase in IL-2R during the late phase response (Table 2).

Surface Marker	Naïve	Day 3	Day 8	Day 14	Day 26
CD25 (IL-2R)	617 ± 178 (5.2)	0 ± 0 (0)	1,738 ± 1,390 (2.3)	2,819 ± 2,313 (2.0)	1,307 (4.3)
CD69	2,443 ± 2,152 (11.8)	304 ± 304 (1.7)	7,829 ± 7,829 (12.7)	11,166 ± 5,128*# (10.1)	0 ± 0 (0)
CD43	9,377 ± 2,799 (68.6)	7,755 ± 744 (51.9)	69677 ± 8,347 (85.1)**	112,897 ± 19,185 (90.1)**	25,746 (84.7)
CD45R B	3,059 ± 2,070 (28.4)	1,421 ± 151 (22)	11,137 ± 1,595* (25.3)	15,085 ± 5,774** (21.2)	1,609 (9.3)
ICAM-1 (CD54)	712 ± 473 (4.1)	185 ± 185 (1.0)	0 ± 0 (0)	3,323 ± 2,715 (2.3)	182 (0.6)
CD 18	9,709 ± 2,240 (73.9)*	6,286 ± 1,101 (42.6)	65,488 ± 7,057** (80)*	118,889 ± 23,457*** (94.3)**	25,716 (84.6)

Table 2:Phenotypic analysis of BAL fluid T lymphocytes at various time points after N.b. infection in ICAM-ko mice. Data are the means ± SEM from 10 to 30 mice/time point, from two to more experiments. Percentages are shown in brackets. Statistically significant differences compared to naïve or day 3 mice, as determined by ANOVA, are indicated by asterix, (* = $p<0.05$, ** = $p<0.01$,*** = $p<0.001$). # indicates a statistically significant difference between ICAM-ko and WT mice ($p<0.05$)

IV. CD69

The expression of CD69 increased slightly during the early phase response and decreased thereafter during the late phase response in WT mice. In contrast CD69 expression in ICAM-ko mice dropped substantially from 11.8% (2.4×10^3) in naïve animals to 1.7% (0.3×10^3) during the early response before rising to 10.1% (11×10^3) during the late response ($p<0.05$).

This represented a significantly greater CD69 expression in ICAM-ko mice compared to WT controls on day 14 pi (Table 1).

V. CD45RB

The percentage of BAL fluid T cells expressing CD45RB increased significantly during larval migration through the lungs in WT mice, rising from 4.5% in naïve animals to 38% 3 days pi ($p< 0.02$), before decreasing back to naïve levels during the late phase response. In contrast, the percentage of BAL fluid T cells expressing CD45RB in ICAM-ko mice remained high throughout the infection, ranging from 28% in naïve animals to 21% on day 14 pi (Tables 1 & 2).

VI. L-selectin (CD62L)

The percentage of T cells expressing L-selectin in WT mice peaked during larval migration through the lungs, rising from 5.1% in naïve animals to 38% on day 3 pi and falling to < 7% during the late phase response ($p< 0.001$)(Figure 7-12). Similarly, the percentage of T cells expressing L-selectin in ICAM-ko mice peaked during larval migration, rising from 7.1% in naïve animals to 20.2% on day 3 pi prior to falling to 8.5% during the late response. There was a significantly greater increase in the percentage of L-selectin$^+$ T cells in WT mice compared to ICAM-ko mice ($p<0.01$)(Figure 7-12). In contrast, the total number of T cells expressing L-selectin, remained unchanged in WT mice but increased significantly in ICAM-ko mice during the late phase response ($p<0.01$)(Figure 7-12). Furthermore, the number of T cells expressing L-selectin in ICAM-ko mice was significantly greater than in WT mice 14 days pi ($p<0.05$)(Figure 7-12).

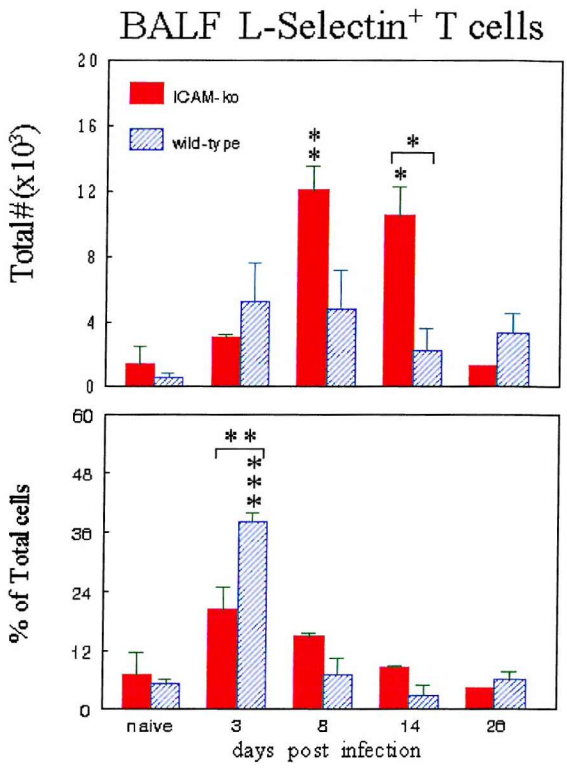

Figure 7-12. The total number and percentage of T cells in the BAL fluid expressing L-selectin in ICAM knockout and wild-type (WT) control mice following N.b. infection.

VII. Pgp-1 (CD44)

The percentage of BAL fluid $CD4^+$ cells in WT mice expressing the hyaluronate receptor, Pgp-1, decreased during the early response, falling from 69% in naïve animals to 41% on day 3 pi, before rising to 82% during the late response (Figure 7-13).

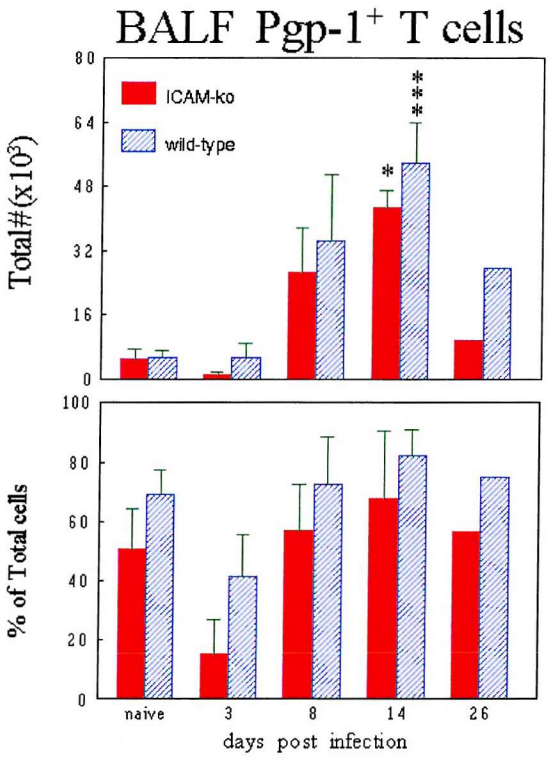

Figure 7-13. The total number and percentage of T cells in the BAL fluid expressing PgP-1 in ICAM knockout and wild-type control mice following N.b. infection.

Similarly, the percentage of BAL fluid $CD4^+$ cells in ICAM-ko mice expressing Pgp-1 decreased from 51% in naïve animals to 15% on day 3 pi, before rising to 68% during the late response. This biphasic pattern was reflected in the changes in the total number of $CD4^+$ T cells expressing Pgp-1 in ICAM-ko mice but was less clear cut in WT mice. In both ICAM-ko mice and WT mice there was a significant increase in the total number of $CD4^+$ cells expressing Pgp-1 during the late phase response ($p<0.05$ and $p<0.001$, respectively)(Figure 7-13).

VIII. CD43

The number of BAL fluid T cells expressing the activation marker CD43 increased significantly in ICAM-ko mice during the late phase response, increasing from 9.4×10^3 in naïve mice to 69.7×10^3 in mice on day 8 pi ($p<0.01$). Total numbers of $CD43^+$ T cells increased still further by day 14 pi to 112.9×10^3 ($p<0.01$), before declining to naïve levels by day 26 pi. The percentage of $CD43^+$ T cells in the airways of ICAM-ko mice increased steadily following N.b. infection. In contrast, the percentage of $CD43^+$ T cells in WT mice remained fairly constant throughout although the total number of T cells expressing this marker did increase following N.b. infection (Tables 1 & 2).

IX. ICAM-1

The total number of T cells in the BAL fluid of WT mice expressing ICAM-1 increased significantly during the late pulmonary response from 7.3 x 10^3 in naïve mice to 40.9 x 10^3 on day 8 pi ($p< 0.0008$). Numbers increased further by day 14 pi to 55.5 x 10^3 ($p<0.03$) before declining slightly by day 26 pi to 27.5 x 10^3 (Table 2). This was not reflected in a significant change in the percentage of cells expressing ICAM-1 (Table 2). The expression of ICAM-1 in ICAM-ko mice was persistently <5% (Table 1). However, the expression of CD18, the counter receptor for ICAM-1, increased significantly in ICAM-ko mice during the late response. Thus the number of T cells expressing CD18 increased from 9.7 x 10^3 in naïve mice to 65.5 x 10^3 on day 8 pi ($p<0.01$) and 118.9 x 10^3 on day 14 pi ($p<0.001$) (Table 1).

X. VLA-4

The expression of VLA-4 on BAL fluid T cells in WT and ICAM-ko mice increased significantly during the late pulmonary response. Thus the number of T cells in WT mice expressing VLA-4 increased from 8.4 x 10^3 in naïve mice to 28.3 x 10^3, on day 8 pi ($p<0.01$). The number of T cells expressing VLA-4 increased still further on day 14 pi to 42.1 x 10^3 ($p<0.001$) before declining to 24.7 x 10^3 on day 26 pi (Figure 7-14). Similarly, the number of T cells in ICAM-ko mice expressing VLA-4 increased from 2.4 x 10^3 in naïve mice to 23.3 x 10^3, on day 8 pi. The number of T cells expressing VLA-4 in ICAM-ko mice increased still further on day 14 pi to 31.3 x 10^3 ($p<0.01$) before declining to 11.0 x 10^3 on day 26 pi (Figure 7-14). These changes were less apparent when expressed as a percentage, with the only significant finding being a much lower expression of VLA-4 on the T cells recovered from the BAL fluid of ICAM-ko on day 3 pi compared to WT control mice ($p<0.05$)(Figure 7-14).

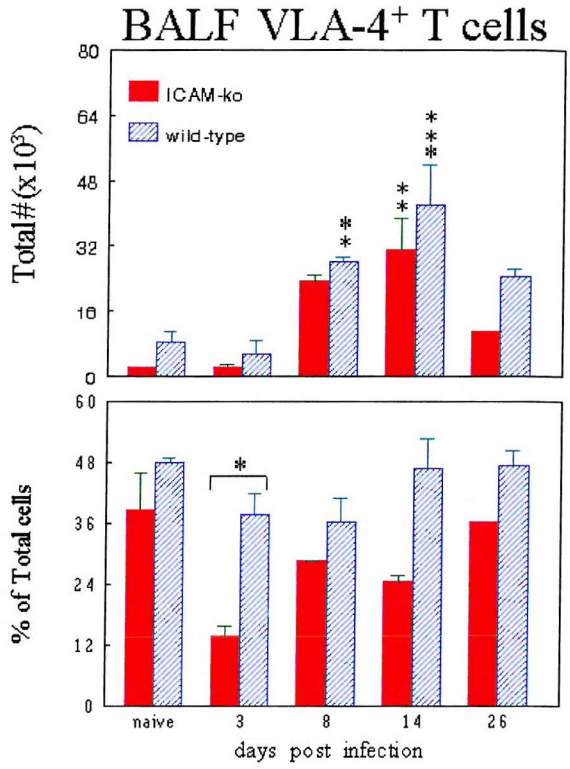

Figure 7-14. The total number and percentage of T cells in the BAL fluid expressing VLA-4 in ICAM knockout and wild-type control mice following N.b. infection.

XI. LFA-1

Similarly the total number of T cells in the BAL fluid in WT and ICAM-ko mice expressing LFA-1 increased significantly during the late response. Thus the number of T cells expressing LFA-1 in naïve ICAM-ko mice increased from 9.9 x 10^3 in naïve mice to 62.6 x 10^3 on day 8 pi ($p<0.05$), and 102.7 x 10^3 on day 14 pi ($p<0.001$). A virtually identical pattern was seen in WT mice with LFA-1^+ T cells increasing naïve WT mice from 4.3 x 10^3 to 58.6 x 10^3 on day 8 pi ($p<0.051$), and 79.9 x 10^3 on day 14 pi ($p<0.001$) (Figure 7-15).

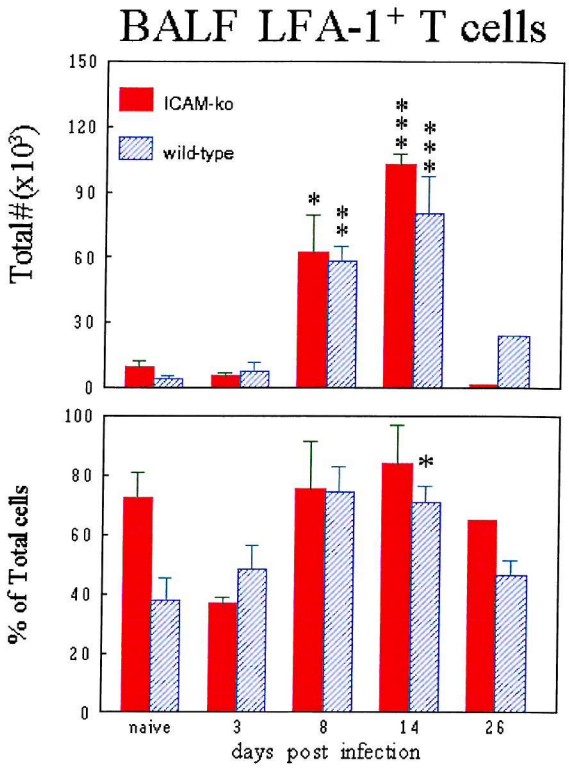

Figure 7-15. The total number and percentage of T cells in the BAL fluid expressing LFA-1 in ICAM knockout and wild-type control mice following N.b. infection.

The percentage of WT BAL fluid LFA-1$^+$ lymphocytes also increased significantly from 38% in naïve animals to 71% on day 14 pi ($p< 0.05$). The high background level of LFA-1 staining in ICAM-ko mice meant that there was a non-significant increase, from 72.5% in naïve animals to 84.1% on day 14 pi, in the percentage of CD4$^+$ cells expressing this adhesion molecule during the late response (Figure 7-15).

7.3.6 Phenotypic analysis of eosinophils in the airway lumen following N.b. infection

I. CD45RB

There was a six-fold increase in the percentage of eosinophils expressing CD45RB in WT mice, rising from 8.7% in naïve animals to 53.6% during the late phase pulmonary response ($p< 0.05$). Similarly the total number of eosinophils recovered from the BAL fluid of WT mice that expressed CD45RB increased from 1.9×10^3 in naïve animals to 800×10^3 during the late phase ($p< 0.05$) (Figure 7-16).

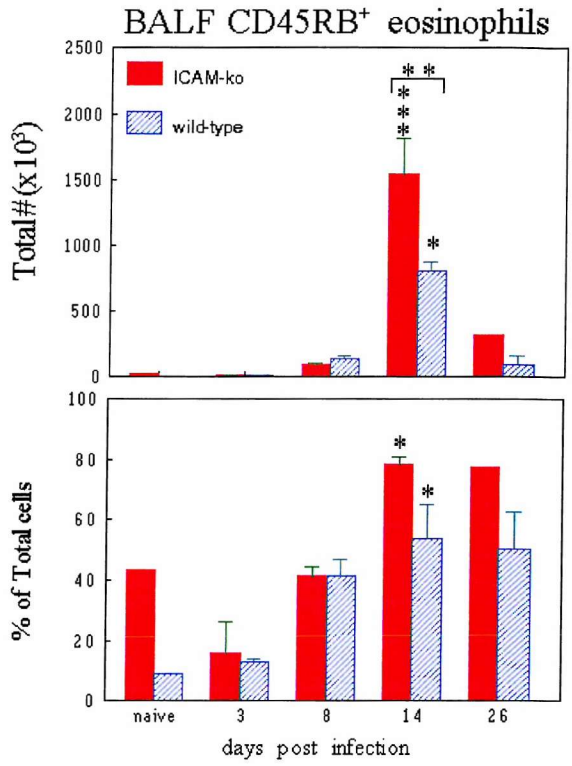

Figure 7-16. The total number and percentage of eosinophils in the BAL fluid expressing CD45RB in ICAM knockout and wild-type control mice following N.b. infection.

In contrast, the percentage of eosinophils expressing CD45RB in ICAM-ko mice rose from 43% in naïve animals to 78.2% during the late phase pulmonary response ($p< 0.05$). The percentage of ICAM-ko BAL fluid eosinophils expressing CD45RB on day 14 pi was not significantly different from that seen in WT mice. In contrast, the rise in total number of BAL fluid eosinophils in ICAM-ko mice expressing CD45RB from 18 x 10^3 in naïve animals to 1.5 x 10^6 during the late phase ($p< 0.001$) was significantly greater than in WT mice ($p<0.01$)(Figure 7-16).

II. CD43

The total number of eosinophils in the BAL fluid in WT mice expressing CD43 also increased significantly following N.b. infection, from 3.7 x 10^3/mouse in naïve animals to a peak of 546 x 10^3/mouse 14 days pi ($p<0.04$)(Figure 7-17).

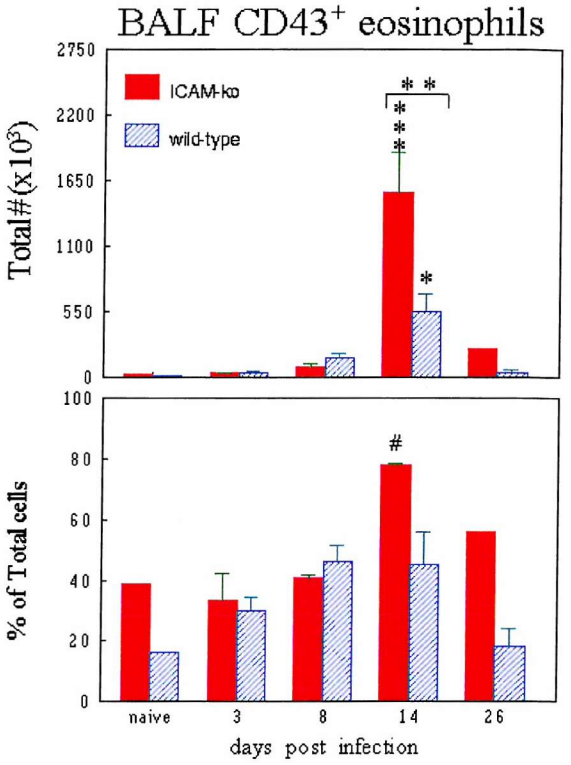

Figure 7-17. The total number and percentage of eosinophils in the BAL fluid expressing CD43 in ICAM knockout and wild-type control mice following N.b. infection.

Similarly, the total number of eosinophils in the BAL fluid in ICAM-ko mice expressing CD43 increased from 16.3 x 10^3/mouse in naïve animals to 1.5 x 10^6/mouse 14 days pi ($p< 0.001$). This increase in the number of eosinophils expressing CD43 in ICAM-ko mice was significantly greater than the number of eosinophils expressing CD43 in WT mice on day 14 pi ($p<0.01$)(Figure 7-17).

The increase in the number of eosinophils expressing CD43 in BAL fluid was reflected in an increased percentage of WT BAL fluid eosinophils expressing CD43 from 16.2% in naïve animals to 45.1% during the late response. Similarly, the percentage of BAL fluid eosinophils in ICAM-ko mice expressing CD43 also rose from a higher background level of 38.8% in naïve animals to 77.8% during the late response ($p= 0.053$). There was no significant difference in the scale of the increase between WT and ICAM-ko mice (Figure 7-17).

III. Pgp-1

The total number of eosinophils in the BAL fluid of WT mice expressing Pgp-1 increased significantly during the late response, rising from 57 x 10^3/mouse (42%) three days pi, to 800

x 10^3/mouse (80%) on day 14 pi ($p<0.01$)(data not shown). ICAM-ko mice showed a significantly greater rise in the total number of BAL fluid eosinophils expressing Pgp-1 ($p<0.01$) compared to WT mice, from 20 x 10^3/mouse (48.5%) in naïve animals to 1.6 x 10^6/mouse (82%) during the late response ($p<0.001$).

IV. ICAM-1

The total number of BAL fluid eosinophils in WT mice expressing ICAM-1 increased significantly during the late pulmonary response, from 6.2 x 10^3/mouse in naïve animals to 832 x 10^3/mouse 14 days pi ($p< 0.05$). This was reflected in an increase in the percentage of eosinophils expressing ICAM-1 from 27% in naïve animals to 65% 14 days pi (data not shown). ICAM expression on BAL fluid eosinophils in ICAM-ko mice remained <4% (data not shown).

V. VLA-4

The total number and percent of eosinophils expressing VLA-4 in the BAL fluid of WT mice increased significantly during the late response. Thus the percentage of eosinophils in WT mice expressing VLA-4 increased from 25% in naïve animals to 61% 14 days pi ($p< 0.001$). This was reflected in a significant increase in the number of eosinophils in WT mice expressing VLA-4 from 5 x 10^3/mouse to 773 x 10^3/mouse ($p< 0.01$)(Figure 7-18).

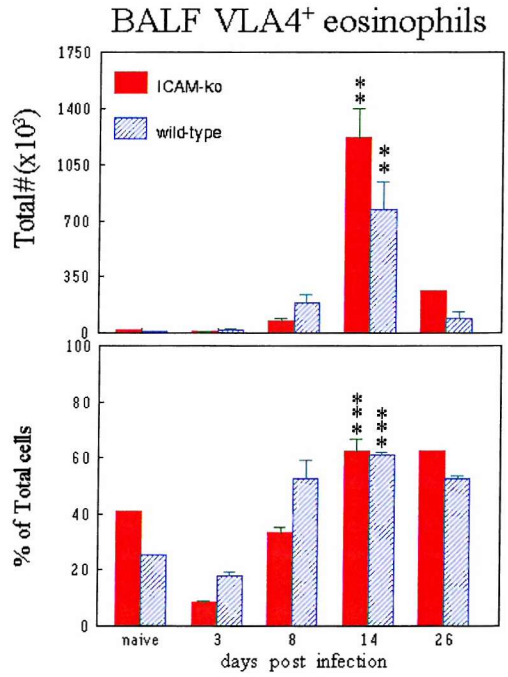

Figure 7-18. The total number and percentage of eosinophils in the BAL fluid expressing VLA-4 in ICAM knockout and wild-type control mice following N.b. infection.

Similarly, the total number and percent of eosinophils expressing VLA-4 in the BAL fluid of ICAM-ko mice increased significantly during the late response. Thus the percentage of eosinophils in ICAM-ko mice expressing VLA-4 increased from 41% in naïve animals to 62.6% 14 days pi ($p< 0.001$). This was reflected in a significant increase in the number of eosinophils in ICAM-ko mice expressing VLA-4 from 17 x 10^3/mouse to 1.2 x 10^6/mouse ($p< 0.01$)(Figure 7-18). There were no significant differences in the total number or percentage of eosinophils expressing VLA-4 between ICAM-ko and WT mice.

VI. LFA-1

The percentage of BAL fluid eosinophils in WT mice expressing LFA-1 increased significantly during the late response from 31% in naïve animals to 85% 14 days pi ($p< 0.05$). This was reflected in an increase in the total number of eosinophils in WT mice expressing LFA-1 from 7.2 x 10^3/mouse in naïve mice to 1.08 x 10^6/mouse on day 14 pi ($p<0.001$) (Figure 7-19).

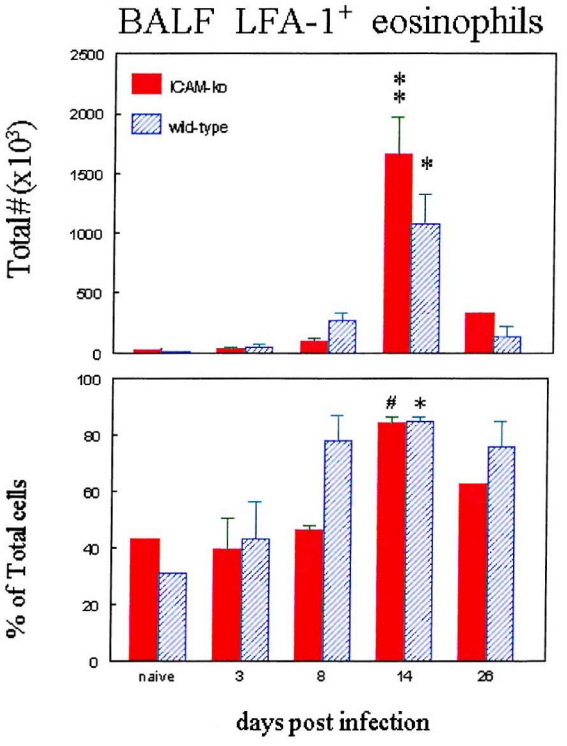

Figure 7-19. The total number and percentage of eosinophils in the BAL fluid expressing LFA-1 in ICAM knockout and wild-type control mice following N.b. infection.

Similarly, the total number and percent of eosinophils expressing LFA-1 in the BAL fluid of ICAM-ko mice increased significantly during the late response. Thus the total number of

eosinophils expressing LFA-1 in ICAM-ko mice increased from 18 x 10^3/mouse in naïve mice to 1.6 x 10^6/mouse on day 14 pi ($p< 0.01$). This was reflected in a near statistically significant increase in the percentage of eosinophils from ICAM-ko mice expressing LFA-1 from 43% in naïve animals to 84.4% 14 days pi ($p< 0.052$)(Figure 7-19).

7.4 DISCUSSION

Investigation of the immune response to parasite antigens has shed considerable light on the pathogenesis of allergic inflammation. The cellular and molecular responses to parasite antigens closely mimic those seen in allergic inflammation and some authors have even speculated that the two are causally related (Moqbel R et al 1990). Of all the experimental parasites *Nippostrongylus* is, perhaps the most relevant since N.b. infection has been shown to override established Th-1 responses and promote Th-2 responses *in vivo* (Rocken M et a;l 1994). Several investigators have utilized N.b. to develop a helminth-based model of antigen-induced airway inflammation in rats (Ramaswamy K et al 1993a/b, Ramaswamy K et al 1991 Egwang TG 1984). We previously reported on the pulmonary and peripheral blood response to primary N.b infection in C57BL/6 mice (Watkins AD et al 1996). This series of experiments demonstrate that there are sufficient similarities in the phenotype and pattern of lymphocyte and eosinophil recruitment to make primary infection of C57BL/6 mice with N.b. a useful animal model of human allergic inflammation.

Here we examined the role of ICAM-1 in the recruitment of lymphocytes and eosinophils to the airways by taking advantage of the availability of ICAM-ko transgenic mice. A recent study using ICAM-ko mice in an ovalbumin model of allergic inflammation reported that the absence of ICAM attenuated bronchial responsiveness to methacholine, significantly reduced eosinophil recruitment to the airways, reduced cytokine levels in BAL fluid, reduced circulating IgE levels and *ex vivo* lymphocyte proliferation (Wolyniec WW et al 1998). However, the data presented here indicated that in a N.b. model of allergic inflammation the absence of ICAM-1 did not attenuate T cell or eosinophil recruitment to the lungs at all.

The importance of leukocyte integrins and the immunoglobulin superfamily to the pathogenesis of pulmonary disorders such as asthma has been extensively documented (Striz

et al 1999, Pilewski et al 1995, Canonica GW et al 1994, Springer TA et al 1994, Pilewski JM et al 1993). *In vitro* studies of the homotypic and heterotypic binding of ICAM-1 to endothelial and matrix cells and the resultant signal transduction events have been well characterised (Krunkosky TM et al 1996, Languino LR et al 1995, Springer TA et al 1994, Cabanas C et al 1993, Hogg N et al 1993). Similarly, numerous studies have been conducted *in vivo* utilizing a variety of blocking antibodies to elucidate the role of ICAM-1 in the recruitment of lymphocytes and eosinophils to the airways (Grunstein MM et al 2000, Tohda et al 1999).

In contrast, the present study employed ICAM-ko mice to determine whether the *in vivo* absence of ICAM-1 would reduce the recruitment of inflammatory cells to the airways in response to a powerful Th-2 stimulus. The use of ICAM-ko mice circumvents many of the problems encountered in the blocking antibody studies, such as antibody delivery, dose response curves, receptor binding avidity, determination of adequate plasma levels and toxicity.

In keeping with our previous studies we demonstrated a biphasic pulmonary response to N.b. infection (Watkins AD et al 1996). This was more marked in the airways than in the peripheral blood. There was significant early leukocyte recruitment on day 3 pi followed by an even greater recruitment of leukocytes, on day 14 pi, to the airways. The late phase response was mirrored by a significant leukocytosis in the blood on day 8 to 14 pi. The cellular provenance of the early phase response differed significantly from that seen during the late phase response.

During the early pulmonary response there was a greater number of neutrophils recruited to the airways of WT mice compared to ICAM-ko mice. When expressed as a percentage of the total number of cells recruited this difference was significant. This is despite a greater resting peripheral blood neutrophil count in ICAM-ko mice and a significantly greater peripheral blood neutrophilia in ICAM-ko mice during the late phase pulmonary response. Taken together these results suggest that the early response to helminth antigens may be more dependent on neutrophils in WT mice than ICAM-ko mice. Alternatively, the peak of the immune response to N.b. may be delayed in ICAM-ko mice and there may have been a greater airway neutrophil response in ICAM-ko mice between day 3 and day 8 pi that went unobserved.

The exact role of neutrophils in allergic inflammation of the airways is unclear. However, an early airway neutrophilia has been shown to occur in asthma 6h after local allergen challenge (Montefort S et al 1994a), and neutrophils have been implicated in early (6 h) rather than late (24-48 h) asthmatic death following an acute asthmatic attack (Sur S et al 1993). The importance of the small early influx of neutrophils seen in WT mice and to a less extent in ICAM-ko mice is unclear. Although, it has been suggested that neutrophils may augment the recruitment of eosinophils and promote resolution of low level N.b. infections (Czarnetski BM et al 1978, Katy SP et al 1976).

During the late pulmonary response we demonstrated a significantly greater peripheral blood neutrophilia and eosinophilia in ICAM-ko mice compared to WT controls, with the neutrophilia occurring slightly earlier on day 8, and the eosinophilia peaking on day 14 pi. In contrast, there was a slightly greater peripheral lymphocytosis in wild type controls during the late response, but this was not reflected in the BAL fluid. Although asthma is not associated with a marked peripheral lymphocytosis or eosinophilia, evidence has suggested that the peripheral blood eosinophil and lymphocyte counts may be correlated with airflow obstruction (Virchow JC et al 1994).

Coincident with the greater peripheral eosinophilia in ICAM-ko mice was a greater airway eosinophilia on day 14 pi. When expressed as a percentage of the total number of cells recruited there was a significantly greater recruitment of eosinophils to the airways of WT mice on day 8 pi, with a significantly greater recruitment of eosinophils on day 14 and day 26 pi in ICAM-ko mice. This suggests that the kinetics of the eosinophil response may differ in ICAM-ko and WT mice, with a later eosinophil recruitment occurring in ICAM-ko mice.

It might be expected that with the greater peripheral blood and airway eosinophilia occurring in ICAM-ko mice that these mice would produce a greater systemic Th-2 response. However, analysis of splenocyte IL-5 and IL-10 production revealed that WT mice produced significantly greater amounts of both cytokines than ICAM-ko mice. This result may be interpreted in a number of ways. Firstly, it suggests that systemic cytokine production may be less important than local cytokine or chemokine production in determining the level of airway eosinophilia. Secondly it is possible that the greater production of Th-2 cytokines in WT mice

on the two days specified might not truly reflect the overall stimulation of eosinophil production during the late phase response. Thirdly it is feasible that the ratio of Th1:Th2 cytokine production may be more important in determining the eosinophil response than the amount of Th-2 cytokines alone, and since Th-1 cytokines were not measured in these experiments a misleading result may have been obtained.

Phenotypic analysis of the lymphocytes and eosinophils recruited to the BAL fluid during the early response revealed a significantly greater percentage of WT lymphocytes expressed the naïve cell surface marker L-selectin compared to ICAM-ko mice. This difference was also reflected in the total number of T cells expressing L-selectin, although this did not reach statistical significance. However, the importance of these changes is unclear since significantly more ICAM-ko T cells expressed L-selectin during the late response. These differences may reflect the small numbers and percentages involved.

The late response in ICAM-ko mice was marked by a significant increase in $Thy1^+$ T cells and more specifically $CD4^+$ cells. However, this influx was no greater than that seen in WT mice, and was consistent with our previous findings which demonstrated increased numbers of $CD4^+$ cells in the BAL fluid and lung tissue during the late response (Watkins AD et al 1996). These findings contradict previous research which suggested that the recruitment of $CD4^+$ cells to the rat airway occurs much later and is associated with a sustained influx of $CD8^+$ cells (Ramaswamy et al 1993a).

The increase in Pgp-1, CD69 and CD43 expression during the late phase response, suggested that the T lymphocytes recruited were predominantly mature, activated cells of the memory phenotype. This recruitment of activated memory T cells was more than seven times greater than the small increase in the number of naïve T cells recruited during the late response.

The significant increase in the number of lymphocytes expressing the adhesion markers LFA-1 and VLA-4 during the late phase suggests that these molecules may be central to the recruitment of lymphocytes to the airways. The lack of the ICAM-1 in the knockout mice did not result in either an increased expression of LFA-1 or VLA-4, or reduced recruitment of lymphocytes to the airways.

We have previously demonstrated that the cellular provenance of the pulmonary inflammatory response in murine lungs closely resembles that previously reported in the rat (Watkins AD et al 1996, Salman SK et al 1980, Taliaferro WFH et al 1939). Comparison of the histopathological response to N.b. infection in ICAM-ko mice to WT mice revealed that there was a greater subepithelial and epithelial infiltration in the airways of ICAM-ko mice than in WT controls.

Our previous study reported on the phenotypic characteristics and cell surface markers of murine eosinophils in inflammatory states (Watkins AD et al 1996). This data confirms work indicating that the late phase pulmonary response is dominated by the recruitment of naïve, activated eosinophils, as indicated by an increase in CD45RB and CD43 expression on eosinophils, which was more prominent in ICAM-ko mice. Although little is known about the significance of these cell surface markers on eosinophils, some authors have suggested that molecules associated with T cell activation may also indicate eosinophil activation (Hartnell 1993). The eosinophils recruited to the airways of ICAM-ko mice also expressed LFA-1 and VLA-4 in equal numbers to WT mice, confirming that the presence of ICAM-1 is not an absolute requirement for eosinophil recruitment to the airways.

In conclusion, we have demonstrated that the loss of ICAM-1 failed to produce an attenuation of the T cell or eosinophil recruitment in the lungs. There are a number of possible explanations for this. Variable expression of the knockout gene or recruitment of compensatory pathways to bypass the deletion, has often been invoked to explain the functional redundancy of gene knockout technology (Thomas et al 1995). However, the very low expression of ICAM-1 on lymphocytes or eosinophils recruited to the airways and the lack of increased expression of other adhesion molecules suggests that gene deletion was effective and there was no significant recruitment of compensatory cell adhesion pathways.

A second possible explanation would be that ICAM-1 does not play a significant role in the recruitment of lymphocytes and eosinophils to the airways. These data and the work of others using blocking antibodies to ICAM-1 (Tohda Y et al 1999, Burke-Gaffney et al 1998) suggest that this explanation is unlikely. We believe that the results reported here best fit a third explanation - an impaired immune response to helminth antigens in ICAM-ko mice that results in prolonged exposure to the potent Th-2 stimuli. This prolonged exposure to helminth

antigens overwhelmed the expected inhibition of cellular recruitment secondary to the loss of ICAM-1. This explanation is supported by our observations that eosinophil recruitment is abolished in ICAM-ko rodents when ovalbumin is the antigenic stimulus (Richards IM et al 1996), and other studies demonstrating that cellular immunity is impaired in ICAM-ko mice (Xu H et al 1994, Sligh JE et al 1993). We conclude that ICAM-1 is involved in cellular recruitment to the airways but that prolonged exposure to powerful Th-2 stimulants can overcome the absence of these adhesion molecules *in vivo*.

CHAPTER 8

CD18-hypomorph Mice

8.1 INTRODUCTION

Allergic inflammation of the airways involves a wide array of cells and a multitude of chemical mediators. Experiments in vitro, in animal models, as well as increasingly numerous studies in atopic human subjects are revealing that there is an orchestrated continuum of cellular activities, which leads to airway inflammation. Furthermore this is set in motion in genetically predisposed individuals at the first exposure to a novel antigen.

This sensitisation step appears to be dependent on the differentiation of and cytokine release by Th-2 lymphocytes. Among Th-2-derived cytokines, IL-4 potently enhances B-lymphocyte generation of immunoglobulin E antibodies. The attachment of these antibodies to specific receptors on airway mast cells sets the stage for an acute inflammatory response on subsequent antigen exposure because IgE cross-linking by a bound antigen activates mast cells to release numerous inflammatory mediators. These mast cell-derived mediators collectively produce acute-phase clinical symptoms by enhancing vascular leak, bronchospasm, and activation of nociceptive neurons linked to parasympathetic reflexes. Simultaneously, some mast cell mediators up-regulate expression on endothelial cells of adhesion molecules for leukocytes (eosinophils, basophils and lymphocytes), which are key elements in the late-phase allergic response.

Chemoattractant molecules released during the acute phase draw these leukocytes to the airways during a relatively symptom-free recruitment phase, where they later release a plethora of cytokines and tissue-damaging proteases that herald a second wave of airway inflammatory trauma (late-phase response). The repetition of these processes, with the possible establishment in the airway mucosa of memory T lymphocytes and eosinophils that are maintained by paracrine and autocrine cytokine stimulation, may account for airway hypersensitivity and chronic airway symptoms (Pearlman DS et al 1999).

The cellular recruitment to the airways is the result of an upregulation of leukocyte CAMs or endothelial CAMs together with the release of chemoattractants. There are three major families of adhesion molecules used by leukocytes and endothelial cells: the immunoglobulin supergene, the selectins and the integrins. Within the integrin family most attention has been paid to the β_1-, β_2-, and β_7-integrins. Expression of β_1- and β_2-integrins

on CD4+ cells is much more common in patients with allergic inflammation of the airways while the β_7-integrins are much more common on CD4+ cells in idiopathic pulmonary fibrosis. In contrast, even in normal individuals, 60% to 90% of BAL fluid CD8+ T cells express β_7-integrins (Rihs S et al 1996) (SeeTable 1).

Table 1. Simplified Schema for the Integrin Family

Leukocyte Adhesion Molecule			**Endothelial & Epithelial Adhesion Molecule**
α chain	**β chain**		
	β_2 integrin	*alternate name*	
CD11a	CD18H	LFA-1	**ICAM**
CD11b	CD18H	Mac-1	
CD11c	CD18H	P150,95	
CD11d	CD18H		
	β_1 integrin	*alternate name*	
CD49 a-e	CD29	VLA 1-6	**VCAM or MadCAM**

The β_2-integrins are membrane glycoproteins and share a common beta subunit, designated CD18H, coupled with one of three alpha subunits, either lymphocyte function associated antigen-1 (LFA-1), macrophage antigen-1 (Mac-1) or p150,95, which are designated CD11a, b and c respectively. The classification and nomenclature of the β_2-integrins is complex as new subunit variants are discovered, however a simplified schema is presented in Table 1. CD11a/CD18H is expressed on the progenitors of all myeloid and erythroid cells lines. In contrast, CD11b/CD18H and CD11c/CD18H are more restricted and are normally only expressed on monocytes, macrophages, neutrophils, eosinophils, basophils and natural killer cells.

The β-integrins mediate homotypic and heterotypic binding between activated leukocytes and other cell types, particularly endothelial cells, via intercellular adhesion molecule (ICAM)-1 in the case of the β_2-integrins and vascular cell adhesion molecule (VCAM) or mucosal addressin (MAd) CAM-1 in the case of the β_1-integrins. There are a number of

complex interactions between leukocyte β-integrins and their counter ligands on epithelial and endothelial cells that not only facilitate transendothelial but also transepithelial migration (Taguchi M et al 1998). This integrin-CAM interaction controls both rolling and firm adhesion and their associations are regulated in a sophisticated manner by the finely balanced interplay of mediators within the microenvironment involving soluble cytokines, extracellular matrix proteins and cell to cell contact (Sriramarao P et al 2000, Spoelstra FM et al 1998). For example recent evidence suggests that there is a basal to apical gradient of RANTES, which coordinates T cell traffic through epithelium (Taguchi M et al 1998). Once the leukocytes have been shed into the sputum the expression of these adhesion molecules is downregulated (Maestrelli P et al 1999), but levels of expression do not correlate with disease activity (Veen JC et al 1998).

For example, it has been demonstrated that specific mediators promote the adhesion of different leukocyte subset to endothelial cells. Thus, IL-5, IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF) and complement (probably iC3b), but not IL-8 tumor necrosis factor (TNF)-α or interferon (IFN)-γ, enhance the adhesiveness of eosinophils to epithelial cells. This is achieved predominantly through an upregulation of CD11b/CD18H when the epithelium is activated. However, when the epithelium is not activated CD11a/CD18H is used (Jagels MA et al 1999, Varsano et al 1994).

Similarly, eosinophil activation may also alter which cell adhesion pathway is utilised. Thus IL-5-stimulated eosinophils predominantly adhered to ICAM-1 through the β_2-integrin CD11b/CD18H, while non-stimulated eosinophils adhered to VCAM-1 through the β_1-integrin VLA-4/CD29 (Zhu Xn et al 1999). The β_1-integrin VLA4/CD29, which is not expressed by neutrophils, is downregulated on eosinophils by IL-5 in favour of the β_2-integrin. Conversely, the counter ligand for β_1-integrin, VCAM, is upregulated by IL-13 (Seminario MC et al 1997). The expression of VLA-4 by eosinophils has recently been shown to correlate with disease severity (Bocchino V et al 2000).

This differential use of CD11a/CD18H, CD11b/CD18H or VLA-4/CD29 by eosinophils, depending on the activation state of the epithelium and or the eosinophil may influence the degree of eosinophil recruitment to the airways (Jagels MA 1999, Walsh GM 1990). Thus in response to CD11/CD18H expression by eosinophils the epithelium predominantly

expresses ICAM-1, and this expression is upregulated by IFN-γ, or to a lesser extent TNF-α or IL-1. Conversely in response to CD49d/CD29 (VLA-4) expression on the leukocyte the epithelium expresses VCAM (Zhu Xn 1999). Integrins appear to not only be important in mediating eosinophil migration and activation in allergic inflammation but their activation may determine the effector functions of the cell. Thus eosinophil adhesion via β_1- and/or β_2-integrin has been shown to alter a variety of functional responses including degranulation and apoptosis (Seminario MC et al 1997) and influence eosinophil survival (Chihara J et al 2000) and consequently the late asthmatic response (Sagara H et al 1997) and airway hyperreactivity (Bloemen PG et al 1996).

The newest member of the β_2 integrin family, alphad, is expressed on human eosinophils in peripheral blood, and surface expression can be upregulated within minutes by phorbol ester or calcium ionophore A23187. Culture of eosinophils, with interleukin 5 (IL-5), leads to a two- to fourfold increase in alphad levels by 3-7 days. This new ligand can function as a partner for VCAM-1 providing an alternative adhesion pathway that may be important for eosinophil recruitment in chronic inflammation (Grayson MH et al 1998).

In contrast to eosinophils, the neutrophil recruitment to the lungs has been shown to be a two-step process. The initial neutrophil influx is mediated by a CD18H-independent mechanism and possibly related to neutrophil deformability followed by a prolonged accumulation of neutrophils in the lung being brought about by a CD18H-dependent mechanism (Doerschuk et al 1992). The adhesion of neutrophils to epithelial cells and subsequent accumulation of neutrophils in the airways is enhanced by IL-8, GM-CSF or C5a (Elliott et al 1993, Yong et al 1992, Walsh et al 1991), and the inhibition of nitric oxide (Kanwar et al 1995), but not IL-5, TNF-α or IFN-γ (Jagels MA et al 1999). Blocking antibody studies indicated that CD11b/CD18H mediated this neutrophil adhesion to endothelial cells. Furthermore, intratracheally administered anti-CD11b significantly attenuated lung injury by reducing BAL fluid TNF-α levels while monoclonal antibodies to CD11b and CD18H but not CD11a suppressed immune complex-stimulated *in vitro* production of TNF-alpha by rat alveolar macrophages (Mulligan et al 1995). The nature of the stimulating antigen (Burns et al 1994, Thomas et al 1992) or provoking agent (Keeney et al 1994, Guha et al 1993) may also determine whether the neutrophil recruitment to the lungs is CD18H-dependent or independent. This differs from neutrophil recruitment to

systemic sites of inflammation, such as the skin or abdominal cavity, which are totally dependent on CD18H (Hellewell et al 1994, Doerschuk et al 1990).

Not all authors agree on the specific cytokines utilised by the different cell types to enhance adhesion and cell recruitment (Sanmugalingham D et al 2000, Morland CM et al 1992). It probably varies depending on the model, species used and provoking stimulus for inflammation. However, the use of different cytokine pathways and different adhesion molecules by eosinophils and neutrophils may go some way to explaining the predominant cell type in certain airway diseases and models of disease (Jagels MA et al 1999, Mulligan et al 1995, Yassin et al 1994, Laurent et al 1994). For example, anti-CD18H antibodies reduced LPS-induced monocyte retention in the lungs of rabbits by up to 90% (Doherty et al 1994), OA-induced neutrophil recruitment in Brown Norway rats (Rabb et al 1994) and numerous other animal models of organ inflammation (Lobb RR et al 1996). Recent data suggests that neutrophil recruitment is more CD18H independent than eosinophil recruitment to the lung parenchyma, which is more CD18H dependent (Schneider T et al 1999).

CD18H has also been implicated in mediating vascular permeability and endothelial injury (Lum et al 1994, Elliot et al 1993), fibrinolysis and phagocytosis of LPS, and adhesion to fibronectin by human monocytes (Simon et al 1994, Kang et al 1992, Owen et al 1992). It has also been implicated in the production of superoxide anions by alveolar macrophages in smokers or in patients with sarcoidosis and idiopathic pulmonary fibrosis (Schaberg et al 1993, Schaberg et al 1995). In studies in guinea pigs sensitized and challenged with sephadex particles or ovalbumin (OA) anti-CD18H antibodies reduced airway hyperresponsiveness in addition to eosinophil recruitment to the bronchoalveolar lavage (BAL) fluid (Das et al 1995, Milne et al 1994). Similarly death from peritonitis was reduced by treatment with an anti-CD18H monoclonal antibody in an animal model of abdominal sepsis (Thomas et al 1992).

Much of the work done investigating the role of CD18H in pulmonary inflammation has relied on blocking antibodies. We adopted a different approach by developing an antigen-induced model of pulmonary inflammation using transgenic mice with an incompletely deleted CD18H gene (CD18H hypomorphs). The use of transgenic CD18H hypomorphs

confers a number of specific advantages over CD18H blocking antibody studies. For example, it circumvents the problems of antibody delivery, construction of dose response curves, the determination of adequate plasma levels, problems of toxicity and CD18H binding avidity to endothelial sites. We set out to test the hypothesis that disruption of CD18-driven cellular recruitment would significantly reduce the pulmonary inflammatory response to N.b. antigens.

Our results indicate that the helminth-induced pulmonary and peripheral eosinophilia in CD18H-hypomorphic mice was not significantly different to that seen in wild type (WT) controls.

8.2 MATERIALS AND METHODS

8.2.1 Larval Culture and Infection

Details of N.b. larval culture are given in chapter 2.

8.2.2 Animals

Details of animal husbandry are given in chapter 2.

8.2.3 Bronchoalveolar lavage and cell preparations

Details of brochoalveolar lavage and cell preparation are given in chapter 2. Data presented throughout this thesis are given per ml of BAL fluid drawn.

8.2.4 Immunofluorescence staining and flow cytometry

Details of cell preparation and the reagents used in flow cytometry are given in chapter 3.

8.2.5 Blood: Leukocyte Subsets

Details of blood acquisition and cell analysis are given in chapter 3. Data presented throughout this thesis are given per ml of blood drawn.

8.2.6 Spleen mononuclear preparations:

Details of spleen cell preparation are given in chapter 5.

8.2.7 Cytokine ELISAs

Details of all the relevant cytokine ELISAs are given in chapter 6.

8.2.8 IgE ELISA

Costar 96 well half area EIA plates (Costar #3690) were coated overnight at 4 ^{0}C with 50µl/well of sheep anti-mouse IgE (Binding Site Ltd, Birmingham, UK) diluted 1:250 in 0.02M carbonate buffer, pH 9.6. Plates were washed with PBS/TW and blocked with 100µl/well of PBS/TW/BSA for 2hr at RT. An IgE standard curve was constructed, using purified mouse IgE (SIGMA D-8406, clone SPE-7) across the range of 250 µg/ml to 1.4 ng/ml, loaded onto the plate at 50µl/well, along with varying dilutions of unknown serum samples, derived from centrifugation of whole blood and plates incubated at RT for 2hr. Plates were washed and incubated for 2hr at RT with 50µl/well of a 1:400 dilution of alkaline-phosphate labeled anti-mouse IgE (Southern Biotechnology Assoc Inc, Birmingham, AL). Plates were washed and incubated for 1hr at 37 ^{0}C with 50µl/well of 1mg p-nitrophenylphosphate (SIGMA 104 phosphatase substrate tablets) per ml substrate buffer. Plates were then read on a microtiter plate reader at 405 nm.

8.2.9 Generation of N.b. specific antigen

Details of N.b. specific antigen generation are given in chapter 5.

8.2.10 Statistics

The data presented in this chapter on wild type C57BL/6 mice are pooled from several experiments conducted throughout this thesis. Thus the peripheral blood and BAL fluid data presented contains, on average, 38 mice per time point. The CD18H data presented were drawn from three separate experiments and therefore the number of mice pooled in each time point averaged was 32 mice. Total and differential cell counts in the BAL fluid, blood splenocyte proliferation and cytokine production were analyzed using a t-test assuming unequal variance and a normal distribution. When this overall test of treatment was found to be significant at the $p<0.05$, this was indicated by an asterisk (*). When the significance level reached $p<0.01$ this was indicated by two asterisks (**), and when the significance level reached $p<0.001$ this was indicated by three asterisks (***). If the result was borderline significant ($p=0.05$), then this was denoted by a # sign.

8.3 RESULTS

8.3.1 Time course of peripheral blood changes following N.b. infection

I. Total Leukocyte Count

Primary infection with third stage N.b. larvae produced a significant increase in the total number of blood leukocytes in CD18H hypomorph (CD18HH) and wild type (WT) mice, peaking on day 14 pi ($p < 0.001$). The total leukocyte count remained elevated up to 22 days pi in both WT and CD18HH mice ($p < 0.001$). A comparison of CD18HH and WT mice indicated that naïve CD18HH mice had higher peripheral leukocyte count prior to infection ($p<0.05$). This difference was still apparent after N.b. infection on day 8 pi ($p<0.05$) and became marked on day 14 pi ($p<0.001$) with the leukocyte count rising from 6.4 $x10^6$ to 12.6 $x10^6$ in CD18HH mice and 5.3 x 10^6 to 8.9 $x10^6$ in WT (Figure 8-1).

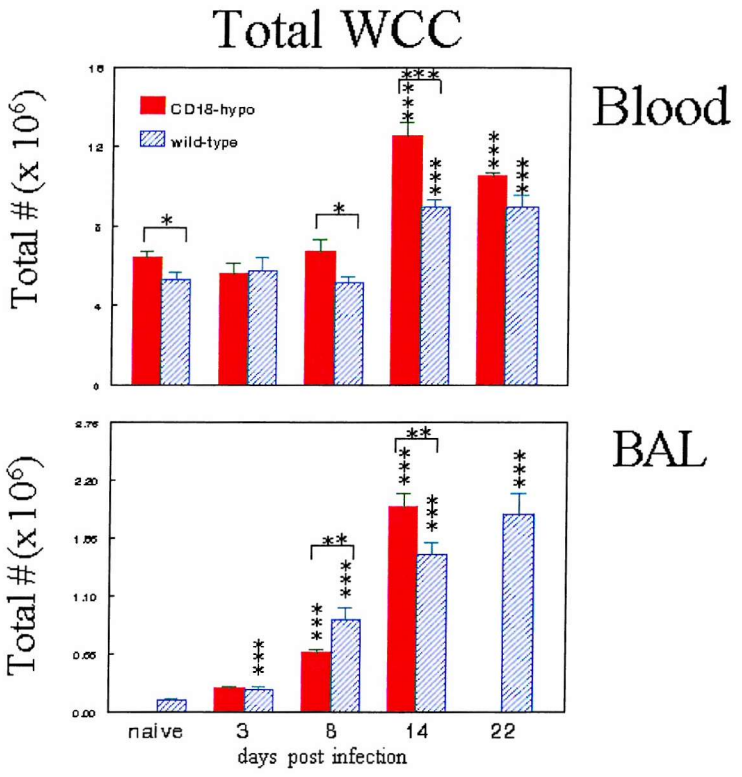

Figure 8-1. The total number of leukocytes recruited to the blood and airways of CD18H hypomorph mice and wild-type control mice following N.b. infection.

II. Lymphocytes

The significant increase in the total leukocyte count following N.b. infection was reflected in the significant increase in the total number of lymphocytes in the peripheral blood in both WT and CD18HH mice. Total lymphocyte count increased from 4.4 $x10^6$ in WT naïve mice

to 6.6 x 10^6 14 days pi ($p<0.001$), and from 5.4 x10^6 in naïve CD18HH mice to 9.2 x10^6 14 days pi ($p<0.001$). This lymphocytosis had not subsided by day 26 pi in either WT or CD18HH mice ($p<0.001$).

In line with the lower total leukocyte count naïve WT mice also had a lower total lymphocyte count compared to naïve CD18HH mice ($p<0.01$). This difference was maintained at day 8 pi ($p<0.01$) and became more marked by day 14 ($p<0.001$) (Figure 8-2).

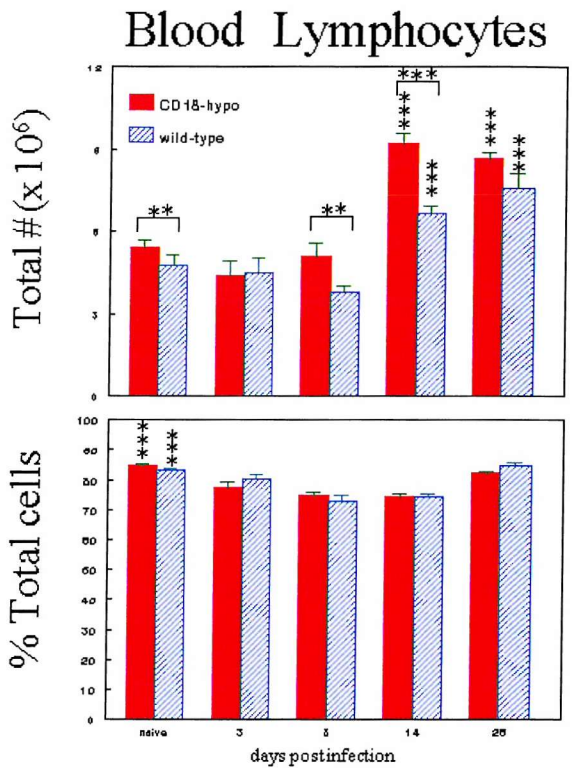

Figure 8-2. The total number and percentage of lymphocytes in the blood of CD18H hypomorph mice and wild-type control mice, following N.b. infection.

In contrast naïve WT and CD18HH mice had a significantly greater percentage of lymphocytes in their peripheral blood than infected animals ($p<0.001$). This was almost certainly due to the significant blood eosinophilia post infection reducing the overall percentage of lymphocytes in the infected animals (Figure 8-2).

III. Neutrophils

Changes in total neutrophil count paralleled changes in total leukocyte and lymphocyte counts. In both WT and CD18HH mice there was significant increase in neutrophil

numbers post N.b. infection. However, the significant increase in neutrophil numbers started earlier, on day 8 pi, in CD18HH mice ($p<0.01$) and in WT mice ($p<0.001$) and peaked on day 14 in CD18HH mice ($p<0.001$) and probably earlier in WT mice ($p<0.001$). Neutrophil number rose from 0.7 $x10^6$ in naïve CD18HH mice to 1.02 $x10^6$ on day 14 pi compared to 0.59 $x10^6$ in naïve WT mice rising to 0.78 $x10^6$ on day 14 pi.

In line with the total leukocyte and lymphocyte counts naïve CD18HH mice had higher neutrophil counts than WT mice ($p<0.05$). In addition, there was a significant difference in the blood neutrophil level in CD18HH and WT mice on day 14 ($p<0.01$) and day 26 pi ($p<0.05$), with CD18HH mice having a greater neutrophilia (Figure 8-3).

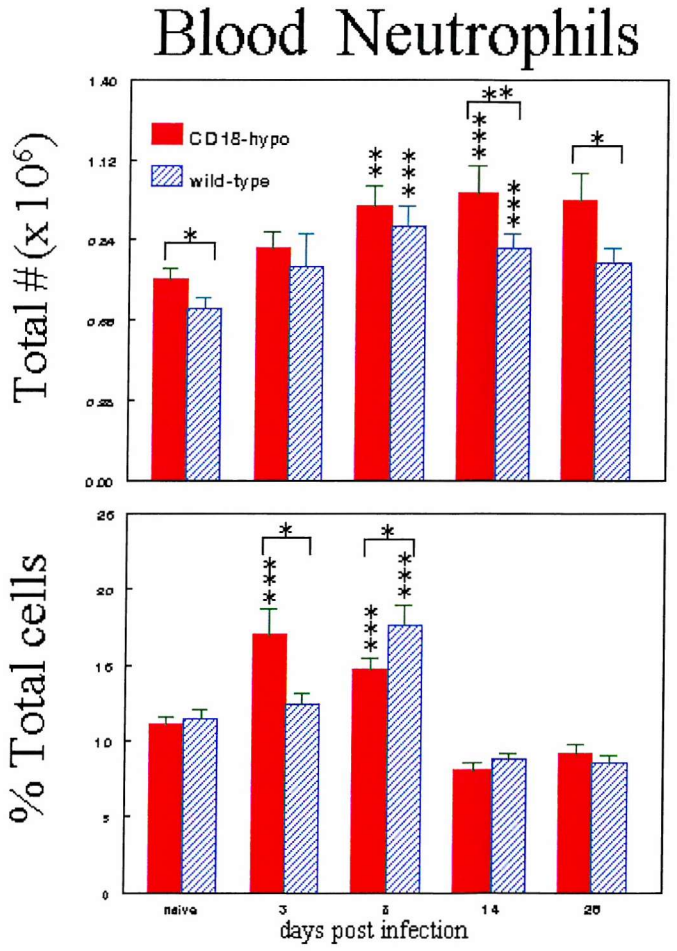

Figure 8-3. The total number and percentage of neutrophils in the blood of CD18H hypomorph mice and wild-type control mice following N.b. infection.

The change in the percentage of neutrophils in the peripheral blood suggested that the blood neutrophilia in the CD18H mice occurred earlier on day 3 pi ($p<0.001$) and although the

neutrophilia persisted until day 8 ($p<0.001$) the percentage change was not so marked. Thus the percentage of neutrophils in the blood rose from 11.1% in naïve CD18H mice to 17% 3 days pi subsiding to 14.7% by day 8 pi before returning to naïve levels by day 14 pi. In contrast the percentage of blood neutrophils did not rise above naïve level until day 8 pi in WT mice, from 12.2% in naïve mice to 17.6% on day 8 pi ($p<0.001$)(Figure 8-3).

These changes were reflected in the differences between CD18H and WT mice. There was a significantly greater percentage of neutrophils in the blood, 3 days pi, in CD18H mice, compared to WT mice, 17% *vs* 12.2% ($p<0.05$), and this ratio reversed by day 8 when there was a significantly greater percentage of neutrophils in WT blood, compared to CD18H mice, 14.7% *vs* 17.6% ($p<0.05$).

IV. Eosinophils

Of all the changes in the peripheral blood the eosinophilia was the most marked. In contrast to the total leukocyte, lymphocyte and neutrophil number there were no differences between naïve CD18H mice and naïve WT mice in the number of eosinophils circulating. However by day 3 pi there was a significantly greater number and percentage of eosinophils in the blood in WT mice compared to CD18H mice ($p<0.05$). By day 14 the number of eosinophils in the blood of both CD18H and WT mice had increased significantly compared to naïve animals ($p<0.001$) and the number in CD18H mice was significantly greater than in WT mice ($p<0.05$). Absolute numbers rose from 0.09 $x10^6$ in naïve CD18H mice to 1.8 $x10^6$ on day 14 pi, while the increase in WT numbers was 0.08 $x10^6$ in naïve mice to 1.2 $x10^6$ on day 14 pi (Figure 8-4).

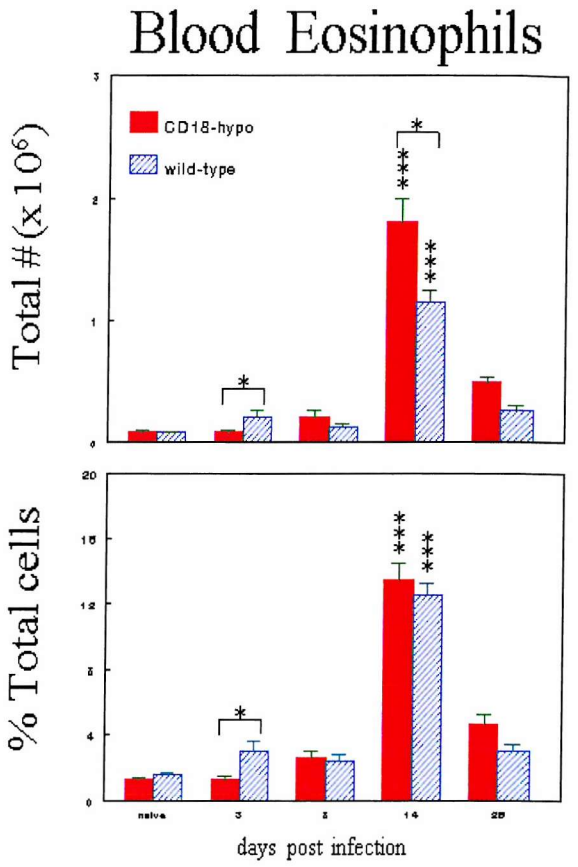

Figure 8-4. The total number and percentage of eosinophils in the blood of CD18H hypomorph mice and wild-type control mice following N.b. infection.

This substantial rise in eosinophil numbers was reflected by a significant rise in the percentage of eosinophils in the peripheral blood in both WT and CD18H mice ($p<0.001$). However there was no difference between WT and CD18H mice in terms of the percentage of eosinophils in the blood on day 14 pi, 12.5% and 13.5% respectively (Figure 8-4).

8.3.2 Time course of leukocyte infiltration into the lung following N.b. infection

I. Total Leukocyte Count

We demonstrated a biphasic cellular response in the BAL fluid following N.b. infection. This consisted of an early cellular influx into the airways during larval migration through the lungs, 3 days pi, followed by a late phase response peaking on day 14 pi. This was most clearly seen when examining the cellular subsets that migrated into the airways. In keeping with the peripheral blood response their was a marked leukocyte recruitment to the airways starting on day 3 pi in WT mice ($p<0.001$) and steadily increasing through day 8, 14 and peaking on day 22 pi ($p<0.001$). We did not examine naïve CD18H mice to enable an

assessment of the day 3 response in CD18H mice compared to naïve controls. However there were significantly more leukocytes recruited to the airways on day 8 and day 14 pi compared to day 3 pi in CD18H mice ($p<0.001$)(Figure 8-1).

Also in keeping with the peripheral blood response, the cellular recruitment to the airways was significantly greater in CD18H mice on day 14 pi than in WT controls ($p<0.01$). In contrast, there were more leukocytes in the airways of WT mice on day 8 pi than in CD18H murine airways ($p<0.01$) suggesting that the cellular recruitment may have peaked slightly later in CD18H mice (Figure 8-1).

II. Lymphocytes

During the late phase response there was a 27-fold increase in lymphocyte numbers in CD18H mice, rising from 8.6×10^3 during the early response to 230×10^3 on day 14 pi ($p<0.001$). Similarly there was a 21-fold increase in lymphocyte numbers in WT mice, rising from 10.3×10^3 during the early response to 216×10^3 on day 14 pi ($p<0.001$). This late phase lymphocyte recruitment to the airways was already well established by day 8 in WT and CD18H mice ($p<0.001$). Interestingly although there was a greater peripheral blood lymphocytosis in CD18H mice on day 8 pi ($p<0.01$), the lymphocyte recruitment to the airways was greater in WT mice than CD18H mice at this time point ($p<0.01$). By day 14 pi there was no difference between the number of lymphocytes in the WT or the CD18H murine airways (Figure 8-5).

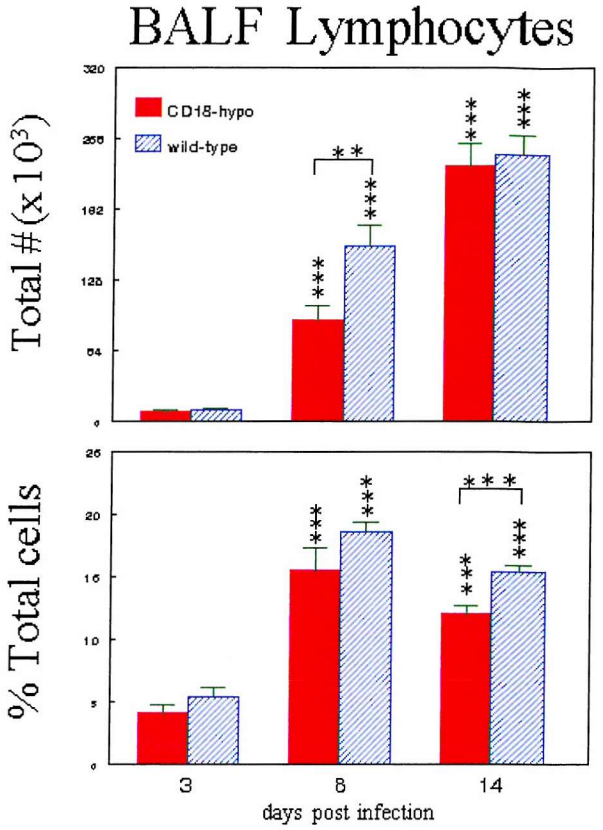

Figure 8-5. The total number and percentage of lymphocytes in the BAL fluid of CD18H hypomorph mice and wild-type control mice following N.b. infection.

The increase in the total number of lymphocytes in the airways during the late phase response was reflected in the percentages of lymphocytes recovered from the BAL fluid. This rose from 4% to 12% in CD18H mice and from 5.4% to 14.8% in WT mice ($p<0.001$)(Figure 8-5). The percentage of lymphocytes recovered from the bronchial lumen of WT mice, on day 14 pi was significantly greater than the percentage of BAL fluid lymphocytes in CD18H mice ($p<0.001$). The lower percentage of lymphocytes in the airways of CD18H mice is probably a reflection of the greater percentage of eosinophils present in the bronchial lumen at this time.

III. Neutrophils

The biphasic cellular recruitment to the airways is characterised by a significant influx of neutrophils to the airways during the "early phase" response on day 3 pi during larval migration through the lungs. There were twice as many neutrophils in the airways during the early response in CD18H mice compared to the late response ($p <0.001$), and four times as many in WT mice during the early response ($p <0.001$). Although there were a greater

number of neutrophils in the BAL fluid in WT mice during the early response, 76.8 x 10^3 compared to 64.7 x 10^3 in CD18H mice, this was not statistically significant. In contrast, there was a significantly greater number of neutrophils in the airways of WT mice on day 8 pi ($p<0.001$), but this subsided by day 14 pi leaving a significantly greater number of neutrophils in the airways of CD18H mice at this time point ($p<0.001$) (Figure 8-6).

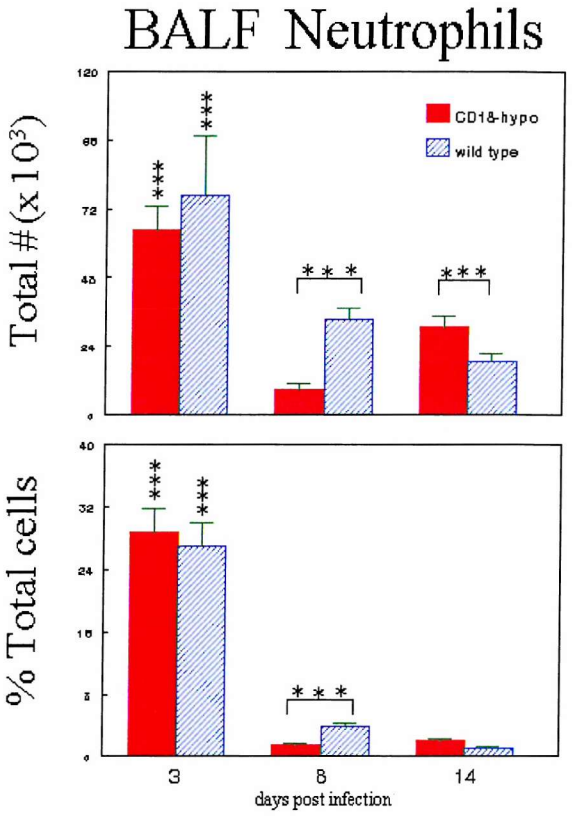

Figure 8-6. The total number and percentage of neutrophils in the BAL fluid of CD18H hypomorph mice and wild-type control mice following N.b. infection.

The changes in airway neutrophil numbers during the early phase response were reflected in the percentages of neutrophils recovered from the BAL fluid. There was a significantly greater percentage of neutrophils in the BAL fluid during the early response than during the late response in both the CD18H and WT controls ($p <0.001$). Neutrophils made up to 29% and 27% of all the leukocytes recruited to the airways during the early response in the CD18H and WT mice, respectively, compared to 2% and 1.4% on day 14 pi. The percentage of neutrophils in the airways of WT mice on day 8 pi was significantly greater than in CD18H mice ($p<0.001$)(Figure 8-6).

IV. Eosinophils

The late phase pulmonary response was dominated by a considerable increase in the number and percentage of eosinophils in the BAL fluid in WT and CD18H mice. In CD18H mice the number of eosinophils in the airways increased from 0.6 $x10^3$ 3 days pi to139 $x10^3$ on day 8 pi ($p<0.001$) and increased still further by day 14 pi to 1.5 $x10^6$ ($p<0.001$). Similarly, the number of eosinophils in the airways of WT mice increased from 3 $x10^3$ 3 days pi to 356 $x10^3$ on day 8 pi ($p<0.001$) and increased still further by day 14 pi to 1.1 $x10^6$ ($p<0.001$). In keeping with the greater number of eosinophils in the peripheral blood 3 days pi there was a significantly greater number of eosinophils in the airways of WT mice compared to CD18H mice at this time point ($p<0.01$). The greater number of eosinophils, in the airways of WT mice on day 3 pi, persisted through day 8 pi ($p<0.001$), but by day 14 pi the number of eosinophils in the airways of CD18H mice significantly exceeded the number of eosinophils recovered from the airway lumen of WT mice ($p<0.01$)(Figure 7). This paralleled the greater peripheral blood eosinophilia in CD18H mice at this time point ($p<0.05$) Figure 8-1).

The changes in airway eosinophil numbers following N.b. infection were reflected in the percentages of eosinophils recovered from the BAL fluid. The percentage of eosinophils in the airways of WT mice increased from 1.5% on day 3 pi to 59.4% by day 8 pi ($p<0.001$) and rose still further by day 14 pi to 65.2% ($p<0.001$). Similarly the percentage of eosinophils recovered from the airways of CD18H mice rose from 0.3% on day 3 pi to 22.8% on day 8 pi ($p<0.001$), rising still further to 71.5% by day 14 pi ($p<0.001$). In line with the changes in the number of eosinophils recovered from the airways the percentage of eosinophils in the airway lumen in WT mice was much greater than CD18H mice at 3 and 8 days pi ($p<0.01$). However, by day 14 the percentage of eosinophils in the airway lumen of CD18H mice was significantly greater than WT controls ($p<0.01$)(Figure 8-7).

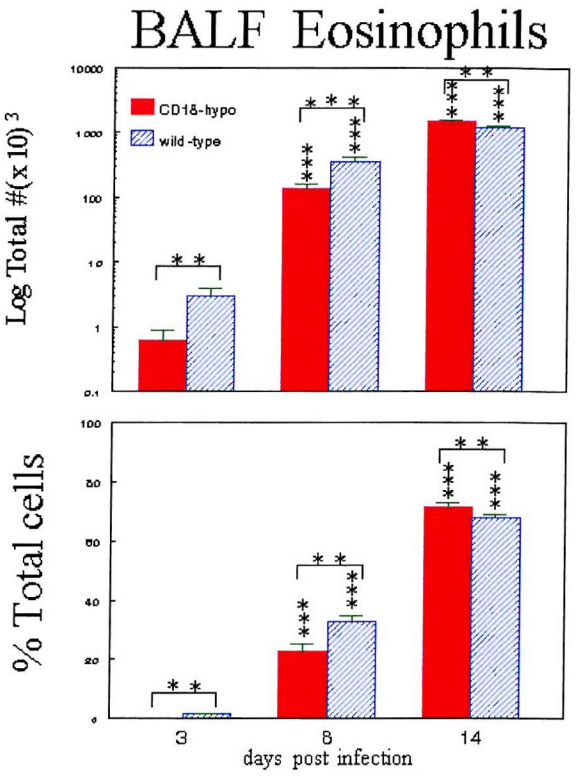

Figure 8-7. The total numbe,r and percentage, of eosinophils in the BAL fluid of CD18H hypomorph mice and wild-type control mice following N.b. infection. Eosinophil counts are given on a log scale for visual representation purposes only.

In addition to the cellular changes outlined above there was a significant increase in the number of mononuclear cells in the BAL fluid in CD18H and WT mice during the late response. Mononuclear cell numbers rose from 122 x10^3 on day 3 pi 201 x10^3 on day 14 pi in WT mice ($p<0.001$) and by roughly the same amount in CD18H mice from 148 x10^3 on day 3 pi to 238 x10^3 on day 14 pi ($p<0.001$). However, this represented a significant decrease when expressed as a percentage of total leukocytes from 66% in CD18H and WT mice during the early response to 14.4% and 18.7% 14 days pi in CD18H and WT mice respectively ($p<0.001$)(data not shown).

8.3.3 Splenocyte proliferation

The splenocyte proliferative response to specific antigen was significantly greater on day 14 pi compared to naïve spleens ($p<0.001$)(data not shown). In contrast, there was no significant difference in the proliferative response to the non-specific mitogens, concavalin A or anti-CD3, between naïve and infected animals (data not shown). In three experiments the day 14 splenocyte proliferative response in WT mice was compared to CD18H mice.

In all cases WT mice had a significantly greater proliferative response to specific antigens than the CD18H mice ($p<0.01$)(data not shown).

8.3.4 Splenocyte cytokine production to specific antigen

Cultured splenocytes were stimulated with varying dose of heterogenous N.b. adult antigens *in vitro* and the production of the Th-2 cytokines, IL-5 and IL-10, were assayed. Both CD18H and WT mice produced a significant increase in the amount of IL-5 and IL-10 detected during the late phase pulmonary response ($p<0.001$). Since intra-experimental controls were only performed in naïve mice and 14 days pi comparisons between CD18H and WT mice were restricted to these time points. These comparisons revealed that during the late response CD18H mice produced significantly more IL-5 than WT controls ($p<0.01$), while WT mice produced significantly more IL-10 ($p<0.001$). Although the latter result may have been a reflection of the greater basal level of IL-10 produced by WT controls as evidenced by the significant difference in the production of this cytokine in naïve CD18H and WT mice ($p<0.05$)(Figure 8-8). The amount of IL-10 produced by CD18H mice on day 3 and day 14 pi was also significantly greater than that produced on day 8 pi ($p<0.001$), suggesting that there may have been a biphasic response in cytokine production as well as cellular recruitment to the airways (Figure 8-8).

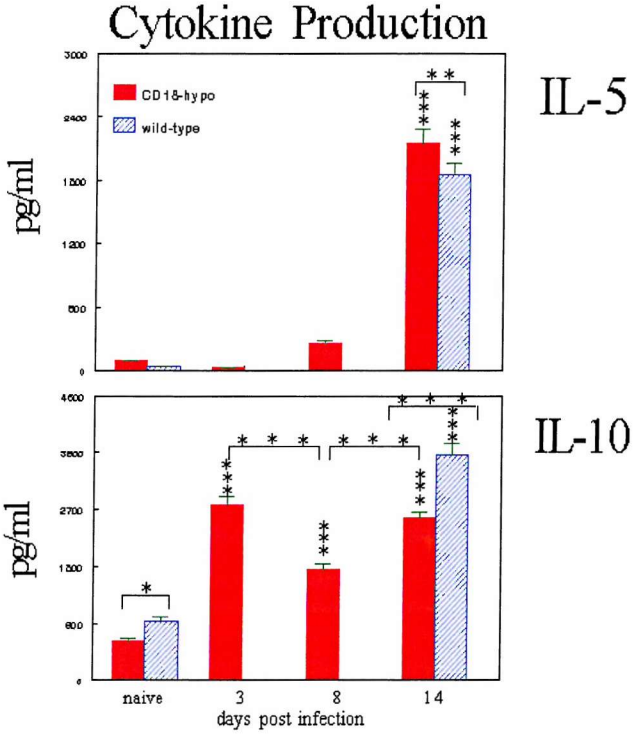

Figure 8-8. Splenocyte cytokine production in response to stimulation with N.b. specific antigen, in CD18H hypomorph mice and wild-type control mice, following N.b. infection.

8.3.5 Serum IgE production

In keeping with the cellular and cytokine response to N.b. infection there was also a significant increase in serum IgE production during the late phase response in both WT and CD18H mice. Serum IgE levels increased significantly from 85 ng/ml in naïve WT mice to 1,539 ng/ml on day 8 pi ($p<0.001$), and increased still further by day 14 pi to 126,506 ng/ml ($p<0.001$). Similarly, serum IgE levels increased in naïve CD18H mice from 65 ng/ml to 970 ng/ml on day 8 pi ($p<0.001$), rising again to 110,053 ng/ml by day 14 pi ($p<0.001$)(Figure 8-9).

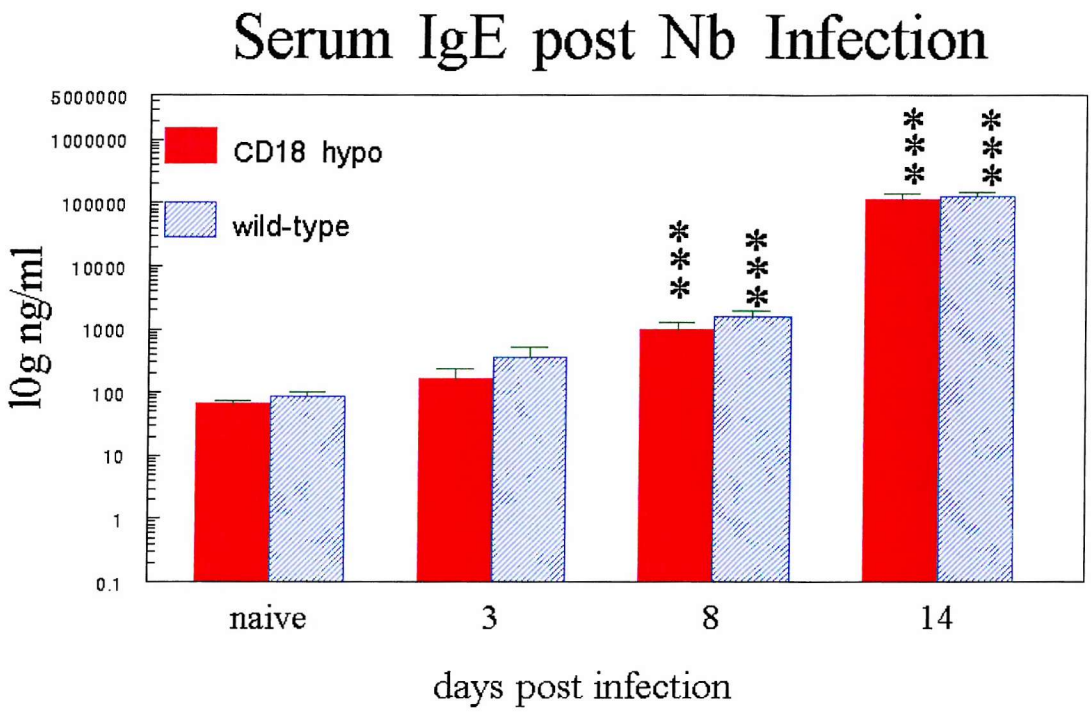

Figure 8-9. The serum IgE levels in CD18H hypomorph and wild-type control mice following N.b. infection.

There were no significant differences between CD18H and WT mice in the amplitude of the serum IgE response. We also compared the serum IgE levels in male and female CD18H mice to ensure that any differences in the number of males and females in each experiment did not skew the response. Although there was a small significant difference in the basal IgE levels in naïve animals, 48.6 ng/ml in males *vs* 84.5 ng/ml in females ($p<0.05$), this difference was not apparent following N.b. infection (Figure 8-10).

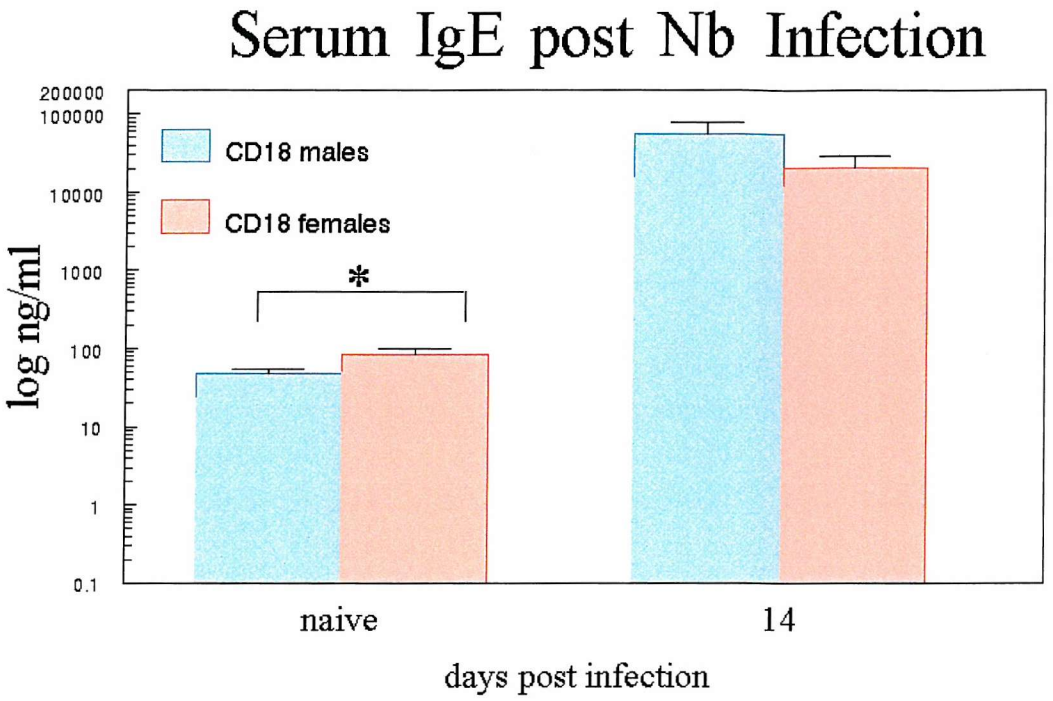

Figure 8-10. The serum IgE levels in CD18H hypomorph male and female mice following N.b. infection.

8.3.6 Immunofluorescence staining and flow cytometry

The gates for lung and BAL fluid lymphocytes were well defined throughout, whereas the absence of a specific eosinophil surface marker made identification of an eosinophil gate difficult, particularly in the BAL fluid of naïve animals and animals 3 days pi. The gated eosinophil population may have been contaminated by other granulocytes, particularly in the BAL fluid, 3 days pi. In contrast, there was a clearly defined lung tissue and BAL fluid eosinophil gate 14 days pi.

8.3.7 Phenotypic analysis of lymphocytes in airway lumen following N.b. infection

I. $CD4^+$

Phenotypic analysis of the BAL fluid lymphocytes revealed a significant increase in the total number of $CD4^+$ lymphocytes in the BAL fluid following N.b. infection in WT and CD18H mice. Specifically there was near eight-fold increase in the number of $CD4^+$ lymphocytes in the airway lumen in CD18H mice, rising from 8.1×10^3 in naïve animals to 63.9×10^3 on day 14 pi ($p<0.05$). The increase in airway $CD4^+$ lymphocytes in WT mice reached significance by day 8 pi ($p<0.05$), but peaked at day 14 pi, from a basal level of 6.3×10^3 in naïve animals to 61×10^3 on day 14 pi ($p<0.05$)(Figure 8-11).

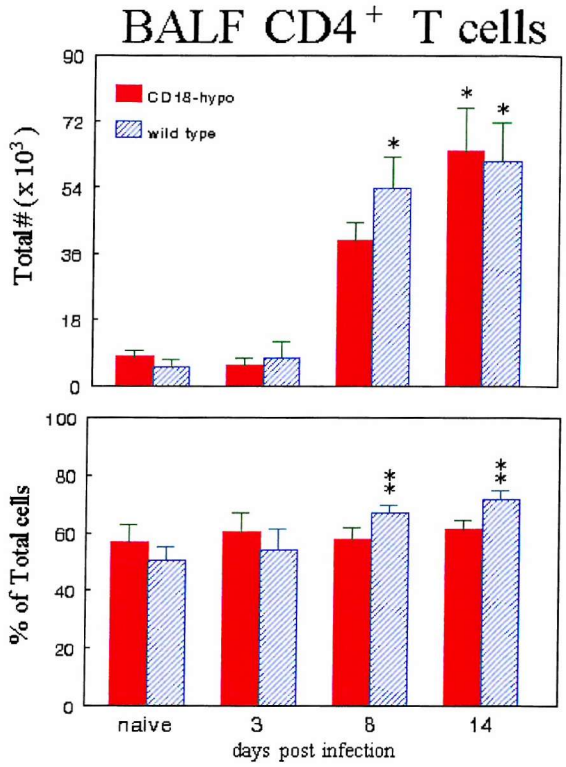

Figure 8-11. The total number, and percentage, of CD4+ T cells in the BAL fluid of CD18H hypomorph and wild-type control mice following N.b. infection.

The changes in the number of $CD4^+$ lymphocytes in the airways following N.b. infection were reflected in an increase in the percentage $CD4^+$ lymphocytes recovered from the BAL fluid in WT but not CD18H mice. The percentage of $CD4^+$ lymphocytes in WT mice increased from 50.4% in naïve animals to 66.8% on day 8 pi ($p<0.01$) rising to 71.6% by day 14 pi ($p<0.01$). The increase in the percentage of $CD4^+$ lymphocytes in CD18H mice was from 56.4% in naïve animals to 61.2% on day 14, but this was not statistically significant (Figure 8-11).

II. $CD8^+$

The number of $CD8^+$ lymphocytes in the airways of CD18H and WT mice also increased following N.b. infection. Thus numbers increased from 1.4×10^3 in naïve mice to 5.9×10^3 on day 14 pi in CD18H mice and from 1.9×10^3 in naïve mice to 6.6×10^3 on day 8 pi and 4.3×10^3 on day 14 pi in WT mice. Due to small sample size and the sample variability these increases were not statistically significant, although they approached significance in WT mice on day 14 pi compared to naïve WT mice ($p=0.05$)(Figure 8-12).

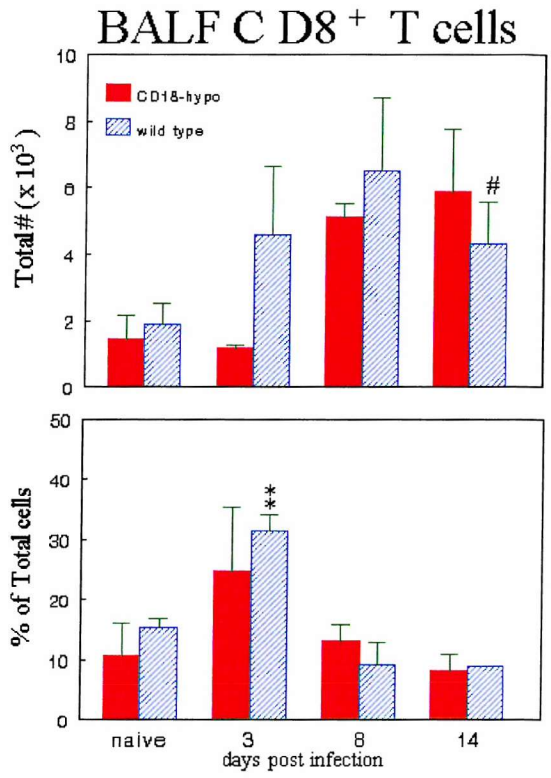

Figure 8-12. The total number, and percentage, of CD8+ T cells in the BAL fluid of CD18H hypomorph and wild-type control mice following N.b. infection.

The changes in the percentages of $CD8^+$ lymphocytes in the airways showed a different pattern. In both CD18H and WT mice there was a substantial increase in the percentage of $CD8^+$ lymphocytes in the airways during the early phase response. In CD18H mice the percentage of $CD8^+$ lymphocytes more than doubled from 10.6% in naïve animals to 24.7% on day 3 pi before subsiding back to basal levels of 8.3% on day 14 pi. But, this increase did not quite reach statistical significance. In contrast, the increase in the percentage of $CD8^+$ lymphocytes in WT mice did reach statistical significance. Thus the percentage of $CD8^+$ lymphocytes doubled from 15.3% in naïve mice to 31.3 on day 3 pi before subsiding during the late response back to a basal level of 5.9% on day 14 pi ($p<0.001$)(Figure 8-12).

III. ICAM-1

The total number of $CD3^+$ lymphocytes in the BAL fluid of CD18H and WT mice expressing ICAM-1 increased substantially during the late pulmonary response. Specifically there was a four-fold increase in the number of $CD3^+$ lymphocytes expressing ICAM-1 in the airway lumen in CD18H mice, rising from 10.4×10^3 in naïve animals to 44.4×10^3 on day 14 pi. Similarly, there was a significant increase in the number of T cells

expressing ICAM-1 in WT mice rising from 5.3 x 10^3 in naïve animals to 37.7 x 10^3 on day 8 pi ($p<0.05$) and 32.2 x 10^3 on day 14 pi ($p<0.001$). The number of ICAM-1 positive T-cells in WT mice may be somewhat reduced since ICAM-1 staining was examined on $CD4^+$ lymphocytes rather than $CD3^+$ lymphocytes as had been done with the CD18H mice. The increase in the number of ICAM-1 positive T-cells in CD18H and WT was of the same order of magnitude, however, due to sample size and variance only the increases in WT numbers achieved statistical significance (Figure 8-13).

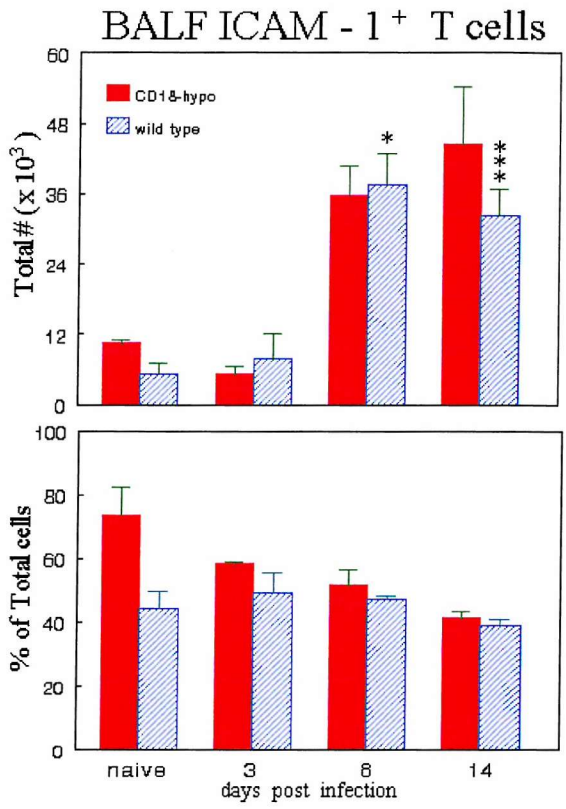

Figure 8-13. The total number, and percentage, of ICAM-1+ T cells in the BAL fluid of CD18H hypomorph and wild-type control mice following N.b. infection.

When expressed as a percentage of the total number of lymphocytes, ICAM-1 staining did not change throughout the infection in WT or CD18H mice suggesting there was no upregulation of this adhesion molecule even in the CD18H hypomorph mice.

IV. VLA-4

The expression of VLA-4 on BAL fluid T cells in WT and CD18H mice also increased substantially during the late phase pulmonary response. There was a near three-fold increase in the number of $CD3^+$ lymphocytes expressing VLA-4 in the airway lumen in

CD18H mice, rising from 7.1 x 10^3 in naïve animals to 19.4 x 10^3 on day 14 pi. Similarly, there was a significant increase in the number of $CD4^+$ lymphocytes expressing VLA-4 in WT mice rising from 6.8 x 10^3 in naïve animals to 28.2 x 10^3 on day 8 pi ($p<0.001$) and 33.6 x 10^3 on day 14 pi ($p<0.01$).

Once again due to the sample size and variance only the increases in VLA-4 expression on WT T cells reached statistical significance (Figure 8-14). Although there were no significant difference in the total number of T cells expressing VLA-4 in WT and CD18H mice the late phase response produced a greater increase in VLA-4 expression in WT mice (Figure 8-14).

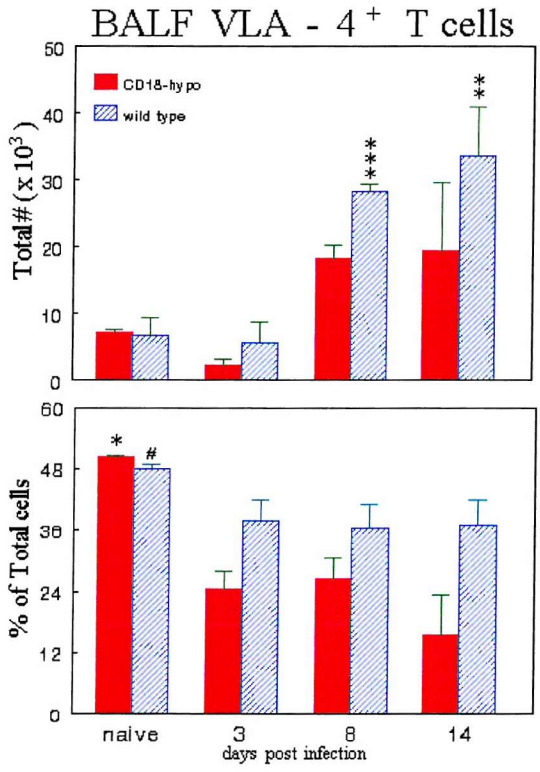

Figure 8-14. The total number, and percentage, of VLA-4+ T cells in the BAL fluid of CD18H hypomorph and wild-type control mice following N.b. infection.

The percentage of lymphocytes expressing VLA-4 was substantially greater in naïve WT and CD18H mice than the percentage of lymphocytes expressing this adhesion molecule following N.b. infection. In CD18H mice the percentage of VLA-4 positive $CD3^+$ lymphocytes dropped from 50.4% in naïve animals to 15.5% on day 14 pi ($p<0.05$). Similarly, in WT mice the percentage of VLA-4 positive $CD4^+$ lymphocytes dropped from 47.9% in naïve animals to 37% on day 14 pi, the latter change not quite reaching statistical significance ($p=0.05$)(Figure 8-14).

V. LFA-1 (CD11a)

The expression of the LFA-1 on lymphocytes recovered from the airway lumen was also assessed. LFA-1 is the alpha subunit of the β_2-integrin CD18H and therefore a very low level of LFA-1 expression was expected in the CD18H hypomorph mice. This expectation was confirmed with the total number cells expressing LFA-1 barely rising above naïve levels in CD18H hypomorph mice and the percentage of CD18H T-cells expressing LFA-1 remaining below 18% throughout the infection.

In contrast, LFA-1 expression on WT T-cells increased significantly from 4.3 x 10^3 in naïve animals to 58.6 x 10^3 on day 8 pi ($p<0.01$) and 64 x 10^3 on day 14 pi ($p<0.001$). This was reflected in a significant increase in the percentage of WT lymphocytes expressing LFA-1, rising from 38% in naïve animals to 74.3% on day 8 pi ($p<0.05$) and rising still further to 76.5% on day 14 pi ($p<0.01$)(Figure 8-15).

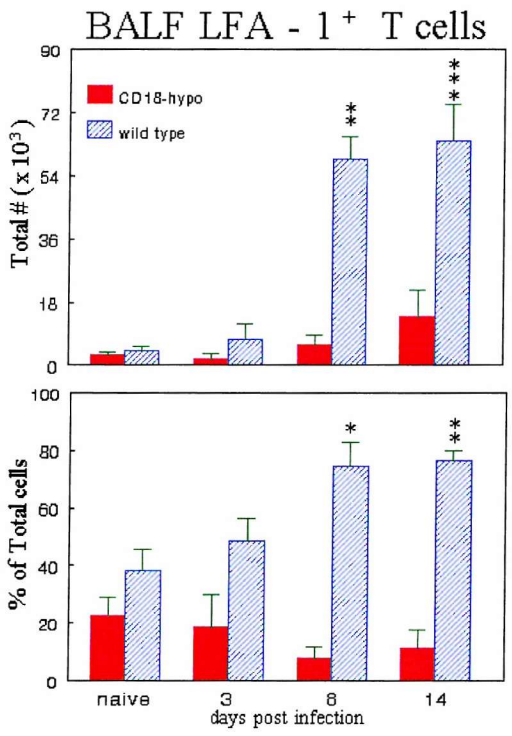

Figure 8-15. The total numbe,r and percentage, of LFA-1+ T cells in the BAL fluid of CD18H hypomorph and wild-type control mice following N.b. infection.

VI. CD43

The number of BAL fluid T cells expressing the activation marker CD43 increased significantly in WT and CD18H mice during the late phase response. Specifically, the number of $CD43^+$ T-cells in CD18H mice rose from 13.6 x 10^3 in naïve animals to

63.7 x 10^3 on day 8 pi ($p=0.05$) and to 100 x 10^3 on day 14 pi ($p<0.05$). Similarly, the number of $CD43^+$ T-cells in WT mice rose from 10.6 x 10^3 in naïve animals to 65.2 x 10^3 on day 8 pi ($p<0.01$) and to 74.3 x 10^3 on day 14 pi ($p<0.001$)(Figure 8-16).

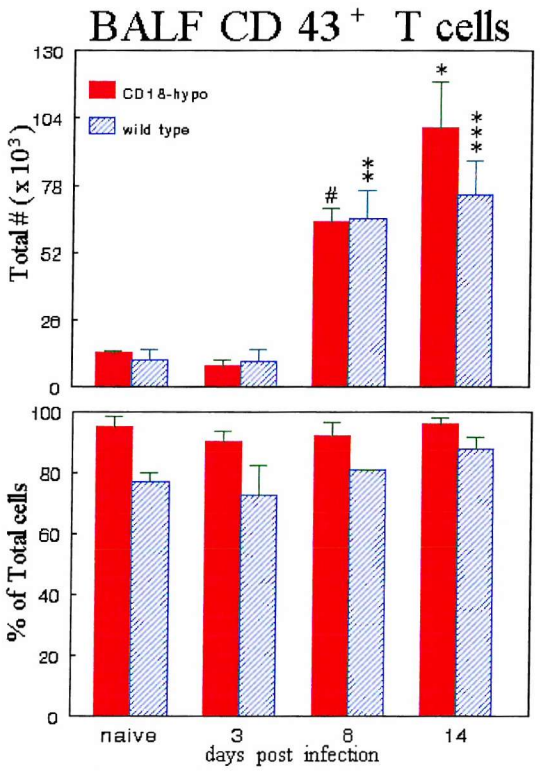

Figure 8-16. The total number, and percentage, of CD43+ T cells in the BAL fluid of CD18H hypomorph and wild-type control mice following N.b. infection.

The percentage of cells expressing this activation marker in CD18H mice never dropped below 90% in naïve or infected animals. In contrast, the percentage of WT expressing this marker was somewhat lower in the 70-80% range (Figure 8-16).

VII. CD45RB

The number of BAL fluid T-cells expressing the memory marker CD45RB increased substantially in WT and CD18H mice following N.b. infection. Specifically, the number of $CD45RB^+$ T-cells in CD18H mice rose from 3.3 x 10^3 in naïve animals to 19.9 x 10^3 on day 14 pi. Because of small sample size and large intra-experiment variation in expression of CD45RB this increase did not reach significance. In contrast, there was a significant increase in the number of WT $CD4^+$ lymphocytes expressing CD45RB, levels rose from 0.3 x 10^3 in naïve animals to 9.9 x 10^3 on day 14 pi ($p<0.01$)(Figure 8-17).

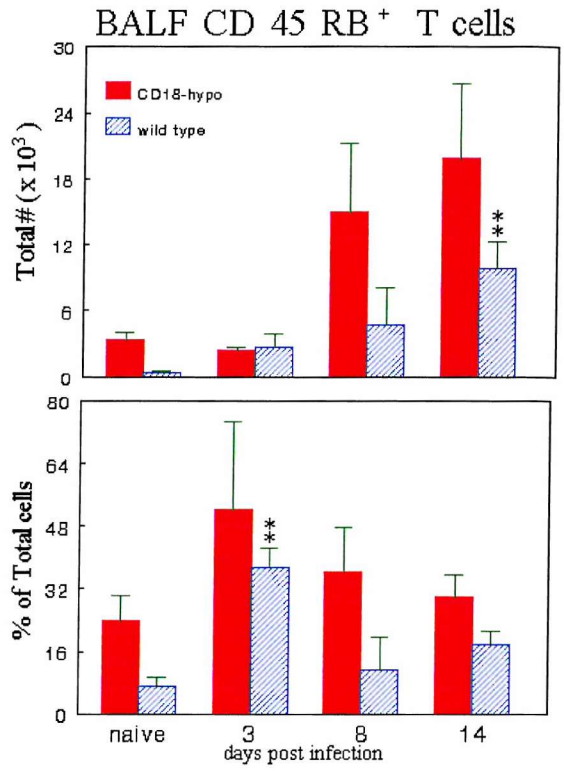

Figure 8-17. The total number, and percentage, of CD45RB+ T cells in the BAL fluid of CD18H hypomorph and wild-type control mice following N.b. infection.

Interestingly the percentage of cells expressing CD45RB increased substantially in CD18H and WT mice during the early response when larvae are migrating through the lungs. In CD18H the percentage of T-cells expressing CD45RB rose form 23.7% to 52.3% on day 3 pi and subsided thereafter. Again because of the small sample size and the variance this increase did not reach statistical significance. In contrast, the percentage of T-cells expressing CD45RB during the early response in WT mice did reach statistical significance, rising from 7.1% in naïve animals to 37.7% on day 3 pi before subsiding ($p<0.01$)(Figure 8-17).

VIII. PGP-1

The number of BAL fluid T-cells expressing the hyaluronate receptor, Pgp-1 (CD44), increased significantly in WT and CD18H mice during the late response. Specifically, the number of Pgp-1$^+$ T-cells in CD18H mice rose from 13 x 10^3 in naïve animals to 50.1 x 10^3 on day 14 pi ($p<0.001$). The rise in Pgp-1 expression on CD4$^+$ lymphocytes in WT mice did not quite reach significance, but increased from 5.2 x 10^3 in naïve animals to 45.3 x 10^3 on day 14 pi ($p=0.05$)(Figure 8-18).

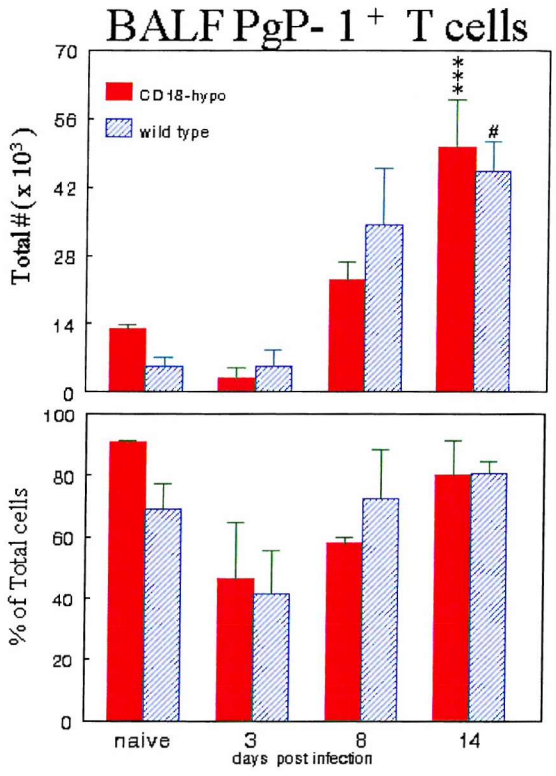

Figure 8-18. The total number and percentage of PgP-1+ T cells in the BAL fluid of CD18H hypomorph and wild-type control mice following N.b. infection.

During the early response the percentage of cells expressing Pgp-1 decreased substantially from 90.8% in naïve CD18H mice to 46.5% on day 3 pi before rising again to 80.4% on day 14 pi. Similarly the percentage of WT T-cells expressing Pgp-1 also decreased substantially during the early response from 69.2% in naïve animals to 41.3% on day 3 pi prior to rising again back to 80.7% on day 14 pi. These changes did not reach statistical significance (Figure 8-18).

IX. L-selectin (CD62L)

The number of BAL fluid T-cells expressing the T cell maturation marker L-selectin remained largely unchanged in CD18H mice. In contrast there was a small and nearly significant increase in the number of WT T-cells expressing L-selectin during the late response ($p=0.05$). However, when examining the percentage of cells expressing this maturation marker there was a substantial increase during the early response in CD18H and WT mice. The percentage of CD18H mice expressing L-selectin rose from 14.1% in naïve mice to 35% on day 3 pi before falling back to 2.8% on day 14 pi. These changes did not reach statistical significance but were of the same order of magnitude as the changes in WT mice that were statistically significant. Thus in WT mice the percentage of T-cells

expressing L-selectin rose from 5.1% in naïve mice to 38% on day 3 pi before levels subsided back to 3.4%on day 14 pi ($p<0.001$)(Figure 8-19).

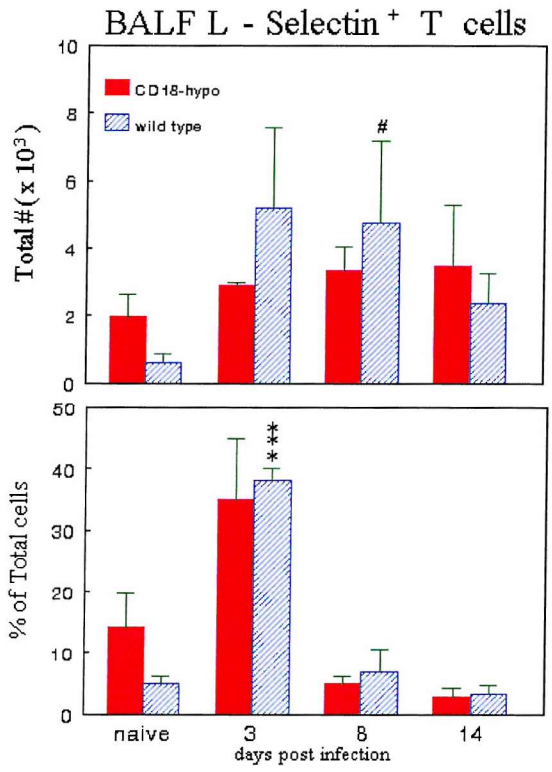

Figure 8-19. The total number, and percentage, of L-Selectin+ T cells in the BAL fluid of CD18H hypomorph, and wild-type control mice, following N.b. infection.

X. IL-2R

The number of BAL fluid T-cells expressing the T cell activation marker IL-2R increased following N.b. infection in CD18H mice however there was extremely wide variation in the expression of this marker in different experiments and therefore no statistical differences between the time points. Similarly there was an increase in the total number of WT T-cells expressing IL-2R during the late response and this did reach statistical significance on day 8 pi ($p<0.01$).

When the percentage of T-cells expressing IL2-R in CD18H and WT mice was examined a more revealing picture emerged. There was a substantial increase in the percentage of T-cells expressing IL-2R during the early response. In CD18H mice the percentage of cells expressing this activation marker rose from 0.5% in naïve animals to 6.3% on day 3 pi. This increase was not as large as that seen in WT mice where the percentage of naïve expressing IL-2R increased from 0.5% to 11.4% on day 3 pi ($p<0.05$).

8.4 DISCUSSION

There are a number of experimental approaches to dissecting the role of specific adhesion molecules in rodent models of pulmonary inflammation. Some studies have employed blocking antibodies to adhesion molecules. For example, monoclonal antibodies to CD18H may block cellular recruitment either by blocking firm attachment of leukocytes to the vascular endothelium after initial capture of the leukocyte by a selectin, or by preventing transmigration across the endothelial barrier. In vitro studies have suggested that CD18H is almost obligatory for endothelial transmigration (Jagels et al 1999, Furie et al 1992). Such studies have highlighted the physiological importance of CD18H with anti-CD11/CD18H antibodies producing a reduction in the guinea pig airway eosinophilia and early phase bronchoconstriction in ovalbumin or sephadex bead-induced models of asthma (Sagara H et al 1997, Bloemen et al 1996, Das et al 1995, Milne et al 1994). This effect on bronchoconstriction may be a direct result of these antibodies on mast cell and basophil cell function rather than a blockade of a small number of β_2-integrin receptors on mast cells and basophils (Rabb et al 1994).

However, experiments employing blocking antibodies require efficient antibody delivery systems of physiologically relevant concentrations of antibody to physiologically relevant compartment, the construction of accurate dose response curves, the determination of adequate plasma levels, the avoidance of toxicity and inappropriate binding to irrelevant tissues. Basically such studies require considerable expertise and substantial amounts of costly blocking antibodies. Therefore other simpler and less expensive approaches have attempted to implicate specific adhesion molecules in airway inflammation. For example, a number of studies have identified specific adhesion molecules in epithelial tissue using techniques such as immunohistochemistry. Thus, neutrophil adhesion to dog tracheal epithelial cell cultures was found to be CD18H and iC3b dependent (Varsano et al 1994). Alternatively, FACS analysis may be used to demonstrate an increase in blood, BAL or epithelial cell adhesion molecule expression during airway inflammation (Yassin et al 1994). A third approach to studying the role of adhesion molecules in airway inflammation is to use genetically engineered animals with reduced or absent expression of specific adhesion molecules. The availability of mice, developed by the Upjohn transgenic breeding program, with an incompletely deleted CD18H gene enabled an investigation of the role of this adhesion molecule in the peripheral blood and pulmonary response to a helminth

antigen.

In keeping with the results presented in earlier chapters the total number of leukocyte recruited to the blood and airways rose gradually throughout Nb. infection. However, on closer examination of the cellular subsets a biphasic response became apparent. The early response, occurring during larval migration through the lungs on day 3 pi, was characterised by significant airway and peripheral blood neutrophilia.

In contrast to the obligatory nature of CD18H involvement in neutrophil recruitment to the skin, recruitment to the airways demonstrates varying degrees of CD18H-dependency according to the nature of the provoking antigen or stimulus. It has been suggested that low levels of neutrophil recruitment may be CD18H-independent, but greater levels of cellular influx are CD18H-dependent (Hellewell et al 1994). The low levels of neutrophil recruitment may be dependent on E-selectin (Mulligan et al 1991) and P-selectin (Till et al 1982).

The significant neutrophilia reported here in CD18H hypomorph mice, during the early phase response, suggests that neutrophil recruitment to the peripheral blood may have been CD18H-independent. Previous research has suggested that CD18H may play a more important role in neutrophil recruitment to the alveolar compartment rather than the vascular compartment (Mulligan et al 1995). However, the experiments reported above indicate that the magnitude of the airway neutrophilia in CD18H hypomorph mice was equivalent to that observed in WT mice suggesting that airway neutrophilia was also CD18H-independent. Thus despite the well recognised role of CD18H in neutrophil chemotaxis, as demonstrated by studies in neonates (Abughali et al 1994), or adults (Springer et al 1986) with low levels of neutrophil CD18H expression, partial deficiency of CD18H seemed to have no effect on neutrophil recruitment to either the vascular or alveolar compartment. These findings support some previous studies demonstrating that neutrophil recruitment to the lungs is more CD18H independent than eosinophil recruitment (Schneider T et al 1999) but contradicts some earlier studies in animal models of pulmonary inflammation suggesting that neutrophil recruitment is CD18H-dependent (Vedder et al 1988, Barton et al 1989).

While the pulmonary and vascular recruitment of neutrophils has been shown to be CD18H-dependent and independent, eosinophil recruitment has been shown to be much more CD18H dependent (Jagels MA et al 1999, Schneider T et al 1999). In contrast the results presented here suggest that eosinophil recruitment to the blood and airways, in response to helminth antigens in this model, is CD18H-independent. We did see a greater eosinophilia in WT mice compared to CD18H hypomorphs on day 8 pi but by day 14 this effect had reversed.

This failure to inhibit eosinophil recruitment to both the blood and the airways in CD18H hypomorph mice may be due to the failure to inhibit neutrophil recruitment since neutrophils may be driving the subsequent eosinophil recruitment (Katy et al 1976, Czarnetzki et al 1978). In fact the total number of eosinophils recruited to the vascular compartment of CD18 hypomorph mice was significantly greater than the number recruited to the blood of WT mice on day 14 pi. This increased recruitment of eosinophils into the blood was mirrored by a significantly greater number of eosinophils recruited to the airways in CD18 hypomorph mice on day 14 pi. However, the greater eosinophil recruitment to both vascular and alveolar compartments did not alter the serum IgE production which was equivalent in both CD18 hypomorph and WT mice.

The fact that there was a reduced recruitment of eosinophils in CD18H hypomorphs on day 8 pi that was overcome by day 14 suggests that there may be an alternative explanation. Thus, given the sophistication of the recruitment strategies employed by eosinophils and the abundance of factors driving eosinophil recruitment (Wardlaw AJ et al 1999), it may have been that partially inhibiting the CD18H recruitment pathway significantly enhanced other recruitment strategies leading to the greater eosinophilia seen here.

It has been suggested that CD18H may play a central role in T cell-dependent and T-cell independent inflammatory reactions (Albelda et al 1990). N.b. infection has been shown to be particularly efficient at promoting a Th-2 driven response (Rocken et al 1994). The results presented here indicate that low expression of CD18H did not inhibit the late phase response to N.b. antigens and failed to inhibit lymphocyte recruitment to peripheral blood. In fact there was a significantly greater lymphocytosis in the peripheral blood in CD18H hypomorph mice compared to WT controls on day 14 pi. The pattern of lymphocyte

recruitment to the airways in CD18H hypomorphs closely resembled that of eosinophils. Thus on day 8 pi there was a significant inhibition of lymphocytes recruited to the airways of CD18H hypomorph mice compared to WT controls but by day 14 pi this effect had disappeared.

N.b. infection is known to effect an isotype switch from Th-1-driven response to a Th-2 driven response, with increases in the production of Th-2 cytokines, IL-5 and Il-10. Although we found no difference in the IgE response of CD18H hypomorph mice and WT controls there was a significant difference in the systemic cytokine response of CD18H hypomorphs and WT mice. It would be expected that WT mice should recruit more eosinophils to sites of inflammation than CD18H hypomorph mice and therefore may generate more vigorous IL-5 responses. However, we found that during the late phase response the CD18H hypomorph mice actually recruited more eosinophils to the blood and airways. Therefore it was not surprising to discover that CD18H hypomorph mice also generated a significantly greater systemic IL-5 response than WT controls. This greater production of IL-5 in CD18H hypomorph mice may help to explain the failure to inhibit eosinophil recruitment to the blood and airways in CD18H mice since IL-5 is known to promote eosinophilia. However, when examining the systemic IL-10 response we found a significantly greater production of this Th-2 cytokine in WT mice compared to CD18H hypomorph mice on day 14 pi.

Taken together the results, and the failure to inhibit IgE production, suggest that N.b. infection produced an effective Th-2 response in the CD18H hypomorph mice. This conclusion is supported by the significant increase in $CD4^+$ lymphocytes recruited to the BAL in CD18H hypomorph mice.

An examination of the adhesion molecules utilized by lymphocytes to access the airway epithelium demonstrated that there was no difference in expression of ICAM-1 or VLA-4 between CD18H mice and WT controls, although CD18H mice clearly had significantly lower levels of LFA-1 expression.

Phenotypic analysis of the lymphocytes recruited to the BAL fluid during the early response revealed a substantial increase in naïve T-cells recruited to the airways as indicated by

increased L-selectin and CD45RB expression in CD18H hypomorph mice and WT controls. The increase in IL2-R expression on both CD18H and WT controls during larval migration through the lungs suggested that a number of these cells were activated.

In contrast the late phase response was characterised by increased numbers of activated memory T-cells as indicated by the increased number of T-cells expressing Pgp-1 (CD44), CD43 and the loss of L-selectin in CD18H and WT mice.

In conclusion, these data indicate that low levels of CD18H expression do not inhibit the cellular, cytokine or humoral response to N.b. infection. These data suggest that in an N.b.-induced model of pulmonary inflammation neutrophil recruitment was either CD18H-independent or that the genetic manipulation failed to inhibit cellular recruitment because it was incomplete or because escape mechanisms were utilised to bypass the genetic defect. Therefore the subsequent late phase response, characterised by a Th-2 driven eosinophilia, appeared to be CD18H independent. It is possible that cellular recruitment to the airways may have occurred through as yet unidentified mechanisms involving glycoproteins or other peptides and therefore deletion of adhesion molecules, partial or otherwise may have only a limited effect. Such a possibility would make adhesion molecule knockout technology less helpful in directing potential therapeutic strategies in the future (David FS et al 1999).

CHAPTER 9

Mast Cell Deficient Mice

9.1 INTRODUCTION

Allergic inflammation of the airways is characterised by an inflammatory cell infiltrate, which consists predominantly of degranulated mast cells, activated T lymphocytes and eosinophils (Azzawi et al 1990, Djukanovic et al 1990b Robinson et al 1993a, Pearlman DS et al 1999). The degree of cellular infiltration is closely related to the disease severity (Wardlaw et al 1988, Walker et al 1991). The central role played by mast cells in this inflammatory process has been recognised for a number of years.

The location of mast cells in the intraepithelial layer of the airways makes them ideally situated to respond to inhaled antigens. During airway inflammation they are primed to secrete an array of preformed and newly generated inflammatory mediators including histamine, neutral proteases and heparin sulphate, prostaglandins and cysteinyl leukotrienes as well as a spectrum of cytokines and chemokines that are involved in leucocyte recruitment and activation. For example mast cells have been shown to synthesise many of the key Th-2 cytokines such as IL-4, IL-5, IL-9 and IL-10 (Wilson SJ et al 2000, Stassen M et al 2000). Their central role in maintaining airway dysfunction in asthma is underpinned by the efficacy of interventions that interfere with mast cell function or activation such as leukotriene antagonists, nuclear factor-kappaB (NF-κB) antagonists and the recently studied E-20 humanised monoclonal antibody that binds to, and removes, IgE (Holgate ST et al 2000, Marquardt DL et al 2000).

Recently mast cells have been specifically implicated in the increased morbidity and mortality in elderly onset asthma (Atsuta R et al 1999) and such studies have re-invigorated the attention given to mast cells as therapeutic targets. For example, the recent discovery of novel inhibitory pathways involving inhibitory motifs (ITIMS) on critical cell surface signalling molecules has opened up new therapeutic lines of attack for preventing mast cell activation (Holgate ST et al 2000).

In the last five years a more sophisticated and complex role for mast cells in the inflammatory process has begun to emerge. For example, it has been suggested that the of volume of pro-inflammatory cytokines produced by mast cells is comparable to that of T-cells implicating mast cells, along with T lymphocytes and eosinophils, in the genesis of

chronic, persistent asthma. In addition, mast cells have been shown to be capable of interacting functionally with B cells to promote IgE synthesis (Yoshikawa T et al 2001), and with T lymphocytes to act as antigen presenting cells (APCs). They are also capable of recognising and ingesting a wide range of bacteria (Abraham SN et al 1997), in addition to their established role in airway remodelling (Rossi GL et al 1997). Therefore, mast cells may be critical for the full expression of certain features of late-phase reactions and may also contribute importantly to clinically relevant aspects of chronic allergic inflammation (Williams CM et al 2000).

Although mast cells are believed to be pro-inflammatory some authors have recently suggested that under certain circumstances mast cells may actually play an anti-inflammatory role, particularly in the intestine, through the production of mast cell cytokines such as tumour necrosis factor (TNF)-alpha and interleukin (IL)-10 (Stenton GR et al 1998). Little is known about what determines whether mast cells play a pro or anti-inflammatory role but nitric oxide, which is not only produced by mast cells but regulates mast cell function, may be involved (Stenton GR et al 1998). Other factors such as IL-10, which is secreted by mast cells and expressed on the mast cell surface, have also been implicated.

The suggestion that mast cells may actually dampen the allergic response has received support from studies with human mast cell lines. In a recent Dutch study, human mast cell lines modulated the proliferation and cytokine production of a human CD8+ T-cell clone in vitro. Thus an activated mast cell line drove this CD8+ T-cell clone towards a more pronounced Th-1 type of response and simultaneously decreased T-cell numbers. This suggested that during the late phase response mast cells might exert negative feedback on Th2-driven IgE production and eosinophilia (de Pater-Huijsen FL et al 1997).

Whether mast cells play a pro or anti-inflammatory role may be partially dependent on the balance of cytokines secreted by surrounding cells or the mast cells themselves (Tachimoto H et al 2000). For example, IL-1 (alpha or beta) increased the production of IL-3, IL-5, IL-6, and IL-9 as well as TNF in activated mouse bone marrow-derived mast cells (Hultner L et al 2000). However, IL-10 has been shown to inhibit nitric oxide production by rodent peritoneal MC (PMC) and potentiate histamine secretion. Although the increased histamine

production is only seen in long term, (24-h) and not in short-term (20-min), incubation with IL-10 this effect of IL-10 is similar to that reported for IL-3 and IL-4 in murine MC (Lin TJ 1997). IL-10 also inhibits TNF-alpha production by PMC in a dose-dependent manner.

In contrast to IL-10, Stem Cell Factor (SCF) has no effect on mast cell nitric oxide production or TNF-alpha production but potentiates histamine secretion in both short term (20-min) and long term (24-h) experiments (Lin TJ et al 1997). Furthermore, following N.b. infection SCF significantly increased rat mast cell protease II (RMCP II) production by rat mucosal mast cell (MMC). A polyclonal sheep anti-rat SCF antibody abolished this effect suggesting that endogenous production of SCF is sufficient to sustain near maximal MMC hyperplasia (Newlands GF et al 1995). Recent evidence from human studies has confirmed the importance of SCF with increased levels of SCF found in the bronchoalveolar (BAL) fluid of allergic asthmatics, particulalry in the pollen season (Olsson N et al 2000). Thus, IL-10 and SCF exert different regulatory effects on MC secretory function with IL-10 potentially playing an autocrine regulatory function (Lin TJ et al 1997).

Another suspect that has been implicated in determining the exact pro-inflammatory role played by mast cells is the Th2-type cytokine IL-9. Recently, IL-9 was identified by genetic mapping analyses as a key mediator that determines the susceptibility to asthma. This has been further supported by data from IL-9-transgenic mice in which the overexpression of IL-9 in the lung causes airway inflammation with eosinophils and lymphocytes predominating. There was also a striking epithelial mast cell hyperplasia, subepithelial deposition of collagen and bronchial hyper responsiveness to inhaled methacholine (Temann UA et al 1998). Murine bone marrow-derived mast cells (BMMC) have been shown to be very potent producers of IL-9, particularly when stimulated with IL-10 (Stassen M et al 2000). In fact many of the Th-2 cytokines such as IL-3, IL-5, IL-6 and IL-9 have been shown to promote mast cell proliferation while the Th-1 cytokine interferon (IFN)-γ suppressed mast cell proliferation (Ochi H et al 1999).

The balance of the cells and cytokines involved in the inflammatory process has recently been explored in rodents. These experiments demonstrated that rodents utilise different effector mechanisms in the expulsion of different gastrointestinal nematodes. Thus, IL-4 can induce the expulsion of N.b. in the absence of B cells, T cells, and mast cells but mast

cells and T cells are required for IL-4 induced expulsion of *T. spiralis* (Urban JF et al 2000). This suggests that resolution of the inflammatory process requires different effector mechanisms depending on the type of inflammatory stimulus. Therefore the subtlety of the mast cell response, and its potential to play both a pro or anti-inflammatory role may be highly relevant in the resolution of allergic inflammation of the airways.

This suggestion received support from a recent study, which examined the effects of Th-1 cytokines on mast cells. Since the balance between Th-1 and Th-2 cytokines may change throughout the allergic response the activation of mast cells may similarly change. Recent evidence suggests that Th-1 cytokines such as IFN-γ increased the expression of the high affinity FcγRI IgG receptor while not affecting FcεRI, FcγRII, and FcγRIII receptors. This IFN-γ induced aggregation of FcγRI on human mast cells led to significant histamine release and increased expression of TNF-α, GM-CSF, IL-3 and IL-13. Therefore mast cell degranulation may not be entirely dependent on cross-linking IgE receptors but may be provoked by cross-linking IgG receptors (Okayama Y 2000). The functional significance of this novel pathway remains to be elucidated.

The potential immunoregulatory role of mast cells in modulating the inflammatory response was highlighted by the suggestion that mast cells may actually change phenotype *in situ* in response to pulmonary inflammation (Arizono N et al 1987). Thus the majority of mast cells recovered from the N.b.-infected rat lung were shown to be mucosal mast cells (MMC) in phenotype but were also shown to produce rat mast cell tryptase (RMCT) mRNA, normally confined to connective tissue mast cells (CTMC)(Tomita M et al 1999). This phenotype switching confirmed an earlier report indicating that MMC could change to CTMC under certain environmental conditions (Nakano T et al 1987), suggesting that both originate from a common progenitor cell (Ochi H et al 1999). In addition, the spectrum of cytokines secreted and the density of high affinity IgE receptors on mast cells have been shown to vary in patients with different allergic conditions (Pawankar R et al 1998).

The role of mast cells in allergic inflammation may not only be dependent on self-regulation through an autocrine process but has also be shown to be dependent other cellular subsets. Thus T cells have been implicated in the mucosal mast cell proliferation that occurs following N.b. infection through their cytokine production (Mayrhofer G et al 1979).

The role of mast cells in allergic inflammation can be investigated through the use of mice that are naturally deficient in mast cells due to an absence of stem cell factor. But these mice tend to be anaemic as well and some of the pathophysiological consequences seen may not be directly related to the mast cell deficiency. A second approach is to use the recently developed mast cell-deficient W/Wv murine strain that possess less than 1% tissue mast cells (Kitamura Y et a 1978). This strain has enabled scientists to probe the role of the mast cell in a variety of airway inflammation models. This mast cell-deficient W/Wv strain was shown to be immunologically robust without significant immune impairment as evidenced by their ability to develop a normal or greater IgM and IgG response to antigens such as sheep erythrocytes (SE), polyvinylpyrrolidone or bacterial lipopolysaccharide compared to syngeneic controls. Similarly, following N.b. infection the serum titres of N.b-specific IgE were higher than normal littermates and their ability to generate delayed-type hypersensitivity (DTH) reactions were also normal (Ha TY et al 1986). The third approach to investigating the role of mast cells in inflammation is to reconstitute mast cell deficient mice with mast cells from syngeneic littermates.

The lack of significant immune impairment in W/Wv mice has meant that this strain has been most widely used to investigate the contribution of mast cells to specific aspects of the inflammatory process. For example mast cells have been shown to be central to the induction of airway hyper-responsiveness independent of pulmonary inflammation (Kobayashi T et al 2000). Thus immunisation of W/Wv mice with alum-adsorbed OVA followed by bronchoprovocation with aerosolised OVA provoked severe eosinophilic infiltration into the airways and an IgE, IgG1 anti-OVA antibody (Ab) response but no airway hyper-responsiveness (AHR). Reconstitution of W/Wv mice with bone marrow-derived mast cells cultured from normal littermates restored the capacity to develop antigen (Ag)-induced AHR, indicating that lack of mast cells was responsible for the failure of W/Wv mice to develop Ag-induced AHR. However, these authors also showed that OVA-immunised W/Wv mice could be induced to develop AHR by increasing the frequency of bronchoprovocations and the actual Ag dose. This suggests that AHR can be induced by two distinct cellular mechanisms one involving mast cell activation and the other, IgE/mast cell independent pathway, involving an eosinophil/IL-5-dependent mechanism (Kobayashi T et al 2000).

We took advantage of this genetic technology and used W/Wv and syngeneic control mice to investigate the role of mast cells in the systemic and pulmonary changes seen in mice following primary infection with Nippostrongylus brasiliensis (N.b.). Previous authors have shown that mast cell-deficient W/Wv mice expelled gastrointestinal nematodes more slowly than syngeneic controls and bone marrow reconstitution accelerated nematode expulsion (Ha TY et al 1983, Nawa Y et al 1987, Khan AI et al 1993). This expulsion has been shown to be independent of immunoglobulin production, even to N.b.-specific antigens (Jacobson RH et al 1977). Mast cell deficiency, in rats, also abolishes N.b.-induced anaphylaxis despite nematode-specific IgE, IgG1 and IgG2a responses and a significant pulmonary eosinophilia (Nishida M et al 1998). This was thought to be due to the lack of mast cell histamine and leukotriene (LT) C4 release and the consequent changes in vascular permeability, which cause anaphylactic death.

However, the results from experiments using genetically manipulated rodent strains must always be interpreted with some caution since they may not accurately reflect the pathophysiological processes seen in medically managed allergic inflammation of the airways in humans. For example, steroid treatment has been shown to block the concentration of the mucosal mast cell protease RMCP II, mast cell numbers and N.b. expulsion in rats, probably by altering T-cell cytokine production. However, mouse mucosal mast cells (MMC) were less T-cell dependent since MMC numbers and concentrations of Mouse Intestinal Mast Cell Protease (MIMCP) in the intestines were unaltered by steroid treatment in some (Newlands GF et al 1990) but not all murine strains (Kennedy MW et al 1980).

We investigated the effect of mast cells on the pulmonary and systemic response in a Th-2-driven model of allergic inflammation using a primary infection with Nippostrongylus brasiliensis. The hypothesis tested was that mast cell deficiency would significantly reduce the pulmonary inflammatory response to N.b. antigens. Thus we studied the cellular populations in the blood and airways following N.b. infection in addition to the splenic cytokine response to N.b. infection.

9.2 MATERIALS AND METHODS

9.2.1 Larval Culture and Infection

Details of N.b. larval culture are given in chapter 2.

9.2.2 Animals

Details of animal husbandry are given in chapter 2.

9.2.3 Bronchoalveolar lavage and cell preparations

Details of brochoalveolar lavage and cell preparation are given in chapter 2. Data presented throughout this thesis are given per ml of BAL fluid drawn.

9.2.4 Blood: Leukocyte Subsets

Details of blood acquisition and cell analysis are given in chapter 3. Data presented throughout this thesis are given per ml of blood drawn.

9.2.5 Spleen mononuclear preparations:

Details of spleen cell preparation are given in chapter 5.

9.2.6 Cytokine ELISAs

Details of specific cytokine ELISAs are given in chapter 6.

9.2.7 Generation of N.b. specific antigen

Details of N.b. specific antigen generation are given in chapter 5.

9.2.8 Statistics

The data presented in this chapter on wild type C57BL/6 mice are pooled from several experiments conducted throughout this thesis. Thus the peripheral blood and BAL fluid data presented contains, on average, 38 mice per time point. The MCD data presented were drawn from two separate experiments with 20 mice pooled in each time point. Total and differential cell counts in the BAL fluid blood splenocyte proliferation and cytokine production were analyzed using a t-test assuming unequal variance and a normal distribution. When this overall test of treatment was found to be significant at the $p<0.05$, this was indicated by an asterisk (*). When the significance level reached $p<0.01$ this was indicated by two asterisks (**), and when the significance level reached $p<0.001$ this was indicated by three asterisks (***). If the result was borderline significant ($p=0.05$), then this was denoted by a # sign.

9.3 RESULTS

9.3.1 Time course of peripheral blood changes following N.b. infection

I. Total Leukocyte Count

Primary infection with third stage N.b. larvae produced a significant increase in the total number of blood white cell count (WCC) in syngeneic control (sync) mice by day 14 pi ($p<0.02$). In contrast, there was no such increase in the total WCC in mast cell deficient (MCD) mice following N.b. infection ($p=0.38$)(Figure 9-1). This increased cellularity in syngeneic controls is in keeping with that seen in wild type (WT) C57BL6 mice (see chapter 3). The lack of a leukocyte response in MCD mice may have been due to the overall blunted response of MCD mice to N.b. infection in addition to the significantly greater baseline peripheral blood leukocyte count in naïve MCD mice compared to their syngeneic controls ($P<0.001$)(Figure 9-1).

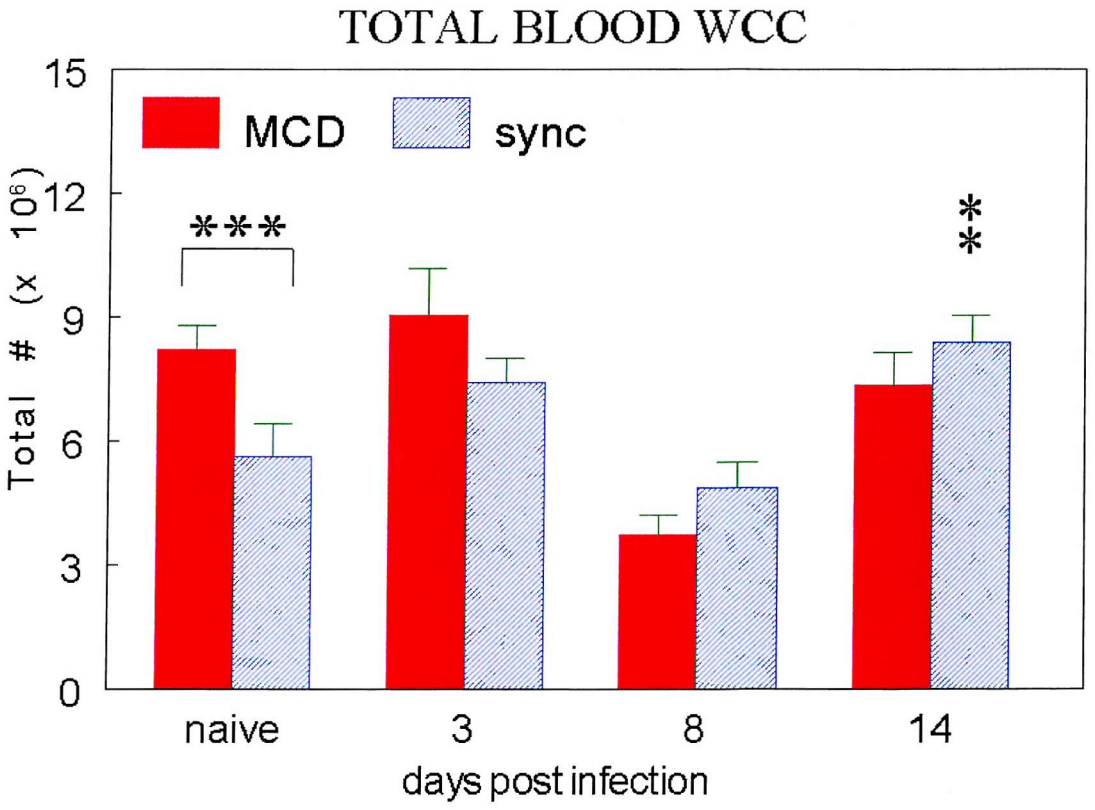

Figure 9-1. The peripheral blood leukocyte response to N.b. infection in mast cell deficient, (MCD, and syngeneic (sync)-type control mice.

Following N.b. infection there were no significant differences in total blood leukocyte count between MCD mice and their syngeneic controls on day 3, 8 or 14 pi.

II. Eosinophils

In keeping with the results seen in wild type, ICAM-deficient and CD18 hypomorph mice the most dramatic cellular changes in the peripheral blood were seen in the eosinophil population.

In contrast to the changes in the total leukocyte count, which just affected sync mice, the number of eosinophils in both MCD and sync mice increased significantly by day 14 pi ($P<0.001$).

There was no difference in the peripheral eosinophil counts between naïve MCD and naïve sync mice 0.08 x 10^6 and 0.11 x 10^6 respectively. However by day 3 there was a much greater eosinophilia in the peripheral blood of sync mice compared to MCD mice. Thus the total number of eosinophils in the blood of sync mice 3 days pi was 0.32 x10^6 compared to 0.15 x10^6 in MCD mice ($p<0.02$). This greater eosinophilia was maintained on day 8 pi with 0.23 x10^6 eosinophils in the peripheral blood of sync mice compared to 0.05 x10^6 eosinophils in the peripheral blood of MCD mice ($p<0.03$). The greater eosinophilia in sync mice compared to MCD mice became even more apparent by day 14 pi. Thus the total number of eosinophils in sync mice 14 days pi was 1.51 x10^6 compared to 0.83 x10^6 in MCD mice ($p<0.03$).

This substantial rise in eosinophil numbers was reflected by changes in the percentage of eosinophils in the peripheral blood in both sync and MCD (Figure 9-2). Specifically there was no difference in the percentage of eosinophils in naïve sync and MCD mice (1.88% and 0.98% respectively). However, the percentage of eosinophils in the blood of sync mice on day 3 pi had risen significantly to 4.33% ($p<0.01$), whereas the percentage of eosinophils in the peripheral blood of MCD mice had not increased significantly by day 3 pi (0.98% in naïve mice and 1.91% three days pi). This significant increase in the percentage of eosinophils in sync mice was reflected in a significant difference between the percentage of eosinophils in sync and MCD mice three days pi, 4.33% and 1.91% respectively ($p<0.02$)(Figure 9-2).

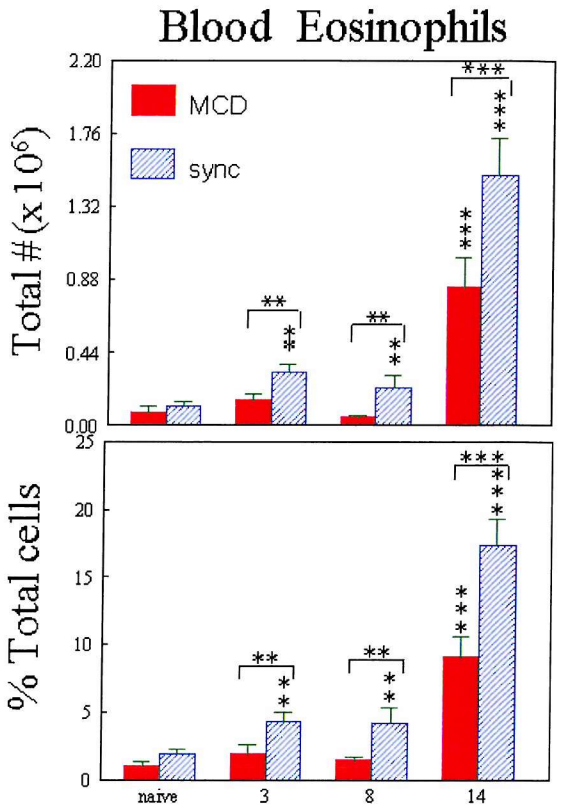

Figure 9-2 The total number and percentage of eosinophils in the blood of mast cell deficient (MCD) and sync-type control mice following N.b. infection.

The peripheral eosinophilia in sync mice on day 3 pi persisted on day 8 with significantly more eosinophils in the peripheral blood of sync mice (4.22%) on day 8 pi compared to naïve sync mice (1.88%)($p<0.02$). Furthermore the greater percentage of eosinophilia in the peripheral blood of sync mice compared to MCD also persisted, with 4.22% eosinophils in the blood of sync mice 8 days pi and 1.46% eosinophils in the peripheral blood of MCD mice ($p<0.03$).

By day 14 pi this greater eosinophilia in sync mice compared to MCD mice was even more marked. The percentage of eosinophils in sync mice had risen to 17.31% by day 14 pi from 1.88% in naïve sync mice ($p<0.001$) and 4.22 on day 8 pi ($p<0.001$). This compared to the rise in the percentage of eosinophils in the peripheral blood of MCD mice from 0.98% in naïve mice to 1.46% by day 8 pi and 9.09% by day 14 pi ($p<0.001$). Consistent with the results seen on day 3 and day 8 pi the peripheral eosinophilia in sync mice on day 14 pi was greater in sync mice compared to MCD mice, 17.31% and 9.90% respectively ($p<0.001$) (Figure 9-2).

III. Neutrophils

The significant increase in the total leukocyte count following N.b. infection was also reflected in the significant increase in the total number of neutrophils in the peripheral blood in both sync and MCD mice. Total neutrophil count increased from 0.57 $x10^6$ in sync naïve mice to 1.04 x 10^6 3 days pi ($p<0.001$), and from 0.44 $x10^6$ in naïve MCD mice to 1.30 $x10^6$ 3 days pi ($p<0.01$). This neutrophilia persisted to day 8 with the number of neutrophils in the peripheral blood of sync mice (1.11 $x10^6$) still being greater than in naïve sync mice ($p<0.01$). Similarly, the number of neutrophils in the peripheral blood of MCD mice on day 8 pi (0.97 $x10^6$) was still greater than in naïve MCD mice ($p<0.01$).

By day 14 pi the number of neutrophils in the peripheral blood of sync mice had subsided to near naïve levels (0.81 $x10^6$)($p<0.13$). Similarly the number of neutrophils in the peripheral blood of MCD mice had also decreased (0.65 $x10^6$) but was still significantly greater than the numbers found in naïve MCD ($p<0.01$)(Figure 9-3). There were no significant differences in the extent of the peripheral neutrophilia following N.b. infection in sync versus MCD mice, and there was no significant differences in the resting neutrophil count in naïve sync or MCD mice.

The peripheral neutrophilia seen in both sync and MCD mice following N.b. infection is in keeping with the peripheral blood response observed in wild type mice (see chapter 3).

The increase in the total number of neutrophils in the peripheral blood of both sync and MCD mice was reflected in the changes in the percentage of neutrophils in the peripheral blood following N.b. infection. Naïve syngeneic controls has a significantly higher percentage of neutrophils in their peripheral blood than naïve MCD mice, 9.78% and 5.43% respectively ($p<0.001$). However, this difference disappeared following N.b. infection with no significant differences being observed in the percentage of neutrophils in the peripheral blood of sync or MCD mice on day 3, 8 or 14 pi.

Nevertheless, the increase in the total number of neutrophils in the peripheral blood of both sync and MCD mice was reflected in a significant increase in the percentage of neutrophils in both sync and MCD mice. Specifically the percentage of neutrophils in sync mice rose from 9.78% in naïve sync mice to 14.29% on day 3 pi ($p<0.001$), 22.34 on day 8 pi ($p<0.001$) before falling back to naïve levels by day 14 pi (9.71%)(Figure 9-3).

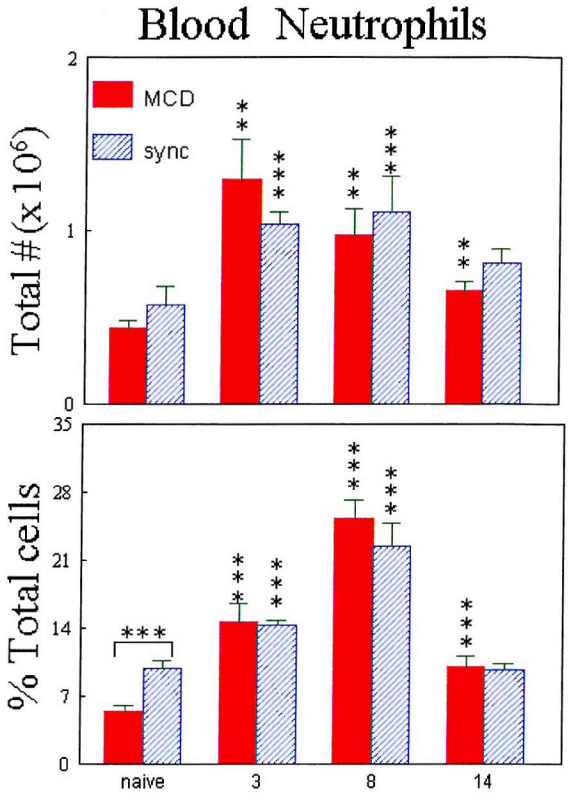

Figure 9-3 The total number and percentage of neutrophils in the blood of mast cell deficient (MCD) and sync-type control mice following N.b. infection.

Similarly the percentage of neutrophils in MCD mice rose form 5.43% in naïve sync mice to 14.66% on day 3 pi ($p<0.001$), 25.38% on day 8 pi ($p<0.001$) before declining although not quite to naïve levels by day 14 pi (10.05% versus 5.43%) ($p<0.001$)(Figure 9-3).

IV. Lymphocytes

In addition to the changes in eosinophil and neutrophil populations N.b. infection caused a significant shift in the number and percentages of circulating lymphocytes. However, the changes seen in MCD and sync mice differed from the pattern seen in the peripheral blood of wild type mice (Chapter 3).

Specifically there was a significant reduction in the number of lymphocytes in the peripheral blood of MCD mice by day 8 and day 14 pi compared to naïve MCD mice. The total number of lymphocytes in the peripheral blood of naïve MCD mice (7.42 $x10^6$) reduced slightly by day 3 pi (7.08 $x10^6$), but then dropped significantly to 2.41 $x10^6$ on day 8 pi ($p<0.001$) and 5.54 $x10^6$ on day 14 pi ($p<0.04$)(Figure 9-4).

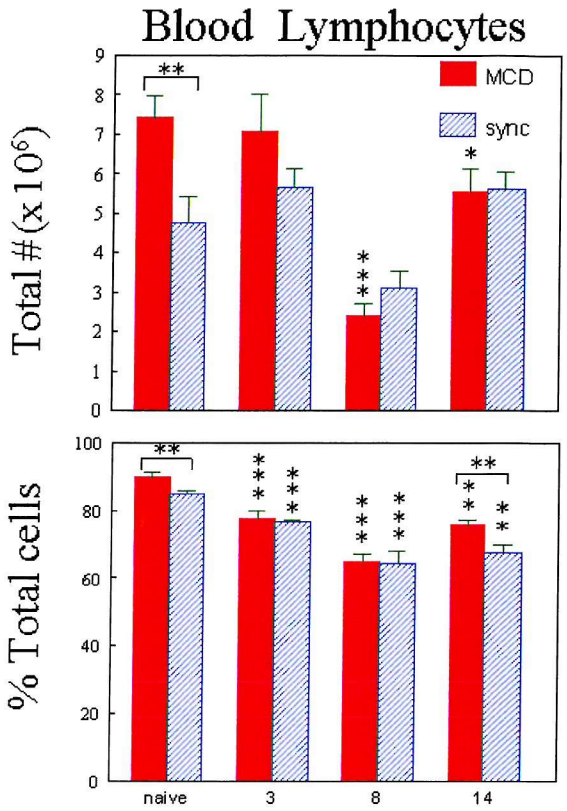

Figure 9-4. The total number and percentage of lymphocytes in the blood of mast cell deficient (MCD) and sync-type control mice following N.b. infection.

Whether the increased eosinophil and neutrophil production in the marrow inhibited a lymphocytosis is unclear.

In contrast, the number of lymphocytes in the peripheral blood of sync mice remained unchanged following N.b. infection. There was a small drop in numbers on day 8 pi but this was not statistically significant and probably an anomalous result ($p<0.06$). In fact lymphocyte numbers increased following N.b. infection from 4.76 $x10^6$ in naïve sync mice to 5.67 $x10^6$ on day 3 pi ($p<0.29$) and 5.63 $x10^6$ on day 14 pi ($p<0.3$). However, this increase was not statistically significant unlike the increase following N.b. infection in WT mice (Chapter 3).

The different response seen in the lymphocyte subset in MCD and sync probably reflected the greater number of lymphocytes in the peripheral blood of naïve MCD mice compared to sync mice, 7.42 $x10^6$ and 4.76 $x10^6$ respectively ($p<0.01$).

The reduction in the total number of lymphocytes in the peripheral blood of MCD mice was reflected in the reduced percentage of lymphocytes in the peripheral blood. Specifically the percentage of lymphocytes in the blood of MCD mice dropped from 89.87% in naïve mice to 77.74% on day 3 pi ($p<0.001$) falling further to 64.93% on day 8 pi ($p<0.001$) before recovering slightly to 75.53 by day 14 pi ($p<0.01$). The changes in the percentage of lymphocytes in the peripheral blood of sync mice followed a very similar pattern. Lymphocyte percentages dropped from 84.66% in naïve mice to 76.46% on day 3 pi ($p<0.001$) falling further to 64.47% on day 8 pi ($p<0.001$) before recovering to 67.74% ($p<0.01$). This reduction in lymphocyte percentages was in keeping with the data seen in WT mice following N.b. infection (Chapter 3).

V. Monocytes

In contrast to the changes in eosinophil, neutrophil and lymphocyte populations, there was very little change in either the number or percentages of circulating monocytes following N.b. infection in MCD or sync mice (Figure 9-5).

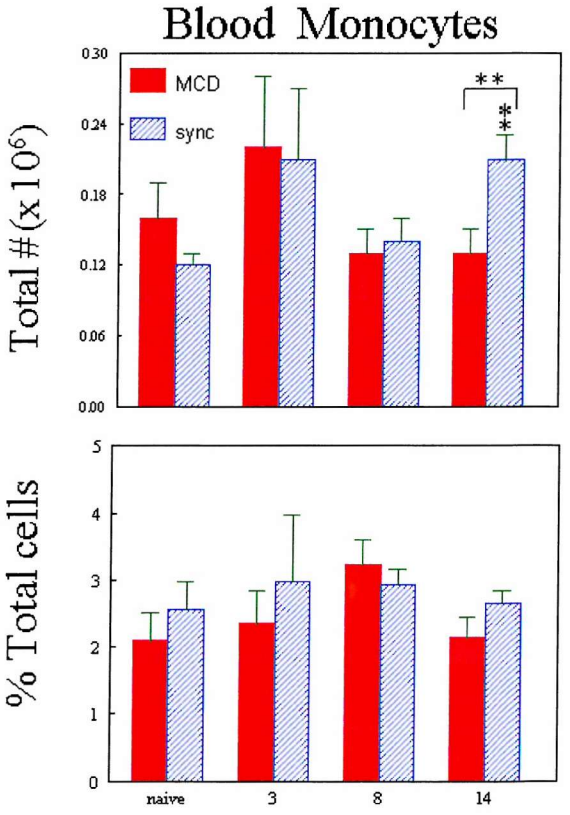

Figure 9-5. The total number and percentage of monocytes in the blood of mast cell deficient (MCD) and sync-type control mice following N.b. infection.

Specifically the very low percentage of monocytes in the peripheral blood of both MCD and sync mice changed very little over time (Figure 9-5). Similarly the total number of monocytes in the peripheral blood of MCD remained unchanged following N.b. infection. There was, however, a significant increase in the number of monocytes in the peripheral blood of sync mice on day 14 pi compared to naïve mice, 0.21 $x10^6$ and 0.12 $x10^6$ respectively ($p<0.001$). This was reflected in a significant difference between the number of monocytes in the peripheral blood of MCD and sync mice on day 14 pi. Specifically there were 0.13 $x10^6$ monocytes in peripheral blood of MCD mice compared to 0.21 $x10^6$ in sync mice ($p<0.01$) (Figure 9-5).

9.3.2 Time course of leukocyte infiltration into the lung following N.b. infection

I. Total Leukocyte Count

In WT mice there was a biphasic cellular response in the BAL fluid following N.b. infection (chapter 3). This consisted of an early cellular influx into the airways during larval migration through the lungs, primarily on day 3 pi, followed by a late phase response peaking on day 14 pi. This was most clearly seen when examining the cellular subsets that migrated into the airways. In contrast to the peripheral blood response, where there was a significant increase in cellularity 14 days pi in sync but not MCD mice, in the lungs there was a significant cellular influx in both MCD and sync mice starting on day 3 pi and peaking on day 14 pi.

Specifically the total number of leukocytes in the airways of MCD mice rose from 1.05 x 10^3 in naïve MCD mice to 2.6 x 10^3 on day 3 pi ($p<0.01$). Leukocyte numbers rose still further by day 8 pi to 4.1 x 10^3 which was significantly greater than the number of leukocytes in the airways of naïve mice ($p<0.005$) but not significantly different from the numbers of leukocytes seen in the airways 3 days after N.b. infection ($p<0.13$).

By day 14 the number of leukocytes recruited to the BALF of MCD mice had risen to 12.5 x 10^3, which was significantly greater than the number of leukocytes in the airways of naïve ($p<0.001$), day 3 ($p<0.001$) or 8 days ($p<0.001$) MCD mice following N.b. infection (Figure 9-6).

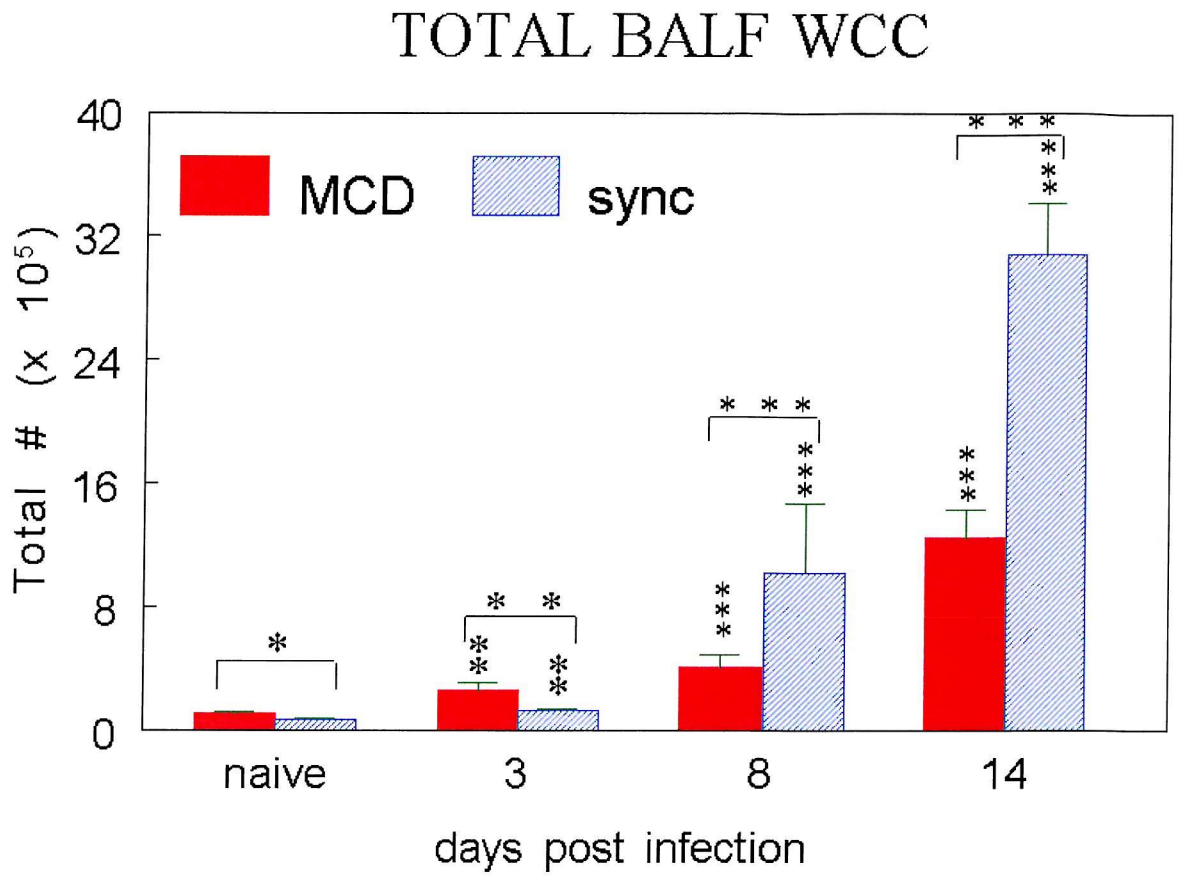

Figure 9-6. The leukocyte recruitment to the BAL fluid in mast cell deficient (MCD) and sync-type control mice following N.b. infection.

II. Eosinophils

The late phase pulmonary response was dominated by a significant increase in the number and percentage of eosinophils in the BAL fluid in sync and MCD mice. In MCD mice the number of eosinophils in the airways increased slightly from 3.8 x 10^3 in naïve mice to 5.5 x10^3 on day 3 pi ($p<0.5$). However, by day 8 pi the influx of eosinophils had increased substantially with numbers rising to 80.1 x 10^3, significantly greater than in naïve ($p<0.001$) or Day 3 pi MCD mice ($p<0.002$). This eosinophil influx peaked at day 14 with over 653 x 10^3 eosinophils in the airway lumen. This represented a 170-fold increase compared to naïve mice ($p<0.001$). The number of eosinophils recovered from the BALF on day 14 pi was also significantly greater than the numbers of eosinophils the BALF on day 3 ($p<0.001$) or day 8 pi ($p<0.001$) (Figure 9-7).

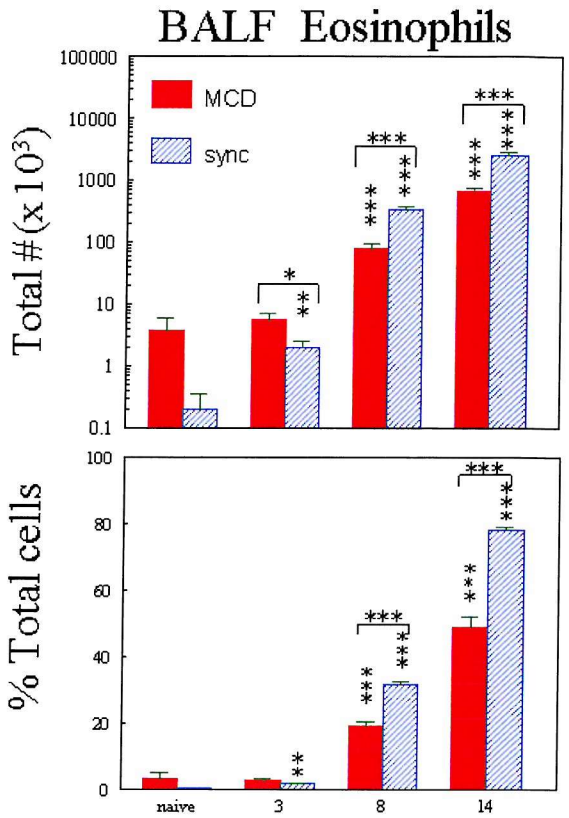

Figure 9-7. The total number and percentage of eosinophils in the BAL fluid of mast cell deficient (MCD) and sync-type control mice following N.b. infection.

In contrast, the late phase eosinophil influx into the BALF on sync mice was considerably greater than in MCD mice. Eosinophil number increased significantly from 0.2 x 10^3 in naïve mice to 2.0 x 10^3 on day 3 pi ($p<0.01$). By day 8 pi the influx of eosinophils had increased still further with numbers rising to 329 x 10^3, significantly greater than in naïve ($p<0.001$) or day 3 pi MCD mice ($p<0.001$). This eosinophil influx peaked at day 14 with over 2.5 x 10^6 eosinophils in the airway lumen. This represented a 12,500-fold increase compared to naïve mice ($p<0.001$). The number of eosinophils recovered from the BALF in sync mice on day 14 pi was also significantly greater than the numbers of eosinophils the BALF on day 3 ($p<0.001$) or day 8 pi ($p<0.001$) (Figure 9-7).

There were substantially more eosinophils in the BALF of naïve MCD mice (3.8 x 10^3) compared to sync mice (0.2 x 10^3) but this did not reach statistical significance ($p<0.12$). By day 3 pi the number of eosinophils in the BALF of sync mice (2.0 x10^3) had risen but it was still statistically less than the number seen in MCD mice (2.02 x10^3 compared to 5.5 x10^3)($p<0.04$). In contrast the number of eosinophils in the airway lumen in sync mice by day

8 pi (329 x 10^3) was significantly greater than in MCD mice on day 8 pi (80.1 x 10^3)($p<0.001$). This much greater late phase response of sync mice compared to MCD mice became even more apparent by day 14 pi with 2.5 x 10^6 eosinophils in the BALF of sync mice compared to 0.7 x 10^6 in MCD mice ($p<0.001$)(Figure 9-7).

The late-phase changes in airway eosinophil numbers following N.b. infection were reflected in the percentages of eosinophils recovered from the BALF. The percentage of eosinophils in the airways of naïve MCD mice and MCD mice 3 day pi were not significantly different, 2.95% and 2.4% respectively. However, by day 8 pi the percentage of eosinophils in the airway of MCD mice had risen significantly to 18.85% compared to naïve and 3 days pi ($p<0.001$). This influx peaked at day 14 with eosinophils making up 49.85% of all the cells recovered from the BALF. This level of airway eosinophilia was significantly greater than that seen in naïve MCD mice ($p<0.001$), or infected MCD mice 3 days or 8 days pi ($p<0.01$).

Similarly the percentage of eosinophils recovered from the airways of sync mice rose significantly from 0.25% in naïve mice to 1.7% on day 3 pi ($p<0.01$). However, by day 8 pi the percentage of eosinophils in the airways had risen to 31.6%, which was a significantly greater percentage when compared to naïve ($p<0.001$), or sync mice 3 days after N.b. infection ($p<0.001$). This significant increase in the percentage of eosinophils in sync mice was reflected in a significant difference between the percentage of eosinophils in the airways of sync and MCD mice on day 8 pi, 18.85% and 31.6% respectively ($p<0.001$)(Figure 9-7).

At the peak of the late phase response, on day 14 pi, the percentage of eosinophils in sync mice airways had increased significantly, to 78.3%, when compared to naïve ($p<0.001$), or sync mice 3 days ($p<0.001$) or 8 days after N.b. infection ($p<0.001$). This was a significantly greater eosinophil influx when compared to MCD mice on day 14 pi, 48.85% and 78.3% respectively ($p<0.001$)(Figure 9-7).

III. Neutrophils

In WT mice a biphasic cellular recruitment to the airways was seen (chapter 3). This was characterised by a significant influx of neutrophils to the airways during the "early phase" response on day 3 pi during larval migration through the lungs. This early phase response was also seen in MCD and sync mice. Specifically the number of neutrophils in the airways of

MCD mice increased significantly from 1.29 x 10^3 in naïve MCD mice to 37.97 x 10^3 three days pi ($p<0.005$). Neutrophil numbers then dropped back during the late phase response to 14.68 x 10^3 ($p<0.02$) on day 8 pi and 13.1 x 10^3 ($p<0.002$) on day 14 pi. These figures were still significantly greater than in naïve MCD mice but the peak neutrophil response occurred on day 3 pi (Figure 9-8).

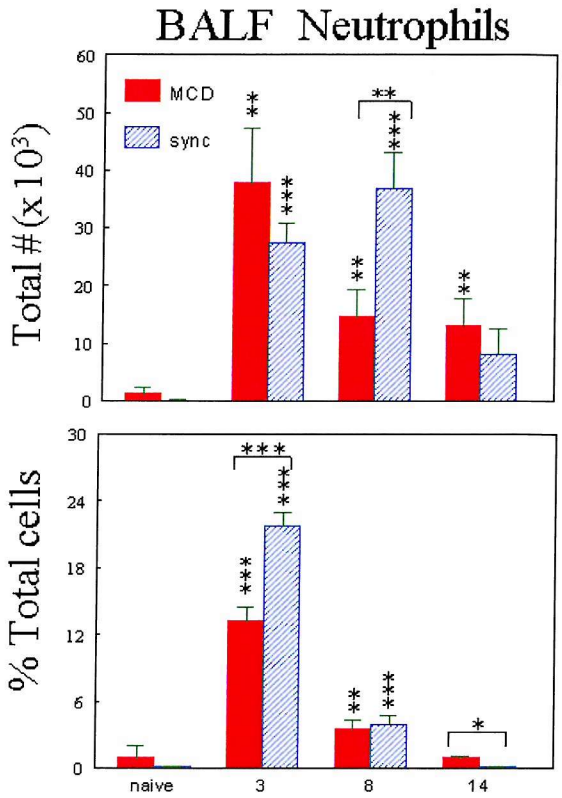

Figure 9-8. The total number and percentage of neutrophils in the BAL fluid of mast cell deficient (MCD) and sync-type control mice following N.b. infection.

In contrast, the number of neutrophils in the airways of sync mice seemed to peak a little later. Neutrophil numbers increased significantly from 0.06 x 10^3 in naïve MCD mice to 27.35 x 10^3 three days pi ($p<0.001$) and remained high, at 36.72 x 10^3, on day 8 pi ($p<0.001$). However, there was no statistical difference seen between the neutrophil numbers in sync mice on day 3 pi and day 8 pi ($p<0.22$). By day 14 pi neutrophil numbers had dropped substantially to 8.16 x 10^3 in sync mice, which was not significantly different from naïve levels ($p<0.08$)(Figure 9-8).

The changes in airway neutrophil numbers during the early phase response were reflected in changes in the percentages of neutrophils recovered from the BALF of MCD. Specifically there was a significant increase in the percentage of neutrophils in the BALF during the early phase response. In naïve MCD mice neutrophils accounted for 3.07% of the cells recovered from the BALF compared to 13.3% on day 3 pi ($p<0.001$) and 3.45% on day 8 pi ($p<0.02$).

By day 14 pi the percentage of neutrophils in the airways of MCD mice had dropped to 0.92%, which was not statistically different from the level seen in naïve MCD mice ($p<0.72$). The percentage of neutrophils in the airways of sync mice by day 14 pi had also dropped to 0.3%, which was not statistically different from the level seen in naïve sync mice ($p<0.23$).

The early phase neutrophil response of sync mice was greater than that seen in MCD mice ($p<0.001$) when comparing the percentage of neutrophils recruited, although the actual number of neutrophils recruited did not differ significantly.

IV. Lymphocytes

During the late phase, but not the early phase response there was a significant increase in lymphocyte numbers in MCD mice. Specifically the number of lymphocytes rose slightly, but not significantly during the early phase response, from 16.06 x 10^3 in naïve mice to 23.61 x 10^3 on day 3 pi ($p<0.32$). However, by day 8 pi lymphocyte numbers had increased significantly in MCD mice to 115 x 10^3 compared to naïve MCD mice ($p<0.001$) and mice 3 days pi ($p<0.001$). Lymphocyte numbers peaked on day 14 pi at 285 x 10^3 compared to naïve MCD mice ($p<0.001$) and mice 3 days ($p<0.001$) and 8 days pi ($p<0.004$)(Figure 9-9).

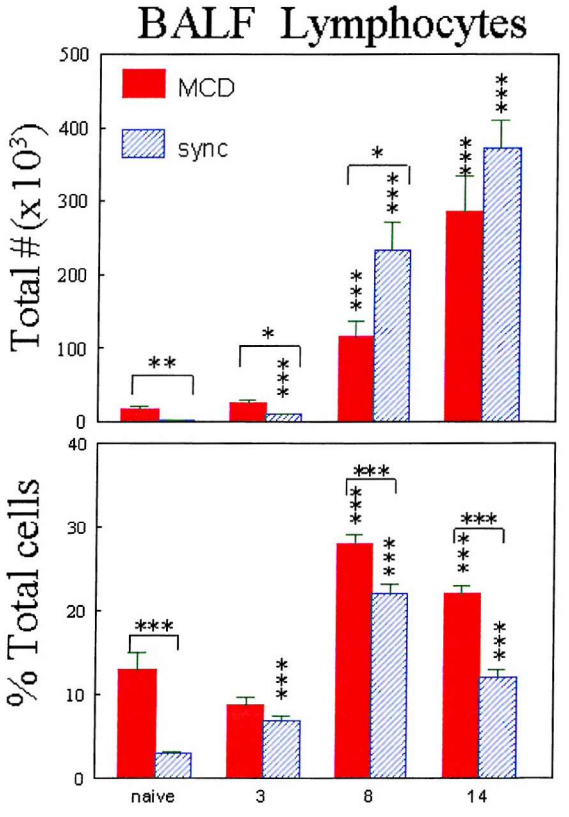

Figure 9-9. The total number and percentage of lymphocytes in the BAL fluid of mast cell deficient (MCD) and sync-type control mice following N.b. infection.

Similarly there was a significant increase in lymphocyte numbers in sync mice following N.b. infection. Lymphocyte numbers increased significantly from 1.9 x 10^3 in naïve mice to 9.08 x 10^3 on day 3 pi ($p<0.001$). There was a much more substantial and significant increase in lymphocyte numbers in sync mice during the late phase response with numbers rising to 232 x 10^3 by day 8 pi compared to naïve mice ($p<0.001$), and day 3 pi ($p<0.001$). By day 14 pi lymphocyte numbers peaked at 372 x 10^3, which was significantly greater than naïve mice ($p<0.001$), day 3 pi ($p<0.001$) or day 8 pi ($p<0.02$)(Figure 9-9).

Comparing lymphocyte numbers in MCD and sync mice there was a greater number of lymphocytes in the airways of naïve MCD mice than sync control mice ($p<0.01$). This difference was still apparent during the early phase response ($p<0.04$), but by the late phase response the lymphocyte recruitment to the airways of sync mice was significantly greater than that seen in MCD mice ($p<0.02$). This difference was still apparent on day 14 pi although actual numbers were not statistically different ($p<0.17$)(Figure 9-9).

The increase in the total number of lymphocytes, in the airways during the late phase response in MCD and sync mice was reflected in changes in the percentages of lymphocytes recovered from the BALF. In naïve MCD mice lymphocytes made up 12.95% of the cells recovered from the BALF. In the early phase response this dropped slightly to 8.8% ($p<0.14$) but during the late phase response the percentage of lymphocytes increased significantly to 27.9% on day 8 pi ($p<0.001$) before reducing slightly on day 14 pi to 22.13% ($p<0.001$). The percentage of lymphocytes in the BALF on day 8 pi was significantly greater than the lymphocyte percentage on day 14 pi ($p<0.03$).

An almost identical pattern was seen in sync mice. There was a significantly lower percentage of lymphocytes in naïve sync mice compared to MCD mice, 2.6% and 12.95% respectively ($p<0.02$). However by day 3 pi this had more than doubled to 6.9% ($p<0.001$). In keeping with the lymphocyte response in MCD mice there was a significant rise in the percentage of lymphocytes recovered from the BALF in sync mice during the late phase response. Specifically the percentage of lymphocytes increased to 22.0% on day 8 pi ($p<0.001$) before falling back on day 14 to 12.4 % ($p<0.001$). Again the percentage of lymphocytes in the BALF on day 8 pi was significantly greater than the lymphocyte percentage on day 14 pi ($p<0.001$). The significantly greater percentage of lymphocytes in naïve MCD mice was also seen during the late phase response with there being a significantly greater percentage of

lymphocytes in MCD BALF on day 8 pi ($p<0.001$) and day 14 pi ($p<0.001$)(Figure 9-9). This may have reflected the significantly smaller airway eosinophilia seen in MCD mice during the late response.

V. Monocytes

Following N.b. infection there was a significant increase in monocyte numbers in MCD mice. Specifically the number of monocytes increased during the early phase response, from 85.9 x 10^3 in naïve mice to 198.3 x 10^3 on day 3 pi ($p<0.01$). In addition, by day 8 pi MCD monocyte numbers had increased still further to 208 x 10^3 which was significantly greater than naïve levels ($p<0.002$), but not significantly greater than day 3 pi ($p<0.85$). Monocyte numbers peaked on day 14 pi at 278 x 10^3, significantly greater levels when compared to naïve MCD mice ($p<0.001$) and mice 3 days ($p<0.04$) but not compared to day 8 pi ($p<0.08$)(Figure 9-10).

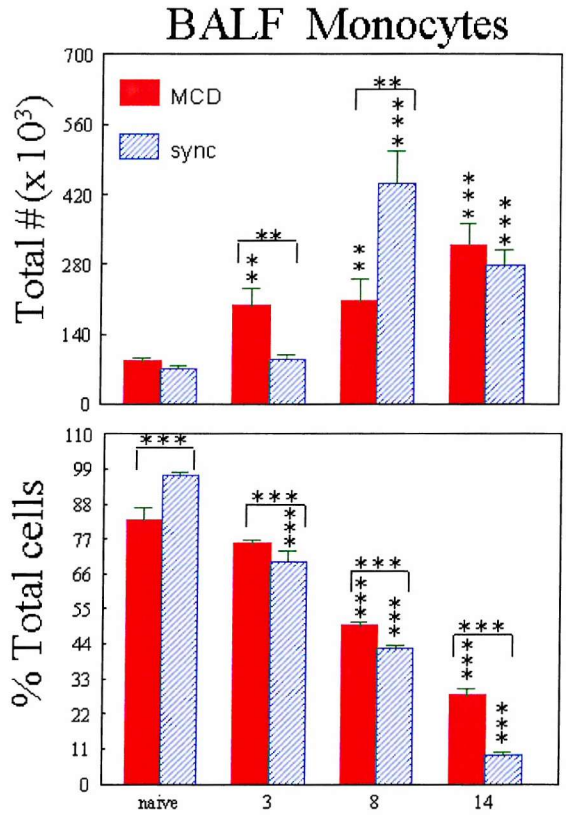

Figure 9-10. The total number and percentage of monocytes in the BAL fluid of mast cell deficient (MCD) and sync-type control mice following N.b. infection.

In contrast monocyte numbers only increased during the late phase response in sync mice. Thus monocyte numbers increased only slightly from 72.9 x 10^3 in naïve mice to 89.2 x 10^3 on day 3 pi ($p<0.19$), but by day 8 pi there was a significant increase to 443 x 10^3. This was significantly greater than the numbers of monocytes seen in either naïve mice ($p<0.001$) or

sync mice 3 days after N.b. infection ($p<0.001$). By day 14 pi monocyte numbers had subsided somewhat to 279 x 10^3, which was still significantly greater than naïve mice ($p<0.001$), and day 3 pi mice ($p<0.001$) but significantly less than the numbers seen on day 8 pi ($p<0.04$)(Figure 9-10).

Comparing monocyte numbers in MCD and sync mice, and in view of the early monocyte response in MCD mice, there were significantly more monocytes in MCD airways 3 days pi compared to sync mice ($p<0.01$). However, by day 8 pi this difference had reversed with significantly greater number of monocytes found in the airways of sync mice compared to MCD mice ($p<0.01$). By day 14 this difference had disappeared ($p<0.47$)(Figure 9-10).

In view of the substantial recruitment of eosinophils and lymphocytes to the airways of MCD mice the percentage of monocytes recovered from the BALF dropped significantly following N.b. infection. Specifically, monocytes constituted 83% of all cells in naïve MCD mice but by day 3 pi this had dropped to 75.5% ($p<0.07$). By day 8 pi the proportion of monocytes in the BALF was significantly reduced to 49.8% ($p<0.001$) and this level dropped still further by day 14 pi to 28% ($p<0.001$). The same pattern was seen in sync mice. In naïve sync mice monocytes made up 97% of all BALF cells but by day 3 pi this had dropped significantly to 69.6% ($p<0.001$). Day 8 pi saw another significant reduction to 42.5% ($p<0.001$) and by day 14 the percentage of monocytes recovered from the BALF of sync mice had fallen to just 9% ($p<0.001$)(Figure 9-10).

When comparing the reduction in the proportion of monocytes recovered from the BALF of MCD and sync mice a clear difference emerged. First of all there was a significantly greater percentage of monocytes in the BALF of naïve sync mice compared to MCD mice ($p<0.007$). Following N.b. infection, and in view of the much greater airway eosinophilia and lymphocytosis seen in sync mice, the proportion of monocytes recovered from the BALF of sync mice at each time point was substantially less than recovered from MCD mice. Specifically on day 3 pi 69.6% compared to 75.5% ($p<0.001$), on day 8 pi 42.5% compared to 49.8% ($p<0.001$) and on day 14 pi 9% compared to 28% in MCD mice ($p<0.001$).

9.3.3 Splenocyte proliferation

I. Splenocyte response to N.b.-specific antigen

Spleen cells were stimulated with a variety of specific and non-specific mitogens. Ranges of specific and non-specific antigen doses were used since the optimum conditions for splenocyte proliferation in response to antigen were unknown. The response of splenocytes to N.b. specific antigen during the early and late phase response was compared in MCD and sync mice (Figure 9-11). This showed that during the early phase response sync splenocytes responded more vigorously to 20mcg/well of N.b. antigen than MCD splenocytes ($p<0.03$). This greater responsivity of sync splenocytes was not seen at 10mcg/well ($p<0.1$), 5mcg/well ($p<0.8$) or 2.5 mcg/well ($p<0.6$).

During the late phase response there was no significant difference between the response of MCD and sync splenocytes at 20 mcg/well ($p<0.4$), 10mcg/well ($p<0.2$), 5mcg/well ($p<0.6$) or 2.5 mcg/well ($p<0.16$).

In addition, there appeared to be a greater splenocyte response to specific N.b. antigen during the late phase in both MCD and sync mice. This difference reached statistical significance for MCD mice at 10 mcg/well ($p<0.04$) and 2.5 mcg/well ($p<0.04$), but not at 20 mcg/well ($p<0.22$) or 5 mcg/well ($p<0.22$). However, the group difference between day 3 pi and day 14 pi remained significant ($p<0.03$). In contrast, although sync splenocyte appeared to proliferate more strongly during the late phase response this did not reach statistical significance at 20 mcg/well ($p<0.16$), 10mcg/well ($p<0.7$), 5mcg/well ($p<0.6$) or 2.5 mcg/well ($p<0.16$) (Figure 9-11).

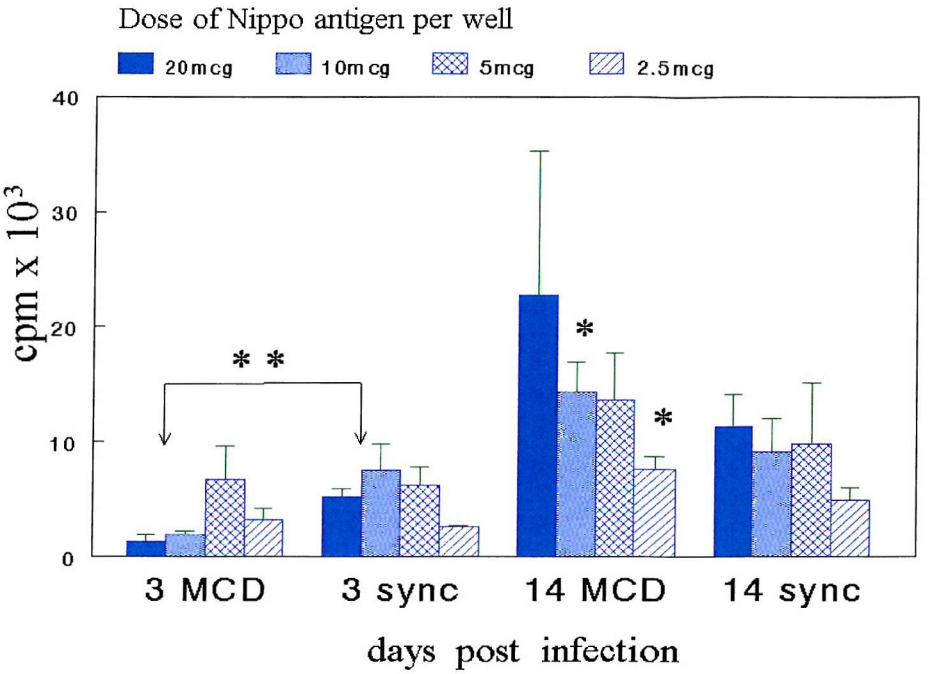

Figure 9-11. Splenocyte proliferative response to stimulation, with N.b. antigen, in mast cell deficient (MCD) and sync-type control mice (WT).

II. Splenocyte response to non-specific antigen.

Spleen cells were stimulated with two different non-specific mitogens, ConA and anti-CD3. Different doses of ConA, but not anti-CD3, were used since the optimum conditions for splenocyte proliferation in response to this mitogen were unknown. The response of splenocytes to ConA during the early and late phase response was compared in MCD and sync mice (Figure 9-12).

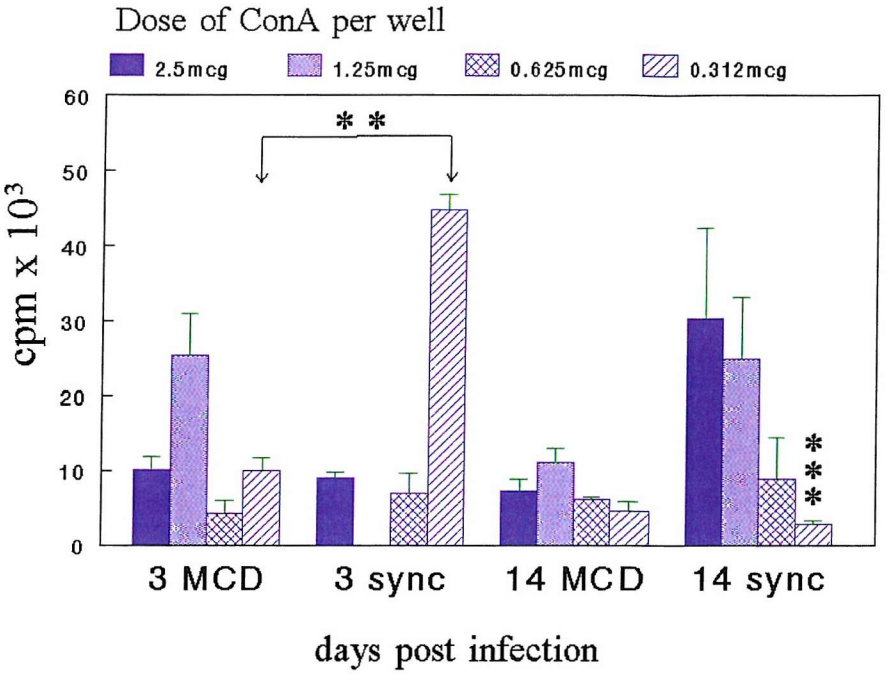

Figure 9-12. Splenocyte proliferative response to stimulation with ConA in mast cell deficient (MCD) and sync-type control mice.

This showed that during the early phase response sync splenocytes responded more vigorously to 0.312mcg/well of Con A than MCD splenocytes ($p<0.0002$). This greater responsivity of sync splenocytes was not seen at 2.5mcg/well ($p<0.6$) or 0.625 mcg/well ($p<0.4$).

During the late phase response there was no significant difference between the response of MCD and sync splenocytes at 2.5mcg/well ($p<0.2$), 1.25mcg/well ($p<0.2$), 0.625mcg/well ($p<0.67$) or 0.312mcg/well ($p<0.34$).

The response of MCD splenocytes to ConA in the early and late phase response did not differ. This was true at 2.5mcg/well ($p<0.2$), 1.25mcg/well ($p<0.13$), and 0.625mcg/well ($p<0.41$). However, there was a greater proliferative response during the early response to 0.312mcg/well of ConA but this did not quite reach statistical significance ($p<0.06$). Similarly at this dose of ConA, 0.312mcg/well, splenocytes from sync mice proliferated more vigorously during the early phase response than the late phase response ($p<0.002$). In contrast there was no difference in proliferative response of sync splenocytes to 2.5mcg/well ($p<0.22$) or 0.625mcg/well ($p<0.77$) of ConA between the early and late response.

The splenocyte proliferative response to α-CD3 in MCD and sync mice was compared during the early and late phase response. While there appeared to be a greater proliferative response in MCD splenocytes harvested on day 14 pi compared to day 3 pi this did not quite reach statistical significance ($p<0.057$). In addition there was no difference in the proliferative response of splenocytes from MCD or sync mice at either time point ($p<0.28$ and $p<0.55$ respectively)(Figure 9-13).

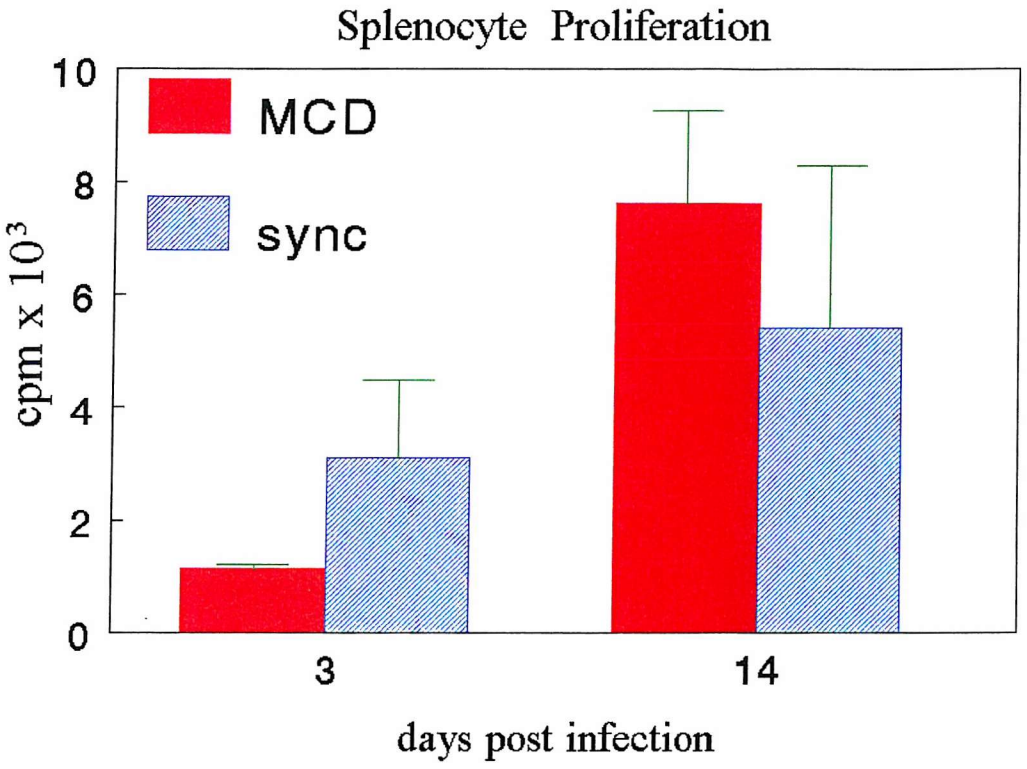

Figure 9-13. Splenocyte proliferative response to stimulation with a-CD3 in mast cell deficient (MCD) and sync-type control mice.

9.3.4 Splenocyte cytokine production in response to N.b.-specific antigen

During the late phase response there was a significant eosinophilia and lymphocytosis in the airways and blood of both MCD and sync mice. During the peak of the late phase response, on day 14, cultured splenocytes were harvested from both strains of mice. These splenocytes were stimulated with varying dose of heterogenous N.b. adult antigens *in vitro* and the production of the Th-2 cytokines, IL-4, IL-5 and IL-10, in addition to the Th-1 cytokines IL-2 and IFN-g were compared in MCD and sync mice.

Syngeneic control mice produced significantly more IL-2 ($p<0.04$), but significantly less IFN-g on day 14 pi compared to MCD mice ($p<0.01$). Interestingly, MCD mice produced significantly more Th-2 cytokines, IL-4, IL-5 and IL-10 compared to sync mice (Table 1) (Figures 9-14 - 9-18).

Table 1.

Th-1 and Th-2 production by MCD and sync mice in response to N.b. specific antigen

Splenocyte population	Th-1 cytokines		Th-2 cytokines		
	IL-2	**IFN-g**	**IL-4**	**IL-5**	**IL-10**
D14 MCD mice (pg./ml)	200	4,122	13.6	1,110	4,103
D14 sync mice (pg./ml)	411	1,332	8.7	404	1,286
p=	**0.04**	**0.01**	**0.03**	**0.01**	**0.01**

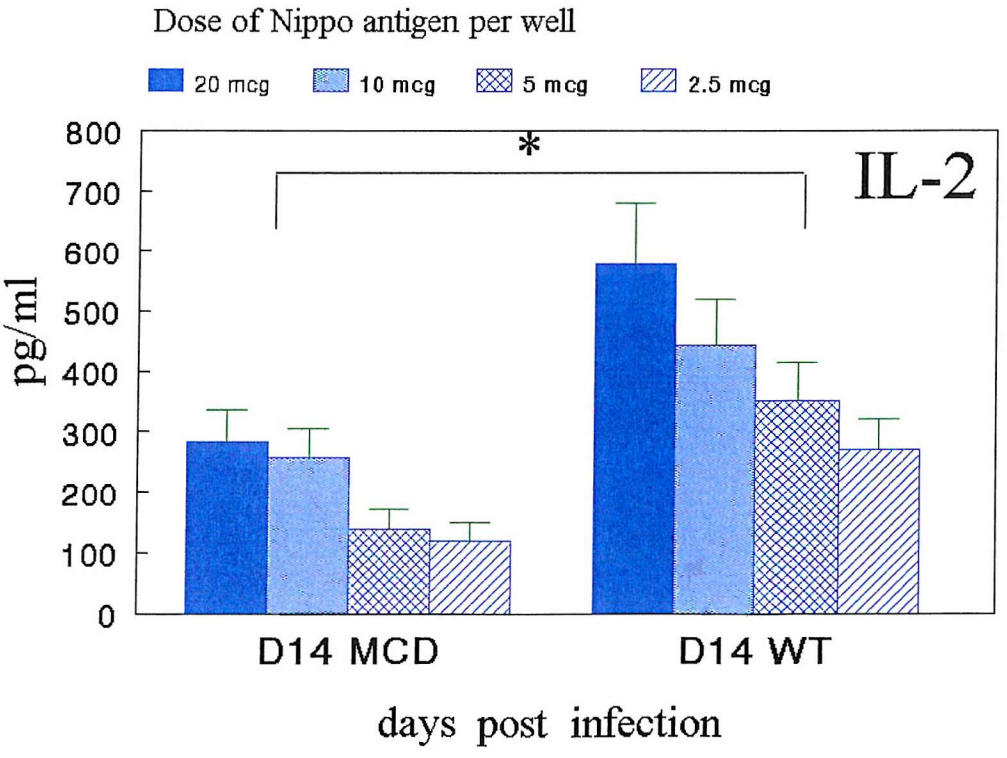

Figure 9-14. Splenocyte IL-2 production in response to stimulation with N.b. specific antigen in mast cell deficient (MCD) and sync-type control mice.

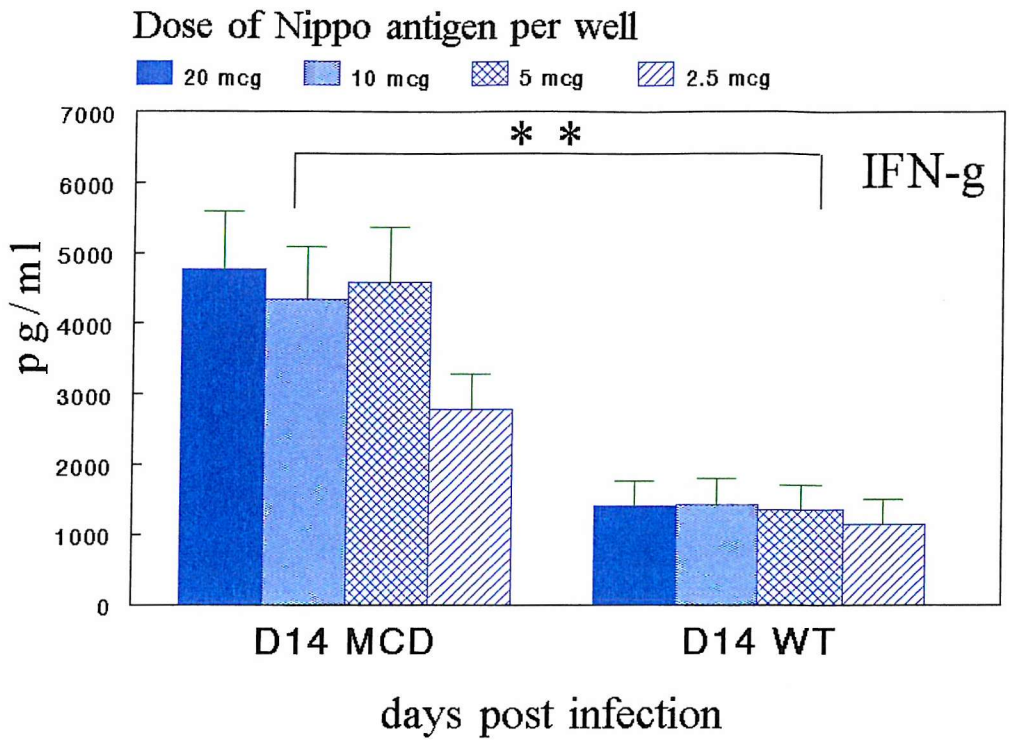

Figure 9-15. Splenocyte IFN-γ production in response to stimulation with N.b. specific antigen in mast cell deficient (MCD) and sync-type control mice.

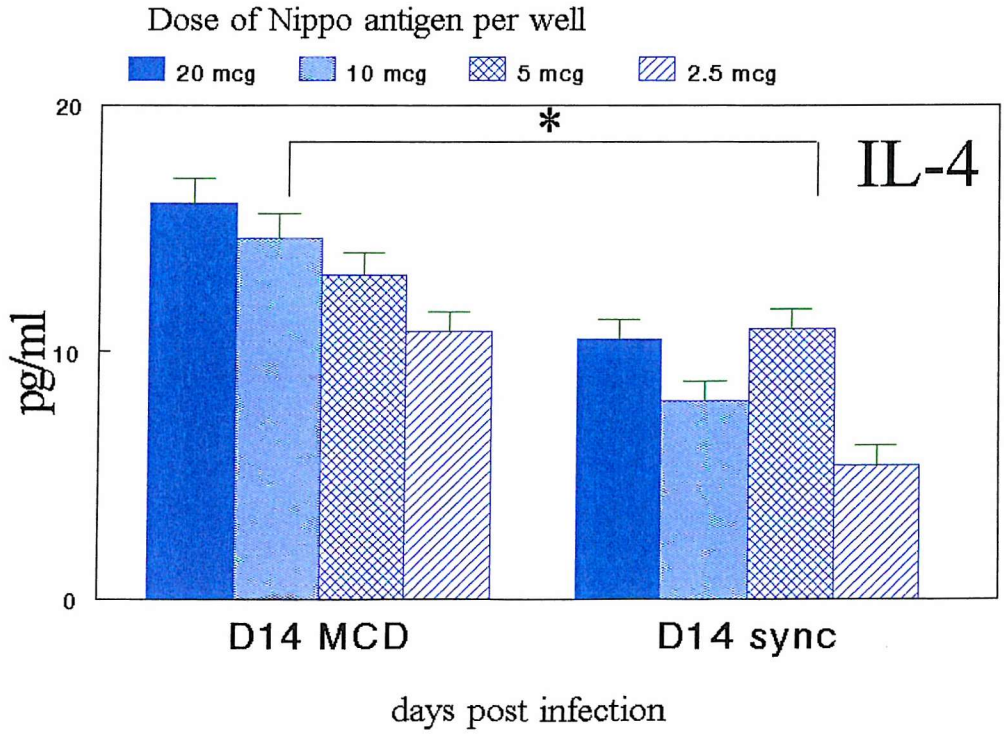

Figure 9-16. Splenocyte IL-4 production in response to stimulation with N.b. specific antigen in mast cell deficient (MCD) and sync-type control mice (WT).

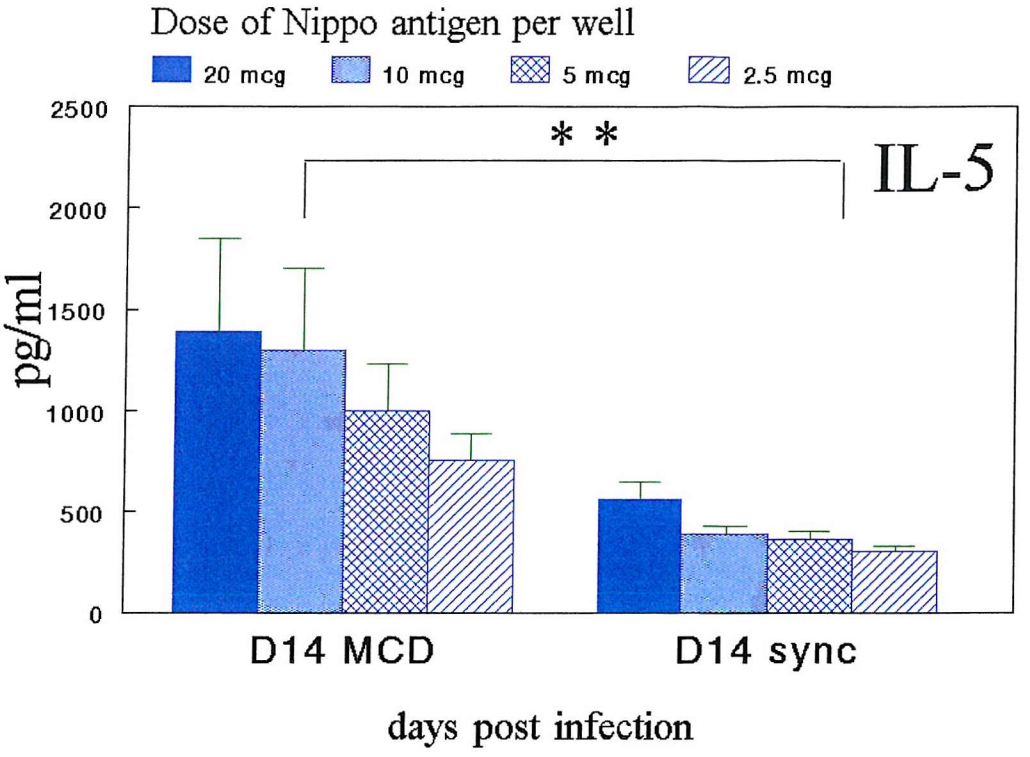

Figure 9-17. Splenocyte IL-5 production in response to stimulation with N.b. specific antigen in mast cell deficient (MCD) and sync-type control mice (WT).

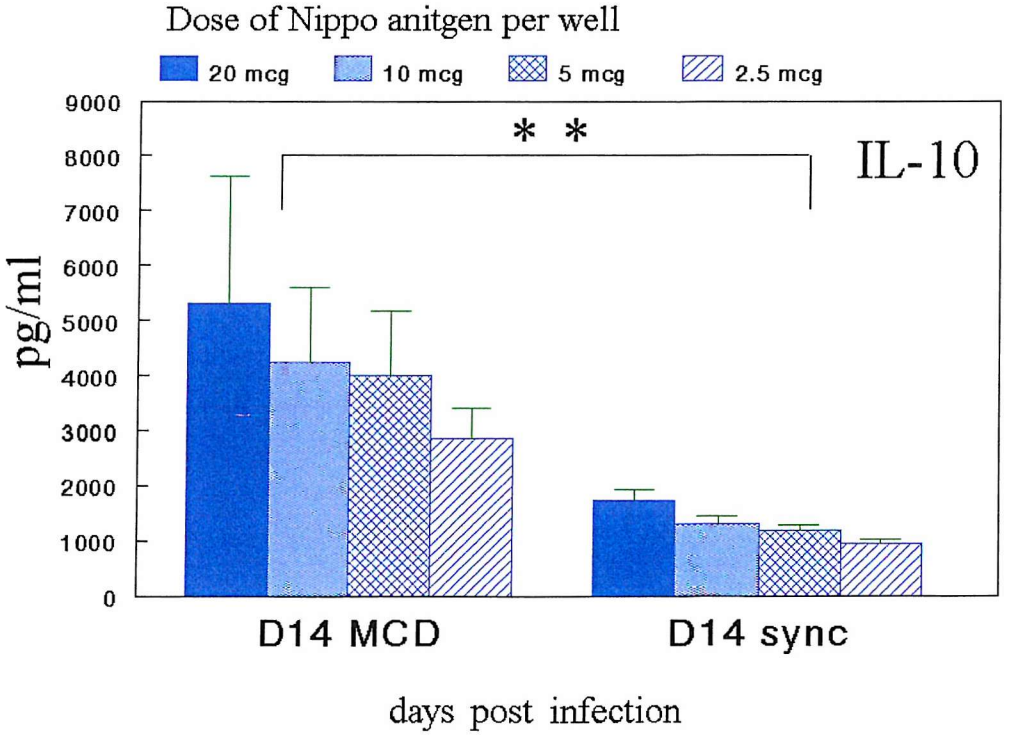

Figure 9-18. Splenocyte IL-10 production in response to stimulation with N.b. specific antigen in mast cell deficient (MCD) and sync-type control mice (WT).

9.4 DISCUSSION

We investigated the role of mast cells on the pulmonary and systemic response in a Th-2-driven model of allergic inflammation. We induced the Th-2 response using primary infection with Nippostrongylus brasiliensis stage 3 larvae. We then explored the kinetics of the cellular response in the blood and airways in addition to the splenic cytokine response to N.b. infection. We discovered that naïve mast cell deficient (MCD) mice had a higher resting white cell count (WCC) in the peripheral blood than their syngeneic controls. However, following N.b. infection the immune response, as reflected in the peripheral leukocytosis, was greater in control mice. This effect was also seen in the lungs with significantly more cells recovered from the airways of naïve MCD mice than controls but a significantly greater cellular recruitment to the airways of control mice during the late phase pulmonary response. Interestingly, this trend was reversed during the early phase response suggesting that MCD mice have a different kinetic response than control mice to the infective challenge. This might suggest that mast cells might be more important in the late phase response rather than the early phase response. The early phase response was well maintained in MCD mice.

We also explored the cellular response in the blood and airways of MCD and control mice. Specifically, we examined the monocyte population in the blood and airways. Monocytes/macrophages make up the largest population of cells in the airways not only of the murine model developed in this series of experiments but also in the airways of allergic asthma patients. The importance of this cellular population in the inflammatory process is still under debate. However, it has been shown recently that mast cell play a central role in monocyte recruitment to the airways by promoting the release of monocyte chemokine chemoattractants such as mast cell protease (MCP)-1 and RANTES (Gordon JR et al 2000, Conti P et al 1999).

Mast cells are also known to induce fibroblasts to express type alpha1 (I)-collagen and they have been implicated in fibroblast-induced monocyte recruitment. Specifically, dermal embryonic fibroblasts have been shown to secrete monocyte chemoattractants following stimulation by mast cell protease–1 (MCP-1) in culture. Furthermore, anti-MCP-1 antibodies neutralised approximately 80% of this fibroblast-induced monocyte chemoattractant activity (Gordon JR et al 2000). This fibroblast-induced monocyte

attraction is believed to involve the mast cell cytokines TGF-β and TNF-α since depletion of TGF-β and TNF-α from the mediator pool secreted by activated mast cells reduces the fibroblast-driven response by 50-80%.

We found that the percentage of monocytes in the peripheral blood varied between 2-4% and did not differ between mast cell (MCD) deficient mice and syngeneic controls and although there was a slight increase in the percentage of monocytes on day 8 pi this was not statistically significant. However, the total number of monocytes in the peripheral blood of syngeneic control mice was statistically greater than number of monocytes in the peripheral blood of MCD mice during the late phase pulmonary response, on day 14 pi. This confirms a role for mast cells in recruitment of monocytes, from the bone marrow to the blood during pulmonary inflammation.

There was a general monocyte recruitment into the airways following N.b. infection and again this was more marked in syngeneic controls than in MCD mice during the late phase response. The kinetics of monocyte recruitment differed in MCD and syngeneic controls in that recruitment occurred a little earlier in MCD mice giving rise to a greater number of monocytes in the BAL fluid during the early phase response than in MCD mice. This suggests that there may be at least two mechanisms for monocyte recruitment to the airways, one is mast cell dependent and the other mast cell independent. There was a significant reduction in the percentage of monocytes in the airways of both MCD and control mice following N.b. infection simply reflecting the marked pulmonary eosinophilia in both groups of mice. The difference between MCD mice and controls, in the percentage of monocytes recovered from the BAL fluid following infection, was assumed to reflect a slightly more vigorous immune response in control mice. It was not considered to reflect any differences in monocyte recruitment since the change in percentage of monocytes recruited did not tally with the number of monocytes recruited.

We also studied the neutrophil and eosinophil response in the blood and airways following N.b. infection. Mast cells are known to play a role in eosinophil recruitment to the airways. For example, eosinophil recruitment induced by eotaxin was reduced and slowed in mast cell deficient W/Wv mice compared to syngeneic controls (Harris RR et al 1997). Other groups have shown that W/Wv mice produce a less vigorous peripheral blood and bone marrow eosinophilia in response to infection with *Toxocara canis* compared to syngeneic

controls. This blunted peripheral eosinophilia was restored by bone marrow transplantation. The bone marrow eosinophilia in W/Wv mice was less impaired than the peripheral blood response and the chemotactic abilities of eosinophils were completely unimpaired. These results suggested that mast cells might play a role in mediating the peripheral blood eosinophilia (Nawa Y et al 1987).

We confirmed this role by demonstrating a much greater peripheral eosinophilia in control mice than in MCD mice following N.b. infection. This was seen not only in the total number of eosinophils in the blood but also in the percentage of eosinophils that were recruited in the blood. There was still a significant eosinophilia in MCD mice but it was delayed and blunted. This peripheral response was reflected in the lungs of MCD and control mice. Thus there was a significantly greater number and percentage of eosinophils recovered from the BAL fluid of control mice compared to MCD mice. However, mast cell deficiency did not ablate the airway eosinophilia suggesting that eosinophil recruitment is facilitated but not dependent on the presence of mast cells. This may be because there are alternative sources of IL-5 in the airways.

We also examined the kinetics of the neutrophil response in the blood and the airways. Neutrophil recruitment has been shown to be associated with acute asthmatic death and neutrophils are one of the first cells to respond to antigen exposure. For example, in a single intranasal ovalbumin challenge model of airway inflammation in BP2 mice hyperreactivity occurred within 1 h after antigen exposure followed by a massive infiltration of neutrophils and eosinophils, plus a mucous-cell metaplasia of the bronchiolar epithelium. Interestingly these neutrophils were shown to express surface IgE (Haile S et al 1999).

Mast cells are thought to play an important role in this neutrophil recruitment to the lung (Abraham SN et al 1997, Ramos BF et al 1990), and may prevent death in situations where a vigorous neutrophil response is required such as bacterial peritonitis (Echtenacher B et al 1996). For example, recent studies in mast cell deficient mice have shown that mast cell-derived TNF-alpha as well as leukotriene (LT)-B4 and LTC4 play a critical role in the recruitment of neutrophils to the sites of infection and inflammation (Abraham SN et al 2000, Wershil BK et al 1991).

We saw a significant increase in neutrophil numbers in the blood of MCD and control mice during the early phase response, 3 days after immune challenge with N.b. infection. The total number of neutrophils in the blood then subsided during the late phase response. However, the neutrophil recruitment to the blood was no greater in control mice than in the MCD mice despite the fact that the percentage of circulating neutrophil was greater in the control mice. In contrast, the percentage of neutrophils recovered from the airways of control mice was greater during the early response than in MCD mice and the numbers recovered remained greater during the late phase response. These data provide partial support for the suggestion that mast cells do play an important role in neutrophil recruitment to sites of inflammation.

Despite the fact that both T cells and mast cells produce a wide range of inflammatory mediators and have both been implicated as regulators of the inflammatory response in the airways the function of mast cells has been shown to be largely independent of T cells (Mayrhofer G et al 1979).

We examined the peripheral and pulmonary T cell response in MCD and syngeneic controls. The total number and percentage of circulating lymphocytes was much greater in naïve MCD mice than in controls and while the percentage of lymphocytes decreased in both following N.b. infection, the total numbers only decreased significantly in MCD mice. The greater number and percentage of lymphocytes in the blood of naïve MCD mice was reflected in the greater number and percentage of lymphocytes in the airways of MCD mice. However, there was a more vigorous airway lymphocytosis in syngeneic controls than MCD mice with significantly greater numbers of lymphocytes recovered during the late phase response. This may have been due to an upregulation of T cell adhesion molecules by mast cell derived cytokines in the control mice compared to the MCD mice. In contrast, there was a greater percentage of T cells in the BAL fluid of MCD mice. However, this may simply reflect the more vigorous eosinophilia in control mice. Taken together these data suggest that there may be a degree of cooperation between T cell and mast cell cytokine release in promoting T cell recruitment to the airways.

We also examined the systemic cytokine response by disaggregating splenocytes and stimulating them with specific and non-specific antigens. While there was a significantly greater proliferative response during the late phase in MCD mice compared to the early phase this was not significantly different from control mice. In fact control mice showed a greater

proliferative response than MCD mice during the early phase response following stimulation with the highest dose of N.b.-specific antigen as well as with non-specific antigen.

The production of the Th-2 cytokines, IL-4, IL-5 and IL-10 by MCD mice was significantly greater during the late phase pulmonary response than seen in syngeneic controls. However, splenocyte production of IFN-γ was also greater in MCD mice. In contrast, the production of the Th-1 cytokine IL-2 was greater in control mice. Apart from the anomalous IFN-γ result these data suggest that MCD mice generally had a greater systemic Th-2 response than their syngeneic controls. This may have been due to the delayed expulsion of N.b. from the gut of infected animals and therefore a more prolonged systemic response in MCD mice.

In summary this series of experiments provide significant support for the role of mast cells in amplifying allergic inflammation of the airways. Specifically, we demonstrated that mast cells may not only play a central role in the early phase of the allergic reaction, but they also participate in the late phase of the allergic reaction (Toru H et al 1998). We provided evidence for mast cells involvement in monocyte and eosinophil recruitment to the blood. In addition, we provided partial support for mast cell involvement in neutrophil recruitment to the airways particularly in the early phase response. We demonstrated that eosinophil recruitment was facilitated but not dependent on mast cells and suggested that mast cells may facilitate T cell recruitment to the airways. Mast cell deficiency is known to prolong worm survival and we demonstrated a greater systemic Th-2 cytokines response in mast cell deficient mice. Exactly why the absence of mast cells impairs peripheral and pulmonary responses to helminth infection requires further investigation. It may be partially due to increased worm survival and therefore prolonged exposure to Th2 cytokines. It may be due to the loss of mast cell secretory products such as histamine or tryptase. Whatever the mechanism the use of W/Wv mast cell deficient mice may be a useful tool for exploring the cellular aspects of a Th-2 model of allergic inflammation of the airways.

CHAPTER 10

Conclusions and Implications

Can We Model Allergic Inflammation of the Airways in Mice?

10.1 Introduction

Medical training teaches you many things about people. There is training in the basic sciences such as anatomy and biochemistry and how the body works in addition to an investigation into the pathophysiology of disease and how to manage illness. There are incredible lessons to be learnt about the nature of human suffering, courage and the capacity of individuals to recover, physiologically mentally and emotionally and indeed lessons on the fragility and meaning of life itself.

However, there is less training in the conditions required for the maintenance of health and there is generally little attention paid to questioning the unconscious embedded assumptions in medical education. The perception is often that there is so much basic information to be learnt that developing a spirit of enquiry and questioning is a luxury that can be ill afforded. Consequently scientific rigour, the ability to evaluate information, test a hypothesis, evaluate the results and generate further questions is often overlooked. In short there is little training in scientific methodology. Consequently the huge benefits of having to plan and execute detailed scientific investigation are rarely learnt. Having gone through such a process with this thesis it is abundantly clear that the lessons learnt would be absolutely invaluable to anyone interested in health and the well being of human beings.

10.2 General Lessons

A thesis cannot be conducted without a sponsor. Finding and identifying a department and a funding agency teaches you many things about the nature of research funding. There are lessons to be learnt about the academic marketplace, scientific fashion and expediency, and the political context in which all research is conducted. There are basic lessons to be learnt about the structuring of a research proposal and the marketing of its importance. There are international lessons that relate to the financial constraints on research in different countries and institutions. While all such lessons are somewhat

peripheral to what is perceived to be the main purpose of a thesis their importance in the development of the career of a physician scientist should not be underestimated.

Having identified a research department, framed a research question that can attract funding, the main work of a thesis can begin. Coming from a medical background there is an initial huge cultural shock and a very steep learning curve that needs to be scaled and scaled quickly if effective research is to commence. The baptism of fire in the first year of a thesis is a make or break time for many physicians who pursue the academic path and the success at negotiating this year will often set the stage for all future progress and career development.

10.3 Specific Research Lessons

In addition to many general lessons there are numerous specific research lessons to be learnt about the nature of experimentation. For example, the importance of precise record keeping or the unwritten rules of the laboratory bench that need to be adhered to in order to ensure that rogue variables are not erroneously introduced into an experiment. The immunological field in which this thesis was conducted generates its own specific lessons. In particular one of the most fundamental lessons is the immense sophistication of the immune system and its regulation. It really should come as no surprise that successful biological systems resist simple analysis for the very reason that they are successful. This lesson has significant implications for the use of all animal models to investigate human disease such as allergic inflammation of the airways.

10.4 Genetic Regulation of the Immune System

There has been a considerable amount of research time and money spent of genome-wide searches for "the asthma gene". But since the somewhat premature and triumphal announcements of the $1.5 billion mapping of the human genome it has become

apparent that the way these genes work is far more complicated than previously thought. Some have even gone so far as to say that there needs to be a fundamental rewriting of the genetic rulebook.

For example, contrary to initial reports we have far fewer genes that originally thought, approximately 30,000, or twice that of a lowly fruit fly. This realisation supports the notion that we are not hard wired in the way that we previously assumed. So there is no "asthma gene" and some authors have called into question the whole wisdom of identifying a few genetic targets for therapeutic intervention (Anderson GG et al 1999). There are numerous potential genetic targets that influence a very wide variety of different immunological mechanisms that are involved in allergic inflammation to various degrees in different people depending on what protein each gene encodes (Ono SJ et.al 2000). Some genes may encode proteins that play a non-specific role in increasing the risk of asthma while others genes encode proteins that may play a more specific role.

However, the importance of any one individual candidate gene, for example the genes encoding the TCR on chromosome 14q, has to be re-evaluated in the light of the understanding that one gene does not just code one protein. In fact it is now clear that one gene may encode up to ten or even fifty proteins, so 30,000 genes may be encoding 300,00 proteins. Therefore it may be more appropriate to look for candidate proteins rather than candidate genes. But with a much greater number of proteins than genes the number of potential targets would increase at least ten fold.

Furthermore, the final protein product itself may depend upon the intracellular as well as the extra cellular environment. Proteins may be encoded by different genes and may differentially cross regulate each other depending on intracellular dynamics. So even if a candidate protein is identified and targeted it will still be necessary to assess the importance and circumstances under which any intracellular cross regulation and post-translational modification of the protein may undermine the effects of therapeutic targeting.

All of this has led some authors to suggest that it is the beginning of the end for genomics, and "proteinomics"; a term already beginning to appear in scientific journals, may be taking centre stage (Bethell T et al 2001).

10.5 Antigenic Challenge

Putting aside the very significant strategic and tactical difficulties in therapeutic targeting of either genes or their encoded proteins, and looking at what might be done downstream, at the level of the immune response itself, it soon becomes apparent that the inherent problems of proteins or genes are echoed in the sophistication of the immune system's response to an antigenic challenge.

Unlike the response to many drugs, the immune response to antigen does not follow a simple dose response relationship. In Figure 10-1 the black line represents standard S-shaped dose response curve where an increasing dose increases the effect but only up to a certain point. Thereafter no further effect is seen despite increasing the dose since the system is responding maximally. However, in many models of allergic inflammation of the airways the immune response to antigen appears to be a threshold effect whereby the immune response flips from a predominantly Th-1 response to a Th-2 response once a certain antigenic load has been crossed. In fact the dose of antigen has been shown to be one of the most important factors in determining the polarisation of T helper cell subsets into Th-1 or Th-2 populations. Thus low dose ovalbumin (OA) can provoke a Th-2 response but a high dose of the same antigen can provoke a Th-1 response (Sakai K et al 1999).

However, the immune response to antigen may not only flip dependent on the antigenic load, but increasing the antigenic load may actually inhibit the immune response, as shown by the red dotted line in Figure 10-1. Thus rather than the response to antigen reaching a plateau with increasing doses having no further effect, an increasing antigenic load may actually start to inhibit the immune response. This may lead to an

overshoot or even toxicity. Such a dose effect may ultimately be repeated generating a continuous dose-response oscillatory cycle. An example of the inhibitory effect of an increased antigenic load has been seen in the specific and non-specific IgE response to N.b. infection. Thus an infection with 10-100 N.b. larvae produces a sustained rise in IgE levels but a much bigger antigenic burden of 2,500 N.b. larvae produces a much less vigorous IgE response (Yamada M et al 1992).

Furthermore, the extent of the immune response may be dose dependent. Thus the immune response to a small antigenic load may be confined to local effects only while a larger antigenic burden may generate a systemic response (Lawrence RA et al 1996).

In addition, there may be a compartmentalisation effect with the pulmonary response to antigen being dose-dependent while the splenocyte response to antigen is dose independent. Thus, the lung cells of Balb/c mice produced Th-1 cytokines at low dose and Th-2 cytokines at high dose while splenocytes produced Th-2 cytokines regardless of dose of antigen (Morokata T et al 2000). This thesis has attempted to identify the optimum conditions for generating a Th-2 response in both the lungs and the periphery.

Furthermore, the dose response effect is influenced by the strain of mouse used in the experiment. Thus a low dose of antigen that induces a Th-2 response in C57BL/6 mice induces a Th-1 response in Balb/c mice. In contrast, a high dose of the same antigen has the opposite effect, inducing a Th-1 response in C57BL/6 but a Th-2 response in Balb/c mice.

The schedule of antigenic exposure has also been shown to influence the direction of the immune response. Thus a single antigenic challenge will produce a different effect to chronic challenge and chronic challenge can dampen or enhance the Th-2 response depending on the frequency of antigen exposure (Janssen EM et al 1999).

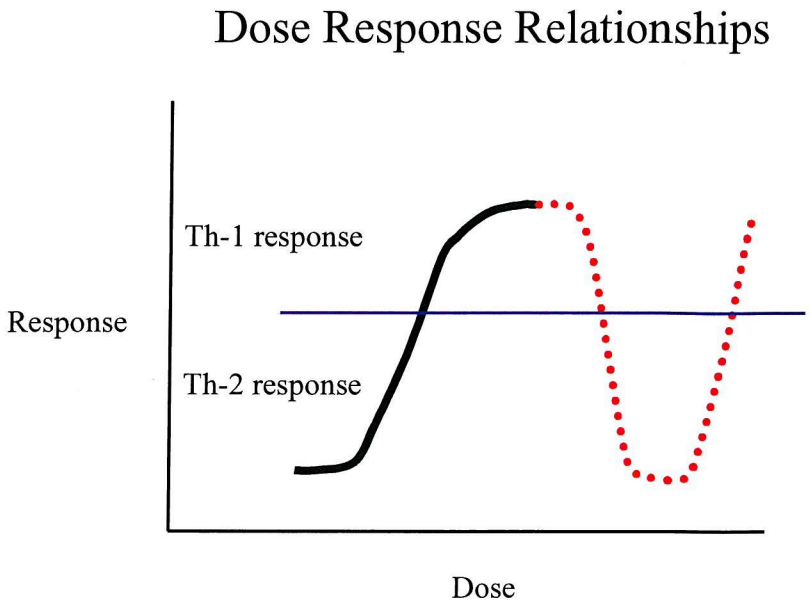

Figure 10-1. Possible relationships between dose of antigen and response to antigen. The effects may be a threshold effect or a cyclical effect (see text).

In conclusion there are many factors (see table 10-1) which influence the immune response to an antigen challenge and therefore determine whether antigen exposure provokes a Th-1 or Th-2 response or induces immunological tolerance (Sakai K et al 1999). Such complexity has been shown to be true of OA and N.b.-induced models of allergic inflammation. Therefore attempts to develop chronic models of inflammation of the airways have often led to a dampening of airway inflammation and the development of tolerance rather than chronic inflammation (Yiamouyiannis CA et al 1999, Murray M et al 1979).

Therefore in developing or comparing the results from different animal models of allergic inflammation it is important to test the assumptions about the nature of the immune response being examined.

Table 10-1 Factors influencing the immune response to antigen.

Antigenic factors	Host factors
physicochemical properties	the murine strain
dose of the stimulating antigen	murine stress level
interval between exposure	nutritional status
chronicity of exposure	housing and environmental conditions
route of administration	age at which sensitised
type of sensitisation regime	
properties of the sensitising agent	
adjuvant used	

10.6 Murine Strain

The wide variety of rodent strains has made it possible to explore the enormous subtlety of the immune response to antigenic challenge. However, the immune response can vary widely depending on the strain chosen. For example, airway sensitivity can vary three-fold depending on the mouse strain involved. Similarly, the percentage of eosinophils recovered from the BAL fluid can vary from 3% to 91%, IgE concentrations in the blood can vary from less than 3 ng/ml to 455 ng/ml and pulmonary inflammation can vary from being undetectable to being widespread and severe in different strains (Brewer JP et al 1999). Therefore the strain chosen may be critical to the optimisation of each model (Uchikawa R et al 1996). For example, in this thesis we demonstrated that despite a greater recruitment of eosinophils to the peripheral blood of Balb/c mice there was a greater recruitment of eosinophils to the airways of C57BL/6 mice.

Such complexity makes it difficult to compare data from seemingly similar studies in the same strain since the immune response can be skewed in completely different directions

depending on the type of antigenic exposure. Therefore it becomes critical to characterize the immune response in each model very carefully including the type and nature of the provoking antigen, the compartment studied and report the methodological details carefully in each case. Such rigour may go a long way to resolving some of the contradictions that currently exist in the research literature such as Balb/c mice generating a stronger (Nakajima H et al 2000), weaker (Morokata T et al 1999) or equivalent (Stadnyk AW et al 1990) Th-2 response to antigen compared to other strains. This thesis has attempted to define the optimizing characteristics of this model and the immunological advantages that it may offer.

10.7 Compartmentalisation of the Immune Response

It is therefore clear that the immunological responses in different body compartments can move in the same or opposite directions depending on the nature of the antigenic challenge and the genetic strain of the mouse studied. However, a compartmentalisation effect has been demonstrated in the same strain and with the same antigenic challenge. For example, Balb/c mice have been shown to produce a vigorous Th-2 response in the spleen while simultaneously producing a Th-1 response in the lungs (Morokata T et al 1999).

This compartmentalisation effect has been increasingly demonstrated in a number of studies. Thus a vigorous systemic Th-2 response may not necessarily provoke a Th-2 response in the lungs. It has been suggested that local cytokine production in the airways may be the critical factor in determining the degree of airway inflammation rather than the systemic cytokine response (Nakajima H et al 2000).

In this thesis we have demonstrated that despite a differentiation in some aspects of the immune response such as eosinophil recruitment other aspects, such as cytokine production, were less well differentiated. For example, Balb/c mice generated a greater eosinophilia in the blood but smaller eosinophilia in the lungs compared to C57BL/6

mice. This suggested a greater systemic Th-2 response in Balb/c mice. In contrast Balb/c splenocytes also produced significantly more Th-1 and Th-2 cytokines suggesting a less clear cut systemic Th-2 response than the peripheral eosinophilia would suggest (see chapter 6).

The sophistication of the immune response goes beyond this uncoupling of different compartments since uncoupling has also been shown to occur within the same compartment. For example, airway hyperresponsiveness (AHR) has been shown to be independent of airway inflammation and IgE responses in numerous murine strains (Wilder JA et al 1999). In fact, under certain circumstances inflammatory changes in the airways can be induced with little or no airway AHR (Haile S et al 1999). Such differences have been shown to be due to subtle differences in the local production of Th-2 cytokines such as IL-4 and IL-5 within the same compartment (Foster PS et al 2000, Dehlawi MS et al 1987).

All these data emphasise the importance of clarifying the immune compartment targeted and characterising the exact nature and time course of the response investigated. Each cellular population can move in different directions in each compartment either independently or in concert with other cellular populations.

10.8 Cellular Redundancy and Phenotypic Switching

In addition to the sophistication of the compartmentalisation effect and the fact that the intracellular environment may modify the peptide modulators of the immune response, the extracellular environment may provoke a phenotype switching of some of the key inflammatory cells. For example, it is well known that IL-12 can effect an isotype switching of Th-2 cells to Th-1 cells. Mast cells have also been shown to be capable of altering their phenotype depending on the surrounding inflammatory conditions (Tomita M et al 1999).

In addition to cells being able to alter their phenotype under certain circumstances some cells are capable of playing a wider role than previously thought. For example, B-lymphocytes and mast cells can act as effective antigen presenting cells under certain circumstances (Abraham SN et al 1997, Morris et al 1994). Mast cells and NK cells have been shown to produce IL-4 even in IL-4 deficient mice (Wilson SJ et al 2000, Noben-Trauth N et al 1997), and the amount of cytokines produced may be equivalent to that generated by lymphocytes (Williams CM et al 2000).

10.9 Cytokine Redundancy

The cellular redundancy highlighted above is mirrored by the redundancy in cytokine function. For example, IL-4 and IL-13 share many common features and receptor components and also play similar roles in resolving mucosal inflammation (McKenzie GJ et al 1998). In addition, the Th-2 population of cells, previously thought to be dependent on IL-4, has now been shown to be capable of developing in the absence of IL-4 (Kropf P et al 1999). Even more surprising is the recent demonstration that IL-4, which had always been thought of as a major driver of allergic inflammation can, under certain circumstances inhibit inflammation. Thus the administration of exogenous soluble IL-4 to mice significantly reduced late phase pulmonary inflammation, blocked airway eosinophil infiltration, VCAM-1 expression, and mucus hypersecretion (Henderson WR Jr et al 2000). Similarly, recent evidence has suggested that the normally anti-inflammatory IL-10 can play a pro-inflammatory role (Yang X et al 2000). In addition, IL-18 has been shown to both inhibit (Yoshimoto T et al 1997) and promote a Th-2 response in different experiments and the induction of a BAL eosinophilia could be reversed by manipulation of the experimental protocol (Wild JS et al 2000).

Taken together these data start to suggest that the intracellular and extracellular microenvironment is important in determining the direction and extent of the immune response. The outcome of the cross regulation of opposing cytokines such as IL-10,

IFN-g, IL-6 and IL-11 may depend on a complex interplay between cytokine, chemokine, hormonal and cellular pro and anti- inflammatory forces. In addition, the timing of the release of inflammatory mediators may be critical to the role the inflammatory mediators actually play

10.10 Temporal Factors

A picture is beginning to emerge of immense sophistication and redundancy not only upstream, in the regulation of the immune system by genes and their encoded proteins, but also downstream within the immune system itself. For example, it is clear that the interaction of antigen presenting cells (APCs) with T cells can provoke a Th-2 response. Effective coupling between APCs and T cells requires the intervention of a costimulatory molecule such as B7-2 and recent evidence suggests that the timing of the costimulatory assistance may be critical in promoting the development of the Th-2 response (Gausse WC1999). Similarly the isotype switching of Th-2 to Th-1 cells by IL-12 is dependent on the timing of the IL-12 administration (Finkelman FD et al 1994).

But it is not only the timing of the assistance provided by cytokine, chemokine, hormonal or other inflammatory and costimulatory molecules that is important, the timing of the changes in the local cellular populations may also be critical in determining the direction and outcome of the immune response. For example, the production of eosinophil chemotactic factor by neutrophils in response to low levels of antigen challenge has been shown to enhance the immune response (Czarnetski et al 1978), whereas larger antigenic burdens that may be expected to occur later in an infective or inflammatory process may override the role of the neutrophil in the early response to immune activation.

10.11 Airway Dysregulation

The highly sophisticated interplay between upstream and downstream factors in allergic inflammation ultimately results in chronic airway epithelial dysfunction and dysregulation. This dysregulation manifests as epithelial fragility and an epithelial phenotype trapped in a permanent cycle of inflammation and repair chronically producing numerous pro-inflammatory mediators and growth factors (Holgate et al 1999). The chronic dysfunction is not confined to airway epithelium or Clara cell remodelling but is also seen in the airway vasculature with persistent angiogenesis and microvascular leakage.

10.12 Summary of Main Results of Thesis

Through a series of thirty or so separate experiments involving over 1,250 mice we set out to develop an animal model of pulmonary inflammation that could be used to investigate the mechanism of airway cellular recruitment and could be developed into a useful model of asthma.

One of the first criteria for success is does the model mimic the disease? Chapter 2 suggested that histopathologically the model looked more akin to Churg Strauss syndrome than asthma, with a very heavy pulmonary eosinophilia. So one of the goals for the future developments of this model would be to increase the histological mimicry of the model.

Chapter 3 suggested that the cellular recruitment to the airways did show some similarity to allergic inflammation of the airways with activated and functionally effective eosinophils and lymphocytes recruited. Most OA-driven models drive the recruitment of 100-200,000 eosinophils to the airways, whereas this model consistently produces a ten-fold greater airway eosinophilia. This may offer some advantage if eosinophils are the main cell under investigation, however, the model could be developed to generate a less florid eosinophil recruitment more akin to asthma.

Chapter 4 attempted to reduce this airway eosinophilia by reducing the number of N.b. larvae introduced. This showed that a much smaller airway eosinophilia could be generated using just 6% of the standard larval burden. Such an approach offers promise in developing a spectrum of severity of airway inflammation and future work may help to identify the optimum dose that provokes a Th-2 response in each compartment.

However, it may be that the most useful aspects of this model may be to offer an alternative antigenic stimulus to driving a Th-2 response. In chapter 5 we attempted to identify whether adult or larval antigens were more important in driving the observed pulmonary inflammation. We discovered that both types of antigens play a role in driving pulmonary inflammation but the adult antigens are probably more effective as Th-2 stimulants, at least in C57BL/6 mice.

Chapter 6 suggested that C57BL/6 mice were probably the optimum strain in which to establish a N.b-driven model of pulmonary inflammation and Balb/c mice conferred no specific advantage.

Chapters 7 and 8 suggested that ICAM-1 and CD18 were not critical to the evolution of the cellular recruitment to the airways since knocking out or partially deleting either molecule did not reduce the pulmonary inflammation. This may have been due to the overwhelmingly powerful nature of the N.b. stimulus and one option would be to repeat such studies with a much smaller infective burden.

In contrast chapter 9 indicated that mast cells were critically important to the development of airway inflammation in this model. This is in keeping with the recent resurgence in interest in this cell as central to allergic inflammation.

As it currently stands this model probably does not offer any specific advantages to an OA-driven model apart from the much greater eosinophilia. It still falls some way short of modelling asthma and requires further work to be done to increase its mimicry.

10.13 Future Developments of this Model

We conducted a number of experiments to explore the effect of N.b. infection on pulmonary function. Although we managed to build a system for measuring pulmonary resistance in mice the time frame of these studies did not allow pulmonary function to be measured consistently and therefore the results of these preliminary studies were not presented here. The effectiveness of any animal model in mimicking asthma should involve a measure of airway resistance and a demonstration of reversible impairment of airway calibre.

We also began to explore other routes of administration of N.b. adult antigens. We began by exploring the effectiveness of direct tracheal installation of adult antigens. Unfortunately this resulted in a 50% death rate of the mice involved in these studies, either from drowning or from the effects of anaesthesia. We concluded that nebulization of mashed adult antigens was perhaps the most viable alternative strategy. The single most important development of this model would be to explore the consequences of nebulized adult antigens to see whether this conferred any specific advantages over nebulized OA. Since OA is cheap and readily available N.b. would have to generate specific advantages to warrant switching from OA-driven models.

If nebulized N.b. antigen could be shown to provoke a less florid subepithelial inflammation rather than an eosinophilic vasculitis then such a model could be subject to therapeutic manipulation. We did conduct a few initial experiments to explore the ability of varying doses of dexamethasone to inhibit the pulmonary inflammation following N.b. infection, with very mixed results (data not presented here), but these studies would have been much more relevant in a model that more closely mimicked asthma.

One of the greatest challenges in the development of animal models of asthma is to develop chronic inflammation. This would have to be a specific goal of any future developments of this model. In most OA-driven models the pulmonary inflammation

subsides after just a few days, therefore a protocol of repeated airway challenge with N.b. antigen would have to be explored to see if a schedule could be developed to provoke the chronic inflammation, which is the holy grail of many animal models of asthma.

10. 14 Advances in Understanding

Prior to this series of experiments little was known about the peripheral or pulmonary response to N.b. infection in mice. Most of the research had been focused on rats. Therefore this series of studies significantly extends existing knowledge in the field and lays down the foundations for any future work using this very powerful Th-2 stimulant in mice.

We established the optimum strain in which to conduct such experiments and demonstrated that a much smaller infective burden would suffice to induce a pulmonary eosinophilia. We also identified the adult antigens as the most important drivers of the pulmonary inflammation, a fact that may be important for future developments of this model. We demonstrated that ICAM-1 and CD18 were not vital to the cellular recruitment in the basic model but mast cell integrity was.

There had been very little work examining the cell surface markers of lymphocytes recruited to the airways following N.b. infection in rats and none in mice. In addition, these were the first series of studies to assess the cell surface markers on eosinophils recruited to the airways in a model of pulmonary inflammation.

In hindsight a number of these studies could have been performed more effectively and a more rapid conversion to a nebulisation model would have been more useful. However, we believe that these series of studies do add significantly to the understanding in the field and offer promise for future development of the model. At the conclusion of these studies my colleagues at Upjohn were refining this model still further and the N.b. model of pulmonary inflammation was still active.

10.15 Conclusions

Over the years attempts to shed light on the pathogenesis and evolution of human disease have relied heavily upon the design, development and analysis of animal models of those diseases. Animal models have enabled scientists to probe the genetic, environmental, cellular and biochemical mechanisms of disease in a way that is not technically possible in humans. The information gathered has provided a mountain of knowledge and provided some novel ideas for therapeutic intervention in addition to providing a test bed for drug discovery programmes.

In investigating allergic inflammation of the airways a number of different animal models have been employed, each has their champions and each confers different experimental advantages. However, despite considerable scientific endeavour, there is no "ideal" animal model and prudent research initiatives will employ a variety of different models to address different experimental questions. In animal models of allergic inflammation of the airways the use of guinea pigs has largely given way to the humble mouse not least because of the immense array of immunological tools available to probe the inflammatory response of mice (Vargaftig BB et al 1999).

This thesis set out to establish an alternative murine model to the popular OA-induced model of allergic inflammation by using primary infection with third stage N.b. larvae. The N.b.-induced model, as it has been described here, may confer considerable advantages over OA-induced model when exploring the role of eosinophils in airway inflammation because of the greater airway eosinophilia generated in the N.b. model. But apart from this specific advantage the N.b.-induced model, as far as it was developed here, offers few other benefits compared to the OA-induced model. However, it is possible that future development of the N.b-induced model, using natural or synthetic N.b. peptides delivered by aerosol, might induce an airway inflammation that more closely mimics human airway inflammation than the OA-induced models. Further work will be needed to clarify the usefulness of N.b. peptides.

Although the use of animal models of allergic inflammation has shed considerable light on the basic immunological mechanisms involved in airway dysfunction it is clear from this thesis, and the work of numerous others, that the immune system is so immensely sophisticated that science should not rely solely on the molecular biological approach to help probe human airway inflammation. There has to be a balance with other avenues of research, such as psychosocial dynamics and the importance of emotions in the regulation of airway function.

The above discussion of immunological complexity has highlighted that the direction and outcome of the immune response can be reversed by even the slightest alteration in the experimental protocol. There is so much redundancy and sophistication built into upstream and downstream immunological regulation of airway function that unless each experiment is conducted with rigorous care and attention to detail an erroneous result could easily be produced. In addition, any extrapolation or comparison of the findings of one experiment to another experiment must be conducted with considerable caution. It is only with this degree of caution and the weighing of bodies of evidence rather than single experiments that we may make genuine progress in the fight against this common and disabling condition.

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