The Role of Eosinophils in the Regulation of $CD4^+$ T helper 2 Regulated Inflammation.

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by

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Statement

I certify that all experiments (barring that which is outlined in Section 4.2.9), represent my own work and have not been previously submitted for a degree or thesis at this or any other university. The experiment outlined in Section 4.2.9 was conducted in close collaboration with Dr Joerg Mattes, visiting fellow of the John Curtin School of Medical Research, Australian National University.

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ABSTRACT

The eosinophil is a leukocyte whose intracellular mediators are considered to play a central role in the pathogenesis of allergic diseases, including allergic asthma, allergic rhinitis and atopic dermatitis, and which is also involved in immunological responses to parasites. Eosinophil differentiation and maturation from bone marrow progenitors is regulated by interleukin-5 (IL-5), which may be secreted by T helper 2 (Th2) T lymphocytes, and is consistently upregulated in allergic conditions. Eotaxin is a potent chemoattractant for circulating and tissue eosinophils, and the production of this chemokine promotes eosinophil infiltration and accumulation within sites of allergic inflammation.

Eosinophils obtained from inflammatory tissues and secretions display an altered phenotype in comparison to peripheral blood eosinophils, with increased surface expression of major histocompatibility complex (MHC) proteins and adhesion molecules (Hansel *et al.*, 1991), and migration across the microvascular endothelium may also increase their capacity to generate an oxidative burst (Walker *et al.*, 1993; Yamamoto *et al.*, 2000). Eosinophils are phagocytic cells, and have been shown to present simple (no requirement for intracellular processing) and complex antigens to MHC-restricted, antigen-specific T lymphocytes (Del Pozo *et al.*, 1992; Weller *et al.*, 1993). Furthermore, eosinophils express the costimulatory molecules required for effective antigen presentation (Tamura *et al.*, 1996), and ligation of costimulatory molecules on the eosinophil cell surface can induce the release of eosinophil derived cytokines (Woerly *et al.*, 1999; Woerly *et al.*, 2002). Therefore the eosinophil may also regulate immune responses.

To date, no studies have demonstrated the ability of eosinophils to modulate activated T lymphocyte function via presentation of relevant antigen in the context of MHC class II (MHC-II), concomitant with Th2 cytokine release. In the experiments described in this thesis, murine eosinophils have been observed to rapidly migrate to sites of antigen deposition within the airways mucosa of naïve mice, suggesting a potential role for this granulocyte in the primary response to inhaled antigen. However, human allergic diseases are often diagnosed after the establishment of allergic responses, and symptom development. Therefore, a murine model of allergic airways disease (AAD) was used to

investigate the ability for eosinophils to participate as antigen presenting cells (APCs), and thereby modulate activated T lymphocyte function both *in vitro* and *in vivo*. Detailed histological analysis of the pulmonary draining lymph nodes following antigen challenge in sensitised mice revealed a rapid infiltration of eosinophils into this tissue, which preceded the accumulation of eosinophils in bronchoalveolar lavage fluid (BALF). This suggested that eosinophils were preferentially translocating to the draining lymph nodes following antigen challenge, and that the subsequent accumulation of these cells in the BALF was a consequence of continued antigen delivery to the lower airways.

Eosinophil trafficking to lymphoid tissue via the afferent lymphatics was substantiated using electron microscopy of lymph node sections and the intravenous (i.v.) transfer of fluorescently labeled eosinophils, which did not traffic to lymph nodes via the blood. During the resolution of AAD, eosinophils were noted for their persistence in the pulmonary draining lymph nodes. These observations suggested a continued modulation of T cell function by lymph node dwelling eosinophils during AAD resolution, particularly in light of recent observations for draining lymph node T cell proliferation following instillation of antigen-pulsed eosinophils into the allergic mouse lung (Shi *et al.*, 2000).

To further investigate the antigen presenting capacity, eosinophils were obtained from the BALF of mice with AAD, and their surface expression of MHC class II (MHC-II) proteins and costimulatory molecules confirmed using flow cytometric analysis. The ability to acquire and process complex antigen both *in vitro* and *in vivo* was also confirmed using naturally quenching fluorescenated ovalbumin (OVA), which is degraded into fluorescent peptides by the action of intracellular proteases. Thus, eosinophil expression of the surface molecules necessary for effective antigen presentation was confirmed, as was their ability to process complex antigen. Further investigations revealed that eosinophils can present complex OVA antigen to $CD4^+$ T lymphocytes obtained from the allergic mouse, and to *in vitro* derived OVA-specific Th2 cells. In the presence of exogenous antigen, eosinophils co-cultured with T lymphocytes were able to induce Th2 cytokine production, and demonstrated an ability for eosinophils to modulate T lymphocyte function *in vitro*.

The ability for eosinophils to act as antigen presenting cells *in vivo* was also investigated. Eosinophils obtained from the antigen-saturated lungs of OVA sensitised and challenged mice were transferred to the peritoneal cavities of naïve host mice. When subsequently challenged with aerosolised OVA, eosinophil recipients developed a pulmonary eosinophilia similar to that of OVA sensitised and challenged mice. To validate this finding, the experimental procedure was altered to accommodate the use of non-allergy derived eosinophils, which were pulsed with OVA *in vitro*, prior to transfer into naïve recipients. When subsequently challenged with aerosolised OVA, eosinophil recipients developed a peripheral blood and pulmonary eosinophilia, and stimulation with OVA induced IL-5 and IL-13 cytokine production from pulmonary draining lymph node cells. Notably, the AAD induced by transfer of antigen pulsed eosinophils did not induce detectable OVA-specific IgG_1, which may be attributed to the lack of soluble antigen required for B cell antibody production.

During the course of these investigations, an OVA T cell receptor (TCR) transgenic mouse (OT-II) was procured with a view to defining the interaction between eosinophils and activated T lymphocytes (Barnden *et al.*, 1998). Despite having specificity for the $OVA_{323-339}$ peptide, an immunodominant epitope that skews naïve T cell responses towards Th2 cytokine release (Janssen *et al.*, 2000), T lymphocytes from the OT-II mouse preferentially secreted IFN-γ in response to stimulation with either OVA peptide or OVA. These mice were further characterised in a mouse model of AAD, and found to be refractory to disease induction and progression, which may be attributed to significant IFN-γ secretion by transgenic $CD4^+$ T lymphocytes during antigen sensitisation. Indeed, these cells were noted for their ability to attenuate pulmonary eosinophilia when transferred to OVA sensitised and challenged wild type mice, although serum OVA-specific IgG_1, peripheral blood eosinophilia levels and airways response to methacholine challenge remained intact.

Knowledge of the biased Th1 phenotype in naïve OT-II provided a unique opportunity to investigate the fate of T lymphocytes bearing high affinity OVA-specific TCRs following neonatal antigen exposure to soluble OVA. In a previous study, subcutaneous (s.c.) administration of soluble OVA to wild type neonatal mice was suspected to have deleted OVA-specific T cells from the T cell repertoire (Hogan *et al.*, 1998a). Using flow cytometry and TCR specific antibody, the delivery of s.c. OVA to OT-II neonates

did not alter transgenic T cell populations in adult mice. Instead, it was surprising to find a skewing towards the Th2 phenotype and loss of IFN-γ secretion following OVA sensitisation and challenge in adult mice. A mechanism for this reprogramming of the transgenic T cell from the Th1 to a Th2 phenotype following OT-II neonatal exposure to soluble OVA is proposed, and further experimentation may validate this hypothesis.

In conclusion, eosinophils residing in the allergic lung have the capacity to interact with activated T cells, both within this tissue and the draining lymph nodes. Despite their relative inefficiency as antigen presenting cells (Mawhorter *et al.*, 1994), eosinophils may participate *en masse* in the serial triggering of activated TCRs, and provide appropriate costimulatory signals that modulate T lymphocyte function. Through the elaboration of Th2 cytokines and stimulation of T cell proliferation, antigen presenting eosinophils may transiently prolong or exacerbate the symptoms of allergic diseases. Alternatively, eosinophils presenting relevant antigens may inhibit T cell activity via degranulation, and such activity has recently been observed in a parasite model (Shinkai *et al.*, 2002). Finally, experiments in the OT-II mouse have provided valuable information to suggest that therapies designed to modulate eosinophil numbers in allergic tissues through the secretion of opposing cytokines such as IFN-γ, may be of limited benefit. The results shown here suggest that airways dysfunction remains intact despite significantly reduced pulmonary eosinophilia.

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LIST OF ABBREVIATIONS

AAD	Allergic Airways Disease
AHR	Airways Hyperreactivity
ANU	Australian National University
APC	Antigen Presenting Cell/s
BALF	Bronchoalveolar Lavage Fluid
BSA	Bovine Serum Albumin
CCR3	CC Chemokine Receptor 3
CFA	Complete Freund's Adjuvant
CFSE	Carboxyflourescein Diacetate Succinimidyl Ester
CPM	Counts per minute
DC	Dendritic Cell/s
DP	Double positive
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
GIT	Gastrointestinal Tract
GM-CSF	Granulocyte/Macrophage Colony Stimulating Factor
HBSS	Hank's Buffered Salt Solution
HEV	High Endothelial Venule
HLA	Human Leukocyte Antigen
ICAM-1	Intercellular Adhesion Molecule 1
IL-	Interleukin-
IES	Immediate Early Signal
IFA	Incomplete Freund's Adjuvant
i.p.	Intraperitoneal
JCSMR	John Curtin School of Medical Research
LALN	Lung Associated Lymph Nodes
LCF,	Leukocyte Chemotactic Factor
LPS	Lipopolysaccharide
MLC	Mixed Lymphocyte Culture (medium)
MHC	Major Histocompatibility Complex
MIP-1α	Macrophage Inflammatory Protein 1α
OVA	Ovalbumin
PAF	Platelet Activating Factor
PBLN	Peribronchial Lymph Node/s
PBS	Phosphate Buffered Saline
PEC	Peritoneal Exudate Cell/s
PMBS	Polymyxin B Sulfate
RANTES	Regulated on Activation, Normal T-cell Expressed and Secreted
RBC	Red Blood Cell
rEOT	Recombinant Eotaxin
s.c.	Subcutaneous
SP	single positive
TCR	T Cell Receptor
Th	T helper
WT	Wild Type

PUBLICATIONS AND CONFERENCE PROCEEDINGS ARISING FROM THIS WORK

CONFERENCE PROCEEDINGS

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PUBLICATIONS

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Chapter 1.
General Introduction

1.0 GENERAL INTRODUCTION

Eosinophils are bone derived effector cells implicated in the pathogenesis of allergic inflammatory diseases such as asthma, and in the defense against some internal parasites (Gleich *et al.*, 1993; Wardlaw *et al.*, 1995). Extensive investigations into the effector roles attributed to eosinophils in the pathogenesis of inflammatory diseases have revealed that this cell may also participate as an immune modulator. In response to inflammatory cytokines, eosinophils can express surface MHC and the co-stimulatory molecules that are necessary for interaction with MHC-restricted T lymphocytes (Weller, 1994; Weller and Lim, 1997). Their relative abundance at sites of allergic inflammation suggests that eosinophils may be significant modulators of T cell function. An extensive study into the ability of eosinophils to perform immunoregulatory functions may define a new role for this cell in addition to the already suspected effector functions in the pathogenesis of atopic diseases.

1.1 EOSINOPHIL BIOLOGY AND ROLE IN ALLERGIC DISEASE.

Eosinophils are terminally differentiated bone marrow-derived leukocytes, noted for their membrane-bound granules and bilobed nucleus. The eosinophilic granules comprise proinflammatory molecules and cytotoxic proteins, including major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (EPO), and in human cells, eosinophil derived neurotoxin (EDN), all of which have been extensively reviewed elsewhere (Gleich and Adolphson, 1986; Wardlaw *et al.*, 1995). Inflammation of the airway mucosa and submucosa is thought to play a major role in the pathogenesis of allergic asthma (Bochner *et al.*, 1994), and the eosinophil is regarded as a key effector cell in the pathogenesis of allergic diseases (Rothenberg, 1998).

1.1.1 Regulation of eosinophilia by IL-5 and eotaxin.

IL-5 is believed to regulate the growth, differentiation, and activation of eosinophils (Sanderson, 1992), although mature eosinophils with normal morphology may be found in the bone marrow, blood, and tissues of IL-5 deficient mice (Kopf *et al.*, 1996), and these cells exhibit functional responses to IL-5 and eotaxin (Mould *et al.*, 1997). In animal models of allergic airways inflammation, allergen inhalation increases the numbers of eosinophil progenitors in the bone marrow (Woolley *et al.*, 1994; Inman *et al.*, 1996; Inman *et al.*, 1999), and in human asthma patients, allergen challenge induces

significant increases in progenitor cells expressing the IL-5 receptor α-chain, which favors the development of eosinophilia (Sehmi *et al.*, 1997). While the bone marrow clearly contributes to blood and tissue eosinophilia (Denburg *et al.*, 1996; Denburg *et al.*, 1997), eosinophil differentiation is not restricted to this compartment, and there is evidence of IL-5 dependent differentiation at local sites of inflammation (Cameron *et al.*, 2000).

Peripheral blood eosinophilia is tightly regulated by IL-5, (Coffman *et al.*, 1989; Kopf *et al.*, 1996), which is thought to be produced primarily by $CD4^+$ T cells in allergic disease (Corrigan *et al.*, 1995; Till *et al.*, 1995; Hogan *et al.*, 1998c). Mice that are deficient for IL-5 display reduced tissue and blood eosinophilia, lung damage and AHR in a mouse model of asthma (Foster *et al.*, 1996), and despite having readily detectable IL-5 in BALF, allergic mice deficient for the IL-5 receptor α-chain have attenuated tissue and BALF eosinophilia (Tanaka *et al.*, 2000). Together, these data highlight the importance of IL-5 in generating a peripheral blood eosinophilia that is necessary for eosinophil recruitment to allergic tissues.

Eotaxin is required for baseline tissue eosinophilia in the healthy state (Matthews *et al.*, 1998), and the gastrointestinal tract (GIT) is the largest compartment for eosinophil accumulation in healthy individuals. Eosinophils reside in the mouse GIT mucosa during embryonic development, and in both germ-free and non germ-free adults, indicating that the recruitment of eosinophils through constitutive eotaxin production by mononuclear cells in the lamina propria is independent of viable intestinal flora (Mishra *et al.*, 1999). Eosinophil recruitment to the duodenal and colonic mucosa can occur independently of IL-5 in a model of allergic gastrointestinal disease (Hogan *et al.*, 2000), and increases in peripheral blood eosinophils resulting from allergic airways disease do not alter mucosal eosinophil numbers in the GIT (Mishra *et al.*, 1999), indicating that recruitment of eosinophils to the GIT and allergic tissues are differentially regulated.

Eosinophil recruitment into allergic tissue is thought to result from the concerted action of IL-5 and eotaxin, which act respectively, to mobilise eosinophils from bone marrow, and selectively recruit eosinophils into tissue. In the guinea pig, systemic administration of IL-5 induces a peripheral blood eosinophilia, which enhances eosinophil

accumulation in response to intradermal eotaxin administration (Collins *et al.*, 1995), while the overexpression of IL-5 in IL-5 transgenic mice enables eotaxin to recruit eosinophils to the airways lumen following intratracheal administration (Rothenberg *et al.*, 1996). In sensitised guinea pigs, eotaxin levels in BALF and lung tissue peak within 6 hours following allergen challenge, and eosinophils appear in lung tissue and the airways lumen 6 and 12 hours following challenge, respectively (Humbles *et al.*, 1997). In this model, the administration of antibodies to IL-5 prior to allergen challenge did not affect eotaxin expression, but significantly attenuated eosinophil numbers in BALF and lung tissue, demonstrating the necessity for IL-5 induced mobilisation of bone marrow eosinophils for eotaxin mediated recruitment to the allergic lung.

Although the expression of IL-5 in the airways epithelium can lead to increased eosinophil numbers in peribronchial tissue and the airways lumen (Lee *et al.*, 1997), circulating, and not lung IL-5 is thought to be necessary for the development of airways eosinophilia in a mouse model of allergic airways disease (Wang *et al.*, 1998).
In sensitised and challenged IL-5 deficient animals, both IL-4 and eotaxin levels produced in the lung are similar to that of wild type mice, yet eosinophil recruitment to the lung is severely attenuated. Circulating IL-5 can restore eosinophil recruitment to the allergic airways, whilst compartmentalised IL-5 expression within the lung does not. (Wang *et al.*, 1998). There is some evidence for extra-pulmonary production of IL-5 by T cells in bone marrow following sensitisation and aeroallergen challenge in mice (Minshall *et al.*, 1998), indicating that Th2 cells secreting IL-5 within the lung and during migration through the blood and bone marrow may contribute to peripheral blood eosinophilia.

1.1.2 IL-4 and IL-13 regulate tissue eosinophilia of the lung.

IL-4 and IL-13 are two cytokines released from Th2 cells that have been implicated in allergic airways disease. Although sensitised and airways challenged IL-4 deficient mice exhibit substantially reduced eosinophils in BALF and attenuated AHR in response to serotonin challenge (Brusselle *et al.*, 1995), IL-4 is not essential for the development of IL-5 producing $CD4^+$ T cells, and attenuated eosinophil recruitment to the lung does not translate to reduced AHR in response to methacholine challenge (Hogan *et al.*, 1997b).

At the receptor level, Th2 driven tissue-eosinophilia and AHR in response to IL-4 receptor (IL-4Rα) signaling is mediated by signal transducers and activators of transcription (STAT)-6 (Akimoto *et al.*, 1998). IL-13 also signals via IL-4Rα (Zurawski *et al.*, 1995), and provides an IL-4 independent pathway for STAT-6 regulation of Th2-regulated eosinophilia (Yang *et al.*, 2001), although the IL-13 specific receptor complex can also signal via STAT-6 independently of IL-4Rα, under some conditions (Mattes *et al.*, 2001).

In OVA sensitised and challenged mice, the removal of IL-4 or IL-13 does not totally abolish tissue eosinophilia, while the depletion of IL-4 in IL-13 deficient mice (but not wild type mice) significantly inhibits residual eosinophil recruitment to the lung (Webb *et al.*, 2000). IL-4 upregulates adhesion molecule expression on the vascular endothelium (Patel, 1998), and both IL-4 and IL-13 can induce eotaxin expression from airway epithelial cells (Li *et al.*, 1999; Matsukura *et al.*, 1999), both of which may influence eosinophil recruitment to lung tissue. The depletion of IL-4 in OVA sensitised and challenged IL-13 deficient mice significantly decreases both IL-5 and eotaxin production in the lung, whilst an elevated peripheral blood eosinophilia is observed (Webb *et al.*, 2000). It is tempting to speculate that in these experiments, IL-5 producing T cells were released from the afferent lymphatics following repeated airways challenge with OVA, and their migration through the blood and bone marrow then promoted eosinophil maturation and differentiation. This idea is supported by observations of extra-pulmonary production of IL-5 by T cells in the bone marrow (Minshall *et al.*, 1998). Thus, newly differentiated eosinophils accumulate in the blood of mice depleted of IL-4 and IL-13, due to systemic IL-5 production and absence of lung derived eotaxin and cell adhesion molecules. In wild type mice, integrated signaling between IL-4 and IL-13 promotes lung IL-5 and eotaxin production, and may also promote adhesion molecule expression on the vascular endothelium, all of which act in concert to promote eosinophil infiltration of allergic tissue.

In summary, peripheral blood eosinophilia seen in allergic disease is regulated by systemic IL-5, and enables integrated signaling processes between IL-4 and IL-13 originating within allergic tissues to promote eosinophil accumulation, which correlates with disease severity and tissue damage (Arm and Lee, 1992; Bochner *et al.*, 1994; Hogan *et al.*, 1997a).

1.2 THE ROLE OF THE EOSINOPHIL IN DEFENSE AGAINST PARASITES.

Elevated levels of serum IgE and eosinophils and intestinal mastocytosis are characteristic of most parasite infections (Butterworth, 1977; Befus *et al.*, 1979; Jarrett and Miller, 1982) and eosinophilia and IgE are hallmark features of *Schistosome mansoni* infection (Capron, 1992). Of the helminths, the parasitic trematode *Schistosome mansoni* is well studied, and responsible for schistosomiasis, a major tropical disease, wherein eggs and egg products become trapped in host tissue (Warren *et al.*, 1967). The two major components of the pathological response to *S. mansoni* infection are the formation of hepatic granulomas and hepatic portal fibroses, and eosinophils are a predominant cell represented in the granuloma (Moore *et al.*, 1977; Phillips and Lammie, 1986).

Early studies on the host response to parasite infection revealed that *in vitro* damage to the larval stage of *S. mansoni* (schistosomula) was an antibody dependent mechanism (Butterworth *et al.*, 1974), and effector function was later attributed to purified eosinophils (Butterworth *et al.*, 1977), whose granule derived major basic protein could kill schistosomula *in vitro* (Butterworth *et al.*, 1979). Following the identification of IgE receptors on human eosinophils (Capron *et al.*, 1981), eosinophils from hypereosinophilic patients were demonstrated to effect the *in vitro* killing of schistosomula in the presence of IgE purified from *S. mansoni* infected sera (Capron *et al.*, 1984). Pre-incubation of eosinophils with IgE immune complexes (but not IgG complexes), or antibody to FcεR (subtype not defined), inhibited eosinophil killing, and demonstrated the specificity for IgE mediated effector function. More recently, the use of antibodies to block FcεRI (α chain) inhibited cytotoxicity to schistosome targets (Gounni *et al.*, 1994), and implicates the high affinity IgE receptor in eosinophil toxicity to schistosomula. The mouse eosinophil has since been shown to lack Fcε receptors, and it is thought that Fcγ receptors may compensate (de Andres *et al.*, 1997).

The elevated peripheral blood eosinophilia seen in helminth infection suggests the involvement of IL-5 in the protective response. An early study revealed that the effector function of eosinophils obtained from patients with eosinophilia (due to schistosomiasis or other infection with other helminths) correlated directly with the patient's peripheral blood eosinophil levels (David *et al.*, 1980). This suggested that enhanced activation *in*

vivo by an (as then) unknown factor or factors, translated to enhanced killing activity *in vitro*. In 1989, IL-5 was shown to be necessary for the induction of both blood and tissue eosinophilia in a mouse model of *Nippostrongylus brasiliensis* infection (Coffman *et al.*, 1989), and in 1990, the regulation of parasite induced eosinophilia in helminth infected patients was attributed to selectively increased IL-5 production by circulating $CD4^+$ T lymphocytes (Limaye *et al.*, 1990). Therefore, IL-5 was proposed to regulate the peripheral blood and tissue eosinophilia seen during helminth infections.

1.2.1 Animal models of parasite infection.

Mouse and other rodent parasite models have provided valuable information on the cell types that are involved in protective immunity to parasites, although there are certain anomalies which may be related to using these non-natural hosts.

Early studies in mice implicated eosinophils in the *in vivo* killing and clearance of *S. mansoni*. In egg-sensitized mice, the administration of anti-eosinophil serum suppressed peripheral blood eosinophils, and ablated eosinophils from granulomatous tissue lesions (Mahmoud *et al.*, 1975a). In comparison to controls, mice infected with 10 cercariae of *S. mansoni* 16 and 32 weeks before challenge with 500 cercariae showed significant reductions in the recovery of schistosomula (40%) and adult worms (60%), indicating an acquired immune response to this parasite. In these immune mice, the administration of anti-eosinophil serum (but not anti-neutrophil or anti-leukocyte serum) increased the worm burden to levels previously seen in non-immune control mice, and suggesting a role for eosinophils in parasite clearance (Mahmoud *et al.*, 1975b). Notably, the transfer of immune sera could confer protection to *S. mansoni* in uninfected mice (Sher *et al.*, 1975), and was inhibited by the administration of anti-eosinophil serum (Mahmoud *et al.*, 1975b). Therefore, immune mouse sera contained transferable humoral components that relied on endogenous eosinophils, in order to confer protection to non-immune mice.

More recently, it has been recognised that the importance for IL-5 and eosinophils in protective immunity to helminth infections varies according to choice of parasite or the host species. For example, IL-5 and eosinophils are not obligatory in the immune resistance to *Mesocestoides corti* (Kopf *et al.*, 1996), *Trichinella spiralis* (Herndon and Kayes, 1992), nor *S. mansoni* (Sher *et al.*, 1990; Brunet *et al.*, 1999), however the elevated eosinophilia seen in IL-5 transgenic mice is detrimental to *S. mansoni*

clearance following immunisation (Dent *et al.*, 1997). Conversely, IL-5 and eosinophils are required for protective immunity against *Strongyloides ratti* (Ovington *et al.*, 1998), *Strongyloides stercoralis* (Rotman *et al.*, 1996), and *Onchocerca volvulus* (Lange *et al.*, 1994), and resistant BALB/c mice display greater IL-5 gene expression and peripheral blood eosinophilia than susceptible C57BL/6 mice, during *Nippostrongylus brasiliensis* infection (Zhou *et al.*, 1996).

Most interesting is the altered dependency for IL-5 in primary and secondary immune responses. C57BL/6 mice are naturally resistant to the filarial parasite *Litomosoides sigmodontis*, which is a natural parasite of rats. Both wild type and IL-5 deficient mice display similar innate immunity to this parasite, as assessed by parasite attrition at 40 days post infection. Immunisation with irradiated larvae confers protective immunity in wild type mice, and no parasites may be recovered following challenge. However, in IL-5 deficient animals, there is no protective immunity afforded by immunisation and these animals display a parasite recovery rate that is no different to non-immunised controls (Le Goff *et al.*, 2000). Alternatively, *Strongyloides ratti* infection leads to lower body weights and larger worm burdens in IL-5 deficient mice when compared to wild types controls, yet IL-5 deficient mice display resistance to challenge infections 3 weeks after the primary infection has been expelled (Ovington *et al.*, 1998).

1.2.1.1 Regulatory roles for eosinophils in primary responses to parasites.

In murine schistosomiasis, granuloma formation is dependent on schistosome egg antigen specific $CD4^+$ T cells (Mathew and Boros, 1986; Iacomini *et al.*, 1995), and there is evidence for a requisite Th1 to Th2 shift in the developing granuloma, which is concomitant with egg deposition (Pearce *et al.*, 1991; Pearce and Reiner, 1995). In the *S mansoni* infected mouse, splenocytes are seen to be dividing, and contain intracellular IL-2, whereas granuloma lymphocytes display an activated phenotype, and contain low yet detectable amounts of intracellular IL-4, and are prone to apoptosis (Rumbley *et al.*, 1998).

Careful analysis of splenic eosinophils reveals a heterogeneous population of activated and hypodense cells, with no detectable intracellular IL-4. In contrast, granuloma eosinophils are characteristically activated, have significant amounts of intracellular IL-4, and may be an important source of this cytokine during the development of Th2

responses to egg antigen (Rumbley *et al.*, 1999). Schistosome eggs are strong inducers of a Th2 response (Sabin and Pearce, 1995), and the injection of eggs into the mouse peritoneum induces rapid IL-4 production by peritoneal eosinophils, which are recruited in response to IL-5 produced by mast cells (Sabin *et al.*, 1996). IL-5 deficient mice are unable to mount a peripheral blood or tissue eosinophilia, yet produce basal numbers of eosinophils with normal morphology (Kopf *et al.*, 1996). While Th2 development following immunisation with schistosome egg antigen is unaffected in IL-5 deficient mice, there is still evidence for a small population of IL-4 producing eosinophils that may participate in establishing Th2 mediated immunity (Brunet *et al.*, 1999). Similarly, migration through the lung by the larval stage of *Nippostrongylus brasiliensis* induces a powerful Th2 response, and eosinophils were recently shown to be a significant source of early IL-4 during this period (Shinkai *et al.*, 2002). Therefore, the eosinophil may be an important source of IL-4 during the induction of Th2 responses to parasites.

Strongyloides stercoralis is a parasitic nematode of humans whose complete life cycle is not supported in the mouse (Grove *et al.*, 1986). The innate response to primary infection leads to larval killing, which correlates with increased serum levels of IL-5, recruitment of eosinophils, and eosinophil peroxidase release adjacent to the parasite. Recently, the presence of eosinophils during immunisation to *S. stercoralis* was shown to be necessary for *S. stercoralis*-specific IgM antibody production, which is a component of the protective immune response to this parasite (Herbert *et al.*, 2000). In this study, Herbert *et al* speculated that eosinophils may be an important source of IL-5 during development of protective immunity, necessary for B cell activation and IgM production.

In summary, the eosinophil may play functionally diverse roles in parasite elimination. In one instance, the eosinophil may be required during primary responses to parasites, whilst having no obligatory role during protective immunity (and vice versa). Alternatively, the eosinophil has the capacity to modulate adaptive responses to parasite infection, through the synthesis of cytokines including IL-4 and IL-5, and may be necessary for certain aspects of protective immunity.

1.3 IMMUNOMODULATORY ROLES FOR EOSINOPHILS.

Eosinophils that line the gastrointestinal and urogenital tracts at baseline may play a role in immune surveillance (Matthews *et al.*, 1998; Mishra *et al.*, 1999). When recruited to sites of parasite infection or to inflamed allergic tissues, eosinophils may collectively act as large reservoirs for various T cell growth factors, costimulatory cytokines and inflammatory mediators (see Table 1). Whilst T and B cells have the capacity to rapidly synthesise and secrete many of these mediators, eosinophils are biologically different, in that they have the ability to synthesise and store these preformed mediators – often in close association with the crystalline granule proteins (Dubucquoi *et al.*, 1994; Moqbel *et al.*, 1995; Bosse *et al.*, 1996). Within tissue, the release of stored mediators such as IL-3 (Moqbel *et al.*, 1995), IL-5 (Dubucquoi *et al.*, 1994) and GM-CSF (Nakajima *et al.*, 1996) may act in an autocrine fashion to promote eosinophil survival, whilst others including co-stimulatory IL-1, IL-6, and either IL-2 or IL-4 may enable the eosinophil to participate in immune regulation of T lymphocyte function during primary and secondary responses to antigen.

1.3.1 TH1 CYTOKINE PRODUCTION BY EOSINOPHILS

1.3.1.1 IL-2.

IL-2 is essential for T cell growth and proliferation *in vitro* (Watson, 1979), and is also chemotactic for eosinophils that express the IL-2 receptor (Rand *et al.*, 1991b). IL-2 expression in human peripheral blood eosinophils may be upregulated in response to GM-CSF (Bosse *et al.*, 1996), and IL-2 associated with the eosinophil crystalloid granule proteins may be released following incubation with immune complexes (Levi-Schaffer *et al.*, 1996). Of particular interest is the unexpected finding that eosinophils express surface CD28 (Woerly *et al.*, 1999), which is normally expressed by T cells and is important in T cell activation (Gimmi *et al.*, 1991; Gause *et al.*, 1997). CD28 ligation induced by antibodies to CD28 promotes the release of biologically active IL-2 from peripheral blood eosinophils (Woerly *et al.*, 1999). Activated T cells in human peripheral blood have been observed to express B7 (CD80) (Azuma *et al.*, 1993),

TABLE 1
Eosinophil Derived Cytokines and Chemokines

Mediator	Eosinophil Origin/Tissue	Molecule Detected	Release Factor/s	Protein Location	References
CYTOKINES					
Interleukin-1α	Human/Blood	mRNA protein	PMA	-	(Weller *et al.*, 1993)
	Mouse/Peritoneal cavity	mRNA protein [#]	LPS calcium ionophore		(Del Pozo *et al.*, 1990)
Interleukin-2	Human/Blood	mRNA protein	GM-CSF &/or calcium ionophore serum coated beads CD28 ligation (IL-10 inhibits)	crystalline granule core	(Bosse *et al.*, 1996) (Levi-Schaffer *et al.*, 1996) (Lacy *et al.*, 1998) (Woerly *et al.*, 1999)
	Mouse/Granuloma	mRNA protein			(Rumbley *et al.*, 1999)
Interleukin-3	Human/Blood	mRNA protein	immune complexes or TNF-α calcium ionophore		(Nakajima *et al.*, 1996) (Kita *et al.*, 1991)
	Human/Gut	protein		granule matrix	(Desreumaux *et al.*, 1996)
Interleukin-4	Human/CD34 Progenitor	mRNA protein		diffuse	(Velazquez *et al.*, 2000)
	Human/Blood	mRNA protein	immune complexes TNF-α serum coated particles calcium ionophore eotaxin, RANTES IL-16 (via eotaxin & RANTES)	 crystalline granule core crystalline granule core	(Nonaka *et al.*, 1995) (Nakajima *et al.*, 1996) (Moqbel *et al.*, 1995) (Bjerke *et al.*, 1996) (Bandeira-Melo *et al.*, 2001) (Bandeira-Melo *et al.*, 2002)
	Human/Lung	mRNA protein		crystalline granule core	(Moller *et al.*, 1996b) (Ying *et al.*, 1997a) (Nonaka *et al.*, 1995)
	Mouse/Granuloma	mRNA			(Rumbley *et al.*, 1999)
	Mouse/Peritoneal cavity	protein	Schistosome eggs		(Sabin *et al.*, 1996)
	Mouse Lung	protein			(Shinkai *et al.*, 2002)

	Eosinophil	Molecule			

Mediator	Origin/Tissue	Detected	Release Factor/s	Protein Location	References
Interleukin-5	Human/BALF	mRNA			(Broide *et al.*, 1992)
	Human/Blood	mRNA	GM-SCF, IL-5, immune complexes	granule matrix	(Dubucquoi *et al.*, 1994)
		protein			(Desreumaux *et al.*, 1993)
					(Lacy *et al.*, 1998)
	Human/Various tissues	mRNA			(Desreumaux *et al.*, 1992)
					(Desreumaux *et al.*, 1993)
		protein		crystalline granule core	(Moller *et al.*, 1996a)
					(Ying *et al.*, 1997a)
				granule matrix	(Desreumaux *et al.*, 1996)
	Mouse/Granuloma	mRNA			(Rumbley *et al.*, 1999)
Interleukin-6	Human/Blood	mRNA			(Melani *et al.*, 1993)
		protein	IFN-γ		(Hamid *et al.*, 1992)
				crystalline granule core	(Lacy *et al.*, 1998)
Interleukin-10	Human/Blood	mRNA		-	(Nakajima *et al.*, 1996)
		protein	sIgA immune complexes		(Woerly *et al.*, 1999)
			FcεRI engagement		(Kayaba *et al.*, 2001)
Interleukin-12	Human/Blood	mRNA	IL-4 or GM-CSF >> IL-5 or IFN-γ	-	(Grewe *et al.*, 1998)
		protein			
Interleukin-13	Human/Blood	mRNA	eotaxin >>IL-5 or GM-CSF		(Schmid-Grendelmeier *et al.*, 2002)
		protein	CD28 ligation	crystalline granule core	(Woerly *et al.*, 2002)
	Human/Nasal polyp	protein			(Schmid-Grendelmeier *et al.*, 2002)
GM-CSF	Human/Blood	mRNA	calcium ionophore/PMA		(Kita *et al.*, 1991)
			immune complexes		(Nakajima *et al.*, 1996)
		protein	LPS (IL-10 inhibits)		(Takanaski *et al.*, 1994)
				crystalline granule core	(Levi-Schaffer *et al.*, 1995)
			c5a, FMLP, calcium ionophore		(Miyamasu *et al.*, 1995)
			CD40 ligation		(Ohkawara *et al.*, 1996)
			TNF-α and fibronectin		(Esnault and Malter, 2001)
	Human/BALF	mRNA			(Broide *et al.*, 1992)
		protein			(Esnault and Malter, 2001)
	Human/Gut	protein		granule matrix	(Desreumaux *et al.*, 1996)

Mediator	Eosinophil Origin/Tissue	Molecule Detected	Release Factor/s	Protein Location	References

Interferon-γ	Human/Blood	mRNA protein	CD 28 ligation (IL-10 inhibits)	-	(Lamkhioued *et al.*, 1996) (Woerly *et al.*, 1999)
	Mouse/Granuloma	mRNA			(Rumbley *et al.*, 1999)
CHEMOKINES					
Interleukin-8	Human/Blood	mRNA protein	calcium ionophore LPS (IL-10 inhibits) c5a, FMLP (G protein dependent) GM-CSF/RANTES, PAF or PMA immune complexes IL-5/immune complexes	-	(Braun *et al.*, 1993) (Takanaski *et al.*, 1994) (Miyamasu *et al.*, 1995) (Yousefi *et al.*, 1995) (Nakajima *et al.*, 1996) (Nakajima *et al.*, 1997)
Interleukin-16 (LCF)	Human/Blood	mRNA protein	 GM-CSF	 cytoplasmic granules	(Lim *et al.*, 1995) (Lim *et al.*, 1996)
Eotaxin	Human/Blood	mRNA protein	IL-3 C5a, calcium ionophore IL-5, TNF-α IL-16	 granule associated	(Garcia-Zepeda *et al.*, 1996) (Nakajima *et al.*, 1998) (Han *et al.*, 1999) (Bandeira-Melo *et al.*, 2002)
RANTES	Human/CD34^{+}	mRNA protein		diffuse	(Velazquez *et al.*, 2000)
	Human/Blood	mRNA	 GM-CSF IFN-γ/serum coated particles IFN-γ	 extra granule associated	(Lim *et al.*, 1995) (Lim *et al.*, 1996) (Ying *et al.*, 1996) (Chihara *et al.*, 1997) (Lacy *et al.*, 1999)

Adapted from Lacey *et al.*, 1997 (Lacy and Moqbel, 1997).

Abbreviations; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; LCF, leukocyte chemotactic factor; LPS, lipopolysaccharide; PAF, platelet activating factor; PMA, Phorbol myristate acetate.

[#] An IL-1α "like-protein" was secreted."-" Not indicated.

and it is possible that any interaction with CD28 expressing eosinophils may elaborate eosinophil derived IL-2, and thus potentiate T cell activation and/or proliferation.

1.3.1.2 IL-12 and IFN-γ.

IL-12 is considered to be a macrophage derived cytokine that drives the differentiation of IFN-γ producing Th1 cells (Brunda, 1994), that selectively express the IL-12 receptor β2 chain (Rogge *et al.*, 1997; Rogge *et al.*, 1999), and in the absence of accessory cells, IL-12 can promote the differentiation of naïve $CD4^+$ T lymphocytes into IFN-γ producing Th1-type cells (Schmitt *et al.*, 1994). The mucosal delivery of IL-12 can abrogate airways eosinophilia hyperreactivity in a mouse model of allergic airways disease (Sur *et al.*, 2000a), and may be mediated by the endogenous production of IFN-γ (Hogan *et al.*, 1998b). Therefore, IL-12 has the potential to abrogate allergic airways disease when delivered to the mucosal surface.

Human peripheral blood eosinophils obtained from both atopic and non-atopic donors synthesise and secrete biologically active IL-12 in response to incubation with IL-4, IL-5 or GM-CSF, which may enable eosinophils to promote a switch from Th2-like to Th1-like immune responses in both atopic and parasitic diseases (Grewe *et al.*, 1998). Human peripheral blood eosinophils may fall into two distinct groups that produce either Th1 (IFN-γ), or Th2 (IL-4, IL-5, IL-10) cytokines (Lamkhioued *et al.*, 1996). Ligation of CD28 on a homogenous mixture of peripheral blood eosinophils promotes IL-2 and IFN-γ release, while secretory IgA complexes induce IL-10 production (Woerly *et al.*, 1999). In the presence of both stimuli (anti-CD28 and secretory IgA-anti-IgA), IL-2, IL-10 and IFN-γ production are all attenuated, suggesting that IL-10 may inhibit IL-2 and IFN-γ release from eosinophils and vice versa. Clearly, there are multiple pathways that may operate during eosinophil synthesis and secretion of Th1 cytokines, and eosinophils may also be categorised into Th1- and Th2-like subpopulations, that may potentially regulate cytokines released by each other.

1.3.2. TH2 CYTOKINE PRODUCTION BY EOSINOPHILS

1.3.2.1 IL-4, IL-13 and IL-10.

IL-4 is an immunoregulatory cytokine of particular importance for T cell activation and differentiation into the Th2 phenotype (Snapper and Paul, 1987; Swain *et al.*, 1990),

and for B cell isotype switching in favour of IgE production (Finkelman *et al.*, 1988b; Finkelman *et al.*, 1990). It may enhance eosinophil accumulation in inflamed tissues by selectively upregulating VCAM-1 expression on the vascular endothelium (Patel, 1998; Sanz *et al.*, 1998a), and through the induction of eotaxin expression (Mochizuki *et al.*, 1998). Human eosinophils also express a functional IL-4 receptor, and priming by IL-4 may enhance eosinophil chemotactic responses to RANTES (Dubois *et al.*, 1998).

In human peripheral blood eosinophils, IL-4 is associated with the crystalline granule core, and may be secreted in response to calcium ionophore, immune complexes (Moqbel *et al.*, 1995; Bjerke *et al.*, 1996), RANTES, eotaxin (Bandeira-Melo *et al.*, 2001), and IL-16 (Bandeira-Melo *et al.*, 2002). In the bronchial mucosa of allergic asthmatics, a population of activated eosinophils display immunoreactivity for IL-4 protein (Moller *et al.*, 1996b), while eosinophils in nasal polyp tissue show even greater immunoreactivity (by comparison) (Nonaka *et al.*, 1995). Interestingly, immunoreactivity for IL-4 may be localised to eosinophils from either atopic, or nonatopic asthmatics, but not to T cells, which contain greater amounts of IL-4 mRNA (Ying *et al.*, 1997a). This may be due to the ability of eosinophils to store pre-formed IL-4 within their secondary granule structures, and eosinophils are unique in that IL-4 starts to accumulate during early maturation and differentiation from $CD34^+$ progenitor cells (Velazquez *et al.*, 2000).

IL-13 shares some common biological functions with IL-4, and IL-13 deficient mice show impaired development of Th2 cells (McKenzie *et al.*, 1998). In a similar fashion to IL-4, IL-13 can enhance endothelial expression of VCAM-1 (Bochner *et al.*, 1995), and P-selectin (Woltmann *et al.*, 2000), which promote eosinophil adhesion via VLA-4 and P-selectin glycoprotein ligand-1 respectively. IL-13 can also induce eotaxin production in nasal tissue fibroblasts (Terada *et al.*, 2000), and airways epithelial cells (Li *et al.*, 1999), although both IL-5 and eotaxin are required for IL-13 induced recruitment of eosinophils to lung tissue (Pope *et al.*, 2001).

IL-13 mRNA cannot be detected in human peripheral blood eosinophils from either atopic or non-atopic individuals, but can be induced following *in vitro* stimulation with IL-5 or GM-CSF (Schmid-Grendelmeier *et al.*, 2002). Interestingly, peripheral blood eosinophils from atopic individuals already contain detectable IL-13 protein, which may be a direct result of differential levels of circulating IL-5 and other cytokines during

their differentiation and maturation. Incubation with IL-5 or GM-CSF can induce IL-13 synthesis by peripheral blood eosinophils, and either eotaxin (Schmid-Grendelmeier *et al.*, 2002) or CD28 ligation (Woerly *et al.*, 2002) can effect IL-13 release.

IL-10 was originally characterised as a factor generated by mouse Th2 cells, which inhibited the cytokine synthesis from Th1 cells (Fiorentino *et al.*, 1989). However, IL-10 has since been shown to down-regulate both Th1 and Th2 inflammatory responses (Pretolani and Goldman, 1997; Muraille and Leo, 1998). IL-10 is constitutively expressed in eosinophils (Nakajima *et al.*, 1996), and secretory IgA complexes can induce IL-10 release from peripheral blood eosinophils obtained from allergic (Woerly *et al.*, 1999), but not normal (Nakajima *et al.*, 1996) subjects *in vitro*. Recently, human peripheral blood eosinophils were shown to express FcεRI (the high affinity receptor for IgE), and cross-linking this receptor using antibodies was shown to induce IL-10 release (Kayaba *et al.*, 2001). It should be emphasised that eosinophils that coexpress IL-4 and IL-5 also coexpress IL-10, but not IFN-γ, identifying two distinct populations of eosinophils which express either Th1 or Th2 cytokines (Lamkhioued *et al.*, 1996).

1.3.2.2 IL-3, IL-5, and GM-CSF.

IL-3, IL-5 and GM-CSF are eosinopoietic cytokines implicated in the inflammation of the airways specifically as seen in asthma, which has been reviewed elsewhere (Arm and Lee, 1992). Each molecule signals through a receptor comprising a ligand-specific α-chain, and a common shared subunit (β_c), and each may prolong eosinophil survival *in vivo* (Corrigan *et al.*, 1995; Till *et al.*, 1995).

In the human gut, IL-3 is associated with the eosinophil-crystalline granule core (Desreumaux *et al.*, 1996), and human peripheral blood eosinophils incubated with calcium ionophore release both IL-3 and GM-CSF, which act in an autocrine fashion to promote eosinophil survival *in vitro* (Kita *et al.*, 1991). IL-5 is a key cytokine involved in eosinophil regulation (Kopf *et al.*, 1996), and it is interesting that this cytokine may be localised to crystalloid granules in eosinophils obtained from blood and gut tissue (Dubucquoi *et al.*, 1994), and from the bronchial mucosa (Moller *et al.*, 1996a). GM-CSF is the main cytokine that enhances eosinophil survival in the airways of symptomatic asthmatics (Park *et al.*, 1998), and like IL-3 and IL-5, has also been localised to the crystalline granules in blood (Levi-Schaffer *et al.*, 1995) and tissue

(Desreumaux *et al.*, 1996) eosinophils. Interestingly, GM-CSF may be released from peripheral blood eosinophils in response to CD40 ligation (Ohkawara *et al.*, 1996), and suggests a role for CD40 ligand expressed by T cells in eosinophil survival *in vivo*.

1.3.3 CHEMOKINE PRODUCTION BY EOSINOPHILS.

1.3.3.1 RANTES.

RANTES (Regulated on Activation, Normal T-cell Expressed and Secreted) is a CC chemokine that is a potent chemoattractant for $CD4^+/CD45RO^+$ memory T cells (Schall *et al.*, 1990). RANTES is also chemotactic for eosinophils *in vitro* (Rot *et al.*, 1992; Alam *et al.*, 1993), and the cutaneous injection of RANTES induces eosinophil recruitment in human subjects, which is more rapid in allergic individuals (Beck *et al.*, 1997). Eosinophils obtained from the peripheral blood of normal subjects constitutively express mRNA and RANTES protein (Lim *et al.*, 1996), and in peripheral blood eosinophils from atopics, constitutive RANTES expression may be upregulated by IFN-γ (Ying *et al.*, 1996).

Recently, RANTES protein was localised to the granule matrix, and also to an extra-granular compartment in human peripheral blood eosinophils. Transitory incubation with IFN-γ (10 minutes) induced rapid release of extra-granular RANTES, while prolonged incubation (60 minutes) reduced RANTES immunoreactivity associated with the granules (Lacy *et al.*, 1999). Eosinophils express functional IFN-γ receptors (Ishihara *et al.*, 1997), and IFN-γ can also modulate eosinophil expression of FcγRIII (low affinity IgG receptor) (Hartnell *et al.*, 1992) and IL-6 (Hamid *et al.*, 1992). Although commonly regarded as a Th1 type cytokine, increased IFN-γ may be found in serum of acute asthma sufferers (Saito *et al.*, 1988; Corrigan and Kay, 1990; ten Hacken *et al.*, 1998), and an expanded population of IFN-γ secreting $CD8^+$ T cells are also found in lung tissue of patients dying of asthma (O'Sullivan *et al.*, 2001). Therefore, eosinophils in acute asthma patients may be exposed to IFN-γ in either the blood, or the lung, which may modulate eosinophil activation and function via release of chemotactic RANTES, IL-6, and Fc receptor expression.

1.3.3.2 IL-16.

IL-16 is increased in the bronchial mucosa of subjects with asthma (Laberge *et al.*, 1997), and in BALF following segmental allergen challenge (Krug *et al.*, 2000). IL-16 is a chemotactic factor for $CD4^+$ T cells (Cruikshank *et al.*, 2000) and human (but not mouse) $CD4^+$ eosinophils cells (Rand *et al.*, 1991a). IL-16 is constitutively expressed in peripheral blood eosinophils from healthy donors, and lysis of these cells releases detectable IL-16 protein (Lim *et al.*, 1995). When cultured *in vitro* with GM-CSF, peripheral blood eosinophils from healthy donors may be induced to secrete IL-16 (Lim *et al.*, 1996), which may act in an autocrine manner to promote RANTES, eotaxin, and eventual IL-4 release (Bandeira-Melo *et al.*, 2002).

1.3.3.3 IL-8.

IL-8 is a member of the C-X-C family of proinflammatory cytokines, initially identified as a chemoattractant for neutrophils (Schroder *et al.*, 1987; Gusella *et al.*, 1993), but which also has chemotactic activity for T cells (Larsen *et al.*, 1989; Gesser *et al.*, 1996) and eosinophils (Sehmi *et al.*, 1993; Schweizer *et al.*, 1994). However, the evidence for IL-8 induced chemotaxis in human peripheral blood eosinophils has recently been contested, and a lack of IL-8 receptors CXCR1 and CXCR2 on peripheral eosinophils suggests that IL-8 may not contribute *in vivo* to the influx of eosinophil granulocytes into sites of allergic inflammation (Petering *et al.*, 1999).

Peripheral blood eosinophils may be induced to release IL-8 in response to various stimuli, including LPS (Takanaski *et al.*, 1994) and immune complexes (Nakajima *et al.*, 1996). Peripheral blood eosinophils from atopic dermatitis and bronchial asthma sufferers express IL-8 mRNA and protein, which is released following priming with GM-CSF, and incubation with either RANTES, PAF or PMA (Yousefi *et al.*, 1995). In this study, IL-8 levels in BALF from asthma sufferers were also found to correlate with eosinophil numbers, suggesting that eosinophils may be a source of this cytokine in the asthmatic lung (Yousefi *et al.*, 1995), and may contribute to neutrophil and T cell recruitment to the airways.

1.3.3.4 Eotaxin.

Eotaxin is a CC chemokine that activates guinea-pig eosinophils *in vitro*, and is a selective eosinophil chemoattractant *in vivo* (Griffiths-Johnson *et al.*, 1993). The purification and partial sequencing of eotaxin from BALF of allergic guinea pigs (Jose

et al., 1994b), has led to the subsequent cloning of guinea pig (Jose *et al.*, 1994a), murine (Rothenberg *et al.*, 1995a), and human eotaxin (Garcia-Zepeda *et al.*, 1996). Eotaxin-2 has also been identified, and is a distantly related protein sharing 39% homology to eotaxin (Forssmann *et al.*, 1997). Eotaxin signals via CC chemokine receptor 3 (CCR3) (Combadiere *et al.*, 1995; Kitaura *et al.*, 1996), and acts in concert with IL-5 to promote tissue eosinophilia (Collins *et al.*, 1995; Mould *et al.*, 1997). Functional CCR3 is also expressed on a subset of T cells having the Th2 phenotype, and enables T cells to migrate in response to eotaxin (Gerber *et al.*, 1997; Sallusto *et al.*, 1998).

In human peripheral blood eosinophils, eotaxin expression may be induced in response to IL-3 (Garcia-Zepeda *et al.*, 1996), and eotaxin protein may be synthesized and stored in association with granule proteins (Nakajima *et al.*, 1998). Complement (C5a), IL-5, and IL-16 can all promote the release of eotaxin from peripheral blood eosinophils *in vitro* (Nakajima *et al.*, 1998; Han *et al.*, 1999; Bandeira-Melo *et al.*, 2002), and identifies a pathway wherein eosinophils may promote their own recruitment into sites of inflammation.

In summary, eosinophils have the ability to synthesise and store a variety of cytokines and pre-formed mediators. These mediators may act in auto- and para-crine fashions to modulate both eosinophil and T cell trafficking into tissues, and in turn affect their activation status, and subsequent responses to various stimuli including antigen.

1.4 ACCESSORY CELL FUNCTION OF EOSINOPHILS.

Eosinophils frequently localise to sites of allergen deposition in allergic disorders such as allergic rhinitis and asthma (Gleich *et al.*, 1993; Martin *et al.*, 1996a), and may reside in the lymph nodes draining allergic and inflamed tissues (Roberts, 1966; Friend *et al.*, 2000; Shi *et al.*, 2000). Their ability to express immunomodulatory cytokines, MHC-II proteins, and costimulatory cytokines suggests that eosinophils may also participate as immune regulators (Weller and Lim, 1997).

1.4.1 MHC expression by eosinophils.

For effective antigen presentation to T lymphocytes, APCs must express MHC-II proteins (Watts, 1997; Weenink and Gautam, 1997). Numerous studies have shown that surface MHC-II expression by eosinophils may be upregulated during disease, and *in*

vitro studies have identified both soluble and endothelial factors that may be responsible for increased MHC-II expression.

1.4.1.1 Enhanced MHC-II expression in vivo.

The recruitment of eosinophils into inflamed tissues is associated with the induction of MHC-II protein expression above that which is seen on peripheral blood eosinophils. There are limited data available for enhanced MHC-II protein expression on mouse eosinophils *in vivo*, although peritoneal cavity eosinophils from mice with acquired resistance to *Brugia malayi* (Th2 mediated) have increased MHC-II expression compared to non-resistant mice (Mawhorter *et al.*, 1993), and BALF eosinophils from the allergic mouse lung are also MHC-II positive (Shi *et al.*, 2000). In humans, the MHC is known as HLA (human leukocyte antigen), of which HLA-DP, DQ, and DR represent functional class II molecules. Eosinophil progenitors initially express HLA-DR (Koeffler *et al.*, 1980; Butterfield *et al.*, 1982), but shed this molecule during differentiation and/or maturation, and normal peripheral blood eosinophils do not express this surface antigen (Hansel *et al.*, 1991; Celestin *et al.*, 2001). Segmental antigen challenge in allergic asthmatics reveals that eosinophils residing in the airways exhibit increased HLA-DR expression when compared with peripheral blood eosinophils (Sedgwick *et al.*, 1992), and this trend has also been reported for non-challenged asthma sufferers (Hansel *et al.*, 1991; Beninati *et al.*, 1993; Mengelers *et al.*, 1994). Furthermore, a direct comparison between allergic and non-allergic individuals has also demonstrated increased HLA-DR expression on peripheral blood eosinophils from asthmatics compared to non-allergic controls (Sakamoto *et al.*, 1996). It is interesting to note that the glucocorticoids dexamethasone and hydrocortisone may synergise with IL-3, IL-5 or GM-CSF to enhance HLA-DR expression on peripheral blood eosinophils from normal healthy subjects (Guida *et al.*, 1994), and the administration of either of these drugs to atopic individuals may be reasonably expected to transiently enhance HLA-DR expression on eosinophils residing in the lung.

1.4.1.2 Induction of MHC-II expression in vitro.

The enhanced expression of MHC-II proteins by eosinophils *in vivo* has prompted many elegant *in vitro* studies to determine what soluble factors may be responsible. In the mouse, peritoneal eosinophils obtained from IL-5 transgenics (Tamura *et al.*, 1996), or mice infected with *M. corti* larvae (Del Pozo *et al.*, 1992) may be induced to express MHC class II following incubation with GM-CSF, which is a prominent cytokine in acute airways inflammation (Arm and Lee, 1992; Ohkawara *et al.*, 1997), and GM-CSF induced expression may either be further enhanced by IL-4, or inhibited by IFN-γ (Mawhorter *et al.*, 1993).

Incubation of human peripheral blood eosinophils with anti-CD3 stimulated and pooled T cell supernatants obtained from either healthy (Hansel *et al.*, 1992) or allergic (Hansel *et al.*, 1991) individuals can induce eosinophil HLA-DR expression. This may be due to a synergistic effect between IL-3 and IFN-γ to induce HLA-DR (Hansel *et al.*, 1992), and synergy between GM-CSF and IFN-γ for HLA-DR expression has also been reported (Guida *et al.*, 1994). When looking at the single cytokine level, IL-3 (Guida *et al.*, 1994; Luttmann *et al.*, 1998; Celestin *et al.*, 2001), IL-5 (Guida *et al.*, 1994), and GM-CSF (Beninati *et al.*, 1993; Guida *et al.*, 1994; Mawhorter *et al.*, 1994; Luttmann *et al.*, 1998; Celestin *et al.*, 2001) can all induce *in vitro* expression of HLA-DR and HLA-DP (Guida *et al.*, 1994) on peripheral blood eosinophils from normal healthy donors. Furthermore, antibodies to either of IL-3 or GM-CSF do not inhibit IL-5 induced HLA-DR expression on human peripheral blood eosinophils, indicating that the autocrine production of either of these two molecules in response to IL-5 does not necessarily regulate IL-5-induced HLA-DR expression (Guida *et al.*, 1994). Interestingly, peripheral blood eosinophils co-incubated with fibroblasts require lower effective doses of either IL-3 or GM-CSF to induce HLA-DR expression (Lucey *et al.*, 1989; Beninati *et al.*, 1993; Weller *et al.*, 1993), suggesting that fibroblasts and extracellular matrix may provide co-stimulatory signals for HLA-DR expression by eosinophils. It is also interesting to note that HLA-DR expression induced by either IL-3 or GM-CSF may be inhibited by TGF-β (Luttmann *et al.*, 1998), which has been identified as a suppressor of immune lymphocyte growth and regulation (Kehrl *et al.*, 1986), and indicates a degree of plasticity for the induction of HLA-DR expression by circulating eosinophils.

1.4.2 Costimulatory molecule expression.

Costimulatory molecules provide necessary second signals for positive T cell activation, and a lack of costimulation during MHC-peptide/TCR interaction can lead to clonal anergy (Schwartz, 1990; Van Gool *et al.*, 1999; Corrigall *et al.*, 2000). CD80 (B7.1) and CD86 (B7.2) are expressed by antigen presenting cells, and provide the most potent costimulatory signals through interaction with CD28/CTLA-4 on the T cell surface (Wu *et al.*, 1997). Following TCR engagement by MHC-II (or MHC-I), the costimulatory signal provided by B7 interaction with CD28 stabilises T cell IL-2 mRNA, and increases IL-2 secretion, which results in T cell proliferation and clonal expansion (Linsley *et al.*, 1991; Freeman *et al.*, 1993). Effective CD80 and CD86 costimulation is necessary for allergic airways disease induction and progression in animal models (Krinzman *et al.*, 1996; Harris *et al.*, 1997; Keane-Myers *et al.*, 1997; Tsuyuki *et al.*, 1997; Harris *et al.*, 2001), and demonstrates the importance of these co-signaling pathways.

1.4.2.1 CD80 and CD86 expression.

Eosinophil isolated from the peritoneal cavity of IL-5 transgenic mice constitutively express both CD80 and CD86, which can be further increased by *in vitro* incubation with GM-CSF (Tamura *et al.*, 1996). Airways eosinophils from mice with AAD express both CD80 and CD86, and may also translocate to the draining lymph node, which is a recognised site of T cell activation (Shi *et al.*, 2000). In humans, peripheral blood eosinophils from normal healthy donors lack constitutive expression of CD80 (Mawhorter *et al.*, 1994), and incubation with either GM-CSF or a synergistic mixture of IFN-γ and GM-CSF can induce HLA-DR, but not CD80 surface expression (Guida *et al.*, 1994). IL-3 has been shown to induce CD86 but not CD80 expression on normal peripheral blood eosinophils (Celestin *et al.*, 2001), while peripheral blood eosinophils from hypereosinophilic donors show some constitutive CD86 but not CD80 expression (Woerly *et al.*, 1999). Another costimulatory molecule known as CD40 can interact with CD40 ligand (CD40L) on the T cell surface (Van Gool *et al.*, 1999), and this molecule is expressed by peripheral blood eosinophils. When CD40 expressed by eosinophils is ligated using anti-CD40 antibodies, eosinophil survival is enhanced by the autocrine production of GM-CSF (Ohkawara *et al.*, 1996), and suggests another putative costimulatory pathway for eosinophils to communicate directly with T cells.

1.4.2.2 Adhesion molecule expression.

Adhesion molecules provide for stable intercellular interactions and cross-communication. In addition to B7 molecules, intercellular adhesion molecule 1 (ICAM-1) expressed on the surface of APCs may interact with leukocyte functional antigen 1 (LFA-1) on T cells to reinforce T cell activation by MHC-peptide/TCR interactions. ICAM-1 is not normally found on peripheral blood eosinophils from normal healthy individuals, but may be upregulated by the mixture of soluble factors in pooled anti-CD3 stimulated T cell supernatants, and increases eosinophil adhesion to autologous T cells (Hansel *et al.*, 1992). At the single cytokine level, peripheral blood eosinophils from healthy (Guida *et al.*, 1994) and atopic (Handzel *et al.*, 1998) individuals increase their surface expression of ICAM-1 in response to GM-CSF incubation, while airways eosinophils obtained from asthmatics show greater ICAM-1 (Hansel *et al.*, 1991; Mengelers *et al.*, 1994) and LFA-3 (Mengelers *et al.*, 1994) expression than peripheral blood eosinophils from the same individuals, and identifies a possible pathway for eosinophil-T cell adhesion and interaction via LFA-1 and CD2, respectively.

1.4.2.3 Costimulatory IL-1α, and IL-6.

IL-1α serves as a costimulatory signal for lymphocyte proliferation (Tosato *et al.*, 1990; Weaver and Unanue, 1990), and IL-6 is an important helper factor in primary antigen-dependent T cell activation and proliferation (Heinrich *et al.*, 1990). Incubation of mouse peritoneal cavity eosinophils with lipopolysaccharide can induce IL-1α mRNA, and an IL-1α "like" protein is released following incubation with calcium ionophore (Del Pozo *et al.*, 1990). In human peripheral blood eosinophils, both mRNA transcripts and IL-1α protein have been identified, and its expression may be upregulated by PMA (Weller *et al.*, 1993). The costimulation afforded by IL-1α ad IL-6 is most effective when given in conjunction with other accessory molecule co-receptors (Joseph *et al.*, 1998), and the ability to express and secrete either IL-1α or IL-6 in conjunction with surface expression of MHC-II and B7 co-stimulatory molecules may enable eosinophils to modulate primary T cell responses to antigen.

1.4.3 Intracellular antigen processing.

The ability to take up and process antigen prior to presentation of antigenic peptides on MHC molecules is essential for effective antigen presentation (Watts, 1997). Superantigens such as *Staphylococcus* enterotoxins do not require any intracellular

processing by APCs, nor significant costimulatory molecule activity in order to stimulate T cells (Dellabona *et al.*, 1990; Damle *et al.*, 1993). While eosinophils are quite capable of presenting superantigens (Mawhorter *et al.*, 1994; Tamura *et al.*, 1996), little is known about the eosinophil's ability to capture and present complex exogenous antigens. Incubation with the lysosomotropic agent chloroquine can inhibit eosinophil uptake and processing of *M. corti* (Del Pozo *et al.*, 1992), and phospholipase A_2 (Hansel *et al.*, 1992) complex antigens, suggesting that eosinophils have the capacity to process intracellular antigens.

In the respiratory tract, inhaled allergens are primarily particulate, and may be carried by small dust particles that act as adjuvants (Ormstad *et al.*, 1998; Ormstad, 2000). Although particulate aeroallergens are processed by macrophages, alveolar macrophages are not effective APCs due to lack of co-stimulatory B7 and adhesion molecules (Chelen *et al.*, 1995), and unlike B cells and dendritic cells (van Rooijen, 1990), eosinophils readily interact with this form of antigen in the presence of immunoglobulin (Kassis *et al.*, 1979; Weller, 1991).

The colocalisation of allergen-specific IgE, IgG, IgA, and eosinophils (which express receptors for these immunoglobulins) in mucosal surfaces may facilitate enhancement of antibody-mediated antigen uptake and presentation (Shi *et al.*, 2000) as is observed for other APCs (Gosselin *et al.*, 1992; Maurer *et al.*, 1995). Therefore, eosinophils may engage, internalise, and process particulate antigens, before presenting them in the context of MHC-II.

1.4.4 Eosinophils functioning as antigen presenting cells.

Our knowledge about the ability of eosinophils to present antigen is growing. It has been suggested that eosinophils have distinct spatial locations in tissues and functional characteristics that endow them with unique roles as APCs in contrast to APCs such as dendritic cells, macrophages, and B cells (Shi *et al.*, 2000). These cells are well placed to encounter exogenous antigen within various tissues and mucosal surfaces (Rytomma, 1960; Moller *et al.*, 1996b; Stokes *et al.*, 1996; Mishra *et al.*, 1999), can express MHC antigens and necessary costimulatory molecules , and are able to translocate to the draining lymph nodes, carrying antigenic peptides (Roberts, 1966; Friend *et al.*, 2000; Shi *et al.*, 2000).

1.4.4.1 Presentation of simple antigens.

Eosinophils from IL-5 transgenic mice treated with GM-CSF and incubated with *Staphylococcal enterotoxin* B superantigen can stimulate the proliferation of both lymph node and thymus T cell populations, in a manner that is dependent on CD80 and CD86 signaling (Tamura *et al.*, 1996). Similarly, GM-CSF treated HLA-DR^+ eosinophils may present *Staphylococcal enterotoxin* A to resting human peripheral blood T cells, but are notably less efficient than macrophages (Mawhorter *et al.*, 1994). Interestingly, dexamethasone synergises with GM-CSF to induce HLA-DR expression on peripheral blood eosinophils from healthy donors. When cultured with influenza hemaglutinin $peptide_{307\text{-}319}$ (does not require processing), increasing numbers of HLA-matched (but not HLA-mismatched) dexamethasone/GM-CSF treated eosinophils promotes increasing amounts of T cell proliferation, demonstrating that eosinophils of the correct haplotype may present relevant peptide antigens to T cell clones (Guida *et al.*, 1994). Although simple antigens may not require extensive intracellular processing by eosinophils, the requirement for functional CD86 signaling remains. Recently, purified peripheral blood eosinophils from normal patients were induced to express HLA-DR and CD86 following incubation with IL-3, and these cells were shown to present tetanus toxoid $peptide_{1273\text{-}1284}$ to peptide-specific T cell clones in a manner that was sensitive to anti-CD86 antibody (Celestin *et al.*, 2001), highlighting the importance of B7 molecules in eosinophil presentation of simple peptide antigens.

1.4.4.2 Presentation of complex antigens by mouse eosinophils.

In 1966, heavily tritiated porcine γ-globulin (^{3}H-PγG) injected into the right hind footpad of the naïve mouse was observed to travel rapidly to the right inguinal and axillary lymph nodes, and eventually the draining popliteal lymph node. A careful comparison of autoradiographs and lymph node tissue sections revealed that ^{3}H-PγG protein was predominantly localised to eosinophils residing in these tissues. These ^{3}H-PγG containing eosinophils were seen to cluster in the medulla and cortex regions, and remained in these areas for the duration of the experiment (24 hours) (Roberts, 1966). These results differed from earlier observations of eosinophil trafficking to lymph nodes following internalisation of antigen/antibody complexes (Litt, 1963, 1964a, 1964b), in that they demonstrated a potential role for eosinophil participation during the primary response to complex antigen

In the late 1900's, eosinophils were identified as phagocytic cells (Lopez *et al.*, 1981), having the ability to secrete costimulatory IL-1α (Del Pozo *et al.*, 1990), and capable of expressing functional IL-2 receptor (Plumas *et al.*, 1991). All of these observations coupled with the (then) recent finding that eosinophils could express MHC-II (Lucey *et al.*, 1989) compelled Del Pozo *et al* to explore the capacity for mouse eosinophils to acts as antigen presenting cells *in vitro* (Del Pozo *et al.*, 1992). They observed that GM-CSF treated eosinophils pulsed with *M. corti* antigen or OVA prior to fixation in paraformaldehyde, were respectively, capable of stimulating the proliferation of *M. corti* or OVA-specific T cell clones. This induced proliferation was MHC-restricted, and required intracellularly processing of the relevant antigens prior to fixation. In particular, incubation with anti I-A^d antibody abrogated OVA pulsed eosinophil ability to induce IL-2 production when co-cultured with OVA-specific T cell hybridomas, demonstrating a necessity for MHC-II proteins in eosinophil presentation of complex antigen.

Elegant *in vitro* experiments have indicated the requirement for CD80 and CD86 costimulation during OVA presentation by GM-CSF treated mouse eosinophils (Tamura *et al.*, 1996). In this study, MHC-II^+/$CD80^+$/$CD86^+$ eosinophils induced proliferation of lymph node derived T cells from OVA sensitised C3H/He mice in the presence of exogenous OVA, which was inhibited by antibodies to CD80, CD86, and I-A^k (but not I-A^b). Thus, mouse eosinophils can present complex antigens to T cells *in vitro*, following antigen processing and presentation of peptides in the context of MHC-II. Furthermore, mouse eosinophils may utilise the costimulatory molecules CD80 and CD86, to enhance antigen-specific T cell proliferation and activation.

These i*n vitro* observations of antigen presentation by mouse eosinophils were recently validated by Shi *et al.*, who have demonstrated MHC-II^+/$CD80^+$/$CD86^+$ eosinophil ability to translocate to the paratracheal lymph nodes following intra-tracheal instillation into the allergic mouse lung (Shi *et al.*, 2000). In this report, GM-CSF treated and OVA pulsed eosinophils derived from the BALF of allergic mice promoted OVA-specific T cell proliferation *in vitro*. When instilled into the mouse trachea, bovine serum albumin (BSA), OVA, or human gamma globulin pulsed eosinophils were shown to stimulate the proliferation of lymph node T cells in only those mice that were previously sensitised to relevant antigen, and further analysis showed that only $CD4^+$

but not $CD8^+$ T cells were proliferating in response to the instillation of antigen pulsed eosinophils (Shi *et al.*, 2000).

1.4.4.3 Presentation of complex antigens by human eosinophils.

Peripheral blood eosinophils express HLA-DR in response to GM-CSF, and can present tetanus toxoid antigen to autologous T cells (Weller *et al.*, 1993). In this study, fixing eosinophils in paraformaldehyde following incubation with antigen was required for T cell proliferation, and fixing in paraformaldehyde prior to incubation with antigen inhibited intracellular processing of tetanus toxoid antigen (Weller *et al.*, 1993). A separate study has demonstrated that live eosinophils expressing HLA-DR in response to pre-treatment with IL-3 and IFN-γ could present phospholipase 2 (PLA_2) to PLA_2 specific autologous T cells (Hansel *et al.*, 1992). In both of these studies, antibodies to HLA-DR significantly inhibited eosinophil mediated T cell proliferation, and a requirement for ICAM-1 during antigen presentation was also identified (Hansel *et al.*, 1992). When compared to macrophages, however, human eosinophils appear relatively inefficient at presenting complex antigen that requires intracellular processing (Mawhorter *et al.*, 1994; Celestin *et al.*, 2001), and quantitative presentation by eosinophils may be more significant than qualitative presentation events.

1.4.4.4 Presentation of viral antigens.

Respiratory virus infections trigger exacerbations of human asthma (Busse and Gern, 1997), and activated virus-specific $CD8^+$ T cells can endure in the lungs for up to several months following respiratory infections (Hogan *et al.*, 2001). Human eosinophils can bind rhinovirus on the cell surface, and subsequently present viral antigens to autologous T cells that produce IFN-γ (Handzel *et al.*, 1998). ICAM-1 expression on airways epithelial cells can be selectively induced by IFN-γ (Look *et al.*, 1992), and rhinovirus induced IFN-γ resulting from eosinophil/$CD8^+$ T cell interactions may therefore modulate inflammatory cell infiltration and contribute to viral exacerbations of asthma. Mouse eosinophils can synthesise and store pre-formed IL-4 (Sabin *et al.*, 1996; Shinkai *et al.*, 2002), and are a predominant feature in the allergic mouse lung (Foster *et al.*, 1996; Hamelmann *et al.*, 1997a). Within the allergic lung, virus-specific $CD8^+$ T cells can switch to IL-5 production, by a mechanism that is dependent on IL-4 (Coyle *et al.*, 1995). Unlike eosinophils, T cells do not have the capacity to store pre-formed IL-4, and it is tempting to speculate that $IL\text{-}4^+$ eosinophils

may drive the switching of $CD8^+$ T cells to IL-5 production during the presentation of viral antigens.

In summary, eosinophils are phagocytic cells with the capacity to process complex antigens, and to present antigenic peptides in the context MHC proteins to T cells. These cells also produce a range of immune regulatory molecules, are key features of allergic inflammation, and translocate to draining lymphoid tissues following antigen provocation. Mouse models of experimental asthma provide valuable tools to study the disease mechanisms that underpin this disorder. Eosinophil recruitment to the mouse lung correlates with the development of pathogenic features that are characteristic hallmarks of asthma in humans (Brusselle *et al.*, 1995; Foster *et al.*, 1996; De Sanctis *et al.*, 1997; Tomkinson *et al.*, 2001), and mouse models may therefore be used to further corroborate postulate roles for eosinophils as modulators of T cell function.

1.5 AIMS OF THIS STUDY.

Eosinophils have a demonstrated ability to process and present complex antigens *in vitro*, which is dependent on MHC-II and costimulatory molecule expression. Numerous eosinophils are recruited to the allergic lung in response to antigen deposition, and obtain an activated phenotype. Within the lung, they may actively sample the antigen-rich extracellular milieu, before modulating $CD4^+$ Th2 lymphocyte function through presentation of relevant antigenic peptides. These modulatory events may occur locally at the site of antigen deposition, or distally within draining lymphoid tissues. Ultimately, eosinophils recruited to sites of allergic inflammation may exacerbate Th2 mediated disease, by regulating memory T cell function.

To date, there has been no demonstration of Th2 cytokine release resulting from eosinophil-T lymphocyte interactions. The general aims of this thesis were to examine eosinophil trafficking into the allergic lung and draining lymph nodes, and to investigate antigen presentation by eosinophils *in vitro* and *in vivo*.

Specific aims:

1. Analyse the temporal and spatial distribution of eosinophils in lung and lymphoid tissues during the induction, progression, and resolution of allergic airways disease.
2. Investigate eosinophil surface molecule expression of MHC-II and costimulatory proteins, and determine their capacity to process complex antigen.
3. Analyse eosinophil antigen presenting function *in vitro*, by measuring proliferative responses and any cytokine production resulting from eosinophil-T cell interactions.
4. Determine eosinophil antigen presenting function *in vivo*, by transferring antigen pulsed eosinophils to naïve mice, and measuring the characteristic parameters of allergic airways disease.

Chapter 2

Antigen Inhalation Promotes Eosinophil Accumulation In The Lungs Of Naïve Mice

2.1 INTRODUCTION

In studies of eosinophil homing to respiratory tissue, past attention has been focused on the effects of inflammatory mediators that regulate eosinopoiesis, and those that promote selective eosinophil recruitment. It is generally accepted that IL-5 regulates the growth, differentiation and activation of eosinophils (Lopez *et al.*, 1988; Yamaguchi *et al.*, 1988; Ingley *et al.*, 1991; Ingley and Young, 1991; Foster *et al.*, 2001), and also regulates eosinophil infiltration, airways hyperreactivity and lung damage in mouse models of AAD (Foster *et al.*, 1996; Hamelmann *et al.*, 1997a). For instance, the compartmentalised expression of IL-5 within the airways epithelium of naïve (and unchallenged) mice leads to eosinophil and lymphocyte recruitment to the lung and airways lumen, with concomitant expansion of bronchus-associated lymphoid tissue, airway hyperresponsiveness to methacholine, and airways tissue remodeling (Lee *et al.*, 1997). GM-SCF is another eosinopoietic molecule (Arm and Lee, 1992), that enhances eosinophil survival in the asthmatic lung (Park *et al.*, 1998). In a mouse model of allergic airway disease, transient expression of this molecule within the respiratory epithelium leads to increased eosinophil recruitment to the lung and to the airways lumen, and alters the resolution kinetics to such an extent that eosinophils persist in lung tissue for a sustained period of time following cessation of antigen challenge (Lei *et al.*, 1998).

Eotaxin is a potent and selective eosinophil chemoattractant that induces eosinophil recruitment to tissues (Rothenberg, 1998). Systemic IL-5 administration in naïve mice has been shown to increase peripheral blood eosinophilia, and acts in synergy with eotaxin to promote eosinophil accumulation into tissues of guinea pigs, and mice (Collins *et al.*, 1995; Mould *et al.*, 1997). In eotaxin deficient mice, eosinophil accumulation in the allergic lung is attenuated (Rothenberg *et al.*, 1997), and collectively, these studies demonstrate the capacity for eosinopoietic molecules and selective chemoattractants to cooperatively promote tissue eosinophilia.

Antigen administration to the airways of naïve mice may closely parallel the processes associated with initial human exposure to environmental allergens, and may provide further understanding of the local and systemic cellular responses generated from within this tissue. In mice, exclusive sensitisation via the airways with OVA induces antigen-

specific T cell responses, IgE production and increased airways hyperreactivity upon re-challenge (Haczku *et al.*, 1997; Hamelmann *et al.*, 1997a; Hamelmann *et al.*, 1997c).

Interestingly, the administration of inhibitory antibodies to IL-5 during airways sensitisation attenuates eosinophil recruitment to lung tissue and airways hyperreactivity to cholinergic stimuli, whilst ultimately leaving T- and B-cell responses intact (Hamelmann *et al.*, 1997a). In another model, airways sensitisation in mice using aerosolized OVA required concomitant GM-CSF transgene expression in the respiratory epithelium (Stampfli *et al.*, 1998). Thus, the depletion of IL-5 - which regulates eosinophil maturation from bone marrow, and the expression of eosinopoietic GM-CSF during airways sensitisation can respectively attenuate or promote sensitisation via the airways. Collectively, these data suggest that eosinophils recruited to the lung during airways sensitisation may participate in the priming for AAD. Eosinophils may be a potential source of early IL-4 during primary responses to antigen (Sabin *et al.*, 1996), and it is interesting to observe that IL-4 deficient mice are resistant to sensitisation via the airways (Herrick *et al.*, 2000).

Of particular interest is the elegant work conducted by Mould *et al*., wherein transient expression of both IL-5 and eotaxin within the lung was shown to synergistically promote a pronounced airways eosinophilia in the absence of complex tissue signals associated with AAD (Mould *et al.*, 2000). In this study, eosinophil degranulation and airways hyperreactivity were absent, however the delivery of OVA to the airways both prior to and in the context of IL-5 and eotaxin transgene expression led to degranulation of infiltrating eosinophils and the development of airways hyperreactivity via a mechanism acutely sensitive to $CD4^+$ T cell depletion. It is tempting to speculate that in this study, lung-recruited eosinophils promoted allergic sensitisation via the airways in a manner not dissimilar to that of Stampfli *et al*., (Stampfli *et al.*, 1998), and provides a rationale for the dependency of degranulation and airways hyperreactivity upon $CD4^+$ T cells. In Mould *et al.*, eosinophil degranulation may have been dependent on OVA-specific serum antibodies (not measured, but noted in similar work by Stampfli *et al*.), or possibly due to bi-directional signaling between recruited eosinophils and the emerging population of OVA-specific $CD4^+$ T cells. Ultimately, the expression of eosinophil active agents (GM-CSF, IL-5, eotaxin), in the lungs of naïve mice, can lead to Th2 mediated responses following airways exposure to antigen, and eosinophils may be implicated in this process.

It is further interesting to note that underlying factors exist to mask Th2-type responses following airways sensitisation. Earlier work in rats (McMenamin and Holt, 1993) shows that a strong airways sensitisation regime can lead to transient $CD4^+$ T cell-mediated OVA-specific IgE antibody production, which is then suppressed by the appearance of MHC-I-restricted OVA specific IFN-γ-producing $CD8^+$ T cells. In this study, prolonged exposure to inhaled antigen led to a tolerant state, wherein rats normally predisposed to high IgE antibody production became unresponsive to subsequent immunisation with OVA in adjuvant. Therefore, a window of opportunity may exist within which Th2 type responses may be generated, and extended exposure of the airways to environmental antigen may lead to the generation of opposing regulatory Th1 type responses.

While the kinetics of cellular – particularly eosinophil infiltration into the allergic mouse airways can be measured following antigen sensitisation and challenge (Brusselle *et al.*, 1995; Foster *et al.*, 1996; Schneider *et al.*, 1997; Walker *et al.*, 1998; Hertz *et al.*, 2001) it is somewhat harder to quantify these events when antigen is deposited in the airways of naïve mice. To date, no experiments conducted by others have documented eosinophil infiltration of the antigen-challenged lung at baseline - i.e. in the absence of transient cytokine expression.

In this Chapter, experiments have been designed to investigate eosinophil responses to antigen deposition within the naïve lung. Naïve wild type and eotaxin deficient mice having a non-allergy driven peritoneal eosinophilia and a low peripheral blood eosinophilia were exposed via the airways to aerosolised OVA. The surprising results suggest that eotaxin has an important role in recruiting eosinophils to the naïve airways following antigen deposition, and that these recruited eosinophils may go on to participate in the priming for AAD.

2.2 MATERIALS AND METHODS

2.2.1 Animals.

Wild type C57BL/6 and eotaxin deficient ($^{-/-}$) mice (129/sv background crossed into C57BL/6 to N10) (Rothenberg *et al.*, 1997) were obtained from the Specific Pathogen Free (SPF) unit of the Australian National University (ANU), and housed in an approved containment facility. All mice (male, 4-8 wk old) were treated according to ANU animal welfare guidelines.

2.2.2 Polymyxin B Sulfate induced peritoneal eosinophilia.

Male 4 wk old wild type and eotaxin$^{-/-}$ mice received twice weekly intra-peritoneal injections of Polymyxin B Sulfate (PMBS, Sigma, 0.2mg in 200 μl saline) to induce a peritoneal eosinophilia (Pincus, 1978). A pronounced infiltration of eosinophils into the peritoneal cavity was observed after 12 injections/6 weeks. To determine total eosinophils in the peritoneal exudate cell (PEC) population, mice were sacrificed by CO_2 asphyxiation and the peritoneal cavity lavaged 3 times with 5 ml ice cold Hank's Buffered Salt Solution (HBSS). After centrifugation at 500 g and 4^{o}C for 10 minutes, the cell pellet was resuspended in HBSS and cell numbers were determined using a standard haemocytometer. PEC cells were suspended at 2 x 10^5/100 μl and cytocentrifuged at 500 g for 5 minutes before air-drying and subsequent differential staining with May-Grunwald-Giemsa solution. Eosinophils were identified by morphological criteria, with 200-250 cells counted on each slide.

2.2.3 Antigen delivery to naïve PMBS-treated mice.

Naïve PMBS-treated (at least 6 weeks of twice weekly injections) wild type and eotaxin$^{-/-}$ mice were exposed to an OVA aerosol (10 mg/ml in 0.9% saline) for 3 x 30 minute periods (at 30 min intervals) on days 1, 3, 5 and 7. The OVA aerosol was generated by a RapidFlo nebuliser bowl (Allersearch, Australia) with an airflow rate greater than 10 Litres/minute in order to produce particles of 2 microns in diameter. Blood samples were drawn from the tail vein for differential cell counts on days 0, 2, 4, 6 and 8, and stained with May-Grunwald-Giemsa solution prior to differential cell counting (~210 cells). Mice were sacrificed by cervical dislocation on day 8 for collection and analysis of peritoneal cavity and bronchoalveolar lavage fluids. In a separate experiment, naïve PMBS-treated (at least 6 weeks of twice weekly injections)

wild type mice were exposed to OVA aerosol (10 mg/ml in 0.9% saline) for 3 x 30 minute periods (at 30 min intervals) on days 1, 3, and 5. Blood samples were drawn at 24 hours following each aerosol, and mice were then sacrificed by cervical dislocation for collection and analysis of peritoneal cavity and bronchoalveolar lavage fluids.

2.2.4 Collection and analysis of bronchoalveolar lavage fluid.

Following cervical dislocation, the trachea was exposed by a ventral midline incision through the skin and subcutaneous tissue, and further separation of the submandibular salivary glands and sternohyoid muscle tissue by blunt dissection. A blunted 18-gauge needle (Terumo, USA) was inserted as a cannula through an incision in the upper trachea, providing a good seal. A 1 ml syringe (Temuro, USA) was then fitted to the cannula and the airways were washed with 2 x 1 ml ice-cold HBSS, giving an average return of 1.6 ml bronchoalveolar lavage fluid (BALF) in total. The BALF from each animal was centrifuged at 500 g and 4^{o}C for 10 minutes and the cell pellet was resuspended in 500 μl red blood cell (RBC) lysis solution and incubated at 37^{o}C for 5 minutes before the addition of 1 ml ice cold PBS. The cells were then centrifuged again at 500 g and 4^{o}C for 10 minutes, and the cell pellet resuspended in 500 μl ice-cold PBS before determining cell numbers in a standard haemocytometer. BALF cells (2 x 10^{5}) were then suspended in 100 μl HBSS and cytocentrifuged at 500 g for 5 minutes before differential staining with May-Grunwald-Giemsa solution. Lymphocytes, eosinophils, macrophage and neutrophils were identified by morphological criteria, with 200-250 cells counted on each slide.

2.2.5 Eosinophil purification by fluorescence activated cell sorting.

PMBS-treated wild type mice were sacrificed by CO_2 asphyxiation and the peritoneal cavity lavaged as in Section 2.2.2. Eosinophils in the PEC population were purified by fluorescence activated cell sorting (FACS) on the basis of size (forward scatter), granularity (side scatter), and the ability to polarise light. The purity of the enriched population of eosinophils was >98% as determined by differential staining with May-Grunwald-Giemsa. Purified eosinophils were diluted as required into RPMI-1640 (RPMI) culture medium.

2.2.6 Calcium signaling in PMBS eosinophils.

Purified PMBS eosinophils were incubated at a density of 5 x 10^5 cells per well in round-bottomed 96 well cell culture plates (NUNC) in a total of 200 μl of Mixed Lymphocyte Culture (MLC) medium containing 10% FCS and in the presence or absence of recombinant murine IL-5 (10^{-11} M) for 12 hours at 37^{o}C. Recombinant murine IL-5 was a generous gift from Professor Ian Young (John Curtin School of Medical Research (JCSMR), ANU) and was expressed and purified from the baculovirus expression system (Ingley *et al.*, 1991). The cells were then harvested, washed in RPMI, and incubated at a density of 2 x 10^6 cells/ml in RPMI medium containing 1% FCS and 5 μM Indo-1 AM (1-[2-Amino-5-(6-carboxy-2-indolyl)phenoxy]-2-(2-amino- 5-methylphenoxy)ethane- N,N,N',N'-tetraacetic acid, pentaacetoxymethyl ester) for 60 minutes at 37^{o}C. Cells were then washed twice in ice cold calcium flux buffer containing (mM) NaCl, 145; KCl, 4; NaH_2PO_4, 1; $MgCl_2$, 0.8; HEPES, 25; glucose, 22; $CaCl_2$, 1.8, (pH 7.4) before being resuspended at 1 x 10^6 cells/ml in flux buffer and stored on ice prior to calcium signaling experiments. For calcium signaling, 1 ml of Indo-1 AM loaded eosinophils were introduced into a water-jacket-heated and stirred (~ 60 rpm) cuvette, contained within a Hitachi F-3000 fluorescent scanning spectrophotometer (Japan), with excitation at 340 nm and emission at 390 nm. The cells were allowed to equilibrate at 37^{o}C (typically taking 30-40 seconds), before addition of exogenous factors. The calcium ionophore A23187 (1 μM in flux buffer) and SDS (10% in flux buffer) were used (respectively) to confirm the ability of Indo-1 AM loaded eosinophils to respond to exogenous stimuli, and to measure maximum attainable signals. Where indicated, recombinant mouse eotaxin (rEOT, 5 μg/ml, a gift from A. Mould, JCSMR, ANU) was added to the stirred cuvette system.

2.2.7 Intraperitoneal IL-5 administration to PMBS-treated wild type mice.

IL-5 is an activator of eosinophil function (Lopez *et al.*, 1988). To study the effects of intraperitoneal (i.p.) IL-5 administration on eosinophil homing to sites of antigen deposition, PMBS-treated wild type mice (as in Section 2.2.2) were given a single dose of recombinant murine IL-5 (200ng i.p. in 200 μl 0.9% saline) or vehicle alone 24 hours prior to the first aerosol with antigen (as in Section 2.2.3). BALF was analysed on day 8 (as in Section 2.2.4).

2.2.8 OVA specific IgG_1 determination by ELISA.

Serum samples were taken from the tail veins of naïve PMBS-treated wild type mice on day 0, 24 hours prior to exposure to antigen. Flat bottomed 96-well microtiter plates (Dynatech, Chantilly, VA) were coated with OVA (100 μg/ml in $NaHCO_3$ buffer, pH 9.6) and incubated overnight at 4^{o}C. Plates were then blocked with 3% skim milk powder/PBS for 1 hour at 37^{o}C. After incubation with dilutions of serum samples and standard murine IgG_1 for 1.5 hours at 37^{o}C, OVA-specific immunoglobulins were detected with biotinylated goat anti-mouse IgG_1 (Biosource International, Camarillo, CA). Plates were then incubated with streptavidin-conjugated alkaline phosphatase (Amersham International plc, Buckinghamshire, UK), for 1 hour at 37^{o}C and alkaline-phosphatase substrate solution (Sigma Chemical Co., St Louis, MO), was added. Reactions were stopped with 0.1 M citric acid and the plates read at 405 nm in a Microplate reader (BIO-TEK Instruments Inc., Winooski, VT).

2.3 RESULTS

2.3.1 Eosinophils migrate to antigen deposition in the lung in an eotaxin dependent manner.

Naïve, PMBS-treated wild type and eotaxin$^{-/-}$ mice were exposed to antigen to investigate whether eosinophils would traffic to the airway mucosal surface in response to antigen deposition. Both wild type and eotaxin$^{-/-}$ mice had a pronounced peritoneal eosinophilia in response to PMBS treatment (Figure 2.1), with 2.5 and 1.5 million recoverable eosinophils located in the peritoneal cavity, respectively. Control mice had substantially lower numbers of recoverable eosinophils (17×10^4). There was no significant difference in eosinophil numbers in the peritoneal cavity between wild type or eotaxin$^{-/-}$ mice.

Surprisingly, wild type mice developed a pronounced peripheral blood eosinophilia following the first exposure to OVA, which peaked at 12.5% of total white blood cells after the third OVA challenge on day 5 (Figure 2.2). This strong response was not seen in either eotaxin$^{-/-}$ mice or untreated wild type controls. Subsequent analysis of BALF revealed a pronounced eosinophil infiltration of the airways lumen in naïve PMBS-treated wild type mice (Figure 2.3a), and to a much lesser extent in eotaxin$^{-/-}$ mice, where inhibition of eotaxin function is known to attenuate antigen induced tissue eosinophilia (Rothenberg *et al.*, 1997). Eotaxin$^{-/-}$ mice had significantly more eosinophils/ml BALF than wild type controls, suggesting that an alternative pathway to eotaxin signaling may have been acting to draw eosinophils into the airways lumen. While not significantly different from each other, both wild type and eotaxin$^{-/-}$ mice had significantly greater lymphocytes/ml BALF than wild type controls (Figure 2.3b).

2.3.2 PMBS eosinophils pre-incubated with IL-5 show enhanced in vitro responses to eotaxin.

Interleukin-5 has been identified as a key regulator of eosinophil development, migration and activation (Lopez *et al.*, 1988; Yamaguchi *et al.*, 1988; Coffman *et al.*, 1989; Walsh *et al.*, 1990; Lee *et al.*, 1997), and regulates CCR3 expression (Tiffany *et al.*, 1998). Recombinant IL-5 was used to demonstrate increased PMBS-derived eosinophil responses to eotaxin *in vitro*. Eosinophils enriched from PMBS-treated mice and cultured overnight in the absence of IL-5 showed slightly increased intracellular calcium levels following the addition of 10 μl of rEOT. This increase in intracellular

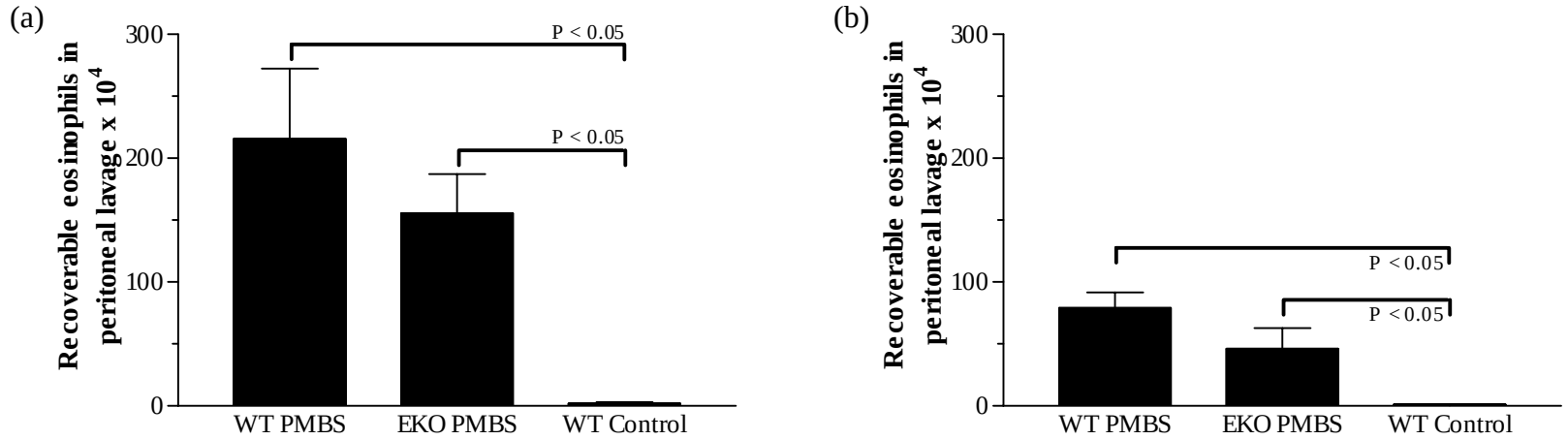


Figure 2.1. Recoverable eosinophils in the peritoneal cavities of naïve PMBS treated mice. (a) Recoverable eosinophils in PMBS treated wild type (WT PMBS), eotaxin$^{-/-}$ (EKO PMBS), and untreated control (WT Control) mice prior to airways exposure to OVA antigen (day 0). Both WT PMBS and EKO PMBS mice had significantly greater numbers of eosinophils residing in the peritoneal cavity than WT Controls. (b) Recoverable eosinophils on day 8, following repeated airways exposure to OVA on days 1, 3, 5, and 7. There was no significant difference in recoverable peritoneal eosinophils between WT PMBS and EKO PMBS mice following repeated exposure to OVA. Data represents mean ± SEM from groups of 6 mice. Significant differences in means were determined by Student's unpaired *t* test ($p < 0.05$), and are indicated by bars (¬).

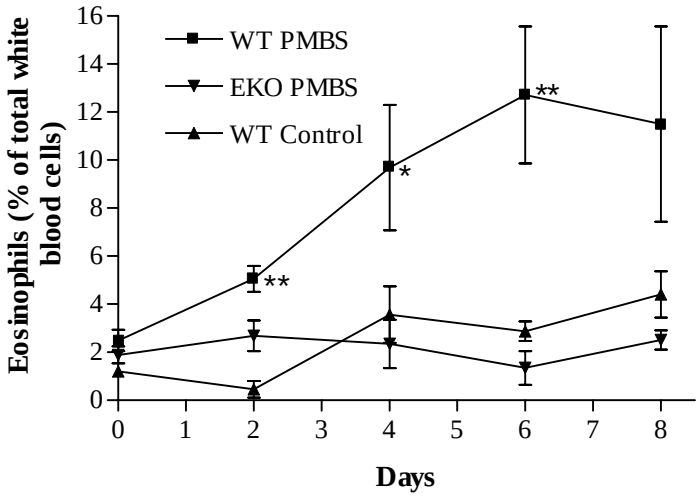


Figure 2.2. Peripheral blood eosinophilia in naïve PMBS treated mice exposed to airways antigen. WT PMBS treated mice developed a pronounced peripheral blood eosinophilia in response to OVA deposition in the naïve airways (days 1, 3, 5, and 7), which was significantly different from EKO PMBS and WT Controls on day 2, and peaked at 12.5% on day 6. Data represents mean ± SEM from groups of 4 mice. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$). * $P < 0.05$ compared to EKO PMBS, and ** $P < 0.05$ compared to EKO PMBS and WT Control mice.

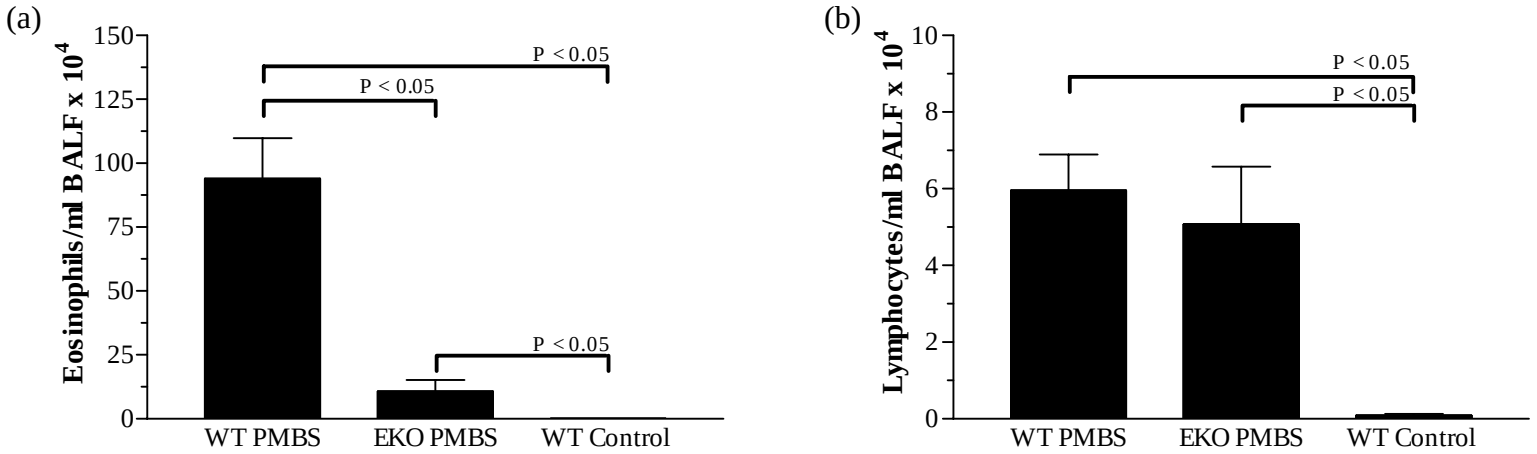


Figure 2.3. Eosinophils and lymphocytes in BALF of naïve PMBS treated mice following airways exposure to antigen. (a) WT PMBS mice had significantly greater eosinophils/ml BALF than EKO PMBS and WT Control mice. EKO PMBS mice had significantly greater eosinophils/ml BALF than WT Control mice. (b) WT PMBS and EKO PMBS mice had significantly greater lymphocytes/ml BALF than WT Controls. Data represents mean ± SEM for groups of 6 mice. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$), and are indicated by bars (¬).

calcium was sustained until the addition of calcium ionophore (Figure 2.4a). The addition of 10 μl rEOT to eosinophils incubated overnight with rMIL-5 resulted in a markedly increased response (Figure 2.4b), which was further sustained until the addition of calcium ionophore, and demonstrates an increased responsiveness to eotaxin for PMBS-derived eosinophils incubated with IL-5.

2.3.3 IL-5 administration in vivo does not enhance PMBS eosinophil homing to the lung after antigen deposition.

IL-5 (200ng) was administered i.p. to PMBS-treated wild type mice ($PMBS^{+IL\text{-}5}$), in an attempt to increase peritoneal eosinophil activation and to accentuate homing to antigen deposition in the naïve lung. For comparison, a subset of PMBS-treated mice received vehicle alone ($PMBS^{+vehicle}$), and untreated wild type mice were used as a control. Recoverable eosinophils were measured in the peritoneum of PMBS-treated mice and untreated wild type control animals prior to and following challenge with OVA. Prior to OVA exposure, PMBS-treated mice had significantly greater numbers of recoverable eosinophils than untreated controls (Figure 2.5a). Following airways exposure to OVA on days 1, 3, 5, and 7, there was no significant difference in recovered peritoneal eosinophils between $PMBS^{+IL\text{-}5}$ and $PMBS^{+vehicle}$ mice (Figure 2.6b). Both $PMBS^{+IL\text{-}5}$ and $PMBS^{+vehicle}$ mice developed a pronounced peripheral blood eosinophilia in response to OVA deposition in the lung (Figure 2.6), with peripheral blood eosinophils peaking at 22% after the third aerosol exposure in $PMBS^{+IL\text{-}5}$ mice. Differences in peripheral blood eosinophils between $PMBS^{+IL\text{-}5}$ and $PMBS^{+vehicle}$ mice were significant on days 2 and 6, which followed aerosol exposures to OVA on days 1 and 5. Analysis of BALF showed no significant differences between $PMBS^{+IL\text{-}5}$ and $PMBS^{+vehicle}$ mice with respect to eosinophils or lymphocytes/ml BALF (Figure 2.7a and b). Both $PMBS^{+IL\text{-}5}$ and $PMBS^{+vehicle}$ mice had significantly greater amounts of eosinophils and lymphocytes/ml BALF than WT Controls.

2.3.4 Eosinophils migrate to the lung in naïve PMBS-treated mice immediately following antigen exposure.

The kinetics of eosinophil migration to sites of antigen deposition were measured using naïve PMBS treated wild type mice exposed to aerosolised OVA on days 1, 3, and 5. Peripheral blood eosinophils were significantly elevated in PMBS treated mice following the 3rd exposure to aerosolised antigen, but not in WT Controls (Figure 2.8). Analysis of BALF revealed a significant infiltration of eosinophils following the first

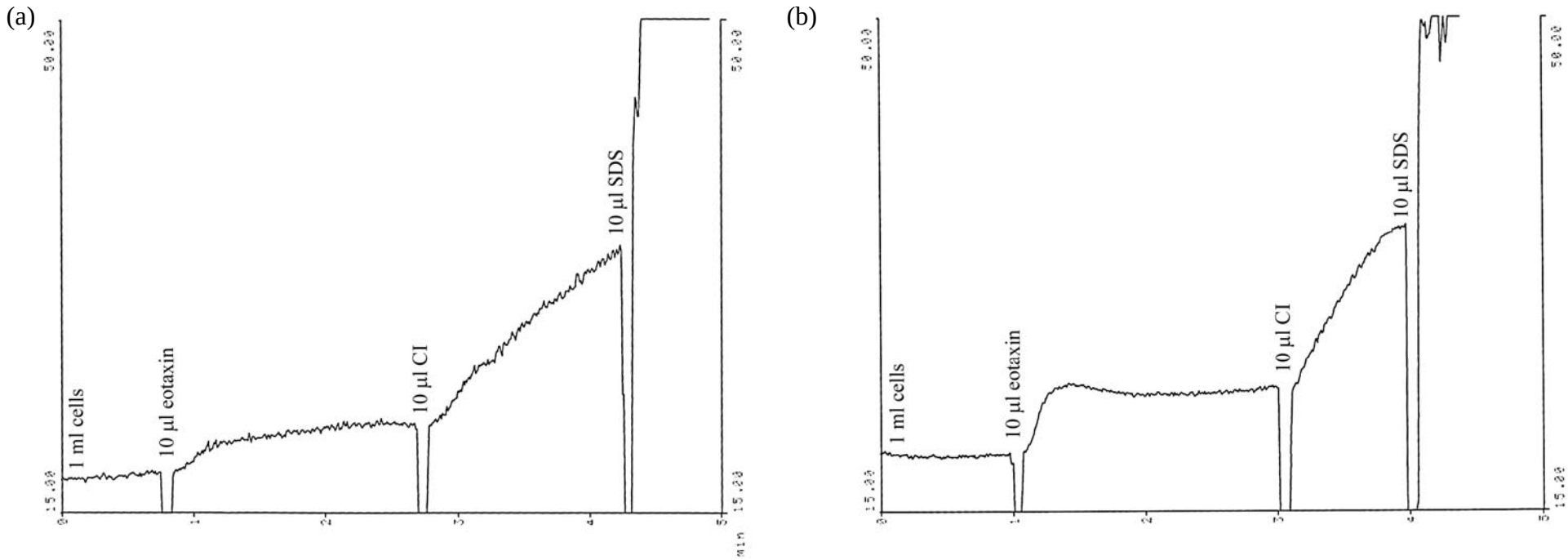


Figure 2.4. Intracellular calcium signaling in FACS sorted PMBS eosinophils pre-incubated in the presence or absence of IL-5 (10^{-11} M). (a) 1 x 10^6 FACS sorted eosinophils were allowed to equilibrate at 37^{o}C prior to the addition of 10 µl (50ng) of eotaxin, which induced a moderate increase in intracellular calcium levels (measured on the Y axis). (b) The addition of 10 µl (50ng) of eotaxin to eosinophils pre-incubated with IL-5 produced a pronounced and sustained increase in intracellular calcium levels. In both instances, the addition of calcium ionophore (CI) further increased intracellular calcium levels, demonstrating cell membrane integrity, and ability of FACS sorted eosinophils to respond to exogenous stimulus. The addition of SDS lysed all cells, giving a maximum attainable signal. Duration in minutes is shown on the x-axis.

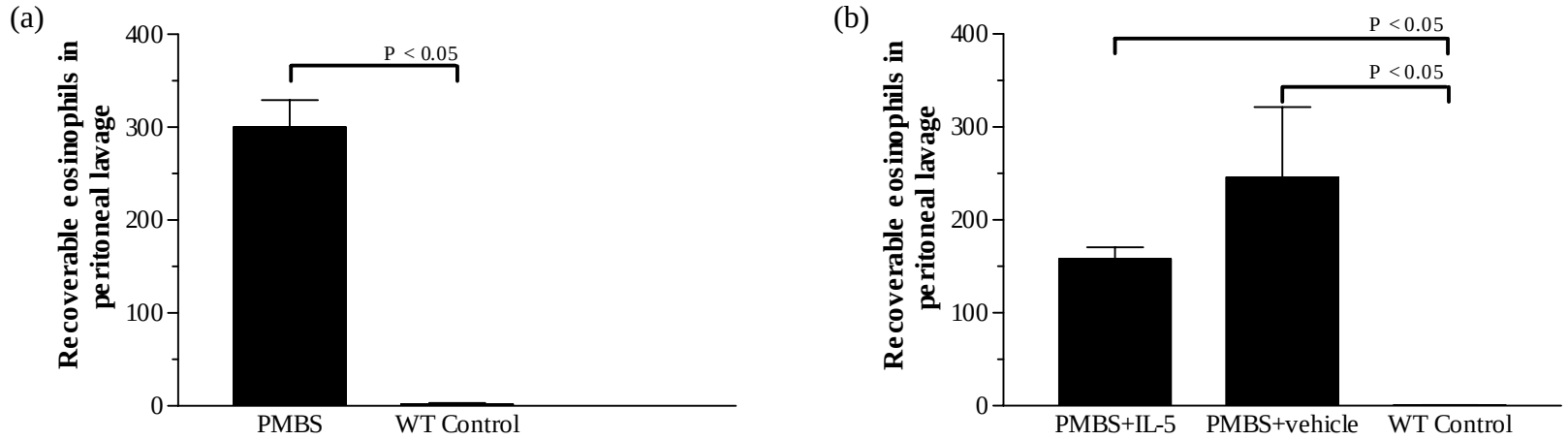


Figure 2.5. Recoverable eosinophils in the peritoneal cavity of naïve PMBS treated mice: effects of i.p. IL-5 administration. (a) Recoverable eosinophils in wild type C57BL/6 PMBS treated mice (PMBS) mice and untreated controls (WT Control) prior to IL-5 administration and airways exposure to OVA antigen. (b) Following exposure to OVA antigen, IL-5 ($PMBS^{+IL\text{-}5}$) and vehicle treated ($PMBS^{+vehicle}$) mice had significantly greater recoverable eosinophils than WT Controls. There was no significant difference in the numbers of recovered peritoneal eosinophils between $PMBS^{+vehicle}$ and $PMBS^{+IL\text{-}5}$ mice exposed to OVA antigen. Data represents mean ± SEM from groups of 4 mice. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$), and are indicated by bars (¬).

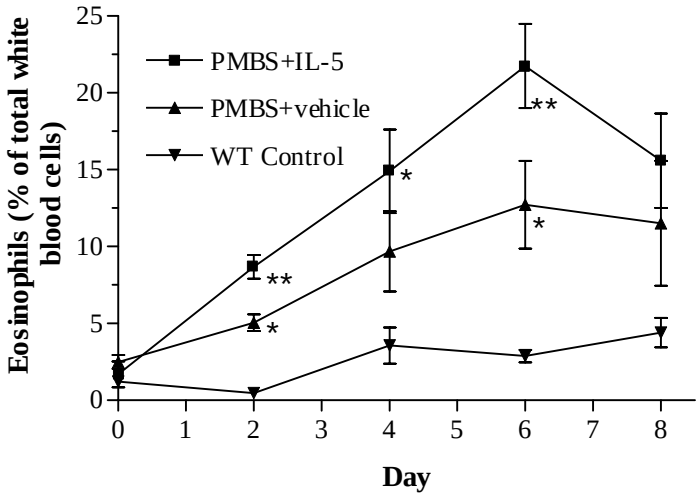


Figure 2.6. Peripheral blood eosinophilia in naïve PMBS treated mice exposed to antigen: effects of i.p. IL-5 administration. Both $PMBS^{+IL\text{-}5}$ and $PMBS^{+vehicle}$ mice developed a pronounced peripheral blood eosinophilia in response to OVA antigen exposure to the airways (days 1, 3, 5, and 7), which was significantly different from WT Control mice following the first antigen exposure on day 1. There was a significant difference in peripheral blood eosinophils between $PMBS^{+IL\text{-}5}$ and $PMBS^{+vehicle}$ treated mice on days 2 and 6. Data represents mean ± SEM from groups of 4 mice. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$). * $P < 0.05$ compared to WT Control; and ** $P < 0.05$ compared to $PMBS^{+vehicle}$ and WT Control mice.

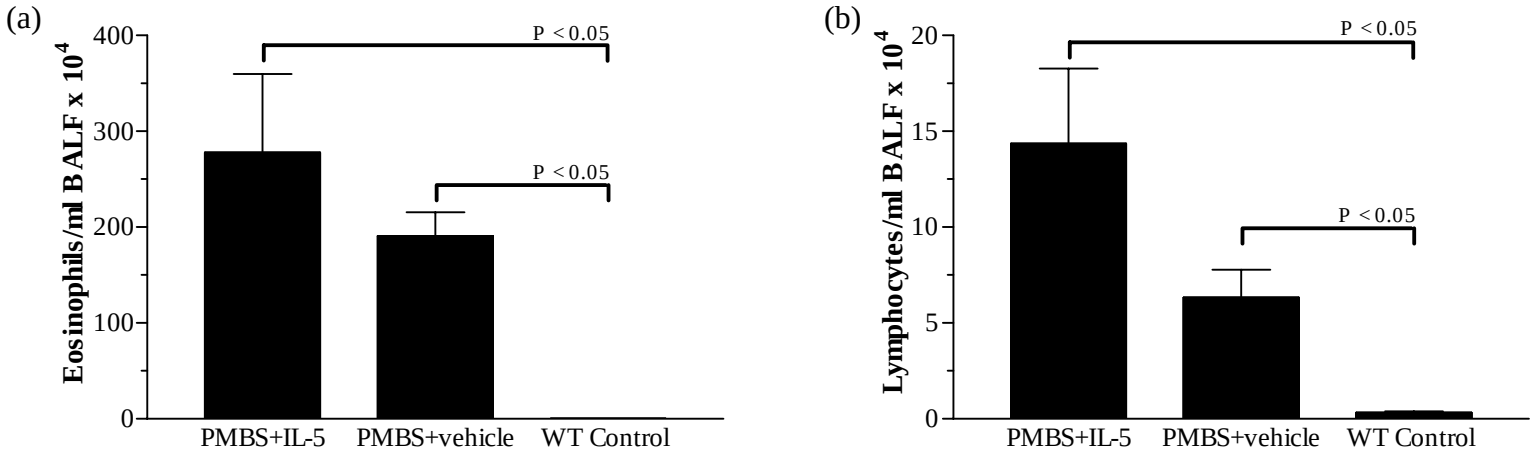


Figure 2.7. Eosinophils and lymphocytes in BALF of naïve PMBS treated mice following airways exposure to OVA antigen: effects of i.p. IL-5 administration. (a) $PMBS^{+IL\text{-}5}$ and $PMBS^{+vehicle}$ mice had significantly greater eosinophils/ml BALF than WT Controls, but were not significantly different from each other. (b) $PMBS^{+IL\text{-}5}$ and $PMBS^{+vehicle}$ mice had significantly greater lymphocytes/ml BALF than WT Controls, but were not significantly different from each other. Data represents mean ± SEM for groups of 4 mice. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$), and are indicated by bars (¬).

OVA aerosol on day 1 (Figure2.9a), with a trend towards greater eosinophil infiltration following each aerosol. Lymphocytes/ml BALF were also measured, and were significantly increased following 2 aerosols (Figure2.9b).

2.3.5 PMBS-treated mice lack detectable serum OVA-specific IgG_1.

To ensure the phenomenon of eosinophil homing to the lung was independent of prior sensitisation to OVA, serum samples from 5-7 mice were analysed for OVA-specific IgG_1 antibodies both prior to and following airways exposure to antigen. In comparison to OVA sensitised and challenged mice (Hogan *et al.*, 1997b), PMBS mice had no detectable OVA-specific IgG_1 above background prior to exposure to OVA (Figure 2.10). Following airways exposure to OVA, PMBS treated mice had detectable serum OVA-specific IgG_1, which was significantly less than the levels seen in OVA sensitised and challenged mice.

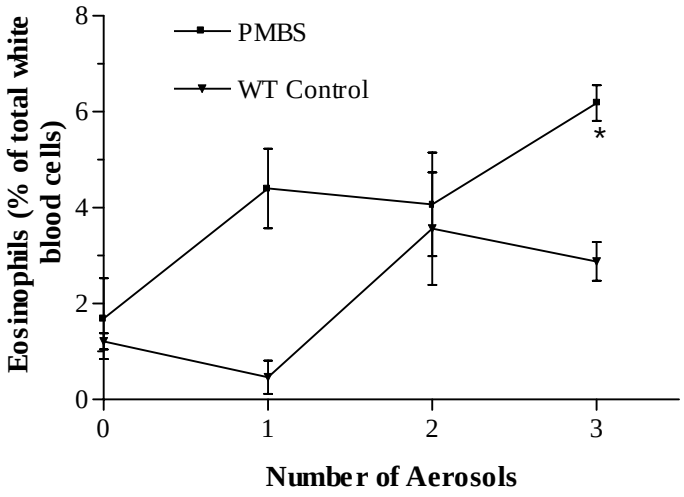


Figure 2.8. Peripheral blood eosinophilia in naïve PMBS treated mice exposed to 0, 1, 2 or 3 OVA antigen aerosols. PMBS treated mice developed a peripheral blood eosinophilia greater than 6% following 3 aerosols, which was significantly different when compared to pre-exposure levels. Peripheral blood eosinophil levels in PMBS mice were not significantly different to WT Controls at any time point. WT Controls did not develop a peripheral blood eosinophilia significantly different to pre-exposure levels. Data represents mean ± SEM from groups of 4 mice. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$). * $P < 0.05$ compared to PMBS treated mice prior to airways exposure to OVA antigen.

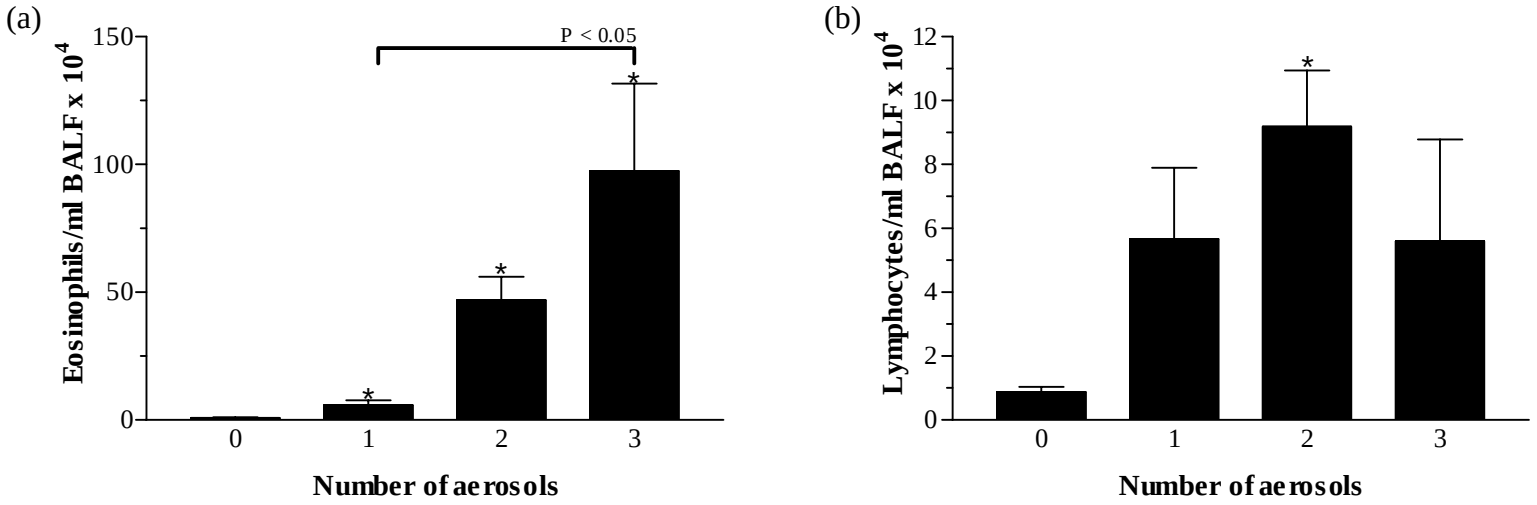


Figure 2.9. Eosinophils and lymphocytes in BALF of naïve PMBS treated mice exposed to 0, 1, 2 or 3 OVA aerosols. (a) PMBS treated mice exhibited significant eosinophil infiltration of the airways following 1 exposure to aerosolised antigen, and cell numbers rose appreciably with each consecutive exposure to OVA antigen. (b) Lymphocytes in BALF were only significantly elevated from pre exposure levels following the 2nd exposure to OVA, but not after the 3rd exposure. Data represents mean ± SEM for groups of 4 mice. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$), and are indicated by bars (¬).

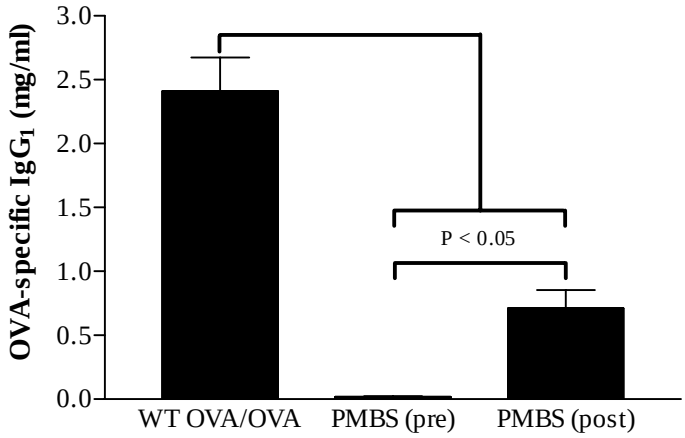


Figure 2.10. OVA-specific IgG_1 antibody titers in OVA sensitised and challenged mice compared to PMBS treated mice. Serum from wild type PMBS treated mice prior to (pre) and following (post) airways exposure to novel antigen was analysed for OVA-specific IgG_1 and compared to that of wild type OVA sensitised and challenged mice. Data represents mean ± SEM for groups of 5-7 mice. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$), and are indicated by bars (¬).

2.4 DISCUSSION

The extent to which eosinophils may participate during the early immune response to antigen is poorly understood, and is currently restricted to the measurement of surface molecules associated with antigen presentation and observations of cellular interactions ex-vivo. While the eosinophil is certainly equipped with the necessary surface molecules required for effective antigen presentation to antigen-specific T cells (Del Pozo *et al.*, 1992; Hansel *et al.*, 1992; Weller *et al.*, 1993; Tamura *et al.*, 1996; Handzel *et al.*, 1998; Celestin *et al.*, 2001), there remains a poor understanding of its ability to interact with naïve (and memory) T lymphocytes at sites of antigen deposition. Furthermore, the participatory role of eosinophils in the early stages of airways sensitisation have not been investigated to date. The experiments presented in this Chapter were designed to evaluate what effect (if any) antigen deposition in the naïve airways had upon a distal pool of eosinophils located within the peritoneal cavity.

Eotaxin is an eosinophil specific chemokine, constitutively expressed in various tissues including small intestines, the colon, heart, kidneys and lung (Jose *et al.*, 1994a; Rothenberg *et al.*, 1995b; Kitaura *et al.*, 1996; Matthews *et al.*, 1998). Eotaxin expression is upregulated in response to IL-4 (Rothenberg *et al.*, 1995a; Mochizuki *et al.*, 1998; Sanz *et al.*, 1998b), and eotaxin expression in the allergic lung has since been linked to both IL-4 and IL-13 secretion from $CD4^+$ T-cells (Mattes *et al.*, 2001; Webb *et al.*, 2001; Foster *et al.*, 2002). In this Chapter, chronic PMBS treatment induced a pronounced peritoneal eosinophilia (Figure 2.1) in both wild type and $eotaxin^{-/-}$ mice, providing an animal model in which to study distal eosinophil responses to local antigen exposure within the airways. When exposed to repeated aerosols of antigen, PMBS-treated wild type mice developed a significant peripheral blood eosinophilia (Figure 2.2). Surprisingly, analysis of BALF on day 8 revealed a pronounced pulmonary eosinophilia in PMBS-treated wild type mice (Figure 2.3a), suggesting that a signaling pathway generated from within lung tissue was acting distally, to induce migration of peritoneal and peripheral blood eosinophils to the lung. This migration was dependent on antigen deposition in the airways, as PMBS-treated wild type mice that were exposed to aerosolized vehicle (0.9% saline) did not generate a BALF eosinophilia (data not shown). When exposed to repeated aerosols of OVA, $eotaxin^{-/-}$ mice did not develop a peripheral blood eosinophilia significantly different from that of untreated wild type controls (Figure 2.2). Analysis of BALF on day 8 revealed a very slight

eosinophil infiltration of the airways lumen in eotaxin$^{-/-}$ mice (Figure 2.3a) which was significantly greater than that of wild type controls, yet 8-fold less than that seen in PMBS-treated wild type mice. This data strongly suggests that distally located eosinophils can respond to antigen deposition in the naïve airways in a manner that is heavily dependent on eotaxin, which is most likely generated within the respiratory epithelium. The small but significant number of eosinophils recruited to the airways seen in eotaxin$^{-/-}$ mice suggests that another factor or factors may be acting in the absence of eotaxin to promote eosinophil recruitment to antigen deposition in the lung.

IL-5 administration has been shown to increase peripheral blood eosinophilia, and to synergise with local eotaxin administration to promote eosinophil accumulation into tissues (Collins *et al.*, 1995; Mould *et al.*, 1997). Peritoneal eosinophils derived from PMBS mice pre-incubated with IL-5 displayed increased intracellular calcium responses to eotaxin *in vitro* (Figure 2.4). This effect of IL-5 upon PMBS eosinophils suggested that IL-5 administration to PMBS-treated mice could augment eosinophil homing to sites antigen deposition in the lung. However, IL-5 administration was shown to have no significant effect upon peripheral blood eosinophilia in PMBS-treated mice exposed to OVA (Figure 2.6), nor were there any increases in the numbers of eosinophils and lymphocytes infiltrating the airways lumen (Figure 2.7), suggesting that there was no synergy between distally acting IL-5 and the locally generated signal from the naïve lung in response to OVA deposition.

In an airways sensitisation model, IL-4 and IL-5 in the BALF can be measured following 5 days of consecutive antigen exposure, albeit in the context of GM-CSF transgene expression (Stampfli *et al.*, 1998). In the results presented in this Chapter, it is unlikely that emerging antigen-specific IL-4, IL-5 and IL-13 secreting CD4^{+} T-cells were responsible for the early induction of eotaxin expression within the lung and subsequent trafficking of eosinophils from the peritoneum to this site. This assumption is based on the kinetics of the response (Figures 2.8 and 2.9), which suggest that an immediate early signal generated in the lung results in eosinophil homing to sites of OVA deposition. Furthermore, the lack of OVA specific IgG_1 in serum of PMBS treated mice prior to antigen exposure does not indicate previous sensitisation to OVA (Figure 2.10), and lymphocytes/ml BALF in wild type and eotaxin$^{-/-}$ mice were typically 5-fold less than those seen in OVA sensitised and challenged mice (Foster *et al.*, 1996).

In summary, antigen deposition in the airways of naïve mice induces an immediate early signal (IES) that can induce eosinophil homing from a distal site to the lung, and these experiments strongly suggest that eotaxin is a major component in the signaling pathway. Thus, at least two mechanisms for eosinophil recruitment may operate in the lung: one that is dependent on antigen-specific T cells, and another IES that is non-T cell dependent and reliant on eotaxin.

Notably, detectable OVA-specific IgG_1 in serum was seen in PMBS treated mice on day 8, following repeated airways exposure to OVA (Figure 2.10). It is possible that eosinophils recruited by the IES to the naïve lung may have participated in the primary response to OVA deposited in the airways. Eosinophil recruitment to the airways lumen preceded observable increases in lymphocyte numbers (Figure 2.9), and eosinophils have previously been identified as a source of IL-4 (Sabin *et al.*, 1996; Rumbley *et al.*, 1999). Mouse eosinophils are known to have some antigen presenting capability (Del Pozo *et al.*, 1992), and provide necessary costimulatory signals during antigen presentation to T cells (Tamura *et al.*, 1996). Therefore, eosinophils that were recruited to the lung soon after antigen deposition may have provided early IL-4, which is necessary for OVA specific IgG_1 and the development of Th2 responses following sensitisation via the airways.

It would be interesting to determine the spatial and temporal aspects of eosinophil trafficking to regions of antigen deposition (airways lumen) and presentation (draining lymph nodes) in sensitised mice following aeroallergen challenge. Monitoring parameters such as eosinophil infiltration into lung tissue, the airways lumen, and possibly the draining lymph nodes may further our understanding of the processes involved in the selective recruitment of eosinophils into allergic tissues following antigen exposure, and may further delineate their participation in airways responses to antigen.

Chapter 3

Analysis Of Tissue Eosinophilia During The Progression And Resolution Of Allergic Airways Disease

3.1 INTRODUCTION

Animal models of allergic airways disease provide a useful tool in which to investigate the cellular and humoral response to allergen deposition within the sensitised organism. An initial i.p. sensitisation using antigen and adjuvant is often necessary for disease induction, although exclusive sensitisation via the airways is possible (Haczku *et al.*, 1997; Hamelmann *et al.*, 1997a; Hamelmann *et al.*, 1997c). Subsequent aeroallergen challenge allows detailed analysis of the selective accumulation of eosinophils and activated $CD4^+$ T cells, which is now accepted as a central event in the progression and maintenance of allergic airways disease (Anderson and Coyle, 1994; Hetzel and Lamb, 1994; Fowell *et al.*, 1997; Hogan *et al.*, 1998c).

Using an OVA sensitisation and aeroallergen challenge model in BALB/c mice, Ohkawara and colleagues (Ohkawara *et al.*, 1997) have measured the kinetics of the eosinophil response in both the blood and lung compartments. They observed inflammatory cell infiltration (predominantly neutrophils, eosinophils, and some lymphocytes) in lung tissue and the airways lumen within 3 and 24 hours of aeroallergen challenge respectively, while peripheral blood eosinophilia also increased significantly within 3 hours of airways challenge. Notably, IL-4 and IL-5 expression were detected in the lung 3 and 5 hours, respectively, following airways challenge. During disease resolution, eosinophils were the predominant leukocyte remaining in the airway lumen (as measured by BALF) for up to 15 days following cessation of aeroallergen challenge, a feature which was also reflected in peripheral blood eosinophilia. Histological analysis revealed a return to a normal bronchial epithelium 21 days after challenge of the airways. Recently Gajewska and colleagues (Gajewska *et al.*, 2001) have expanded upon this body of work and monitored the expansion of APC populations and further detailed the expression of cytokines following aeroallergen challenge. Using flow cytometrical analysis, macrophage and dendritic cell populations were greatly increased in lung tissue 24 hours following airways challenge, and accompanied a significant increase in MHC-II/B7.2 double positive cells in draining thoracic lymph nodes. Measurement of cytokine expression in the lung revealed a significant increase in mRNA for IL-4, IL-13, IL-5 and IL-6 in as little as 3 hours following airways challenge, and modest yet significant increases in message for each of these cytokines in draining lymph nodes. It is of particular interest to note that in these two very similar models, lung IL-4 expression was detected within 3 hours of

antigen deposition of the airways. IL-4 has been shown to induce eotaxin expression (Rothenberg *et al.*, 1995a; Mochizuki *et al.*, 1998), and this may have been a contributing factor to the rapid infiltration of eosinophils into the allergic lung and airways lumen (Ohkawara *et al.*, 1997).

In these and other described models, airways challenge with OVA would ultimately lead to a mixture of soluble antigen, lung derived cytokines and antigen bearing APCs entering the draining lymphatic vessels and progressing towards the draining thoracic lymph nodes. Soluble OVA has been shown to enter the draining lymphatics and to penetrate beneath the subcapsular sinus, before associating with the reticular network and eventually reaching the high endothelial venules (HEVs) (Gretz *et al.*, 2000), a site where naïve T cells may gain access to the lymph node (Gretz *et al.*, 1996; McEvoy *et al.*, 1997). The arrival of soluble OVA into the draining lymph nodes would then allow resident APCs such as interdigitating dendritic cells which actively sample the extracellular milieu to internalise and process OVA, prior to presenting antigenic OVA peptides to resident naïve T cells in the context of MHC-II. Alternatively, OVA deposited in the airways may be taken up in situ by resident professional APCs such as immature dendritic cells, which then undergo a maturation process involving increased B7.1/B7.2 and MHC-II expression and concomitant migration to the draining lymph nodes and eventual stimulation of naïve T cells (Lambrecht, 2001).

Eosinophils have been observed to present superantigens (Weller *et al.*, 1993; Mawhorter *et al.*, 1994; Tamura *et al.*, 1996; Celestin *et al.*, 2001), viral antigens (Handzel *et al.*, 1998), and antigen requiring intracellular processing (Del Pozo *et al.*, 1992; Hansel *et al.*, 1992; Tamura *et al.*, 1996) to antigen specific T cells in a B7.2 dependent manner (Tamura *et al.*, 1996; Celestin *et al.*, 2001). Eosinophils in the allergic lung and airways lumen are well situated to sample the extracellular milieu and to traffic via the afferent lymphatics to the draining lymph nodes, where antigen may either be presented to resident naïve T cells or be sequestered by professional APCs and subsequently presented to naïve T cells. Eosinophil recruitment to lymph nodes after antigen loading is not a recent observation and dates back at least to 1966 (Roberts, 1966), and during helminth infection in mice, eosinophils have been observed to translocate to the draining lymph node during the recovery phase (Friend *et al.*, 2000). Ultimately, eosinophils carrying soluble antigen to the draining lymph nodes in allergic

airways disease models may exacerbate established Th2 responses, by interacting with naïve T cells and generating memory T cells with an OVA specific Th2 phenotype.

In this Chapter, the kinetics of eosinophil trafficking and spatial distribution into the lung and airways lumen are studied following antigen deposition, as well as monitoring the resolution of eosinophilia in both pulmonary and thoracic lymphoid tissues. The results presented here suggest that eosinophils can migrate to draining lymphoid tissue following antigen deposition in the airways, and then dwell in draining lymphoid tissue for extended periods of time during resolution of allergic airways inflammation.

3.2 MATERIALS AND METHODS

3.2.1 Animals.

Wild type and IL-5 transgenic mice (Dent *et al.*, 1990) of the C57BL/6 strain (male, 4-8 wk old) were obtained from the SPF unit of the ANU and housed in an approved containment facility. Mice were treated according to ANU animal welfare guidelines.

3.2.2 Induction of allergic airways inflammation in wild type mice, collection and analysis of peripheral blood and BALF.

Male wild type C57BL/6 mice were sensitized by i.p. injection with 50 µg OVA/1 mg Alhydrogel (alum, Commonwealth Serum Laboratories, Parkville, Australia) in 0.9% sterile saline on days 0 and 12 (Figure 3.1a). To qualitatively measure subtle increases in resident eosinophil numbers within BALF, the mice were then subjected to a very mild antigen challenge regime. On days 24, 25, and 26, the mice were aeroallergen (OVA, 10 mg/ml in 0.9 % saline) challenged for 30 minutes using a RapidFlo nebuliser bowl. BALF (see Section 2.2.4) was analysed at 0, 3, 6, 9, 24, 48 and 72 hours from the first aerosol on day 24. Blood samples were drawn from the tail vein for differential cell counts at the same time points, and stained with May-Grunwald-Giemsa solution prior to differential cell counting (~210 cells).

3.2.3 Airways and peribronchial lymph node (PBLN) histology in allergic wild type mice.

To measure eosinophil migration into the airways and also into PBLN tissue, sensitised and challenged mouse lungs (Section 3.2.2) were immediately removed and fixed in 10% phosphate-buffered formalin solution, pH 7.0 for at least 2 hours. Later, sections representing the central (bronchi-bronchiole) and peripheral (alveolar) airways were cut and immersed in 10% phosphate buffered formalin solution for a minimum of 72 hours. PBLN were harvested concomitantly with lung tissue, and fixed whole in 10% phosphate buffered formalin for a minimum of 72 hours. The samples were paraffin-embedded, sectioned and stained with Lendrum's carbolchromotrope stain (Lendrum, 1944). Leukocytes were identified by morphological criteria and quantified. Eosinophils per high-powered field (40x objective) were counted from histological sections of tissue. As a control, brachial lymph nodes were also sampled at all time points to compare eosinophil trafficking in extra-pulmonary lymphoid tissue.

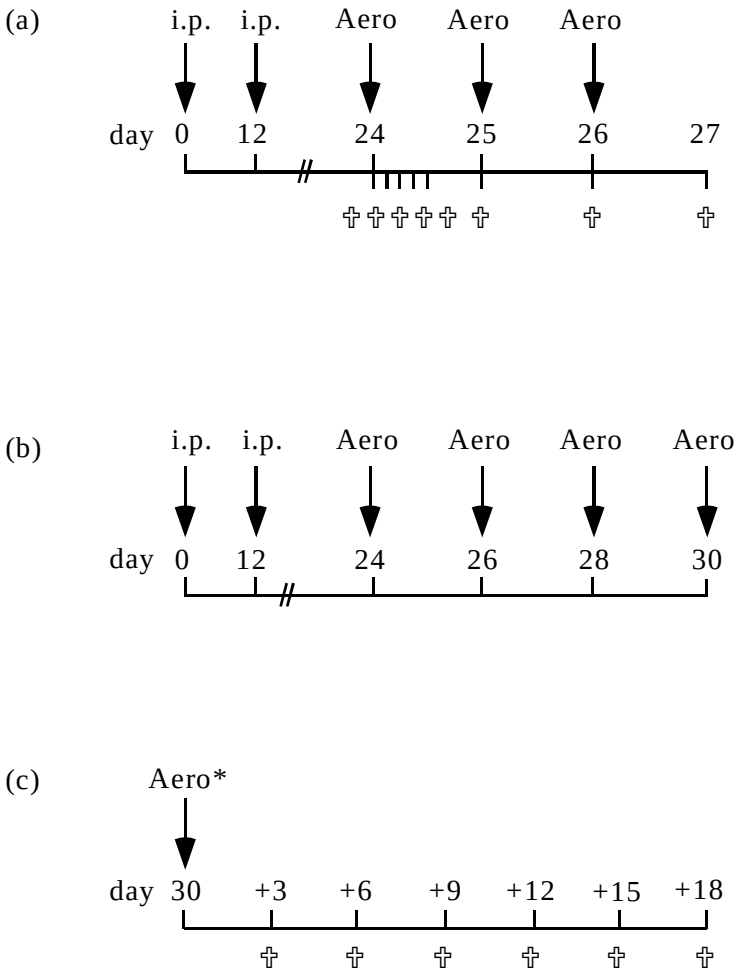


Figure 3.1. Sensitisation and aerosol challenge regimes. (a) To study eosinophil migration into allergic tissue, mice were given intraperitoneal injections of OVA in alum on days 0 and 12 (i.p.), and challenged with an OVA aerosol (Aero) on days 24, 25 and 26. Mice were sacrificed (✞) at 0, 3, 6, 9, 12, 24, 48 and 72 hours following the first aerosol on day 24, with BALF, PBLN and peripheral blood samples taken at these times. (b), to generate a strong allergic response with which to monitor resolution of tissue eosinophilia, mice were i.p. sensitised with OVA in Alum on days 0 and 12, and challenged with 3 OVA aerosols on days 24, 26, 28 and 30. (c). To study the resolution of tissue eosinophilia in the allergic lung and draining lymphoid tissue, mice from regime (b) were sacrificed 3, 6, 9, 12, and 15 days after the last aerosol (Aero*) on day 30, and appropriate tissues sampled.

3.2.4 Visualisation of eosinophils in PBLN using electron microscopy.

Peribronchial lymph nodes from sensitised and challenged mice (Section 3.2.2) were immersed in a fixative consisting of 2% glutaraldehyde in 0.1-M cacodylate buffer, pH 7.4. The specimens were fixed overnight and then post-fixed in 1% osmium tetroxide for 90 minutes. They were then dehydrated in graded acetone solutions and embedded in Spur's Resin (ProSciTech, Australia). For high-resolution light microscopy, semi-thin (1 µm) sections of the embedded tissue were cut and stained with Toludine Blue. These sections were used to select areas of interest for electron microscopic examination. Ultra-thin sections (80-85 nm) were cut on a Reichert-Jung Ultracut E ultramicrotome (Germany) and routinely contrasted with uranyl acetate and lead citrate. The sections were examined using a Hitachi 7000 transmission electron microscope (Japan).

3.2.5 Cessation of allergic airways inflammation in wild type mice, collection and analysis of peripheral blood and BALF.

Male wild type C57BL/6 mice were sensitized by i.p. injection with 50 µg OVA/1 mg Alhydrogel (Commonwealth Serum Laboratories, Parkville, Australia) in 0.9% sterile saline on days 0 and 12 (Figure 3.1b). On days 24, 26, 28, and 30, the mice were aeroallergen challenged (OVA, 10 mg/ml in 0.9% saline) for 3 x 30 minutes at half hour intervals using a RapidFlo nebuliser bowl. BALF and PBLN (as in sections 2.2.4 and 3.2.3) were sampled at 3, 6, 9, 12, 15 and 18 days following the last aerosol challenge on day 30 (Figure 3.1b and c). Blood samples were drawn from the tail vein for differential cell counts on the same days, and stained with May-Grunwald-Giemsa solution prior to differential cell counting (~210 cells). This antigen delivery regime induced maximal eosinophil recruitment to BALF and PBLN, and changes in lymph node diameter by day 31, providing a satisfactory model to investigate resolution of eosinophilia from these compartments. As a control, brachial lymph nodes were also sampled at all time points to compare eosinophil trafficking in extra-pulmonary lymphoid tissue.

3.2.6 Eosinophil purification by flow cytometry.

IL-5 transgenic C57BL/6 mice (Dent *et al.*, 1990) were sacrificed by CO_2 asphyxiation and the peritoneal cavity lavaged as in Section 2.2.2. Eosinophils were purified by FACS as in Section 2.2.5. The purity of the enriched population of eosinophils was >98% as determined by differential staining with May-Grunwald-Giemsa. Purified eosinophils were diluted as required into RPMI-1640.

3.2.7 Fluorescent labeling of IL-5 transgenic eosinophils and analysis of trafficking to lung and lymphoid tissues in vivo.

FACS sorted IL-5 transgenic eosinophils (>98% purity) were stained *in vitro* with the fluorescent nuclear dye Hoechst 33342 (H33342, 20 μg/ml, Molecular Probes) in PBS (1% BSA) at a concentration of 5 x 10^7 cells/ml for 30 minutes at 37^{o}C. The cells were then washed (x3) in ice cold PBS and placed on ice. Stained cells (4x 10^6) were then i.v. injected into OVA sensitised and challenged mice following the third aerosol challenge (as described above in Section 3.2.5). At each step, the labeled cells were protected from incident light. At 2 and 20 hours post-transfer, peripheral blood, BALF, lung, PBLN, spleen and gut (jejunum) were sampled. Mice were rendered unconscious with ether, and respective tissues were excised following cardiac perfusion with PBS. The tissues were separately homogenised (the jejunum was irrigated with ice cold PBS prior to homogenisation) in HBSS/10% FCS at 4^{o}C, and the resulting cell suspensions were then filtered through sterile nylon mesh (70μM) before centrifugation at 500 g for 5 minutes at 4^{o}C. Each pellet was then resuspended in PBS. The cell homogenates were protected from incident light during each step. Fluorescent cells in each tissue were then detected with a Becton and Dickinson FACScan® flow cytometer in conjunction with Cellquest (Becton and Dickinson, San Jose, CA) and WinMDI software packages (kindly provided by J. Trotter, Scripps Research Institute, La Jolla, CA).

3.3 RESULTS

3.3.1 Eosinophils migrate to lung and PBLN tissue following aeroallergen challenge in sensitised mice.

Ovalbumin aerosol was periodically delivered to the sensitised airways over a period of 72 hours, and eosinophil numbers characterized in the blood, airways lumen (as measured by BALF), and PBLN compartments. Blood eosinophilia peaked at 6 hours following the first aerosol challenge on day 24, and was sustained for the duration of the experiment (Figure 3.2a). Eosinophils in BALF were significantly elevated at 48 hours, i.e. following exposure to aerosolised OVA on days 24 and 25 (Figure 3.2b). Interestingly, eosinophil numbers in PBLN were significantly elevated with 3 hours of the first exposure to aerosolised OVA on day 24, and were elevated again at 48 hours following the second exposure on day 25 (Figure 3.2c). Microscopic analysis of carbolchromotrope stained PBLN sections indicated that eosinophils infiltrated the lymph node, residing predominantly within the medullary sinus (Figure 3.3a-c), and were notably absent from follicular structures. Electron microscopy analysis of PBLN tissue sections at 48 hours revealed eosinophils residing in the draining lymphatic duct and within tissues beneath the subcapsular sinus of the lymph node (Figures 3.4a and b). In some sections, eosinophils were observed in close proximity to lymphocytes (Figure 3.4b) and displayed characteristics of activation, including loss of granule matrix and crystalloid core. Although these cells showed loss of secondary granules, the majority showed no signs of degranulation nor condensation of nuclear chromatin, which are indicative of the late stages of apoptosis.

3.3.2 Eosinophil numbers in PBLN, lung and BALF return to basal levels 18 days after cessation of aeroallergen challenge.

The kinetics of resolution of eosinophilia in the lung and lymphoid tissues during the refractive phase of allergic inflammation is poorly defined. To characterize the clearance of eosinophils, a strong aeroallergen challenge regimen was used to generate large numbers of eosinophils in pulmonary compartments and in the blood of allergic mice. The BALF, lung tissue, PBLN and blood were then characterized for 18 days following cessation of aeroallergen exposure. Allergic mice were sacrificed every 3 days, beginning after the last aerosol challenge at day 30. Eosinophil numbers in the blood steadily declined over 12 days to baseline levels (Figure 3.5a). Eosinophils/HPF for each tissue were counted and compared with residual numbers of eosinophils in the

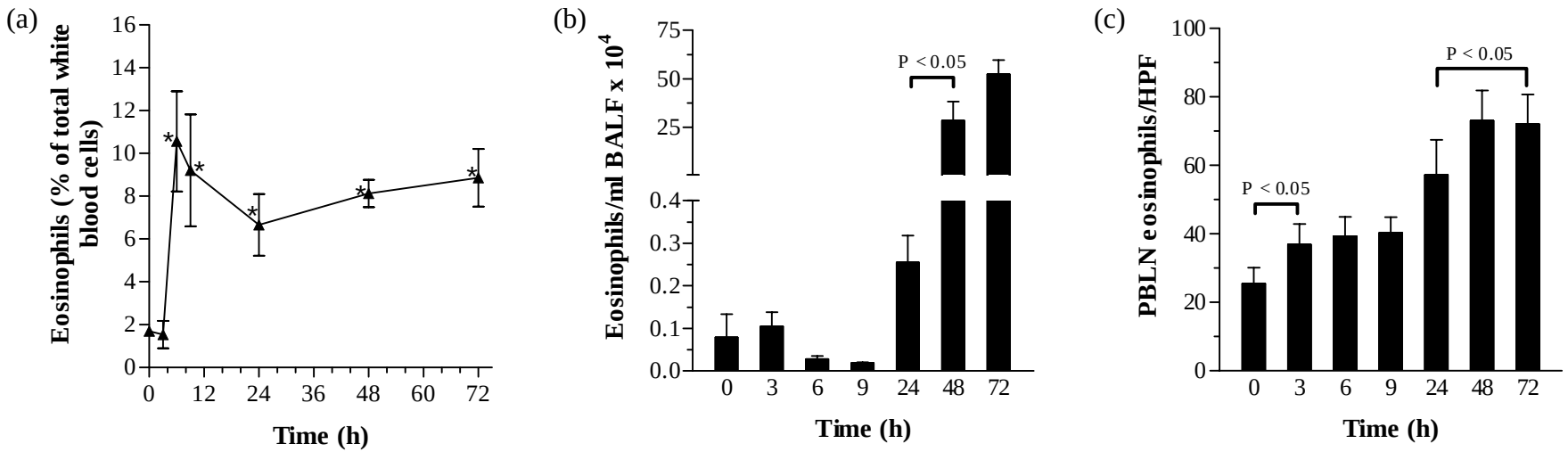


Figure 3.2. Eosinophil numbers in Blood, BALF and PBLN compartments following antigen provocation in allergic mice. (a) A significant rise in peripheral blood eosinophils was observed at 6 hours after the first aerosol and persisted for the duration of the experiment. (b) Significant numbers of eosinophils appeared in BALF at 48 hours, and remained elevated at 72 hours. (c) Eosinophils were significantly increased in PBLN 3 hours following the initial aeroallergen challenge, and were greatly increased at 48 hours. Data represents mean ± SEM for groups of 4 mice. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$), and are indicated by bars (¬).* $P < 0.05$ compared to 0 and 3 hours.

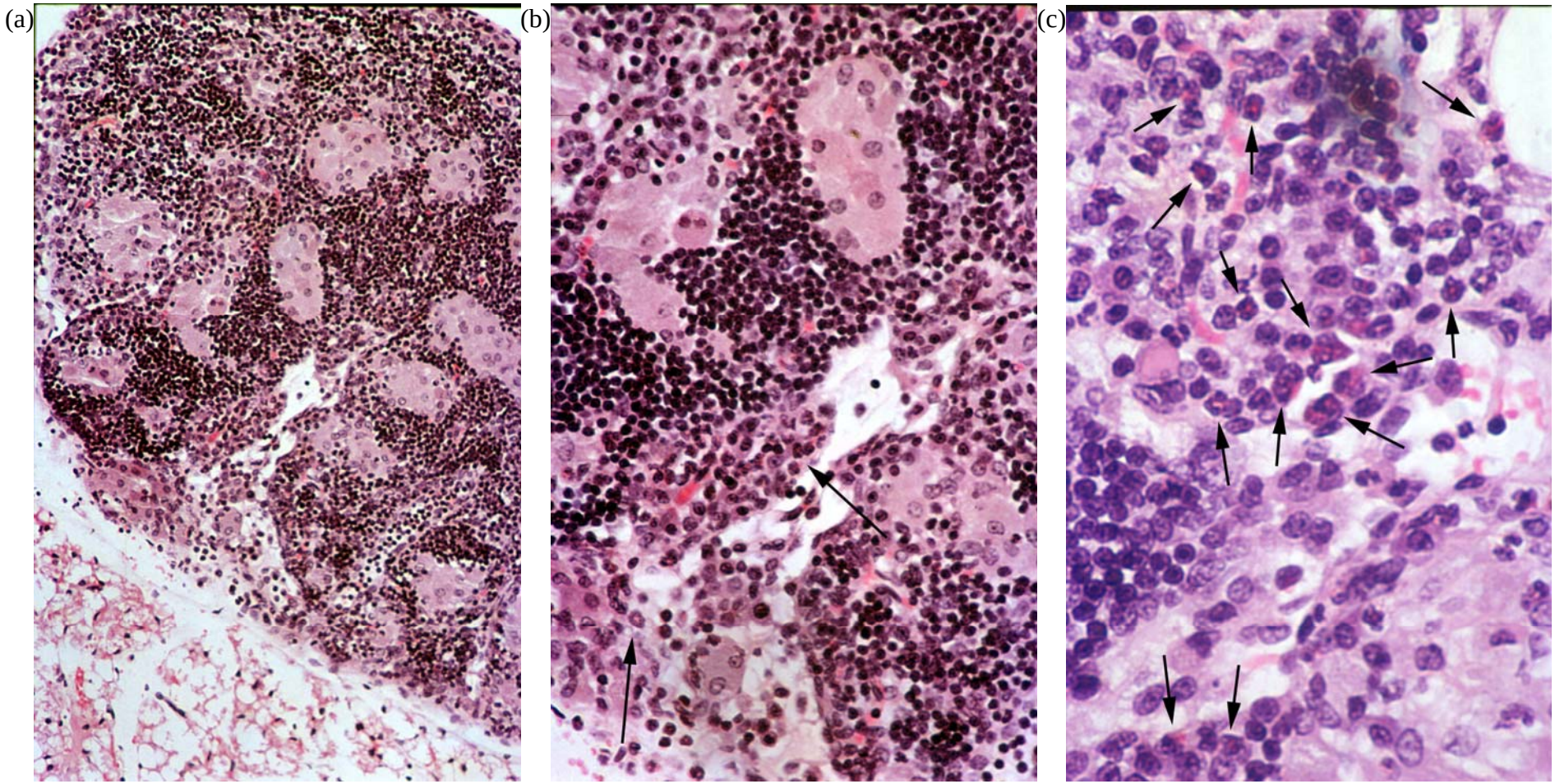
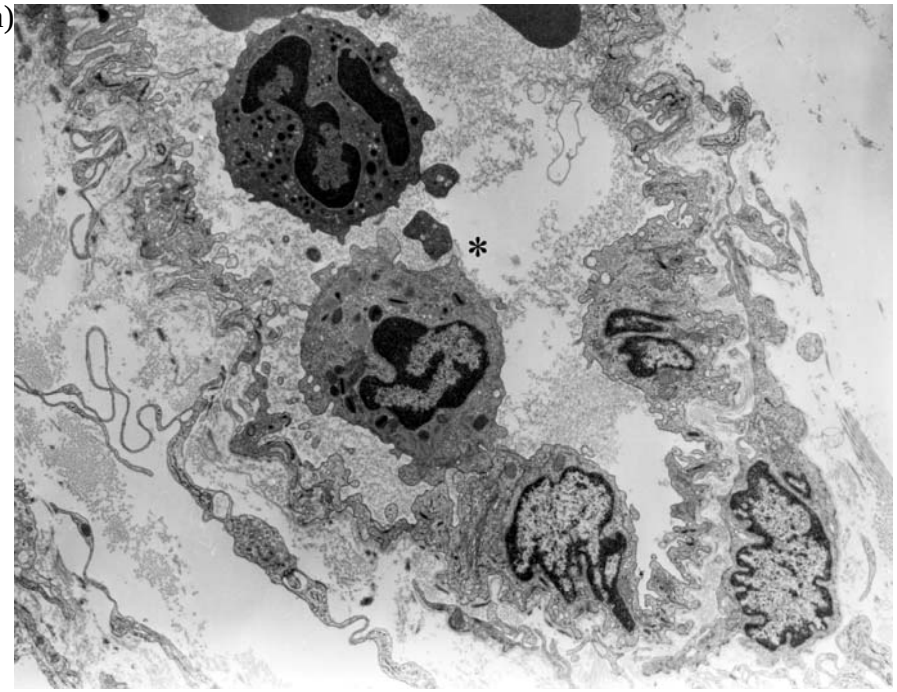


Figure 3.3. Eosinophils infiltrate the lymph node medulla following antigen deposition in the airways of sensitised mice. (a) Carbolchromotrope stained sections of the peribronchial lymph node showing follicle development (10x). (b) Eosinophils can be seen in the medullary sinus (arrowed) but not in follicular structures (20x). (c) Eosinophils can be observed in discrete clusters within the cortex (arrowed, 40x).

(a)



(b)

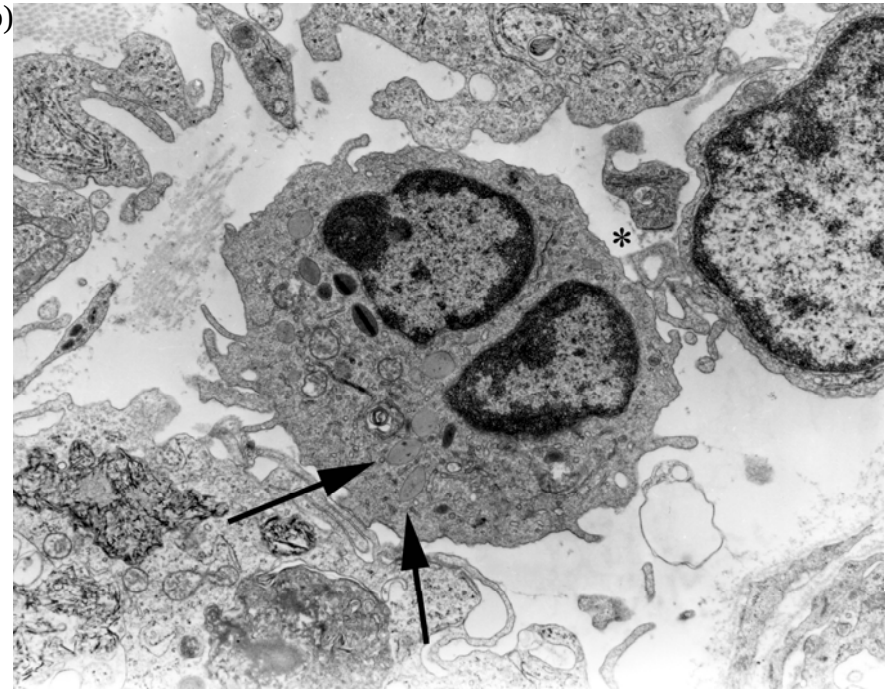


Figure 3.4. Eosinophils localise to PBLN after antigen provocation of the lung. (a) following antigen provocation in allergic mice, electron microscopy showed that eosinophils (∗) were caught in transit within the draining lymphatic duct. (b) Electron microscopy showed that eosinophils localised within the subcapsular sinus region in PBLN of allergic mice. Some evidence of activation was noted (arrowheads) by the loss of crystalloid core and matrix of eosinophil granules. Here an eosinophil appears to be interacting with a lymphocyte, as evidenced by extended processes (∗).

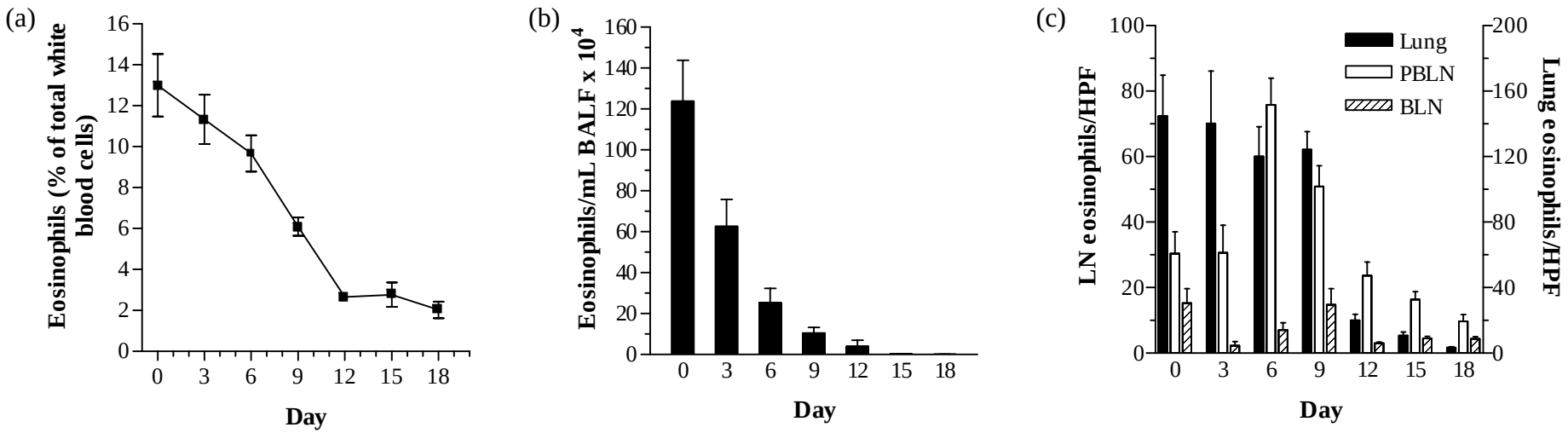


Figure 3.5. Resolution of eosinophilia from blood, lung and lymphoid tissues following cessation of aeroallergen challenge. (a) At the end of the aerosol regime, peripheral blood eosinophilia was 13%, and gradually declined to basal levels within 12 days of cessation of aeroallergen challenge. (b) Eosinophil numbers in BALF declined rapidly after cessation of aerosol and were undetectable by day 15. (c) Eosinophils per high powered field (HPF) in lung tissue (plotted on second Y axis), were elevated for 12 days after cessation of aeroallergen challenge, while eosinophils in PBLN did not peak until day 6, before gradually declining. Brachial lymph node eosinophil numbers did not change significantly. Data represents mean ± SEM for groups of 4 mice.

BALF (Figures 3.5b and c). Brachial lymph nodes were also sampled for comparative purposes. BALF eosinophil numbers rapidly declined after cessation of allergen exposure (~50% within 3 days), returning to baseline levels by day 15 (Figure 3.5b). By contrast, the number of eosinophils residing within the tissue (Figure 3.5c) did not significantly decline until day 12, when the pool of eosinophils in the airways lumen was almost completely depleted (Figure 3.5b). PBLN eosinophil numbers continued to increase to day 6 after cessation of allergen exposure and then fell in parallel with tissue levels over the next 12 days to baseline levels (Figure 3.5c). In all compartments, eosinophil levels had returned to baseline within 18 days of the last allergen exposure. Eosinophil numbers in brachial lymph nodes did not change significantly in response to allergen provocation, suggesting that eosinophil trafficking to the lung and airways-draining lymph nodes was specifically related to allergic inflammation of the airways.

3.3.3 Eosinophils do not directly enter PBLN from the blood following allergen provocation of the allergic lung.

To further identify the migration pathway of eosinophils to pulmonary compartments during allergen provocation, 2 x 10^6 fluorescently labeled eosinophils from IL-5 transgenic mice were injected i.v. into allergic wild type mice following the third aeroallergen challenge. Within 2 hours of transfer, labeled cells were detected in the lung, spleen, and blood, but not in BALF, PBLN tissue, nor the gut (Figures 3.6 and 3.7). By 20 hours, labeled eosinophils were still migrating into the lung tissue, and fluorescent cells were still undetected within PBLN tissue (not shown). These results suggest that eosinophils do not passage directly via the blood and high endothelial venules to PBLN following allergen provocation, but migrate via the pulmonary parenchyma and draining lymphatics.

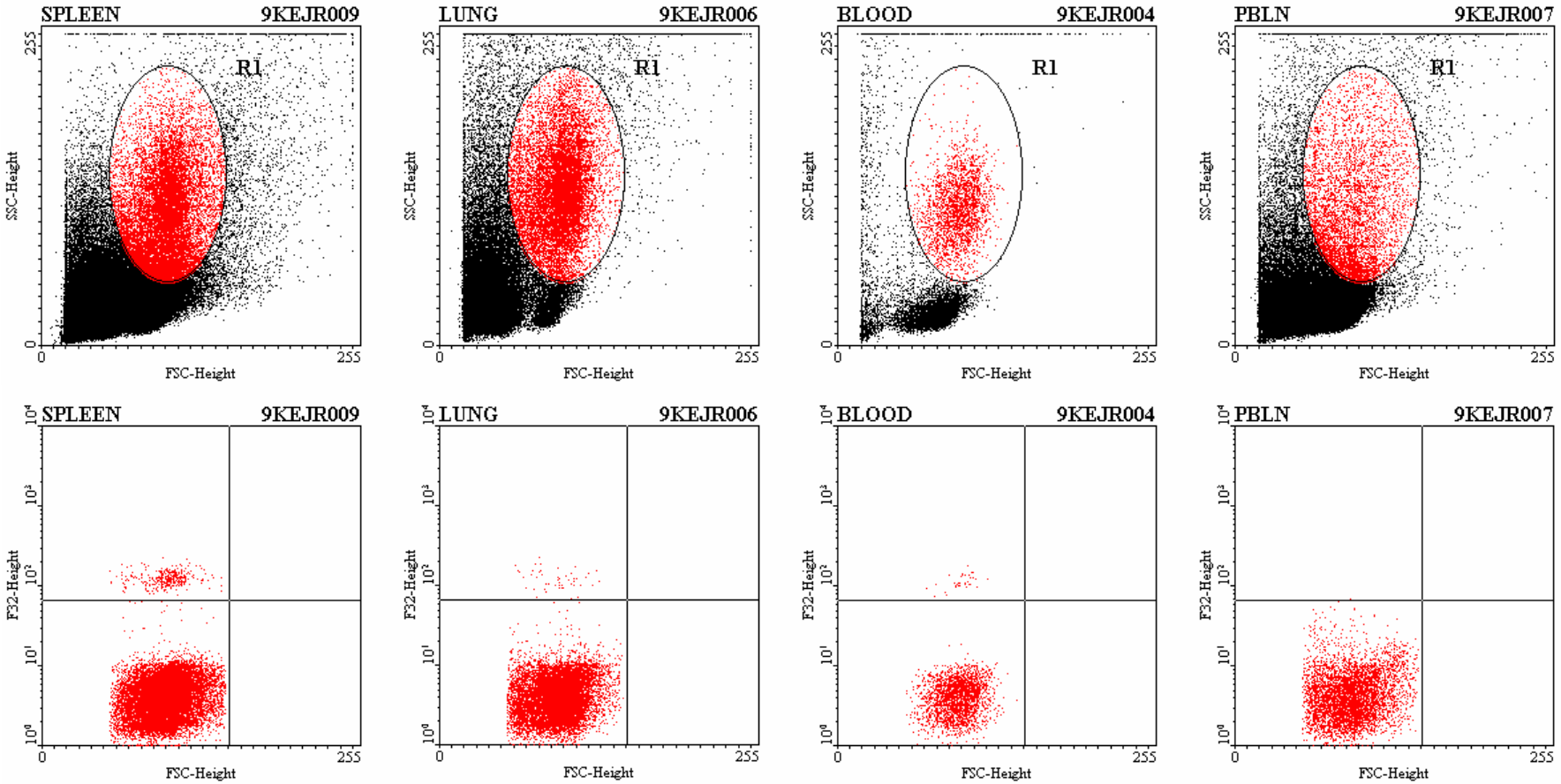


Figure 3.6. Trafficking of labeled eosinophils to pulmonary and lymphoid tissue following allergen provocation. Within 2 hours of i.v. transfer, labeled cells were detected in spleen, lung and blood, but not in PBLN tissue. Region 1 (R1) represents the area in which eosinophil would fall based on forwards (FSC-Height) and side scatter (SSC-Height). Detection of H33342 fluorescence (F32-Height) for each tissue is given below each window, and is gated for cells within R1 only. Fluorescent cells are represented in the upper left quadrant.

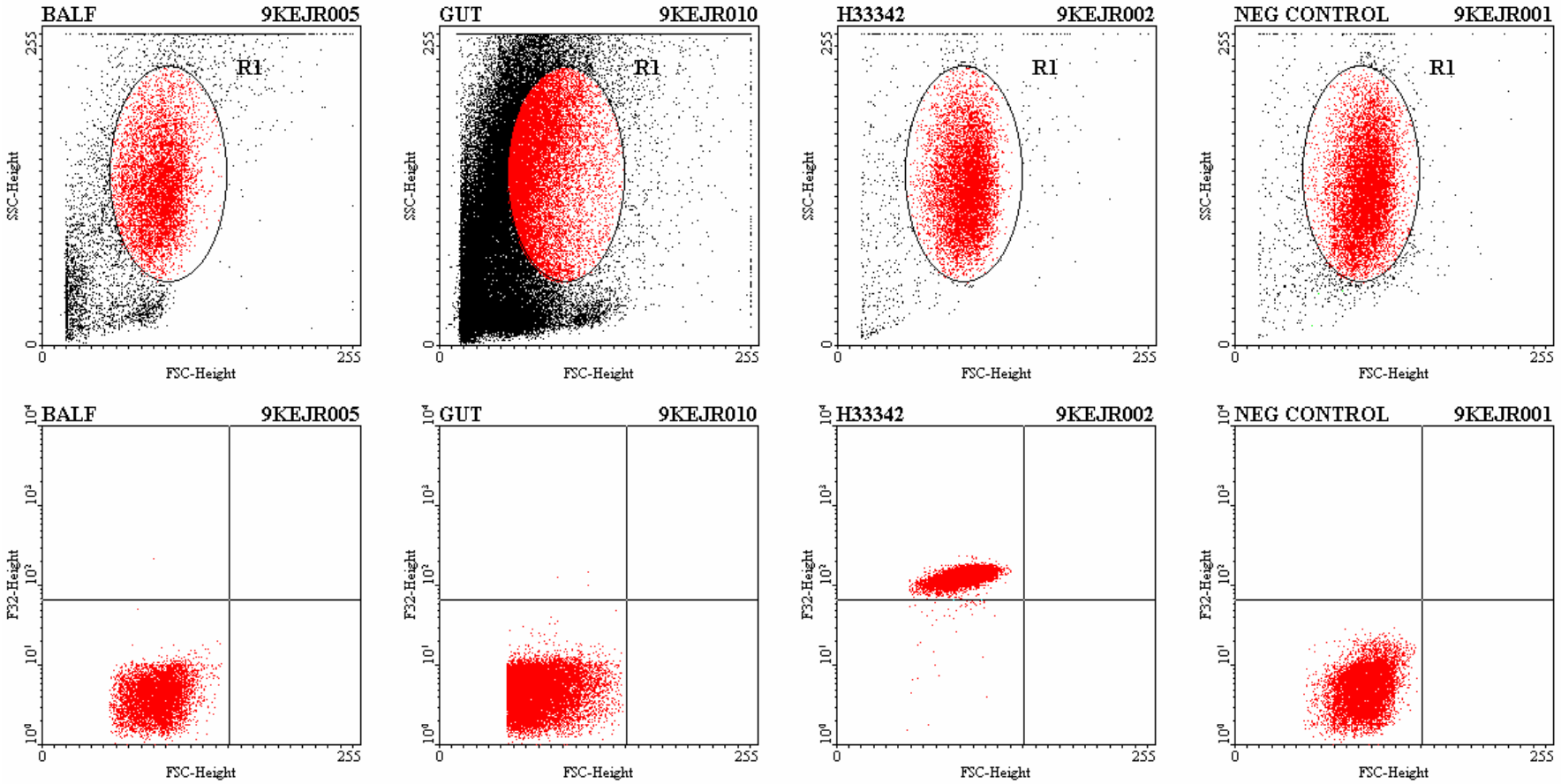


Figure 3.7. Trafficking of labeled eosinophils to pulmonary and lymphoid tissue following allergen provocation. Within 2 hours of i.v. transfer, labeled cells were not detected in BALF nor the small intestines (GUT). Region 1 (R1) represents the area in which eosinophil would fall based on forwards (FSC-Height) and side scatter (SSC-Height). Detection of H33342 fluorescence (F32-Height) for each tissue is given below each window, and is gated for cells within R1 only. Fluorescent cells are represented in the upper left quadrant. Eosinophils stained with H33342 dye prior to transfer (H33342) are shown in comparison to unstained cells (NEG CONTROL).

3.4 DISCUSSION

In the experiments described in Chapter 2, it was shown that antigen deposition in the naïve lungs of PMBS-treated mice could induce eosinophil accumulation in the airway lumen via an eotaxin dependent mechanism. Eosinophils are prominent features in allergic mucosal surfaces of the lung and gastrointestinal tract (Gleich *et al.*, 1993; Rothenberg, 1998), and these cells are currently thought to act as effector cells, that induce pathological changes through the release of granular proteins and proinflammatory mediators. The degranulation status of mouse eosinophils in allergic airways disease models is currently under intense scrutiny (Stelts *et al.*, 1998; Persson and Erjefalt, 1999; Denzler *et al.*, 2001; Malm-Erjefalt *et al.*, 2001), however the results presented in this Chapter do not attempt to delineate a pathological role for eosinophils. Rather, attention has been focused on other potential roles for eosinophils in the regulation of allergic airways disease through characterisation of their spatial and temporal distribution during the development and resolution of allergic inflammation of the lung.

Detailed analysis of eosinophil trafficking to the lung and draining lymphoid tissue during both the progression and resolution of allergic airways disease has provided a valuable insight into the potential role eosinophils may play in disease regulation. In this Chapter, eosinophils were observed to traffic to sites of antigen deposition (airways lumen) and T cell expansion (PBLN) in response to antigen provocation of the airways. An increase in peripheral blood eosinophils was observed in as little as 6 hours following antigen provocation (Figure 3.2a), and a significant increase in eosinophils in the airways lumen was observed within 48 hours (Figure 3.2b). These results agree with those of Ohkawara *et al*., who have also used a mouse model of allergic airways disease to measure the kinetics of eosinophil migration into pulmonary tissues (Ohkawara *et al.*, 1997). In their model, Ohkawara *et al*. observed eosinophil adhesion to pulmonary endothelial cells in as little as 3 hours following airways challenge, which paralleled an increase in peripheral blood eosinophilia and subsequently led to infiltration of the airways lumen within 24 hours.

Antigen specific lymphocytes circulating through the allergic mouse lung can quickly respond to relevant antigen deposition through rapid synthesis and secretion of IL-4, IL-5 and IL-13 (Gajewska *et al.*, 2001), which act in concert to promote tissue

eosinophilia. In this Chapter, a significant increase in the numbers of eosinophils residing in the draining PBLN was noted 3 hours following the initial exposure to antigen, and preceded the accumulation of eosinophils in the BALF (Figures 3.2b and c). It is tempting to speculate that eosinophils may preferentially translocate to the lymph node during the early phase of airways inflammation, and that repeated exposure to antigen then promotes the accumulation of eosinophils in the lung and BALF. This effect may be seen in Figures 3.2b and c, wherein eosinophils accumulate in both draining PBLN and the airways lumen following repeated exposure to OVA on days 25 and 26 (i.e. at 48 and 72 hours following the initial exposure).

Following the cessation of antigen provocation, airways eosinophils become apoptotic and may be engulfed by airway macrophages (Kodama *et al.*, 1998), or small airway epithelial cells (Walsh *et al.*, 1999). This may be directly related to the loss of antigen induced inflammatory cytokines such as IL-5, IL-3 and GM-CSF (Stern *et al.*, 1992; Simon and Blaser, 1995), and suggests a mechanism whereby eosinophils are removed from lung tissue following the cessation of antigen provocation. In this Chapter, peripheral blood eosinophil levels and the numbers of eosinophils in BALF returned to baseline within 12 and 15 days, respectively, following the cessation of airways challenge with OVA (Figures 3.5a and b). Notably, the numbers of eosinophils residing in lung tissue remained elevated until the pool of eosinophils in the airway lumen were depleted (Figures 3.5b and c). These results correspond to the observations made by Ohkawara *et al*., who in a similar model, have noted resolution of BALF and blood eosinophil levels at 15 and 21 days, respectively, and a return to a normal bronchial epithelium by day 21 (Ohkawara *et al.*, 1997).

Interestingly, PBLN eosinophil numbers continued to increase until day 6 following the cessation of antigen challenge, before falling in parallel with lung tissue levels over the next 12 days and finally to baseline levels by day 18 (Figure 3.5c). Mouse eosinophils traffic to the draining lymph nodes during the recovery phase of helminth infection (Friend *et al.*, 2000), and in conjunction with data presented in this Chapter, suggests a potential role for eosinophils in the regulation of draining lymph node function during the resolution of Th2 cytokine driven diseases. In the rat, peripheral lymph node high endothelial cells express VCAM-1, the expression of which is upregulated by cytokines (May *et al.*, 1993). Antibodies to the cognate receptor VLA-4 have been shown to reduce eosinophil recruitment to tissue *in vivo* (Weg *et al.*, 1993), and thus identifies a

possible route of entry for peripheral blood eosinophils into the lymph node via high endothelial venules. In this Chapter, fluorescently labeled eosinophils were not observed to enter draining thoracic lymph nodes via the blood (Figures 3.6 and 3.7), suggesting that the afferent lymphatics are the major entry point for eosinophils found to reside in peribronchial lymphoid tissue following antigen challenge of the airways.

Eosinophils are phagocytic cells with antigen presenting capacity (Del Pozo *et al.*, 1992; Weller *et al.*, 1993), and the ability of eosinophils situated in the airways lumen to migrate via the lymphatics to sites of antigen presentation has recently been confirmed (Shi *et al.*, 2000). Thus, eosinophils that are recruited to the antigen challenged lung in sensitised mice may then sample the extracellular milieu, and phagocytose endogenous antigen. Within inflamed lung tissue, eosinophils may then process and present endogenous antigen to activated memory T lymphocytes, and thereby modulate local T cell function and disease progression/resolution. Alternatively, recruited eosinophils may sample the contents of the airway lumen, before translocation back across airways epithelium and subsequent trafficking to the draining lymph nodes. In these tissues, eosinophils may reside for prolonged periods of time following antigen challenge in the sensitised organism (Figure 3.5c), and may actively present airways derived antigens to antigen-specific T lymphocytes. Thus, the numerous eosinophils that are recruited to the antigen challenged lung in the sensitised organism are well placed to modulate T lymphocyte function within local tissues and distally via the draining lymphatics. It is interesting to note that low numbers of eosinophils were present in the blood, airways lumen, and draining lymphoid tissue prior to antigen inhalation (Figure 3.2, zero time points), which suggests a role for baseline eosinophils in immunological responses associated with immune surveillance and potentially, T cell expansion.

It would be interesting to assess the surface expression of MHC-II and associated costimulatory molecules, and to further investigate any antigen presenting function attributable to lung eosinophils. This may further our understanding of the potential for eosinophil/T cell interactions wherein eosinophils may regulate T cell function during chronic antigen exposure to the lung.

Chapter 4

Eosinophils As Antigen Presenting Cells *In Vitro*

4.1 INTRODUCTION

In Chapter 2, mouse eosinophils were shown to localise to sites of antigen deposition, a phenomenon specific to non-allergic mice having a large pool of responsive eosinophils. In Chapter 3, eosinophils were observed to populate the draining lymph nodes of antigen sensitised and challenged mice during disease progression, and persisted in this compartment until well after the cessation of antigen exposure. These data showed that eosinophils are well placed to interact with antigen-specific lymphocytes during these stages of disease (priming and induction).

The antigen presenting capacity of eosinophils has been partially explored by others, and has tended to focus on the surface expression of MHC-II (mouse), HLA-DR (human), and costimulatory molecules CD80/B7.1 and CD86/B7.2. Class II MHC molecules bind exogenous peptides during their biosynthetic maturation prior to their appearance on the cell surface (Watts, 1997; Weenink and Gautam, 1997), and subsequent recognition by the TCR provides the first signal necessary for T cell activation. Mouse eosinophil expression of MHC-II can be upregulated by *in vitro* incubation with GM-CSF (Del Pozo *et al.*, 1992; Tamura *et al.*, 1996), IL-4 (Mawhorter *et al.*, 1993), or by an *in vivo* disease state, such as parasite infection (Mawhorter *et al.*, 1993) or AAD (Shi *et al.*, 2000). Human eosinophils can be induced to express surface HLA-DR in response to GM-CSF (Lucey *et al.*, 1989), IL-3 (Celestin *et al.*, 2001), and this molecule is upregulated *in vivo* on airways, but not peripheral blood eosinophils from allergic asthmatics (Hansel *et al.*, 1991; Sedgwick *et al.*, 1992; Mengelers *et al.*, 1994; Sakamoto *et al.*, 1996).

The second signal required for T cell activation is an interaction between counterreceptors on the surface of antigen presenting cells with the costimulatory receptors on T cells (June *et al.*, 1994). The B7-CD28/CTLA-4 costimulatory pathways have been shown as essential for development of AAD in mice (Krinzman *et al.*, 1996; Keane-Myers *et al.*, 1997). The interaction between CD80 and CD28 is essential for eosinophil recruitment to the lung (Harris *et al.*, 1997), and lack of costimulatory activity is linked to a defect in IL-4 and IL-5 production by T cells (Harris *et al.*, 2001). Alternatively, blocking CD86 but not CD80 function using monoclonal antibodies during sensitisation attenuates AAD development in mice (Tsuyuki *et al.*, 1997; Keane-Myers *et al.*, 1998). The expression of CD80 and CD86 by mouse eosinophils can be

upregulated by incubation with GM-CSF (Tamura *et al.*, 1996), and has also been noted on activated airway eosinophils from mice with AAD (Shi *et al.*, 2000). In human eosinophils, there is less data available for B7 expression, although resting cells can express CD86 (but not CD80) in response to IL-3 incubation (Celestin *et al.*, 2001), and increased expression by activated eosinophils from eosinophilic donors has also been noted (Woerly *et al.*, 1999).

Research using artificial antigen presenting cells has established the minimum requirements for T cell activation. Prakken *et al*. have shown that liposomes bearing MHC-II/peptide complexes are engaged by MHC-II restricted T cells, and leads to physiological capping of the T cell receptor (Prakken *et al.*, 2000). Alternatively, cells infected with recombinant vaccinia virus (rVV) encoding both MHC-II and antigen can induce $CD4^+$ T cell proliferative responses, which are further enhanced by the co-expression of CD80 and CD86 (Oertli *et al.*, 1997). Mouse eosinophils express surface MHC-II and B7 costimulatory molecules, and have a demonstrated ability to present antigen. Paraformaldehyde-fixed eosinophils that were previously activated with GM-CSF and incubated with complex antigen, are able to stimulate the proliferation of T cell clones and hybridomas in a manner sensitive to anti-MHC-II antibody (Del Pozo *et al.*, 1992). The expression of CD80 and CD86 by murine eosinophils obtained from IL-5 transgenic mice is reported to be essential for antigen presentation to OVA-primed lymph node T cells (Tamura *et al.*, 1996). Similarly, live airway eosinophils pre-cultured with GM-CSF can stimulate allergic T cell proliferation in the presence of OVA (Shi *et al.*, 2000). At the completion of the investigations in this thesis, antigen presentation by eosinophils had previously been limited to measurement of *in vitro* T cell proliferation and IL-2 production only, with no reports of Th2 cytokine release in response to antigen presentation by either mouse or human eosinophils.

In this Chapter the capacity for effective antigen presentation by mouse eosinophils *in vivo* and *in vitro* is investigated, by (1) examining eosinophil surface expression of both MHC-II and co-stimulatory molecules, (2) assessing the eosinophils ability to take up and process antigen both *in vitro* and *in vivo*, and (3) demonstrating T lymphocyte proliferation and cytokine release in response to antigen presentation by mouse eosinophils.

4.2 MATERIALS AND METHODS

4.2.1 Animals.

Wild type and IL-5 transgenic mice (Dent *et al.*, 1990) in the C57BL/6 and BALB/c backgrounds (N10, male, 4-8 wk old), and C57BL/6 T cell receptor OVA transgenics (OT-II, N10) (Barnden *et al.*, 1998), were obtained from the SPF unit of the ANU and housed in an approved containment facility. Mice were treated according to ANU animal welfare guidelines.

4.2.2 Surface expression of class II MHC by eosinophils isolated from the allergic mouse lung.

BALB/c mice* (H-2^d) were OVA sensitised and airways challenged as in Section 3.2.5, airways lavaged as in Section 2.2.4, and BALF eosinophils were purified by FACS as in Section 2.2.5. Purified BALF eosinophils (10^5 cells) were then incubated with biotinylated M5/114 anti-I-A/I-E antibody (a kind gift from A Gautam, JCSMR, ANU) in HBSS for 30 minutes on ice before fetal calf serum underlay to remove any dead cells. The cells were then centrifuged at 500 x g for 5 minutes at 4^oC, and the supernatant removed. The cell pellet was then resuspended in HBSS with streptavidin-phycoerythrin and incubated for a further 30 minutes on ice. In a separate tube, purified BALF eosinophils (10^5 cells) were incubated with FITC-conjugated conformation-dependent anti-I-A (MKD6) (Germain and Hendrix, 1991) for 30 minutes on ice. All cells were then underlaid with fetal calf serum and centrifuged at 500 x g for 5 minutes at 4^oC prior to removal of the supernatant and resuspension of cell pellets in ice cold HBSS. All tubes were protected from incident light at all times, and kept on ice before prompt analysis of fluorescence by Becton and Dickinson FACScan® flow cytometer using Cellquest (Becton and Dickinson, San Jose, CA). Data was further analysed using WinMDI software (kindly provided by J. Trotter, Scripps Research Institute, La Jolla, CA), and eosinophil-gated regions determined based on forward and side scatter signals. Fluorescent cells (MHC II^+) were detected in the FL-1 (MKD6-FITC) and FL-2 (MHC II-PE) channels, and compared to cells incubated with streptavidin-PE alone, or unlabelled control cells.

* At the time, only the M5/114 antibody was available to detect surface MHC-II (H-2^d), which restricted this particular aspect of the experimental procedures to BALB/c mice.

4.2.3 Surface expression of CD80 and CD86 by eosinophils isolated from the allergic mouse lung.

C57BL/6 mice were OVA sensitised and airways challenged as in Section 3.2.5, airways lavaged as in Section 2.2.4, and BALF eosinophils were purified by FACS as in Section 2.2.5. Purified C57BL/6 BALF eosinophils (10^5 cells) were then separately incubated in HBSS with PE-conjugated anti-mouse CD80 or CD86 (BD PharMingen, San Diego, CA) for 30 minutes on ice before FCS underlay to remove any dead cells. As a control, disaggregated mouse spleen cells (erythrocytes lysed) were also incubated (10^5 cells) in HBSS with PE-conjugated anti-mouse CD80 as for purified eosinophils. Cells were then centrifuged at 500 x g for 5 minutes at 4°C, and the supernatant removed. Cell pellets were resuspended in ice cold HBSS. Labeled cells were protected from incident light at all times, and kept on ice before prompt analysis of fluorescence by Becton and Dickinson FACScan® flow cytometer using Cellquest (Becton and Dickinson, San Jose, CA). Data was further analysed using WinMDI software (kindly provided by J. Trotter, Scripps Research Institute, La Jolla, CA), and eosinophil-gated regions determined based on forward and side scatter signals. Fluorescent cells ($CD80^+$ or $CD86^+$) were detected in the FL-2 channels and compared to unlabelled control cells.

4.2.4 Eosinophil internalisation and processing of OVA in vitro.

Purified eosinophils from C57BL/6 IL-5-transgenic mice were used to demonstrate cellular internalization and processing of OVA *in vitro*. Briefly, IL-5-transgenic mice were sacrificed by CO_2 asphyxiation and the peritoneal cavity lavaged as in Section 2.2.2. Eosinophils in the PEC population were then purified by FACS as in Section 2.2.5, and diluted as required into HBSS and kept on ice. Eosinophils (10^5) were incubated with 1 mg/ml OVA (Sigma) or DQ-OVA (Molecular Probes) in 1 ml of HBSS for 30 or 240 minutes at 37°C. Cells were then washed three times in 10 ml of PBS before fixing in 4% paraformaldehyde in PBS. Analysis of fixed cells was performed using a Becton and Dickinson FACScan® flow cytometer using Cellquest (Becton and Dickinson, San Jose, CA), and data further analysed using WinMDI software (kindly provided by J. Trotter, Scripps Research Institute, La Jolla, CA). Eosinophil-gated regions were determined based on forward and side scatter signals and fluorescent cells ($DQ\text{-}OVA^+$) detected in the FL-1 channel were compared with cells incubated with unconjugated OVA.

4.2.5 Eosinophil internalisation and processing of OVA in vivo.

To demonstrate cellular internalization and processing of OVA *in vivo*, wild type C57BL/6 mice were sensitized and airways challenged with OVA as in Section 3.2.5. Following airways challenge on days 24, 26, 28, and 30, mice were given 1 x intranasal doses of OVA or DQ-OVA (1 mg) in HBSS. The DQ-OVA challenges were done in this manner to directly coincide with each individual aerosol. Twenty-four hours after the last aerosol challenge and DQ-OVA administration, mice were sacrificed by cervical dislocation and their airways were lavaged as in Section 2.2.4. Intracellular processing of DQ-OVA by BALF cells was detected using flow cytometry as in Section 4.2.4.

4.2.6 OVA transgenic splenocyte proliferation in response to co-incubation with OVA peptide-pulsed and paraformaldehyde treated eosinophils.

To generate antigen loaded eosinophils, C57BL/6 mice were OVA sensitised and airways challenged as in Section 3.2.5, and eosinophils in BALF (Section 2.2.4) were then purified by FACS as in Section 2.2.5. Purified BALF eosinophils (5×10^6) were then incubated in PBS containing 1 mg/ml $OVA_{323-339}$ peptide (ISQAVHAAHAEIN-EAGR) at 37^{o}C for 15 minutes, before being centrifuged at 500 x g for 5 minutes at 4°C, and the supernatant removed. Cell pellets were resuspended in ice cold PBS containing 4% paraformaldehyde for 30 minutes on ice, before being centrifuged at 500 x g for 5 minutes at 4°C, and the supernatant removed. Cell pellets were then incubated overnight in 20ml PBS at 4^{o}C to leech out any residual paraformaldehyde. Cells were again centrifuged at 500 x g for 5 minutes at 4°C, and the supernatant removed, before resuspension in MLC medium containing 10% FCS. Freshly isolated BALF eosinophils were also fixed in paraformaldehyde as $OVA_{323-339}$ peptide free controls.

OT-II transgenic mice (male) were chosen for their specific responses to OVA, and in particular $OVA_{323-339}$ peptide (Barnden *et al.*, 1998; Robertson *et al.*, 2000). Mice were sacrificed by cervical dislocation and their spleens excised. Splenocytes were disaggregated using sterile wire gauze (0.5mm) and suspended in ice cold PBS. Erythrocytes were then lysed, and the washed splenocytes resuspended at 10^7 cells/ml in MLC medium containing 10% heat-inactivated FCS, 2 mM L-glutamine, and 50 mg/L neomycin sulfate. Fixed and $OVA_{323-339}$ peptide-pulsed eosinophils were co-cultured with 2×10^6 OT-II splenocytes at the following concentrations. A; no stimulus control, B-E; 1×10^4, 5×10^4, 1×10^5, 5×10^5 peptide-pulsed eosinophils, F; 5×10^5

peptide-free eosinophils. OT-II splenocytes were also incubated with $OVA_{323-339}$ peptide as a control (G). Cultures were in 200 μl final volume, in 96-well tissue culture plates. At 48 hours, the tissue culture plates were centrifuged at 300 x g for 5 minutes at RT, and spent media replaced with fresh media (200 μl) containing tritiated thymidine (^{3}H-thymidine, 1μCi/well). At 72 hours of co-culture, the contents of each well were harvested onto a glass fibre filter (Packard, Canberra) using a Filtermate 196 cell harvester (Packard, Canberra). Fibre mats were then assembled in the appropriate cassette with MicroScint-O fluid (Packard, Canberra) and tritiated thymidine incorporation as represented by counts per minute (CPM) measured using a Packard TopCount NXT (Packard Instrument Company INC, IL). Data are presented as means ± SEM from triplicate wells.

4.2.7 Allergic splenocyte proliferation in response to soluble OVA: co-incubation with eosinophils.

C57BL/6 mice were OVA sensitised and airways challenged as in Section 3.2.5, and eosinophils in BALF (Section 2.2.4) were then purified by FACS as in Section 2.2.5. For co-incubation, excised spleens from OVA sensitised and airways challenged C57BL/6 mice were disaggregated using sterile wire gauze (0.5mm) and splenocytes suspended in ice cold PBS. Erythrocytes were then lysed, and the washed splenocytes resuspended at 10^7 cells/ml in MLC medium containing 10% heat-inactivated FCS, 2 mM L-glutamine, and 50 mg/L neomycin sulfate. Purified BALF eosinophils (1 x 10^5) were then co-incubated with allergic splenocytes (2 x 10^6) in 96 well tissue culture plates with or without added OVA (100 μg/well). At 48 hours, the tissue culture plates were centrifuged at 300 x g for 5 minutes at RT, and spent media replaced with fresh media (200μl) containing tritiated thymidine (1μCi/well). At 72 hours of co-culture, the contents of each well were harvested and ^{3}H-thymidine incorporation measured as in Section 4.2.6.

4.2.8 Isolation of in-vivo generated OVA specific $CD4^+$ T cells.

C57BL/6 mice were OVA sensitised and airways challenged as in Section 3.2.2, and excised spleens from these mice were disaggregated using sterile wire gauze (0.5mm) and, splenocytes suspended in ice cold PBS. Erythrocytes were then lysed, and the washed splenocytes (~ 1 x 10^8) were suspended in 90 μl HBSS/5% FCS with 10 μl anti CD4 mAb (L3T4) coated microbeads and incubated for 20 minutes at 6-12^{o}C.

Suspensions were then centrifuged at 500 x g for 5 minutes at 4^{o}C and the resultant cell pellet resuspended in 1 ml PBS/0.5 % BSA. Bead-bound CD4^{+} cells were then isolated using high-gradient magnetic MiniMacs separation columns (MACS separation, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) as per manufacturers instructions. Isolated cells were then washed and resuspended in MLC medium/10% FCS. The CD4^{-} fraction (unbound cells) were retained as a source of antigen presenting cells. The purity of the enriched CD4^{+} cell fraction were then determined by flow cytometry.

4.2.8.1 Assessment of isolated CD4^{+} T cell purity by flow cytometry.

MACS enriched CD4^{+} cells (10^5) from Section 4.2.8 were resuspended in HBSS containing PE-conjugated anti mouse CD4 (CD4^{+}-PE) (PharMingen, San Diego, CA, clone L3T4) for 30 minutes on ice and underlaid with foetal calf serum. The cells were then centrifuged at 500 x g for 5 minutes at 4^{o}C. The supernatant was then removed, and the cell pellet resuspended in HBSS at 4^{o}C and promptly analysed by flow cytometry, using a Becton and Dickinson FACScan® flow cytometer and Cellquest software (Becton and Dickinson, San Jose, CA). Raw data was further analysed using WinMDI software (kindly provided by J. Trotter, Scripps Research Institute, La Jolla, CA).

4.2.8.2 Cytokine production from isolated OVA-specific CD4^{+} T cells incubated with eosinophils and soluble OVA.

C57BL/6 mice were OVA sensitised and airways challenged as in Section 3.2.5, and eosinophils in BALF (Section 2.2.4) were then purified by FACS as in Section 2.2.5. Purified CD4^{+} T cells (5 x 10^5 cells/well) were incubated in 96-well tissue culture plates with purified BALF eosinophils (3 x 10^4, 5 x 10^4, and 1 x 10^5 cells/well) in MLC medium/10% FCS with 200 µg/ml OVA. Cell-free culture supernatants were collected 96 h later and stored in aliquots at -70°C before measurement of IL-4, IL-5, and IL-13. For comparison, CD4^{+} cells (5 x 10^5 cells/well) were also cultured under the same conditions with mitomycin C (25 µg/ml)-treated splenocytes (5 x 10^5 cells/well, the CD4^{-} fraction of Section 4.2.8) as a source of conventional antigen presenting cells. Supernatants were collected 96 hours later for cytokine analysis.

4.2.9 In vitro generation of OVA-specific CD4$^+$ Th2 cells.

OVA-specific CD4$^+$ T cells were derived from BALB/c mice (6–8 wk of age). Mice were sensitized by i.p. injection with 50 µg OVA/1 mg Alhydrogel (Commonwealth Serum Laboratories, Parkville, Australia) in 0.9% sterile saline. Six days after sensitization, donor mice were sacrificed by cervical dislocation, the spleens were excised, and the splenocytes were disaggregated. Erythrocytes were lysed, and the washed splenocytes were resuspended at 5 x 10^6 cells/ml in complete tissue culture medium consisting of HL-1 (BioWhittaker, Walkersville, MD) with 10% heat-inactivated FCS, 2 mM L-glutamine, and 50 mg/L neomycin sulfate. Splenocytes were then cultured for 4 days at 37°C in the presence of 200 µg/ml OVA, recombinant murine IL-4 (20 ng/ml), and anti-IFN-γ antibody (clone R46A2, 40 µg/ml) to generate Th2 cells. CD4$^+$ Th2 cells were then isolated using high-gradient magnetic MiniMacs separation columns as described in Section 4.2.8. The purity of the enriched CD4$^+$ T cell fraction was uniformly above 95% as determined by flow cytometry (as in Section 4.2.8.1, result not shown).

4.2.9.1 Eosinophil incubation with OVA-specific CD4$^+$ Th2 cells generated in vitro.

In vitro derived Th2 cells were cocultured with and without eosinophils, to determine their ability to act as antigen presenting cells. Eosinophils enriched from the peritoneal cavities of BALB/c IL-5-transgenic mice (as for PMBS mice in Section 2.2.5), were cultured in equal numbers with Th2 cells (5 x 10^5 cells/well) in complete medium in the presence of 200 µg/ml OVA in 96-well plates (250 µl/well). For comparison, Th2 cells (5 x 10^5 cells/well) were also cultured under the same conditions with mitomycin C (25 µg/ml)-treated splenocytes (5 x 10^5 cells/well) (as a source of conventional antigen presenting cells). Supernatants were collected 96 hours later for cytokine analysis.

4.2.10 Cytokine analysis.

IL-13, IL-4 and IL-5 concentrations were determined in the supernatants from OVA-stimulated CD4$^+$ T cells by ELISA. Briefly, round bottomed immunoassay plates were coated with 50µl anti-mouse IL-13 (R&D Systems, Minneapolis, MN), anti-mouse IL-4 (BD PharMingen), or anti-mouse IL-5 (TRFK5) and incubated at 4^{o}C overnight. Plates were then blocked with 2% skim milk powder in PBS-Tween20 (0.04%) for 1 hour at 37^{o}C, and incubated with serial dilutions of cultured supernatants or standard IL-13, IL-4 or IL-5. Mouse IL-13, IL-4 and IL-5 were detected with biotinylated anti-mouse IL-

13 (R&D Systems, Minneapolis, MN), anti-mouse IL-4 (BD PharMingen), or anti-mouse IL-5 (BD PharMingen) respectively. Plates were then incubated with streptavidin-conjugated alkaline-phosphatase (Amersham International plc, Buckinghamshire, UK) for 1 hour at 37^{o}C, washed, and alkaline-phosphatase substrate solution (Sigma Chemical Co. St Louis, MO) was added. Where necessary, reactions were stopped with 1M citric acid, or promptly analysed. Plates were read by a Microplate reader (BIO-TEK Instruments Inc., Winooski, VT) at 490 nm with reference to 405 nm.

4.3 RESULTS

4.3.1 Eosinophils from the allergic lung express surface MHC-II and costimulatory molecules CD80 and CD86.

Eosinophil expression of molecules necessary for presentation of exogenous antigens to T cells was assessed using M5/114 and MKD6 antibodies, which recognise mouse MHC-II. Eosinophils from allergic BALB/c mice (H-2^d) were chosen for MHC-II analysis in view of the fact that MKD6 is an anti-Aβ^d antibody, having demonstrated affinity for peptide loaded MHC-II (Germain and Hendrix, 1991). The M5/114 antibody is specific for both I-A and I-E subregion-encoded glycoproteins (I-A^b, I-A^d, I-A^q, I-E^d, I-E^k, not I-A^f, I-A^k, or I-A^s) (Bhattacharya *et al.*, 1981; Germain *et al.*, 1982). Eosinophils purified from the BALF of allergic BALB/c mice expressed MHC-II as measured by M5114 antibody binding (Figure 4.1e), and further expressed peptide-loaded MHC-II molecules as measured by MKD6 antibody binding (Figure 4.1f). The surface expression of costimulatory molecule by eosinophils was also assessed. Eosinophils obtained from the lung of allergic C57BL/6 mice had detectable CD80/B7.1 (Figure 4.2c) and CD86/B7.2 expression (Figure 4.2d).

Collectively, these data confirm recent observations of eosinophil MHC-II and B7 molecule expression (Tamura *et al.*, 1996; Keane-Myers *et al.*, 1997; Tsuyuki *et al.*, 1997; Shi *et al.*, 2000), which are intimately involved in antigen presentation and delivering costimulatory signals during T cell priming with antigen.

4.3.2 Eosinophils can acquire and process exogenous antigen in vitro and in vivo.

The uptake of exogenous antigen by mouse eosinophils was investigated using the self quenching antigen DQ-OVA, which is OVA that has been heavily labeled with the fluorescent dye 3-10 Bodipy-FL, resulting in almost total quenching of the conjugate fluorescence (Santambrogio *et al.*, 1999). It was hypothesised that upon uptake and subsequent intracellular proteolysis, Bodipy-labeled fluorescent peptides would be released, and any measurable increase in cellular fluorescence would imply cellular uptake and processing of exogenous antigen. The ability of eosinophils from BALF of allergic animals to take up and process DQ-OVA *in vitro* was investigated and the results shown in Figure 4.3. It is clear that eosinophils can take up and process exogenous DQ-OVA, as demonstrated by increased fluorescence observed with longer

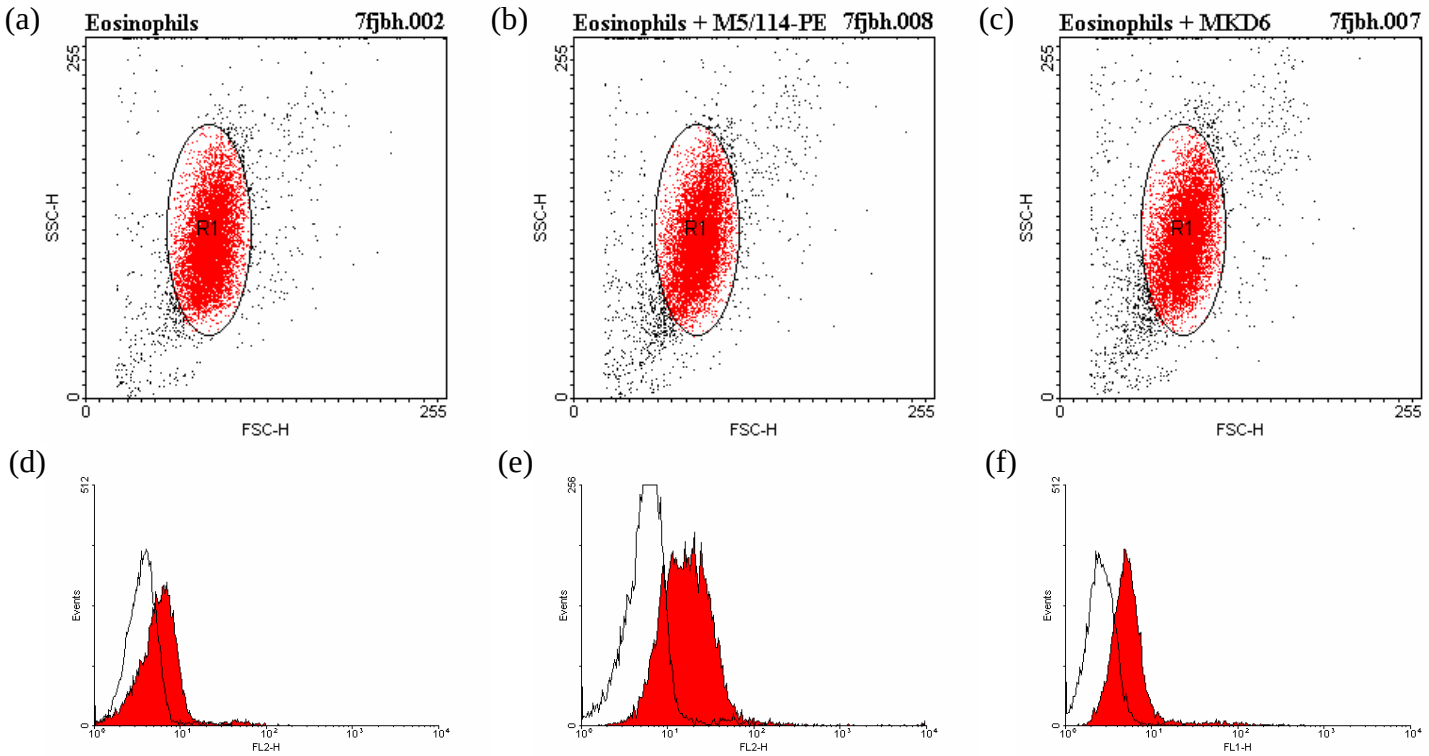


Figure 4.1. Eosinophils express surface MHC-II. Enriched BALF eosinophils from BALB/c mice with AAD (a) were gated (R1) on the basis of forwards and side scatter, and auto fluorescence (d, open histogram) compared with streptavidin-PE incubated eosinophils (d, red). Eosinophils stained with anti I-A/I-E antibody (b) show increased fluorescence intensity (e, red) in comparison to eosinophils incubated with streptavidin-PE (e, open histogram). To determine surface expression of MHC-II bearing cognate peptide, eosinophils were incubated with MKD6-FITC (c), and increased fluorescence intensity (f, red) demonstrated in comparison to control cells (f, open histogram).

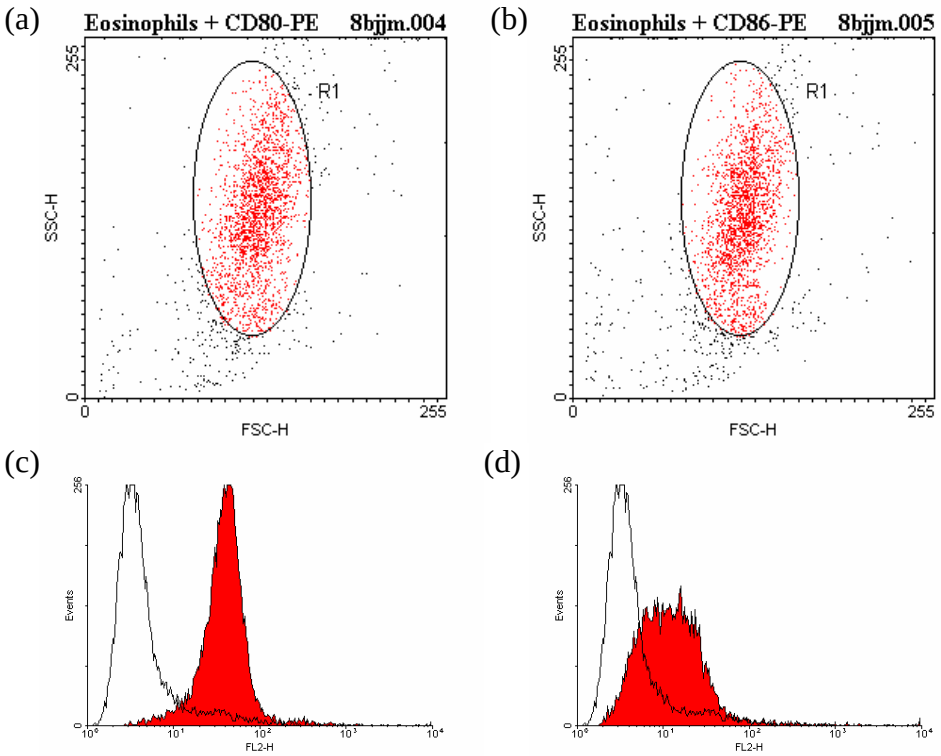


Figure 4.2. Eosinophils express CD80 and CD86 costimulatory molecules. Enriched eosinophils from C57BL/6 mice with AAD (a) stained with anti-CD80-PE were gated (R1) on the basis of forwards and side scatter, and auto fluorescence of unstained cells (c, open histogram) compared with $CD80^+$ cells (c, red). Enriched eosinophils (b) stained with anti-CD86-PE were gated (R1) on the basis of forwards and side scatter, and auto fluorescence of unstained cells (d, open histogram) compared with $CD86^+$ cells (d, red).

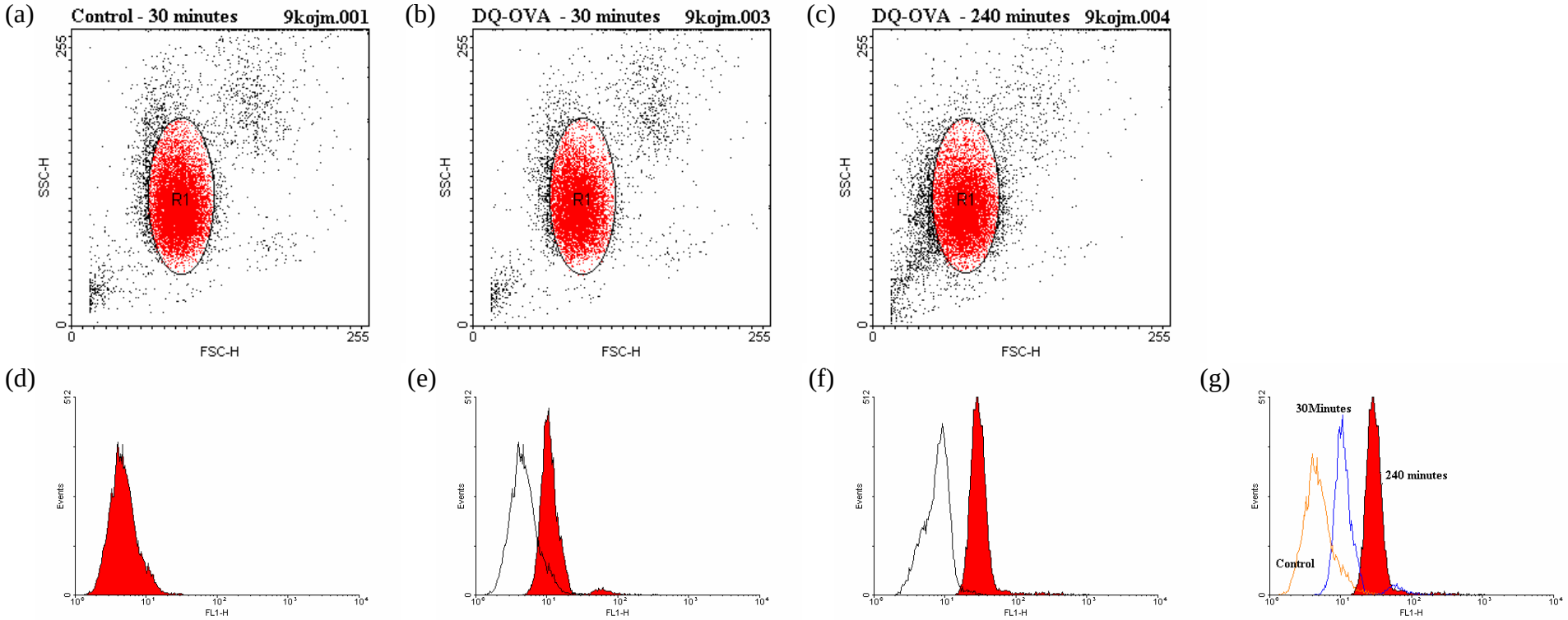


Figure 4.3. Eosinophils take up and process exogenous antigen in vitro. Enriched BALF eosinophils mice with AAD were incubated in vitro with DQ-OVA^{tm} for intervals of 30 and 240 minutes. Control cells (a) were gated on the basis of forwards and side scatter, and auto fluorescence of unstained cells illustrated (d, red). Eosinophils incubated with DQ-OVA^{tm} for 30 minutes (b) showed increased fluorescence (e, red) when compared to control cells (e, open histogram). Eosinophils incubated with DQ-OVA^{tm} for 240 minutes (c) showed further increased fluorescence (f and g, red) when compared to control cells (f, open histogram), and to cells incubated for 30 minutes (g, open histogram in blue).

incubation times (Figure 4.3d-g). To investigate this phenomenon *in vivo*, mice with allergic airways inflammation were administered either DQ-OVA or OVA via the intranasal route following each exposure to aeroallergen. On investigation, BALF eosinophils were shown to have increased fluorescence when compared to controls (Figure 4.4), indicating uptake and processing of this exogenous antigen *in vivo*. Increased fluorescence was not detected in the lymphocyte population of BALF (not shown).

4.3.3 Peptide pulsed and fixed eosinophils promote ^{3}H-thymidine incorporation in OT-II splenocytes.

Previous work by Del Pozo *et al*. has demonstrated the ability of fixed mouse eosinophils to promote antigen specific T cell proliferation *in vitro* (Del Pozo *et al.*, 1992). Similarly, human eosinophils pulsed with tetanus toxoid can promote T cell proliferation *in vitro*, in a manner sensitive to anti HLA-DR antibodies (Weller *et al.*, 1993). In this section, OT-II mice (Barnden *et al.*, 1998), were chosen for their OVA specificity and ease in preparation of uniform populations of responder cells. Eosinophils from BALF of allergic mice were incubated with OVA peptide ($OVA_{323-339}$) and fixed prior to co-incubation with OT-II splenocytes. Figure 4.5a shows that peptide loaded eosinophils promoted ^{3}H-thymidine uptake in OT-II splenocytes in a dose and peptide dependent manner, suggesting BALF eosinophils have the capacity to present OVA peptides to OVA specific T cells. However, subsequent attempts to further investigate eosinophil/OT-II interactions using live eosinophils, isolated $CD4^{+}$ transgenic splenocytes, and whole OVA were unsuccessful, for reasons that are further explored in Chapter 6.

4.3.4 Eosinophils augment ^{3}H-thymidine incorporation by allergic splenocytes incubated with soluble OVA.

The ability of live eosinophils to augment ^{3}H-thymidine uptake in splenocytes from allergic mice was investigated. Previous attempts by others (Del Pozo *et al.*, 1992) suggested that live eosinophils may degranulate during co-incubation unless fixed. To overcome this, low numbers of eosinophils (1×10^{5}/well) were co-incubated with responders (2×10^{6}/well) giving a final ratio of 20:1 for responders to eosinophils. At this concentration, live eosinophils were found to supplement ^{3}H-thymidine incorporation in splenocytes in the presence of soluble antigen (Figure 4.5b), suggesting a possible role for eosinophils in antigen presentation to splenocytes from allergic mice.

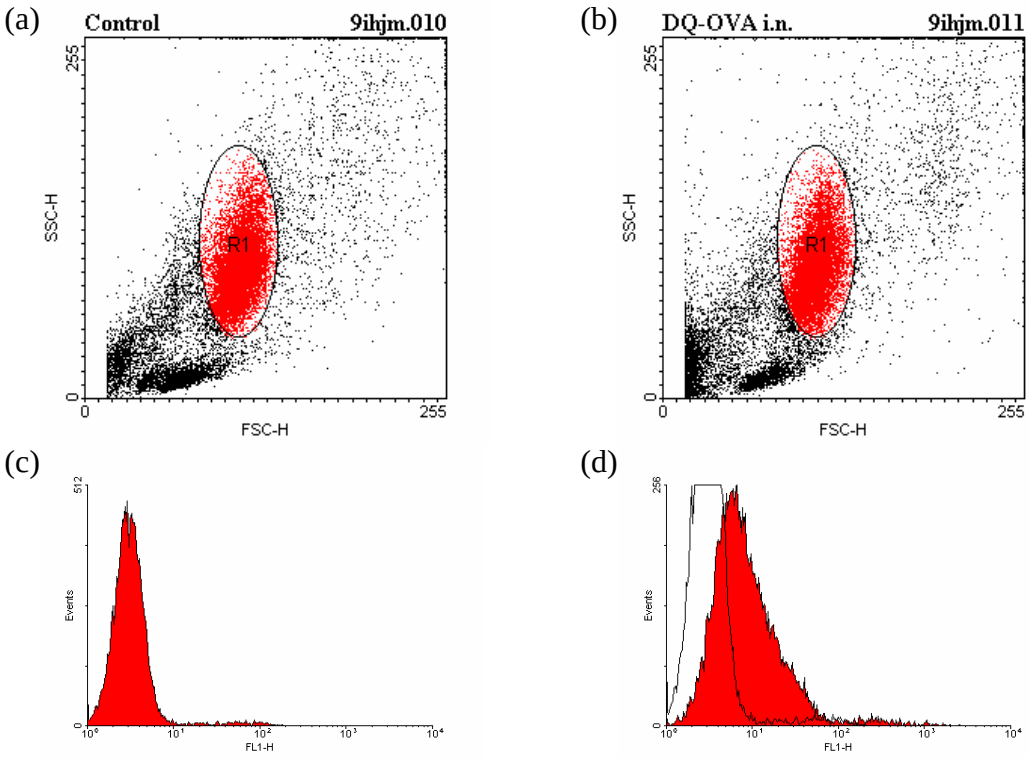


Figure 4.4. Eosinophils take up and process exogenous antigen in vivo. OVA sensitised and challenged mice were administered intranasal DQ-OVA^{tm} or unlabelled OVA in HBSS following each exposure to aerosolised allergen. BALF from control mice (a) that received unlabelled OVA were gated on the basis of forwards and side scatter, and auto fluorescence of control eosinophils is illustrated (c, red). BALF from DQ-OVA^{tm} treated mice (b) show increased fluorescence (d, red) when compared to control cells (d, open histogram).

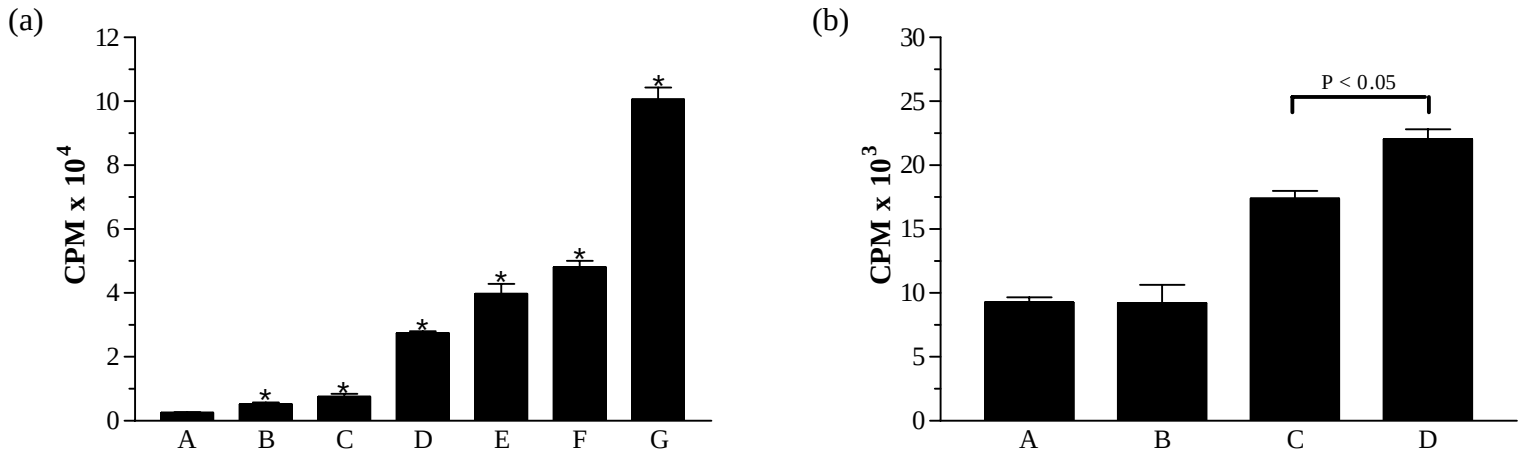


Figure 4.5. Eosinophils promote splenocyte proliferation. (a) Fixed and $OVA_{323-339}$ peptide-pulsed eosinophils were co-cultured with 2 x 10^6 OT-II splenocytes at the following concentrations. A; no eosinophils, B; 5 x 10^5 peptide-free eosinophils, C-F; 1 x 10^4, 5 x 10^4, 1 x 10^5, 5 x 10^5 peptide-pulsed eosinophils, and G; splenocytes incubated with $OVA_{323-339}$ peptide only. (b) Eosinophils (1 x 10^5) purified from BALF of mice with AAD were co-incubated with splenocytes (2 x 10^6) from AAD mice in the presence or absence of 100 μg OVA. Incubation conditions were as follows A; splenocytes alone, B; splenocytes and eosinophils, C; splenocytes and 100 μg OVA, D; splenocytes, eosinophils and 100 μg OVA. Data represents mean ± SEM for triplicate wells. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$), and are indicated by * (when compared to condition A) or by bars (¬).

4.3.5 *Eosinophils promote IL-5 production from CD4$^+$ T cells isolated from mice with AAD.*

Purified CD4$^+$ T cells (Figure 4.6) were used to investigate the antigen presenting capacity of BALF eosinophils *in vitro*. Purified eosinophils from the BALF of mice with AAD were co-incubated with MiniMacs enriched OVA-specific CD4$^+$ T cells in the presence of soluble OVA, and IL-4, IL-5, and IL-13 secretion measured as an indicator of antigen presentation. Figure 4.7 shows that increasing the numbers of eosinophils from 3 x 10^4 to 1 x 10^5/well in the presence of soluble OVA resulted in greater IL-5 production by CD4$^+$ T cells. Very small amounts of IL-4 and IL-13 were detected, but were not significantly different between the different treatment conditions (not shown), and prompted the search for a more tightly controlled *in vitro* culture condition whereby Th2 cytokine production could be investigated.

4.3.6 *Eosinophils promote IL-4, IL-5 and IL-13 production from Th2 cells generated in vitro.*

Purified eosinophils from IL-5-transgenic mice were able to induce IL-4, IL-5, and IL-13 production in Th2 cells in an antigen specific manner (Figure 4.8), and in amounts comparable to mitomycin C treated APCs. The amount of IL-5 production (~189 ng/ml) was greater than that seen when using C57BL/6 CD4$^+$ splenocytes (Figure 4.7, ~8-11 ng/ml), and detectable levels of IL-4 and IL-13 justified the choice of using BALB/c eosinophils and *in vitro* derived Th2 cells. Thus, murine eosinophils are able to induce proinflammatory cytokine secretion from CD4$^+$ Th2 cells polarized *in vitro*.

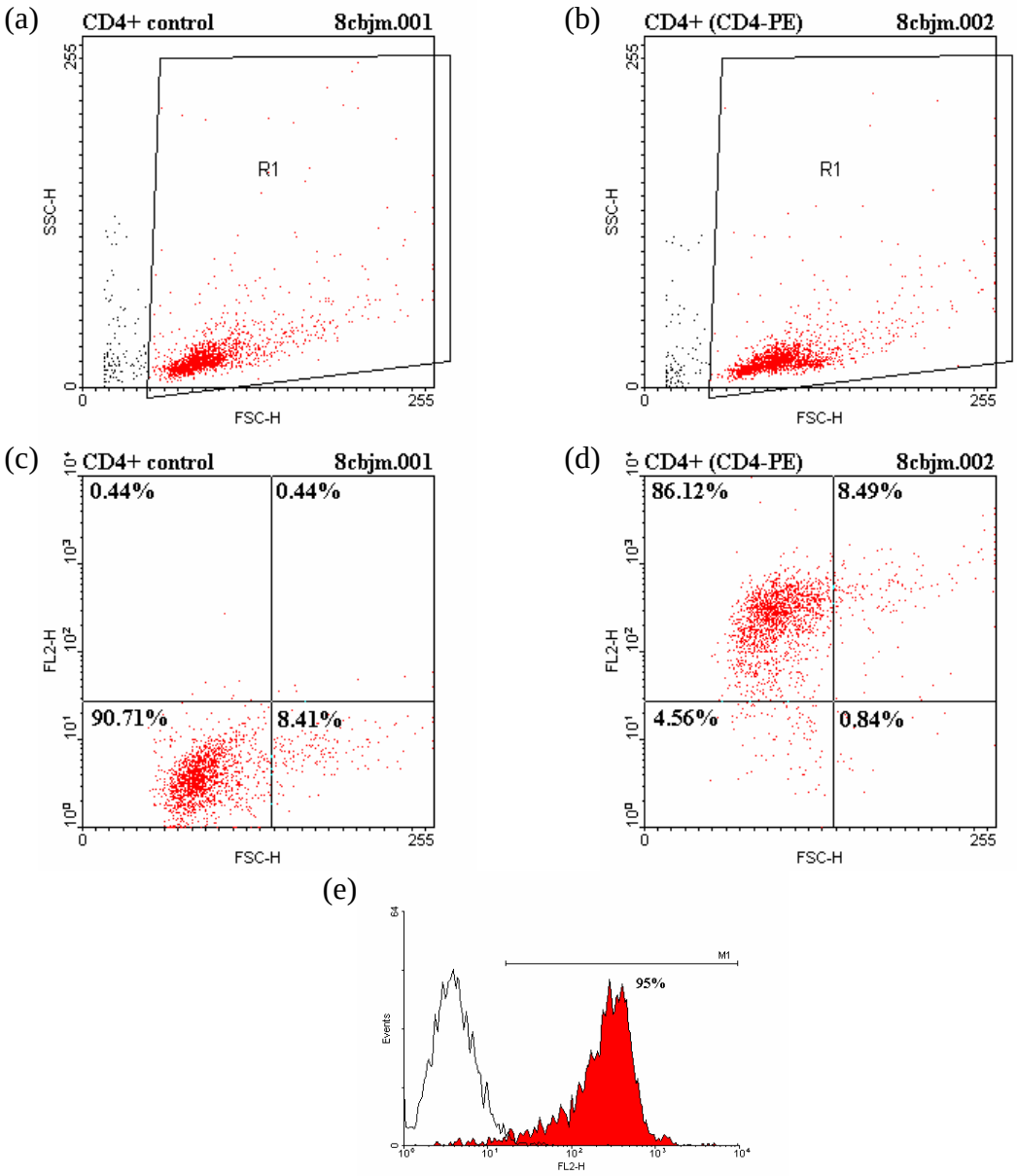


Figure 4.6. Flow cytometry analysis of $CD4^+$ splenocytes purified by high-gradient magnetic MiniMacs separation columns. (a) Representative dotplot of MiniMacs enriched $CD4^+$ splenocytes from C57BL/6 mice with AAD incubated in HBSS alone, and autofluorescence (c) illustrated below. The lymphocyte gated region (R1) was determined based on forward- and side-scatter signals. (b) Representative dotplot of MiniMacs enriched $CD4^+$ splenocytes from allergic airways disease (AAD) mice stained with PE-cojugated anti-mouse CD4, and fluorescence (d) shown below. (e) Representative histogram of MiniMacs enriched $CD4^+$ splenocytes from AAD mice. Filled histogram shows that $CD4^+$ cells were consistently >95% (under marker, M1) of total cells. Open histogram represents unstained $CD4^+$ splenocytes.

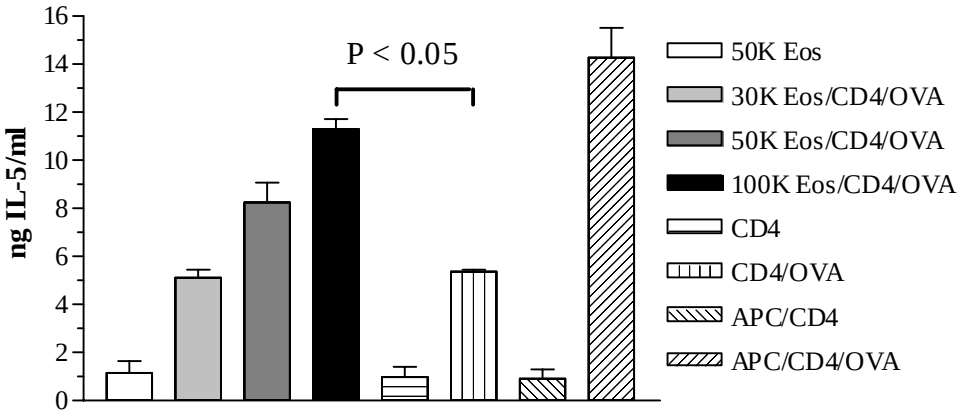


Figure 4.7. Eosinophils induce IL-5 production when co-incubated with $CD4^+$ splenocytes. Enriched BALF eosinophils from C57BL/6 mice with AAD stimulated IL-5 secretion from MiniMacs purified $CD4^+$ splenocytes in the presence of exogenous OVA (100μg/well) (EOS/CD4/OVA). Increasing the numbers of eosinophils from 3 x 10^4 (30K) to 1 x 10^5 (100K) resulted in greater production of IL-5 by $CD4^+$ T cells. For comparison, $CD4^+$ T cells from allergic C57BL/6 mice secreted IL-5 when stimulated with APCs (1x10^5, $CD4^-$ fraction) in the presence of exogenous OVA (100μg/well) (CD4/APC/OVA). For controls, 5 x 10^4 eosinophils (50K Eos) and 2 x 10^6 $CD4^+$ splenocytes (CD4) were plated individually with no other stimuli. Data represents mean ± SEM for triplicate wells. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$).

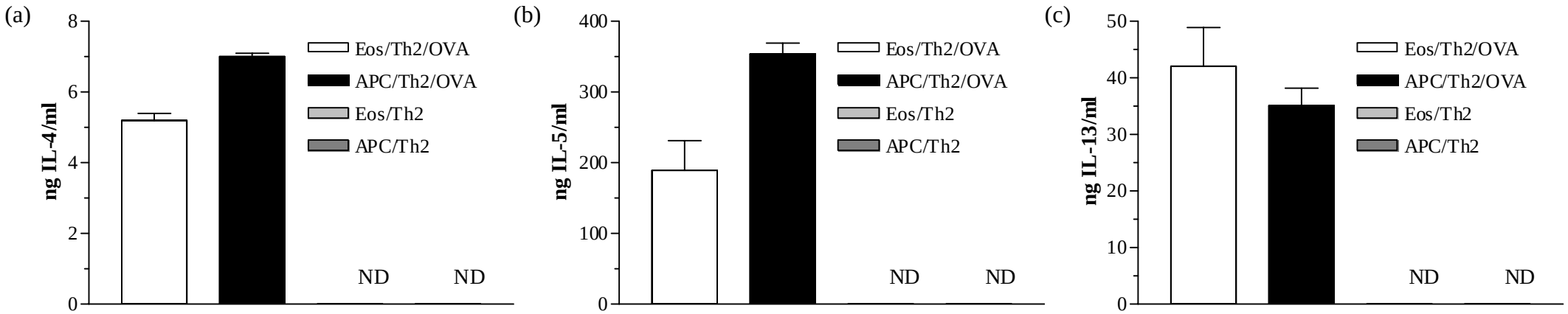


Figure 4.8. Eosinophils promote IL-4 and IL-5 production from *in vitro* derived Th2 cells. Co-culture of eosinophils (5 x 10^5 cells/well) isolated from IL-5-transgenic BALB/c mice with in vitro-polarized Th2 cells (5 x 10^5 cells/well) resulted in elevated levels of IL-4 (a), IL-5 (b), and IL-13 (c). Th2 cells incubated with eosinophils (Eos/Th2/OVA) or mitomycin C-treated splenocytes (Th2/APC/OVA) in the presence of OVA produced similar levels of cytokines. Th2 cells incubated with eosinophils (Eos/Th2) or mitomycin C-treated splenocytes (Th2/APC) without OVA did not promote measurable production of cytokine (ND: not detected). Data represents mean ± SEM for triplicate wells.

4.4 DISCUSSION

In this Chapter, the capacity for mouse airway eosinophils to present antigen *in vitro* was assessed, in an attempt to establish the potential for these cells to exacerbate AAD *in vivo*. During the maintenance of AAD, the inflamed lung and draining lymphoid tissue provides an ideal microenvironment rich in IL-5 (Jarjour *et al.*, 1997; Ohkawara *et al.*, 1997; Gajewska *et al.*, 2001), which is conducive to eosinophil survival and persistence (Yamaguchi *et al.*, 1988). In Chapter 3, eosinophils were shown to populate the lung and draining lymphoid tissues during AAD maintenance and disease resolution, leaving them well placed to present exogenous antigen to T cells and to modulate disease progression. Although eosinophils are regarded as relatively inefficient in comparison to professional APCs (Del Pozo *et al.*, 1992; Mawhorter *et al.*, 1994), their abundance in allergic airway and draining lymphoid tissues may permit a cumulative and *en masse* presentation of antigen to responding T cell populations, irrespective of their relative efficiency as APCs.

Expression of functional MHC-II on APCs is necessary for effective antigen presentation to T lymphocytes (Martin *et al.*, 1996b), and eosinophils from mice with AAD were consequently investigated for MHC-II expression. Airway eosinophils from mice with AAD express surface MHC-II (Figure 4.1), which agreed with the results of Shi *et al*, who have also demonstrated expression of these molecules in BALB/c mice (Shi *et al.*, 2000). In Figure 4.1f, airway eosinophils are stained with MKD6, which reportedly recognises the altered tertiary structure of surface MHC-II combined with cognate peptide (Germain and Hendrix, 1991), and suggests that airway eosinophils in mice with AAD may associate exogenous antigens with MHC-II dimers, prior to their appearance on the cell surface.

The costimulatory molecules CD80 and CD86 enhance T cell responses to antigen presentation (Oertli *et al.*, 1997), and are important for AAD induction and progression. In a mouse model of AAD, use of CTLA4-IG fusion protein to block CD28-B7 costimulatory interactions prior to sensitisation (Keane-Myers *et al.*, 1997), and during exposure to aeroallergen (Krinzman *et al.*, 1996), inhibits inflammatory infiltrate, airways hyperreactivity and T cell responses to antigen. In Figure 4.2, airway eosinophils from C57BL/6 mice with AAD are shown to express both CD80 and CD86 co-stimulatory molecules to an extent comparable with reported levels for allergic

BALB/c mice (Shi *et al.*, 2000). Thus, in addition to expression of MHC-II, airway also express the co-stimulatory molecules necessary for effective antigen presentation.

However, expression of surface MHC-II and costimulatory molecules does not automatically infer antigen presenting capability of eosinophils. Prior to MHC-mediated presentation at the cell surface, exogenous proteins must be subjected to intracellular processing by endosomal and lysosomal proteases, before stable association with MHC-II molecules within MHC-II compartments (Weenink and Gautam, 1997). In the allergic mouse lung, eosinophils may constitute 60-70% of the inflammatory infiltrate responding to repeated aeroallergen challenge. Although this granulocyte population express MHC-II and the costimulatory molecules effective for antigen presentation to T cells, their ability to take up and process aeroallergen in situ is unknown. Self quenching fluorescent OVA (DQ-OVA) (Santambrogio *et al.*, 1999) was therefore used to demonstrate antigen loading and processing by airway eosinophils from OVA sensitised mice. In Figure 4.3, freshly isolated airway eosinophils from allergic C57BL/6 mice displayed increased fluorescence as a function of time following incubation with DQ-OVA. This denotes an ability of airway eosinophils to process DQ-OVA, presumably by the action of endosomal and lysosomal proteases, which in turn generate fluorescent intracellular fragments of the parent protein. Upon investigation, eosinophils isolated from the allergic lung both incorporated and processed DQ-OVA (Figure 4.4), suggesting that eosinophils have the capacity to sample the extracellular milieu, and thereby take up and process aerosolised antigens within the airways.

Prior to these experiments, eosinophils were already known to present antigen to T cells *in vitro*. Del Pozo *et al*., have previously demonstrated the ability of murine eosinophils to stimulate T cell clones and hybridomas following incubation with both simple (no requirement for intracellular processing) and complex (requirement for processing) antigens (Del Pozo *et al.*, 1992). In the results presented in this Chapter, eosinophils from BALF of AAD C57BL/6 mice were initially tested for their ability to present the OVA peptide $OVA_{323-339}$ (no requirement for intracellular processing) to OT-II splenocytes (Figure 4.5a). Peptide loaded and fixed eosinophils were shown to stimulate OT-II splenocyte proliferation in a dose dependent manner, however subsequent attempts to use live eosinophils to detect cytokine secretion were unsuccessful (not shown), and the reasons for this are further explored in Chapter 6.

Logically, the next step was to investigate live eosinophil interactions with T cells from allergic mice. Initially, airway eosinophils from mice with AAD were incubated with allergic splenocytes in the presence of exogenous antigen, and shown to augment splenocyte proliferation (Figure 4.5b). This is not the only reported augmentation of T cell responses attributable to eosinophils. Since this work was completed, the adoptive transfer of eosinophils to allergic IL-5/eotaxin$^{-/-}$ mice has been shown to restore IL-13 production in T cells, possibly via a direct interaction as antigen presenting cells, and involving eosinophil release of IL-18 into the inflammatory microenvironment (Mattes *et al.*, 2002).

The ability of eosinophils to supplement allergic splenocyte responses shown in Figure 4.5b prompted a more detailed evaluation of the interaction between eosinophils and antigen specific T cells, to determine if eosinophils could interact directly with responding T cells as antigen presenting cells. Eosinophils from the BALF of C57BL/6 mice with AAD were co-cultured with purified CD4^{+} splenocytes from the same mice (Figure 4.8). In the presence of exogenous OVA, increasing numbers of eosinophils induced increasing amounts of IL-5 secretion, although significant levels of IL-13 and IL-4 were not detected under these culture conditions.

It has previously been reported that both IL-4 and IL-5 production in splenocyte cultures from C57BL/6 mice are lower than those of BALB/c mice when stimulated with OVA *in vitro* (Morokata *et al.*, 1999). Therefore the final experiment described in this Chapter was conducted using IL-5 transgenic eosinophils (Dent *et al.*, 1990), and *in vitro* derived Th2 cells from the BALB/c background. This experiment was designed to determine Th2 cytokine production (IL-4, IL-5 and IL-13) from *in vitro* derived CD4^{+} T cells, using an enriched population of eosinophil APCs isolated from non-allergic mice, thus ensuring that the only available antigen was that which was added to the cultures. Figure 4.8 shows the ability of eosinophils acting as APCs to induce secretion of the Th2 cytokines IL-4, IL-5 and IL-13, in amounts comparable to that induced by mitomycin C treated splenocytes (as APC controls). To the author's knowledge, this was the first observation of *in vitro* Th2 cytokine release when using eosinophils as APCs and *in vitro* derived Th2 cells. However, it is pertinent to note that eosinophils may also express these proinflammatory cytokines (Desreumaux *et al.*, 1993; Dubucquoi *et al.*, 1994; Lamkhioued *et al.*, 1995; Nonaka *et al.*, 1995; Bjerke *et al.*, 1996; Lamkhioued *et al.*, 1996; Moller *et al.*, 1996a; Moller *et al.*, 1996b), and may

have contributed to cytokine release from antigen-reactive T cells. Interestingly, ligation of CD28 expressed by human eosinophils has recently been shown to induce the release of granule associated IL-13 (Woerly *et al.*, 2002). Activated T lymphocytes in human peripheral blood express CD80 (B7/BB1) (Azuma *et al.*, 1993), and identifies a possible mechanism for IL-13 release from CD28 expressing eosinophils interacting with $CD80^+$ T lymphocytes. Ultimately, TCR engagement by MHC-II on eosinophils may promote Th2 cytokine release into the inflammatory microenvironment.

In this Chapter, the capacity of eosinophils to act as antigen presenting cells has been investigated. It has been established that eosinophils from the allergic airway express MHC-II and costimulatory molecules necessary for effective antigen presentation to T cells (Figures 4.1 – 4.2), and that airway eosinophils are able to sample the extracellular milieu and to process exogenous antigen into smaller antigenic fragments (Figures 4.3-4.4). Furthermore, eosinophils present OVA to $CD4^+$ T cells, and promote IL-4, IL-5 and IL-13 Th2 cytokine secretion in amounts similar to that of other (splenic) APCs (Figures 4.7-4.8). Collectively, these *in vitro* results show the capacity for eosinophils to act as APCs to antigen-specific T cells. By inference, eosinophils that are recruited to the allergic airways and draining lymphoid tissue during AAD progression may sample the extracellular milieu, and ultimately present OVA peptides to resident OVA-specific T cells. This putative interaction may be enhanced by increasing numbers of eosinophils that are recruited to this tissue, which promote T cell release of IL-4, IL-5, and IL-13 and thereby exacerbate airways inflammation following repeated exposure to aeroallergen.

Chapter 5

Eosinophils As Antigen Presenting Cells *In Vivo*

5.1 INTRODUCTION

In mouse models of allergic airways disease, conventional sensitisation via i.p. injection with antigen/adjuvant and subsequent airways challenge with relevant antigen produces hallmark characteristics of asthma, including the appearance of antigen specific immunoglobulins in serum, peripheral blood eosinophilia, inflammatory cell infiltration and remodeling of the airways, and bronchospasm in response to methacholine challenge (Foster *et al.*, 1996; Temelkovski *et al.*, 1998; Wang *et al.*, 1998; Inman *et al.*, 1999; Kumar *et al.*, 2002). Non-conventional sensitisation to relevant antigen can also be achieved using the individual humoral and cellular components of allergic airway disease. For example, the passive transfer of allergen-specific IgE producing B cells to naïve mice can induce allergen-induced airway hyperresponsiveness (Lack *et al.*, 1995), and transfer of allergen-specific IgE antibody alone is sufficient to induce both airway hyperresponsiveness and lung eosinophilia following airways exposure to relevant antigen (Oshiba *et al.*, 1996). Alternatively, the transfer of antigen-specific IL-5 producing T cell clones to naïve mice can induce lung eosinophilia and airways remodeling (Kaminuma *et al.*, 1997), and also airways hyperresponsiveness in the absence of serum IgE (Li *et al.*, 1998). Furthermore, IL-5 producing $CD4^+$ T cells may be obtained from sensitised donor mice, and used to promote aeroallergen induced lung eosinophilia and airways hyperresponsiveness in naïve wild type (Wise *et al.*, 1999) and also in IL-5 deficient recipients (Hogan *et al.*, 1998c).

Passive sensitisation with components of the allergic response provides invaluable insight into the participatory role of each component towards allergic airways disease progression and maintenance. Active sensitisation using antigen loaded cells may be used to identify those cells having the ability to prime for and to regulate Th2 lymphocyte function, and which may potentially exacerbate allergic airways disease. For instance, macrophages loaded with antigen *ex vivo* can induce pulmonary eosinophilia when transferred (i.p.) to naïve recipients, in a manner which is sensitive to CD4 and CD8 blocking antibodies (Lambert *et al.*, 1996). Similar elegant studies using antigen loaded dendritic cells injected into the airways of naïve mice have demonstrated the ability of these professional APCs to educate the naïve immune system and induce characteristic features of allergic airways disease including lung eosinophilia, generation of Th2 cytokines (IL-4, IL-5, and IL-13), airways remodeling (Lambrecht *et al.*, 2000) and also airways hyperresponsiveness (Sung *et al.*, 2001).

Human eosinophils obtained from the asthmatic lung display an altered phenotype when compared to blood eosinophils, including upregulation of surface HLA-DR, intercellular adhesion molecules, and activation markers including CD11b (Hansel *et al.*, 1991; Sedgwick *et al.*, 1992; Mengelers *et al.*, 1994). *In vitro* evidence suggests that eosinophils acquire this phenotype during transendothelial migration, which induces autocrine GM-CSF production that may potentiate eosinophil survival (Yamamoto *et al.*, 2000), and also upregulate HLA-DR expression (Lucey *et al.*, 1989). Despite their capacity to express HLA-DR, there has been no evidence for the regulation of T cell function by airways eosinophils in allergic asthmatics, and similarly, no reported instances of the ability for antigen loaded eosinophils to prime for allergic disease of the lung in any animal model. As discussed in Chapter 4, eosinophils derived from the allergic mouse lung are known to express surface MHC class II, co-stimulatory B7.1 and B7.2 molecules, and to take up and process relevant antigen *in vivo*. They also have the capacity to activate T cells, and to elaborate Th2 cytokine production. It was thus hypothesised that lung eosinophils derived from the allergic airways would be competent APCs, having the capacity induce Th2 mediated allergic airways disease when transferred to naïve mice.

In the experiments described in this Chapter, eosinophils derived from the allergic mouse lung are shown to induce allergic airways disease when transferred to naïve mice, without any requirement for additional antigen loading *ex vivo*. Antigen loaded eosinophils derived from the IL-5 transgenic mouse are also shown to promote allergic airways disease in naïve recipient mice via altered Th2 lymphocyte function.

5.2 MATERIALS AND METHODS

5.2.1 Animals.

Wild type and IL-5 transgenic (Dent *et al.*, 1990) C57BL/6 mice (male, 4-8 wk old), were obtained from the SPF unit of the ANU and housed in an approved containment facility. Mice were treated according to ANU animal welfare guidelines.

5.2.2 Preparation of eosinophils.

In order to measure their antigen presenting capacity *in vivo*, large numbers of eosinophils were obtained from allergic and non-allergic mice in the C57BL/6 background prior to their adoptive transfer to naïve recipients.

5.2.2.1 Preparation of eosinophils from the allergic lung.

Male wild type C57BL/6 mice were sensitised and challenged with OVA as in Section 3.2.5. BALF (see Section 2.2.4) was collected on day 31, and eosinophils were purified by FACS as in Section 2.2.5. The purity of the enriched population of eosinophils was >98% as determined by differential staining with May-Grunwald-Giemsa. Prior to transfer, eosinophils were resuspended in HBSS at a cell concentration of 1×10^6/100 μl and kept on ice.

5.2.2.2 Preparation of non-allergy derived eosinophils.

Male 4 wk old wild type C57BL/6 mice were treated with PMBS as in Section 2.2.2, and eosinophils purified from the PEC population using FACS as in Section 2.2.5. The purity of the enriched population of eosinophils was >98% as determined by differential staining with May-Grunwald-Giemsa. Prior to transfer, eosinophils were resuspended in HBSS at a cell concentration of 1×10^6/100 μl and kept on ice.

In other experiments and where indicated, IL-5 transgenic C57BL/6 mice (Dent *et al.*, 1990) were sacrificed by CO_2 asphyxiation and the peritoneal cavity lavaged as in Section 2.2.2, and eosinophils purified by FACS as in Section 2.2.5. The purity of the enriched population of eosinophils was >98% as determined by differential staining with May-Grunwald-Giemsa solution. As indicated, IL-5 transgenic eosinophils were incubated in HBSS containing 1 mg/ml OVA for 30 minutes at 37°C, before being washed (five times) in excess HBSS to remove any unincorporated OVA. Prior to

transfer, eosinophils were resuspended in HBSS at a cell concentration of 1 x 10^6/100 μl and kept on ice.

5.2.3 Transfer of allergy derived eosinophils into naïve C57BL/6 recipient mice.

Purified eosinophils (98% pure) from the airways of OVA sensitised and challenged C57BL/6 mice (Section 5.2.2.1) were injected into the peritoneal cavities of naïve C57BL/6 mice on days 0 and 7 (AAD EOS, 2 x 10^6). As a control, purified eosinophils from PMBS injected mice (Section 5.2.2.2) were also injected into the peritoneal cavities of separate naïve C57BL/6 mice on days 0 and 7 (PMBS EOS, 2 x 10^6). On days 0, 1, 8, 11 and 19, the peritoneal cavities from 3-4 mice in each group were lavaged following CO_2 asphyxiation, and recoverable eosinophils were enumerated (as in Section 2.2.2). All eosinophil recipient mice were exposed to aerosolised antigen (OVA, 10 mg/ml in 0.9 % saline) for 3 x 30 minutes using a RapidFlo nebuliser bowl on days 12, 14, 16, and 18. Peripheral bloods were taken on days –1, 1, 4, 6, 8, 11, 13, 15, 17, and both BALF and peripheral bloods taken for analysis on day 19.

5.2.4 Transfer of non-allergy derived eosinophils into naïve C57BL/6 recipient mice.

Purified C57BL/6 IL-5 transgenic mouse eosinophils (98% pure) incubated with OVA from Section 5.2.2.2 (Ag^+ EOS, 2 x 10^6) were injected into the peritoneal cavities of naïve C57BL/6 mice on days 0 and 9. As a control, purified eosinophils that were not incubated in antigen (Ag^- EOS, 2 x 10^6) were also injected into the peritoneal cavities of separate naïve C57BL/6 mice on days 0 and 9. All eosinophil recipient mice were then exposed to aerosolised antigen (OVA, 10 mg/ml in 0.9 % saline) for 3 x 30 minutes using a RapidFlo nebuliser bowl on days 15, 17, 19, and 21. Peripheral bloods were taken on days 14, 16, 18, 20, and peripheral blood, sera, BALF, lung and lymph node tissues collected for analysis on day 22.

5.2.5 Collection and analysis of blood and bronchoalveolar lavage fluid.

Peripheral blood films were stained with May-Grunwald-Giemsa solution, and 200-300 leukocytes identified by morphological criteria. On the last day of each experiment, mice were sacrificed by cervical dislocation, and bronchoalveolar lavage performed as in Section 2.2.4.

5.2.6 Histological analysis of lung tissue from IL-5 transgenic-eosinophil recipients.

Mouse lungs from Section 5.2.4 were immediately removed and fixed in 10% phosphate-buffered formalin solution, pH 7.0 for at least 2 hours. Later, sections representing the central (bronchi-bronchiole) and peripheral (alveolar) airways were cut and immersed in 10% phosphate buffered formalin solution for a minimum of 72 hours. The samples were paraffin-embedded, sectioned and stained with Lendrum's carbolchromotrope stain (Lendrum, 1944). Leukocytes were identified by morphological criteria and photographed.

5.2.7 Lymph node cell culture.

Peribronchial lymph nodes from Section 5.2.4 were harvested into ice cold HBSS and physically disaggregated. The isolated cells were centrifuged at 500 g at 4^{o}C for 10 minutes and the cell pellet was resuspended in 500 μl RBC lysis solution and incubated at 37^{o}C for 5 minutes before the addition of 1 ml ice cold HBSS. The cells were then centrifuged again at 500 g at 4^{o}C for 10 minutes, and the cell pellet resuspended in 500 μl ice-cold PBS. Cell numbers were determined using a standard haemocytometer, and lymph nodes cells were then resuspended at 5 x 10^5 cells/100 μl in MLC medium containing 10% FCS. Lymph node cells were cultured at a density of 5 x 10^5/well for 96 hours in the presence or absence of 200 μg OVA, in a total volume of 200 μl/well. Culture supernatants were then taken and assayed for IL-5 and IL-13.

5.2.8 Cytokine analysis.

IL-13 and IL-5 concentrations were determined in the supernatants from OVA-stimulated lymph node cells by ELISA. Briefly, round bottomed immunoassay plates were coated with 50μl anti-mouse IL-13 (R&D Systems, Minneapolis, MN), or anti-mouse IL-5 (TRFK5) and incubated at 4^{o}C overnight. Plates were then blocked with 2% skim milk powder in PBS-Tween20 (0.04%) for 1 hour at 37^{o}C, and incubated with serial dilutions of cultured supernatants, and IL-13, or IL-5 standards. Mouse IL-13 and IL-5 were detected with biotinylated anti-mouse IL-13 (R&D Systems, Minneapolis, MN), and anti-mouse IL-5 (BD PharMingen) respectively. Plates were then incubated with streptavidin-conjugated alkaline-phosphatase (Amersham International plc, Buckinghamshire, UK) for 1 hour at 37^{o}C, washed, and alkaline-phosphatase substrate solution (Sigma Chemical Co. St Louis, MO) was added. Where necessary, reactions

were stopped with 1M citric acid, or promptly analysed. Plates were read by a Microplate reader (BIO-TEK Instruments Inc., Winooski, VT) at 490 nm with reference to 405 nm.

5.2.9 Ovalbumin specific IgG_1 determination by ELISA.

Serum samples were taken from the tail veins of eosinophil recipients from Section 5.2.4 on days 0 and 22. OVA specific IgG_1 was measures as in Section 2.2.8.

5.3 RESULTS

5.3.1 Purified eosinophils from the allergic lung promote allergic disease of the lung when transferred to naïve C57BL/6 recipients.

Eosinophils obtained from the lungs of mice with AAD or from mice with PMBS induced eosinophilia ($2x10^6$) were transferred to the peritoneal cavities of naïve recipients on days 0 and 7. Following the first i.p. injection on day 0, there was a significant difference in recoverable eosinophil numbers from the peritoneal cavities of AAD and PMBS eosinophil recipients (Figure 5.1a). In PMBS eosinophil recipients, about 6×10^5 eosinophils were recovered, suggesting 1.4×10^6 eosinophils were either adhering to tissues and organs within the peritoneal cavity, or were no longer residing in this compartment. This phenomena was more striking in AAD eosinophil recipients, in which only about 3×10^4 of transferred eosinophils were recovered. Following the second i.p. injection on day 7, there were more recoverable eosinophils in peritoneal lavage from PMBS recipients than from AAD recipients, however, this was no longer significantly different. Peripheral blood data (Figure 5.1b) for days 1 and 8 does not indicate that either population of eosinophils entered the blood stream following i.p. administration on days 0 or 7.

Subsequent exposure to aerosolised OVA on days 12, 14, 16, and 18 did not induce any significant differences in peripheral blood eosinophil levels between AAD and PMBS eosinophil recipients at any time point (Figure 5.1b). It was noted that AAD eosinophil recipients had significantly increased peripheral blood eosinophils (5.7%) on day 19 in comparison to day –1 (1.5%). The repeated exposure of eosinophil recipients to aerosolised OVA induced a pulmonary eosinophilia in AAD eosinophil recipients only, as reflected by increased eosinophils/ml BALF (Figure 5.1c). No other cell types were significantly elevated between the two groups, although increased macrophages in BALF from PMBS eosinophil recipients was suggested

5.3.2 Purified eosinophils from IL-5 transgenic mice promote allergic disease of the lung when transferred to naïve C57BL/6 mice.

Antigen loaded (Ag^+ EOS) and antigen negative (Ag^- EOS) eosinophils from IL-5 transgenic mice ($2x10^6$) were transferred i.p. to naïve C57BL/6 wild type recipients on days 0 and 9, and the recipients were subsequently exposed to aerosolised OVA on days 15, 17, 19, and 21. Exposure to aerosolised OVA induced significant differences in

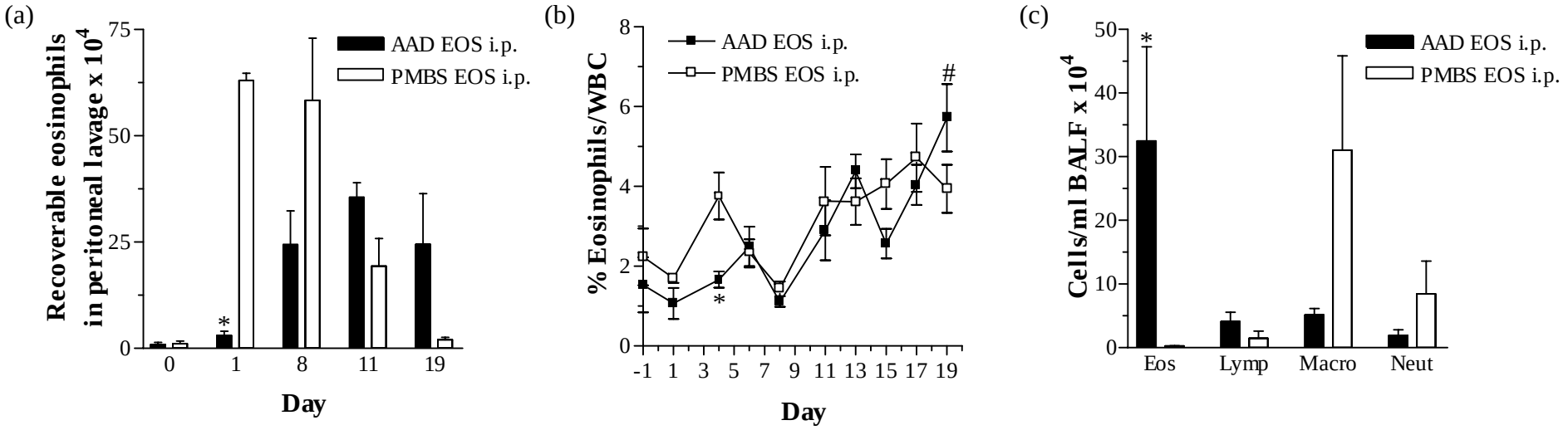


Figure 5.1. Eosinophil numbers in peritoneal lavage, peripheral blood and BALF of naïve eosinophil recipients. (a) Recoverable eosinophils in the peritoneal cavities of mice that received eosinophils from the lungs of mice with AAD (AAD EOS i.p.), and those that received PMBS induced eosinophils (PMBS EOS i.p.). (b) Peripheral blood eosinophils in AAD EOS i.p. and PMBS EOS i.p. recipients. (c) Cellular composition of BALF on day 19. Data represents mean ± SEM for samples taken from 3-5 mice on days 0-18, and 5-8 mice on day 19. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$). * $P < 0.05$ compared to PMBS eosinophil recipients. $^{\#}$ $P < 0.05$ for AAD eosinophil recipients on day 19 compared to day –1.

peripheral blood eosinophil levels between Ag^+ and Ag^- eosinophil recipients at all measured time points (Figure 5.2a), and Ag^+ eosinophil recipients had significantly increased peripheral blood eosinophils at all time points following exposure to OVA when compared to day 14. The repeated exposure of eosinophil recipients to aerosolised OVA on days 15, 17, 19 and 21 induced a pulmonary eosinophilia in Ag^+ eosinophil recipients only, as reflected by increased eosinophils/ml BALF (Figure 5.2b). In addition, macrophages and neutrophils in BALF from Ag^- eosinophil recipients were significantly elevated over Ag^+ eosinophils. Notably, lung sections from Ag^+ but not Ag^- eosinophil recipients were characterized by a pronounced eosinophil infiltrate in and around the airways, mucus plugging, and cellular disruption of the airways epithelium (Figures 5.3 and 5.4)

Draining lymph node cells from Ag^+ and Ag^- eosinophil recipients were harvested on day 22 and stimulated with exogenous OVA. Lymph node cells from Ag^+ eosinophil recipients produced significantly greater amounts of IL-5 (230 ng/ml) and IL-13 (14 ng/ml) in response to the addition of exogenous OVA than did Ag^- eosinophil recipients (0 and 0.5 ng/ml respectively) (Figure 5.5a and b). Lymph nodes cells from Ag^+ eosinophil recipients secreted both IL-5 and IL-13 without addition of exogenous OVA (condition C, Figure 5.5a and b), which was probably carried over from the previous exposure to aerosolised OVA on day 21 *in vivo*.

5.3.4 Priming action of eosinophils does not induce antigen-specific IgG_1 antibodies.

Serum from mice with AAD (Section 5.2.2.1), and from allergic lung (Section 5.2.3) and IL-5 transgenic eosinophil recipients (Section 5.2.5) was analysed for OVA specific IgG_1 following repeated exposures to aerosolised antigen (OVA). Despite the pulmonary eosinophilia seen in AAD and Ag^+ eosinophil recipients (Figures 5.1 – 5.4), only mice sensitised by i.p. injection of OVA in alum had any detectable serum OVA-specific IgG_1 (2.5 ±0.12 mg/ml) (data not shown).

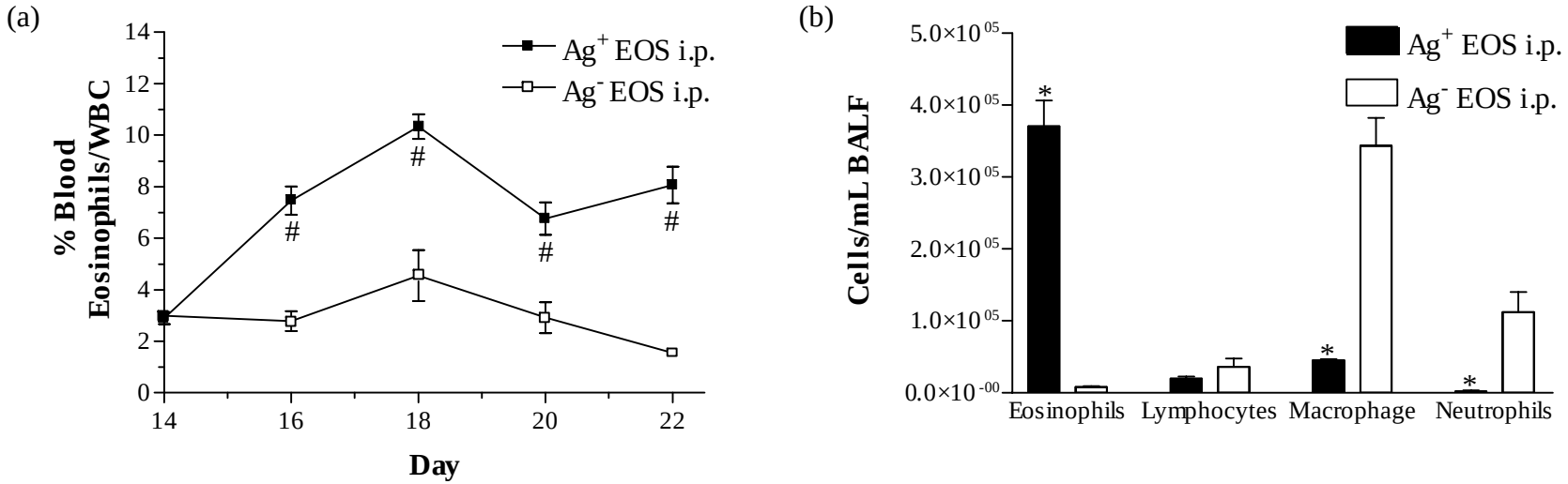
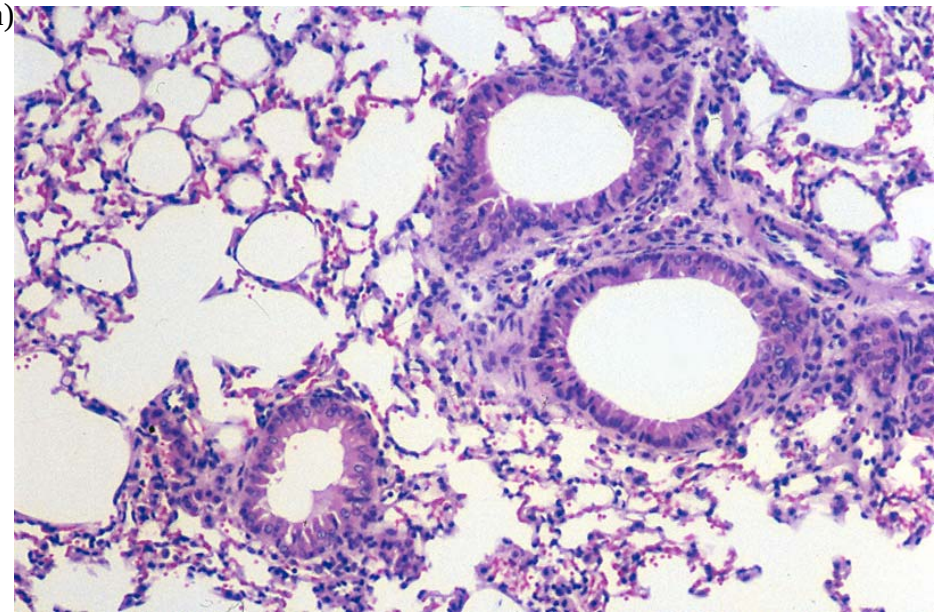


Figure 5.2. Antigen pulsed eosinophils from IL-5 transgenic mice promote allergic disease of the lung in naïve recipients. (a) Peripheral blood eosinophils in naïve mice that received OVA-pulsed (Ag^+) or OVA-free (Ag^-) IL-5 transgenic eosinophils i.p. on days 0 and 9, and which were then exposed to aerosolised OVA on days 15, 17, 19 and 21. (b) Numbers of eosinophils/ml BALF in naïve mice that received Ag^+ or Ag^- IL-5 transgenic eosinophils on day 22. Numbers of eosinophils, macrophage and neutrophils were significantly different between Ag^+ and Ag^- eosinophil recipients. Data represents mean ± SEM for groups of 4 mice. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$). $^{\#}$ $P < 0.05$ compared to Ag^- recipients on days 16-22, and Ag^+ recipients on day 14. * $P < 0.05$ compared to Ag^- recipients.

(a)



(b)

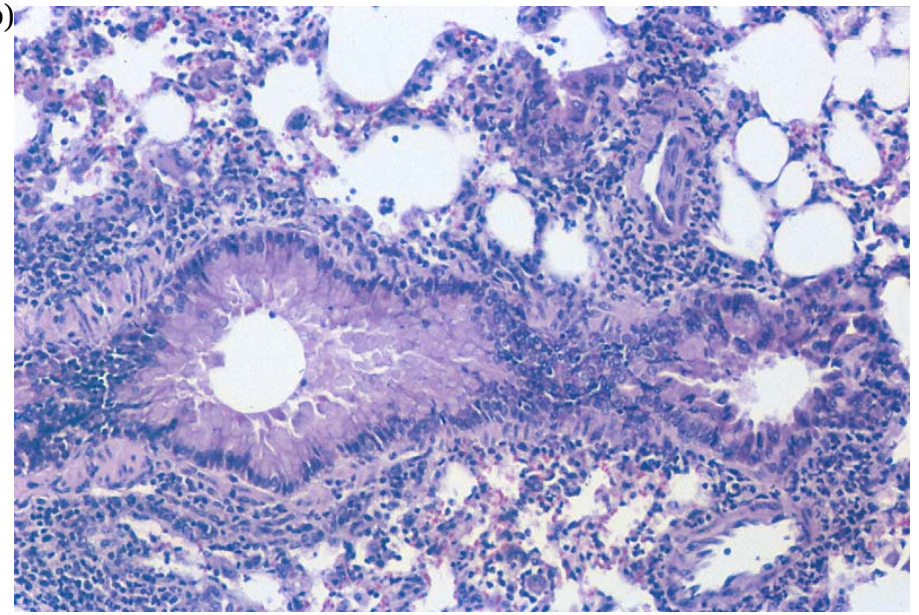
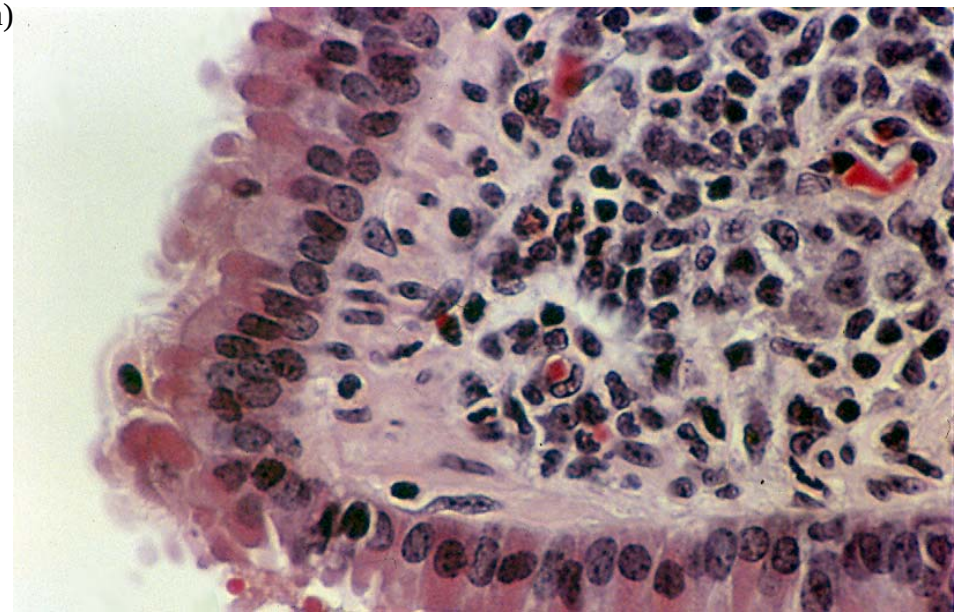


Figure 5.3. Histological analysis of lung sections from antigen-pulsed IL-5 transgenic eosinophil recipients. (a) Histological section (10 x objective) typical of mice that received OVA-free (Ag^-) IL-5 transgenic eosinophils on days 0 and 9, and which were subsequently challenged with OVA on days 15, 17, 19 and 21. There is little evidence of airway plugging nor inflammatory infiltrate. (b) Histological section (10 x objective) typical of OVA-pulsed (Ag^+) eosinophil recipient mice. Note the pronounced peribronchial and perivascular infiltrate surrounding the airways and blood vessels, and occluded airway typical of goblet cell hyperplasia. Lung tissue was stained with Lendrum's carbolchromotrope stain.

(a)



(b)

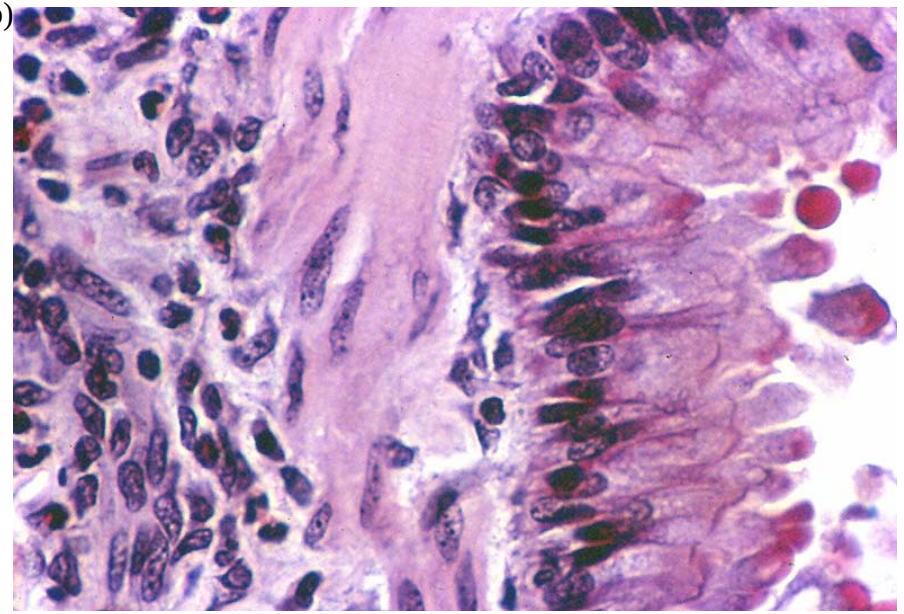


Figure 5.4. Histological analysis of lung sections from antigen-pulsed IL-5 transgenic eosinophil recipients. (a) Histological section (40 x objective) typical of mice that received OVA-free (Ag^-) IL-5 transgenic eosinophils on days 0 and 9, and which were subsequently challenged with OVA on days 15, 17, 19 and 21. There was no airway plugging with eosinophils nor mucus, however a lone eosinophil may be seen in the peribronchial tissue underlying the band of smooth muscle surrounding this airway. (b) Histological section (40 x objective) typical of OVA-pulsed (Ag^+) eosinophil recipient mice. Eosinophils were found to populate the peribronchial tissue underlying the airways smooth muscle. Lung tissue was stained with Lendrum's carbolchromotrope stain.

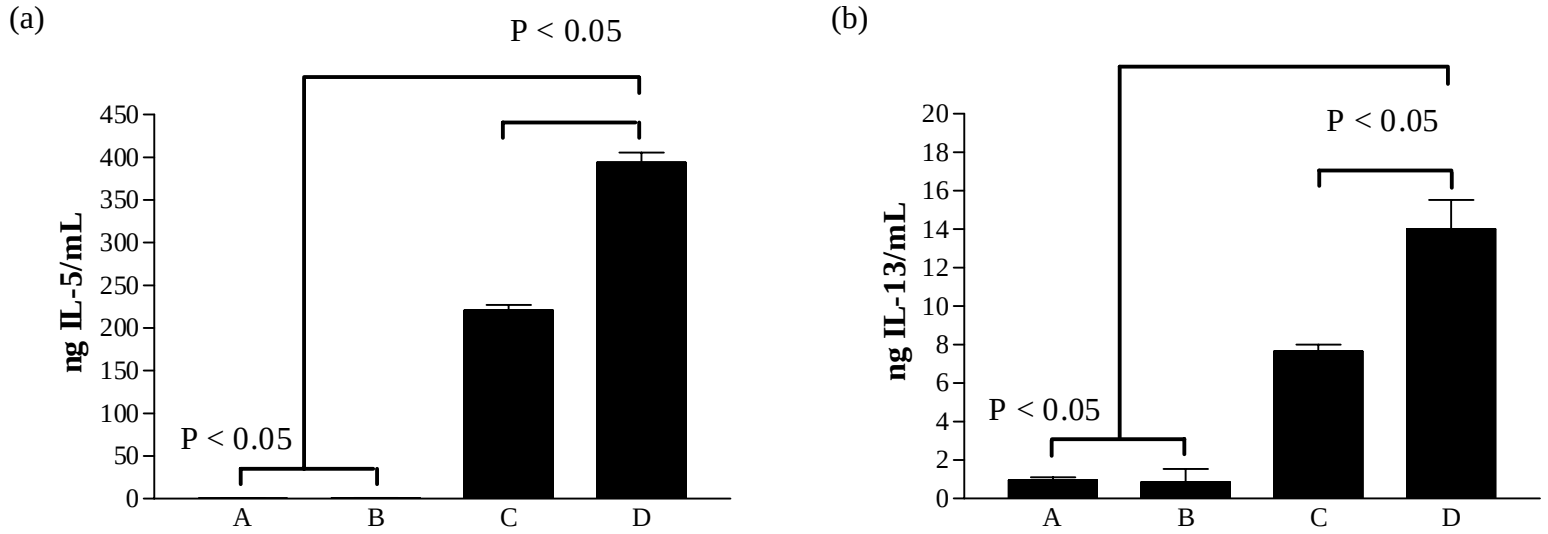


Figure 5.5. Transfer of antigen pulsed eosinophils to naïve mice promotes antigen specific cytokine production airways draining lymph nodes following exposure to aerosolised antigen. Lymph node cells from OVA-pulsed (Ag^+) and OVA-free (Ag^-) IL-5 transgenic eosinophil recipient mice were cultured according to the following conditions: A, Ag^- eosinophil recipients, no exogenous OVA; B, Ag^- recipients + exogenous OVA; C, Ag^+ recipients, no exogenous OVA; D, Ag^+ recipients + exogenous OVA. (a) IL-5 cytokine release from OVA stimulated lymph node cells. (b) IL-13 cytokine release from OVA stimulated lymph node cells. Data represents mean ± SEM for triplicate wells of pooled lymph node cells from 4 mice. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$), and are indicated by bars (¬).

5.4 DISCUSSION

In this Chapter, the capacity for antigen loaded eosinophils to induce allergic disease of the airways in naïve recipient mice has been evaluated. Eosinophils located in allergic human tissues have previously been shown to contain intracellular IL-5 (Broide *et al.*, 1992; Moller *et al.*, 1996a), and IL-4 (Nonaka *et al.*, 1995; Moller *et al.*, 1996b), and in mice, eosinophils may be responsible for the initial burst of IL-4 necessary for subsequent Th2 response to parasite infection (Sabin *et al.*, 1996). Eosinophils obtained from the BALF of mice with AAD were initially chosen in the experiments described here, as these cells were known to express the necessary costimulatory molecules for effective antigen presentation (Chapter 4 and Figures 4.1-4.2), and to take up and process antigen (OVA) deposited in the allergic airways (Chapter 4 and Figure 4.4).

Naïve recipient mice received 2 x 10^6 eosinophils from the allergic lung or from PMBS treated mice (antigen free) on days 0 and 7. When recoverable eosinophils in the peritoneal cavity were analysed on day 1, it was surprising to note that PMBS eosinophil recipients had significantly greater numbers of eosinophils residing in the peritoneal cavity (Figure 5.1a) than did AAD eosinophil recipients. This may have been due to AAD derived eosinophil trafficking from this cavity, although peripheral blood levels on day 1 did not suggest removal via blood. Peripheral blood levels did not differ greatly between the two groups (Figure 5.1b), although it is noted that only peripheral blood levels in AAD eosinophil recipients were significantly greater on day 19 following the aerosol regime, than when compared to day –1. Analysis of BALF (Figure 5.1c), revealed that AAD eosinophil recipients had increased eosinophil infiltrate of the airways following exposure to aerosolised antigen, and mimics the findings of lambert *et al.*, who transferred antigen pulsed APCs (1x10^6 macrophages/mouse i.p. on days zero and 6) to naïve mice, which subsequently developed CD4-dependent allergic disease of the lung (Lambert *et al.*, 1996). The results described in this Chapter are considered to be the first observation of antigen loaded eosinophils ability to prime for allergic disease of the lungs in naïve mice.

The experimental model was lengthened by 3 days to address the lack of a peripheral blood eosinophilia observed when using eosinophils obtained from the allergic lung to prime for allergic airways disease in naïve mice. Moreover, the use of non-allergy derived eosinophils would also ensure that the observed results were a true reflection of

the eosinophils ability to prime for T cell responses, and not due to contaminating T lymphocytes from BALF of allergic mice (typically less than 0.1%). Figure 5.2a shows that this improved model produced a peripheral blood eosinophilia that was significantly greater in Ag^+ compared to Ag^- recipients at each time point following exposure to aerosolised antigen. On day 22, only Ag^+ eosinophil recipients had a pronounced pulmonary eosinophilia as measured by BALF (Figure 5.2b), while Ag^- recipients had significantly greater numbers of macrophage and neutrophils. Stimulation of lymph node cells harvested on day 22 with relevant antigen (OVA) showed appreciable amounts of IL-5 and IL-13 release from Ag^+ recipients when compared to Ag^- controls (Figure 5.5). Secretion of both IL-5 and IL-13 without addition of OVA was noted in Ag^+ recipient lymph node cells, and was probably carried over from recent exposure to antigen *in vivo*. The priming of T cell responses *in vivo* using professional APCs (dendritic cells, DC), pulsed *ex vivo* with antigen has been reported (Lambrecht *et al.*, 2000; Sung *et al.*, 2001). Lambrecht *et al*. injected $1x10^6$ OVA-pulsed DC into the airways of naïve mice that were subsequently exposed to aerosolised OVA on days 14-20, and noted pulmonary eosinophilia, pronounced cellular infiltrate within histological sections, and also IL-4 and IL-5 secretion from mediastinal lymph node cells cultured with OVA. The eosinophil content of BALF, and peribronchial and perivascular eosinophil-rich infiltrates observed in OVA-pulsed DC recipients exposed to aerosolised OVA is similar to that seen in this Chapter for Ag^+ eosinophil recipients (Figures 5.2 – 5.4), and typical of wild type mice i.p. sensitised with OVA/alum and subsequently challenged via the airways (Foster *et al.*, 1996).

In the experiments presented in this Chapter, the transfer of antigen carrying eosinophils to naïve recipients subsequently exposed to aerosolised antigen did not generate detectable antigen specific IgG_1 in serum at any of the time points indicated. The use of OVA-pulsed DC to promote allergic disease of the lung in mice also fails to induce serum OVA-specific IgG_1 (Sung *et al.*, 2001) and IgE (Lambrecht *et al.*, 2000), and may be attributed to the lack of free OVA available for stimulation of naïve B cells.

In summary, eosinophils obtained from the allergic airway have been observed to prime for allergic disease of the lung in naïve mice, without any need for additional antigen loading *ex vivo*. The ability to prime the naïve immune system has further been confirmed using antigen pulsed eosinophils obtained from IL-5 transgenic mice, which led to pronounced inflammatory infiltrate of the airways and measurable Th2 cytokine

secretion from draining lymphoid tissues, in levels similar to those seen when mice are conventionally immunised and challenged with relevant antigen. These data and that of Chapters 2-4 suggest that eosinophils recruited to inflammatory tissues may actively sample the local microenvironment, and present processed antigen to tissue resident memory T cells within the lung and the draining lymphoid tissues. This *en masse* serial triggering of T lymphocytes by cells that are traditionally viewed as non-professional APCs can further promote Th2 inflammatory cytokine production including IL-5 and IL-13 release, and potentially exacerbate already established allergic responses. It is interesting to note that eosinophils have since been implicated in the regulation of IL-13 production from $CD4^+$ T cells and induction of airways hyperresponsiveness. This regulation is currently thought to be mediated by eosinophil derived IL-18 (Mattes *et al.*, 2002), and further supports a fundamental link between the innate (eosinophils) and adaptive (T cells) immune responses for the regulation of Th2 cytokine production.

Chapter 6

Characterisation Of The OT-II Transgenic Mouse In A Mouse Model Of Allergic Airways Disease.

6.1 INTRODUCTION

In Chapter 4, eosinophils acting as antigen presenting cells were shown to induce proliferation of OT-II transgenic splenocytes in an antigen and dose dependent fashion. Subsequent experiments designed to measure cytokine secretion resulting from such interactions failed to show any production of IL-13 nor IL-5 in cell culture supernatants. Instead, minimal amounts of IFN-γ were surprisingly detected (results not shown). The OT-II transgenic TCR recognises $OVA_{323-339}$ peptide, which has been identified as an immunodominant T cell epitope that preferentially skews naïve T cell responses towards Th2 cytokine secretion (Janssen *et al.*, 1999; Janssen *et al.*, 2000). Therefore, it was decided to characterise the OT-II mouse using an established mouse model of AAD, in an attempt to define both *in vivo* and *in vitro* responses to relevant antigen.

The OT-II transgenic mouse was engineered using both genomic and complimentary-DNA from a $CD4^+$ MHC class II $I\text{-}A^b$-restricted $OVA_{323-339}$ specific T cell hybridoma, originally derived from a C57BL/6 mouse that was primed *in vivo* with whole OVA in complete Freund's adjuvant (CFA) (Barnden *et al.*, 1998). The TCR expressed by OT-II $CD4^+$ T cells comprises Vβ5 and Vα2 elements, and like the T cell clone from which they were derived, are responsive to $OVA_{323-339}$ stimulation. Critical T cell contact residues for OT-II transgenic T cell receptors have been mapped to amino acids 331, 333, and 336, of which 333 (E) is the primary TCR contact residue located in the C terminus of $OVA_{323-339}$ (Robertson *et al.*, 2000).

Little is known about the phenotype of the OT-II transgenic mouse, although OT-II $CD4^+$ T cells have been utilised for their ability to specifically recognise $OVA_{323-339}$. For instance, OT-II transgenic cells have been used to study cross-presentation efficiency of cell-associated versus soluble OVA in mice (Li *et al.*, 2001), to investigate the role of splenic dendritic cells in priming for adaptive $CD4^+$ T cell responses to OVA (Castiglioni *et al.*, 2003), to study the influence of suppressor of cytokine signaling-1 (*SOCS-1*) on T cell activation and homeostasis (Cornish *et al.*, 2003), and to study negative selection in the thymus (Zhan *et al.*, 2003). To the best of the author's knowledge, the OT-II transgenic mouse has not been characterised in any allergy model.

In this Chapter, the OT-II transgenic mouse is partially characterised in a mouse model of AAD, and is found to be refractory to Th2 mediated allergic disease induction and progression. On further investigation, OT-II $CD4^+$ T cells carrying the transgenic TCR are found to be programmed for IFN-γ secretion in response to stimulation with OVA, and can be used to attenuate airways eosinophilia when transferred to allergic wild type mice. This ability to reduce airways eosinophilia does not however, translate to lowered tissue eosinophilia, airways hyperreactivity nor reduced OVA-specific serum immunoglobulin (IgG_1).

6.2 MATERIALS AND METHODS

6.2.1 Animals.

C57BL/6 (male, 4 wk old) and OT-II mice (N10 C57BL/6, male*, 4 wk old) (Barnden *et al.*, 1998), were obtained from the SPF unit of the ANU and housed in an approved containment facility. Mice were treated according to ANU animal welfare guidelines. *All OT-II males are transgenic, as the transgene is linked to the Y-chromosome.

6.2.2 Sensitisation and airways challenge of OT-II transgenic mice.

Both wild type C57BL/6 and OT-II transgenic mice were sensitised as in Section 3.2.5 (OVA i.p.), with the following changes: a subset of non sensitised control mice were injected with only saline and Alhydrogel (SAL i.p.). All mice were then airways challenged as in Section 3.2.5. Peripheral blood smears were collected on days 23, 25, 27, 29, and 31, and analysed as in Section 5.2.5. All mice were sacrificed on day 31, with BALF collected and analysed as in Section 2.2.4, serum collected for OVA specific IgG_1 analysis as in Section 2.2.8, and spleens excised for cytokine analysis. Spleens were disaggregated using sterile wire gauze (0.5mm) and suspended in ice cold PBS. Erythrocytes were then lysed, and the washed splenocytes were resuspended at 10^7 cells/ml in MLC medium containing 10% heat-inactivated FCS, 2 mM L-glutamine, and 50 mg/L neomycin sulfate. Splenocytes from each group were then plated out at a density of 2 x 10^6 cells/well in round-bottom tissue culture plates, with added exogenous OVA (100 μg/well). Following 72 hours of culture, cell-free supernatants were removed and stored at –70^{o}C for later cytokine analysis.

6.2.3 Isolation and stimulation of OT-II $CD4^+$ splenocytes.

OT-II transgenic mice were sacrificed by cervical dislocation and their spleens excised. Spleens were then disaggregated using sterile wire gauze (0.5mm) and splenocytes suspended in ice cold PBS. Erythrocytes were then lysed, and the washed splenocytes (~ 1 x 10^8) were suspended in 90 μl HBSS/5% FCS with 10 μl anti CD4 mAb (L3T4) coated microbeads and incubated for 20 minutes at 6-12^{o}C. Suspensions were then centrifuged at 500 x g for 5 minutes at 4^{o}C and the resultant cell pellet resuspended in 1 ml PBS/0.5 % BSA. Bead-bound $CD4^+$ cells were then isolated using high-gradient magnetic MiniMacs separation columns (MACS separation, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) as per manufacturers instructions. Isolated cells were

then washed and resuspended in MLC medium/10% FCS at 5 x 10^7 cells/ml. The $CD4^-$ fraction (unbound cells) were retained as a source of antigen presenting cells (APCs). Briefly, $CD4^-$ cells (5 x 10^7 cells/ml) were incubated in the presence or absence of $OVA_{323-339}$ peptide (100mM, ISQAVHAAHAEINEAGR), and mitomycin C (25μg/ml) in MLC medium/10% FCS for 30 minutes at 37°C. Cells were then washed 3 times in fresh media, and resuspended at 5 x 10^7 cells/ml. Isolated OT-II $CD4^+$ cells (1 x 10^6) were then co-cultured with non- and peptide-loaded APCs (5 x 10^5) in 96-well flat-bottom tissue culture plates (Nunc), in a total volume of 200 μl MLC medium/10% FCS at 37°C for 72 hours. As additional controls, OT-II $CD4^+$ cells were incubated (a) alone, (b) with 100mM added $OVA_{323-339}$ peptide, or (c) with immobilised anti-CD3 monoclonal antibody (100μg/well). Supernatants were then collected for cytokine analysis.

To measure $CD4^+$ splenocyte proliferative responses to $OVA_{323-339}$ peptide, identical tissue culture plates (as above) were centrifuged at 300 x g for 5 minutes at RT following 48 hours of co-culture, and spent media replaced with fresh media (200 μl) containing tritiated thymidine (^{3}H-thymidine, 1μCi/well). At 72 hours of co-culture, the contents of each well were harvested onto a glass fibre filter (Packard, Canberra) using a Filtermate 196 cell harvester (Packard, Canberra). Fibre mats were then assembled in the appropriate cassette with MicroScint-O fluid (Packard, Canberra) and tritiated thymidine incorporation as represented by counts per minute (CPM) measured using a Packard TopCount NXT (Packard Instrument Company INC, IL).

6.2.4 Adoptive transfer of OT-II $CD4^+$ cells to wild type allergic mice.

Wild type C57BL/6 mice were sensitised and challenged as in Section 3.2.5, with the following changes. Mice were i.p. sensitised on day 0 only, and challenged via the airways on days 12, 14, 16, and 18. A subset of mice were adoptively transferred with 2 x 10^6 $CD4^+$ splenocytes from OT-II transgenic mice (enriched as in Section 6.2.3) via the tail vein (total volume 200 μL in PBS) on day 0. Peripheral blood smears were collected on days 11, 13, 15, 17, and 19, and analysed as in Section 5.2.5. All mice were sacrificed on day 19, with BALF collected and analysed as in Section 2.2.4, serum taken for OVA specific IgG_1 analysis as in Section 2.2.8, and lung sections taken for histological analysis as in Section 5.2.6.

Spleens were also excised on day 19 and disaggregated using sterile wire gauze (0.5mm) and, splenocytes suspended in ice cold PBS before lysis of erythrocytes. Whole spleen cell preparations were then incubated in flat-bottom 96-well tissue culture plates (Nunc) at a density of 2 x 10^6 cells/well, in a total volume of 200µl MLC medium/10% FCS at 37^{o}C for 72 hours. Prior to incubation, antigen (OVA) was added at different concentrations (0, 1, 10 and 100 µg/well). Immobilised anti-CD3 monoclonal antibody (100 µg) was also added to a subset of wells as a non-specific positive control. Supernatants were then collected for cytokine analysis.

6.2.5 Measurement of airways hyperreactivity.

Individual mice were placed in a whole body barometric plethysmograph (BUXCO Electronics, Troy, NY) interfaced with a personal computer through a differential pressure transducer. Mice were exposed to an aerosol of sterile double distilled water for 2 minutes before a 3 minute recording period to obtain a baseline P_{enh} (enhanced pause) response. Changes in airways reactivity were measured as P_{enh} using the function as described (Hamelmann *et al.*, 1997b):

$$P_{enh} = [(T_e/0.3T_r)-1] \times [2P_{ef}/3P_{if}]$$

Where
- P_{enh} enhanced pause
- T_e expiratory time (seconds)
- T_r relaxation time (seconds)
- P_{ef} peak expiratory flow
- P_{if} peak inspiratory flow

After the first (water) dose, mice were exposed to increasing concentrations of β-methacholine in ddH_20 (3.125 – 50 mg/ml) using the 2 minute exposure and 3 minute recording periods as described above for baseline. For each individual mouse, P_{enh} recordings were obtained and averaged before collating with other mice in each group.

6.2.6 Detection of adoptively transferred OT-II CD4$^+$ cells in wild type allergic mice.

Wild type C57BL/6 mice were sensitised and challenged and adoptively transferred with OT-II CD4$^+$ cells as in Section 6.2.4 with the following changes. Prior to adoptive transfer, OT-II CD4$^+$ cells (5 x 10^7/ml in PBS) were incubated in carboxyflourescein

diacetate succinimidyl ester (also known as CFSE, 5 μM – a kind gift from C.R. Parish, JCSMR, ANU) for 15 minutes at RT in 1 ml PBS (Weston and Parish, 1990; Parish, 1999). The cells were then washed in PBS/10% FCS to inhibit further dye loading, before resuspension in PBS and adoptive transfer to wild type allergic hosts. Mice were sacrificed on day 19, and lungs, spleens, and peribronchial lymph nodes were individually excised. The tissues were separately homogenised in HBSS/10% FCS at 4^{o}C, and the resulting cell suspensions were then filtered through sterile nylon mesh (70μM) before centrifugation at 500 g for 5 minutes at 4^{o}C. Following red blood cell lysis, 1 x 10^5 cells from each tissue were then incubated with phycoerythrin (PE)-conjugated anti-mouse CD4 (clone L3T4) for 30 minutes on ice. Cells were then FCS underlaid, centrifuged at 500 × g for 5 min at 4° C, and the resulting cell pellets resuspended in ice-cold PBS before analysis by flow cytometry. The cell homogenates were protected from incident light during each step. Adoptively transferred fluorescent cells in each tissue were then detected with a Becton and Dickinson FACScan® flow cytometer in conjunction with Cellquest (Becton and Dickinson, San Jose, CA) and WinMDI software packages (kindly provided by J. Trotter, Scripps Research Institute, La Jolla, CA).

6.2.7 Cytokine analysis.

IL-13 and IL-5 concentrations were determined as in Section 5.2.8. For IFN-γ detection, round bottomed immunoassay plates were coated with 50ng anti-mouse IFN-γ (clone R46A2), and incubated at 4^{o}C overnight. Plates were then blocked with 2% skim milk powder in PBS-Tween20 (0.04%) for 1 hour at 37^{o}C, and incubated with serial dilutions of cultured supernatants, or IFN-γ standard. IFN-γ was then detected with biotinylated rat anti-mouse IFN-γ (clone XMG1.2, BD PharMingen). Plates were then incubated with streptavidin-conjugated alkaline-phosphatase (Amersham International plc, Buckinghamshire, UK) for 1 hour at 37^{o}C, washed, and alkaline-phosphatase substrate solution (Sigma Chemical Co. St Louis, MO) was added. Where necessary, reactions were stopped with 1M citric acid, or promptly analysed. Plates were read by a Microplate reader (BIO-TEK Instruments Inc., Winooski, VT) at 490 nm with reference to 405 nm.

6.3 RESULTS

6.3.1 OT-II transgenic mice fail to develop characteristic markers of AAD when subjected to a mouse model of asthma.

Following sensitisation and airways challenge with OVA, OT-II transgenic mice did not exhibit the characteristic peripheral blood eosinophilia (greater than 5%), nor pulmonary eosinophilia (as measured in the BALF) typically seen in wild type mice (Figure 6.1), although eosinophils in BALF from both OVA sensitised and saline control OT-II mice were significantly greater than that of wild type saline controls (Figure 6.1b). Elevated numbers of lymphocytes in the BALF of OT-II mice were also noted following airway challenge, irrespective of sensitisation to OVA, and were also significantly greater than that of WT saline controls (Figure 6.1b).

Both IL-13 and IL-5 secretion were significantly reduced in OT-II splenocytes stimulated with OVA, in comparison to allergic wild type mice (Figure 6.2a and b), and serum OVA-specific IgG_1 levels were virtually undetectable in sensitised OT-II mice (Figure 6.2c).

6.3.2 OT-II transgenic $CD4^+$ splenocytes secrete IFN-γ when stimulated with $OVA_{323-339}$ peptide.

Significant cell proliferation (as measured by ^{3}H-thymidine incorporation) was observed when OT-II $CD4^+$ splenocytes were co-incubated with mitomycin C inactivated APCs previously loaded with $OVA_{323-339}$ peptide (Figure 6.3a). Under identical culture conditions, appreciable amounts of IFN-γ were released, while both IL-13 and IL-5 were both below detection limits (Figure 6.3b). In both instances, incubation with anti-CD3 monoclonal antibody induced both non-specific proliferation and release of IFN-γ from OT-II $CD4^+$ cells.

6.3.3 IFN-γ producing $CD4^+$ splenocytes from OT-II mice can attenuate pulmonary eosinophilia when adoptively transferred to wild type allergic mice, and reside in the spleen and draining lymphoid tissues.

An attenuated model (1 x i.p. sensitisation with OVA) was chosen to identify any subtle changes in the characteristic markers of allergic airways disease that may be attributed to adoptively transferred OT-II $CD4^+$ cells. Analysis of peripheral blood showed no

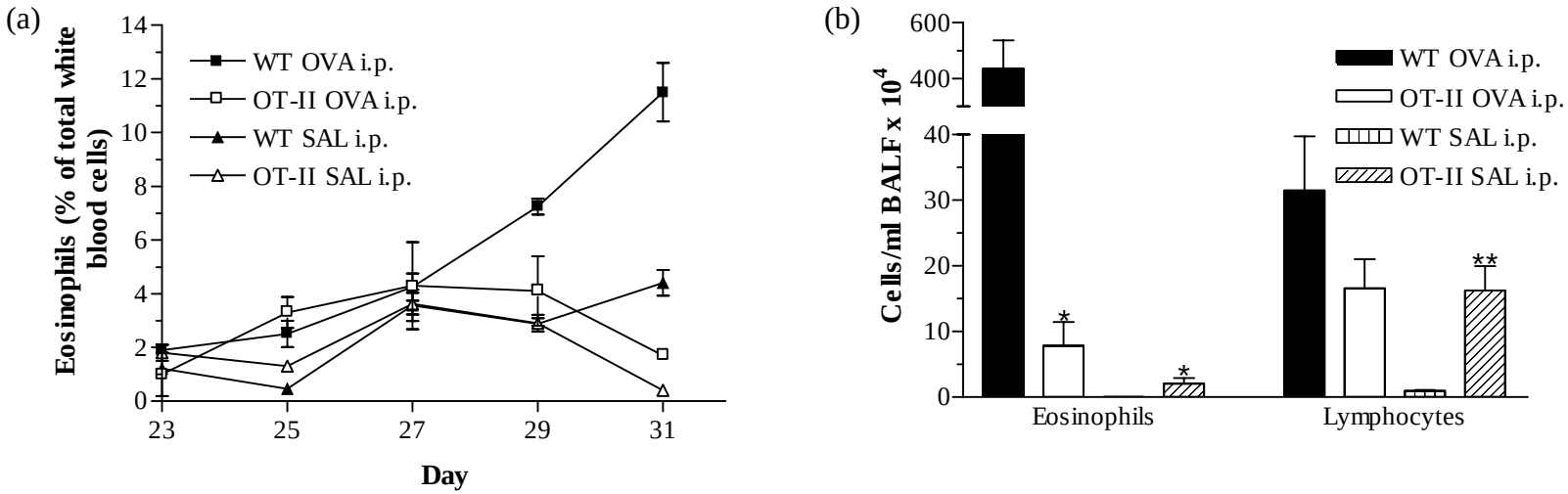


Figure 6.1. OT-II transgenic mice fail to develop characteristic markers of allergic airways disease. (a) Peripheral blood eosinophils in OVA sensitised and challenged wild type (WT OVA i.p.) and OT-II mice (OT-II OVA i.p.). Wild type (WT SAL i.p.) and OT-II (OT-II SAL i.p.) saline controls are shown for comparison. (b) Eosinophil and lymphocyte composition of BALF of WT OVA i.p. and OT-II OVA i.p. mice. Data represents mean ± SEM for groups of 7-9 mice. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$). * $P<0.05$ when compared to WT OVA i.p. and WT SAL i.p. ** $P<0.05$ when compared to WT SAL i.p.

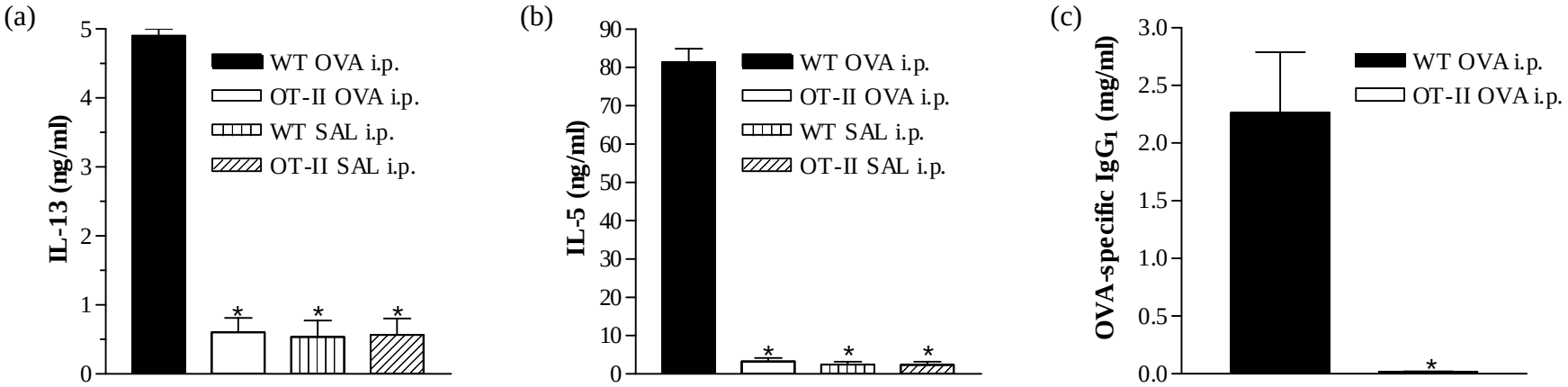


Figure 6.2. Spleen cell responses to OVA, and serum OVA-specific IgG_1 in OVA sensitised and challenged OT-II mice. (a) OVA stimulation of whole splenocytes from OVA sensitised and challenged OT-II mice (OT-II OVA i.p.) reveals a lack of significant IL-13 secretion (not above background) when compared to OVA sensitised and challenged wild type mice (WT OVA i.p.). (b) IL-5 secretion from WT OVA i.p. and OT-II OVA i.p. splenocytes stimulated with OVA. (c) WT OVA i.p. mice have detectable OVA-specific IgG1, which is virtually absent in OT-II OVA i.p. mice. Serum OVA-specific IgG_1 was below detectable limits in wild type (WT SAL i.p.) and OT-II (OT-II SAL i.p.) saline controls. Data represents mean ± SEM for groups of 4 mice. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$). * $P<0.05$ when compared to WT OVA i.p. mice.

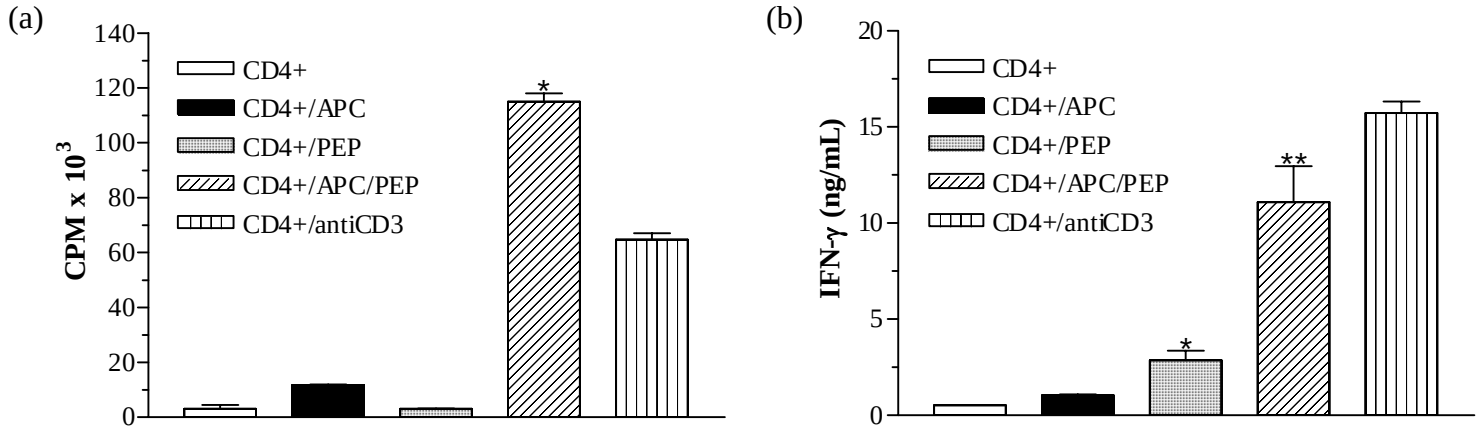


Figure 6.3. $CD4^+$ T lymphocytes from naïve OT-II transgenics release IFN-γ in response to $OVA_{323-339}$ peptide stimulation. (a) Tritiated thymidine incorporation (as represented by counts per minute, CPM) in enriched OT-II $CD4^+$ T cells incubated in the presence of mitomycin treated APCs (APC) and/or $OVA_{323-339}$ peptide (PEP). Cells have also been stimulated with anti-CD3 as a positive control. (b) IFN-γ release from enriched OT-II $CD4^+$ T cells incubated in the presence of mitomycin treated APCs and/or $OVA_{323-339}$ peptide. Cells have also been stimulated with anti-CD3 as a positive control. Of the measured cytokines (IL-5, IL-13 and IFN-γ), only IFN-γ was detected. Data represents mean ± SEM for triplicate wells. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$). * $P<0.05$ when compared to CD4+, CD4+/APC, and CD4+/PEP. ** $P<0.05$ when compared to $CD4^+$.

effect for adoptively transferred OT-II $CD4^+$ cells until day 19, when OT-II recipients had significantly greater levels of eosinophils in peripheral blood compared to allergic controls (Figure 6.4a). Analysis of BALF revealed a significant reduction in eosinophils, but no significant change in total lymphocyte numbers when OT-II $CD4^+$ cells were adoptively transferred to allergic mice (Figure 6.4b). Nor was there any significant change in airways response to methacholine challenge (Figure 6.4c). Serum OVA-specific IgG_1 levels were similar between wild type allergic mice and OT-II $CD4^+$ recipients (Figure 6.5a), indicating that in this model at least, OT-II $CD4^+$ T cells could not influence OVA-specific IgG_1 production.

When whole splenocytes were incubated with exogenous OVA *in vitro*, only OT-II $CD4^+$ recipients displayed detectable IFN-γ release in response to stimulation (Figure 6.5b), which suggested that the adopted OT-II T cell population retained the functional response to OVA previously observed *in vitro*. Analysis of histological sections revealed that lung sections from OT-II $CD4^+$ recipients were virtually indistinguishable from allergic controls (Figure 6.6).

In a parallel experiment, the labeling of OT-II $CD4^+$ splenocytes with CFSE prior to adoptive transfer revealed that OT-II $CD4^+$ splenocytes migrated to the host spleen and peribronchial lymph nodes, following sensitisation and airway challenge (Figure 6.7). Very low numbers of adoptively transferred OT-II $CD4^+$ splenocytes could also be detected in lung homogenates (results not shown).

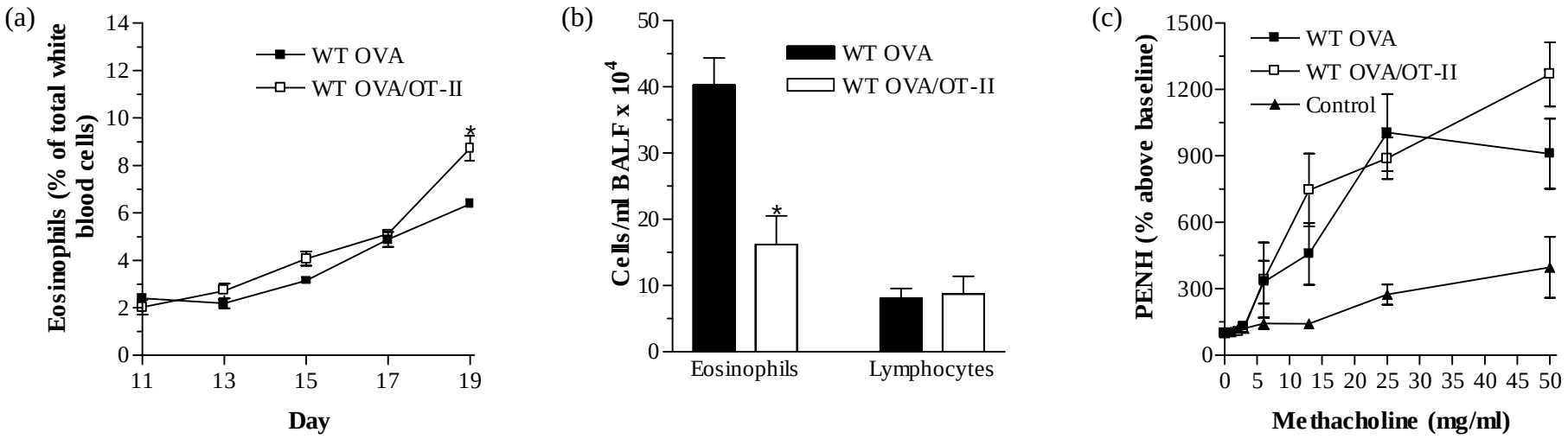


Figure 6.4. Adoptive transfer of OT-II $CD4^+$ T cells to OVA sensitised and challenged mice: attenuation of pulmonary eosinophilia. (a) Peripheral blood eosinophils in wild type OVA sensitised and challenged mice (WT OVA) and OT-II $CD4^+$ T cell recipients (WT OVA/OT-II).
(b) Eosinophil and lymphocyte composition of BALF in WT OVA and WT OVA/OT-II mice. (c) Airways hyperreactivity to methacholine in WT OVA and WT OVA/OT-II mice. A non allergic mouse (Control) is shown for comparison. Data represents mean ± SEM for groups of 6-7 mice. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$). * $P<0.05$ when compared to WT OVA mice.

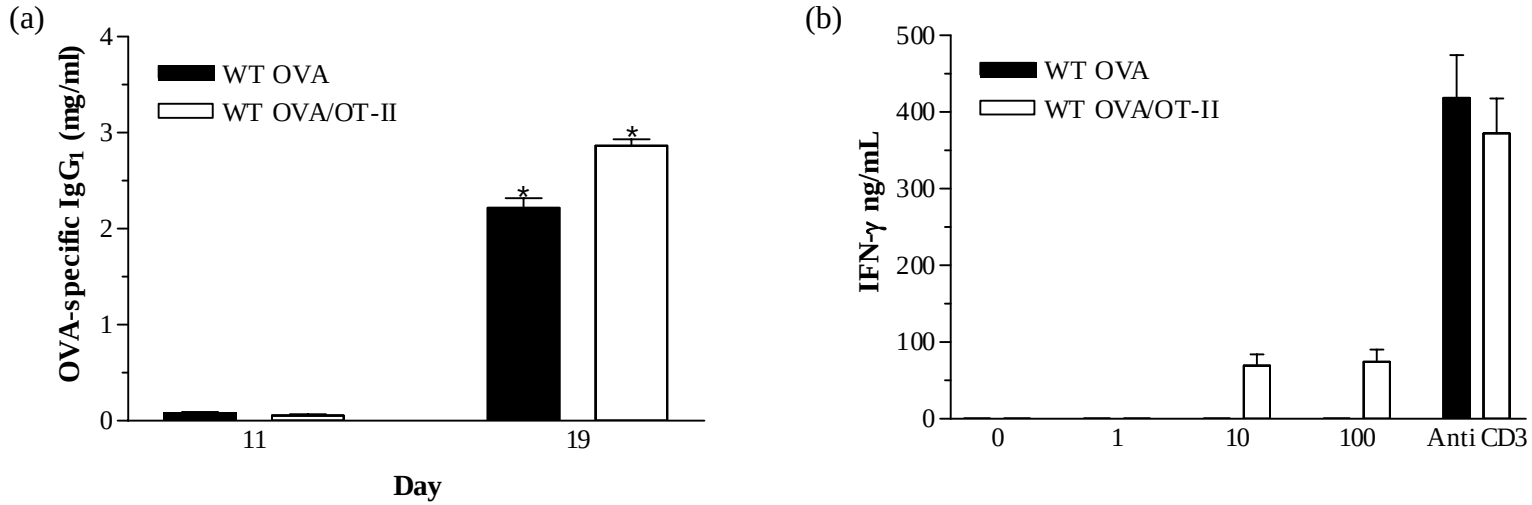
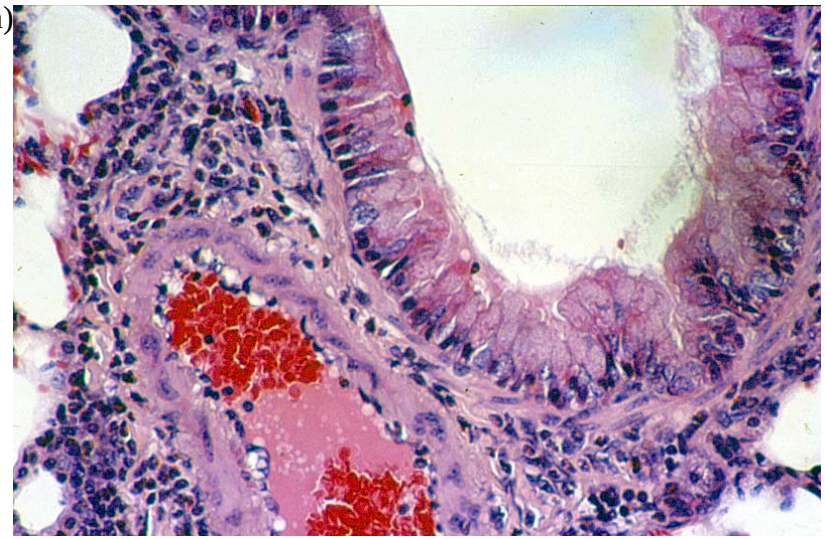


Figure 6.5. Adoptive transfer of OT-II $CD4^+$ T cells to OVA sensitised and challenged mice: effects on OVA-specific IgG_1 and IFN-γ secretion in the spleen. (a) Serum OVA-specific IgG_1 levels in OVA sensitised and challenged wild type (WT OVA) and OT-II $CD4^+$ T cell recipients (WT OVA/OT-II). Data represents mean ± SEM for groups of 4 mice. (b) IFN-γ secretion from whole spleen cell preparations stimulated with OVA (μg/well) or immobilised anti-CD3 monoclonal antibody. Data represents mean ± SEM for triplicate wells. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$)* $P<0.05$ when compared to day 11.

(a)



(b)

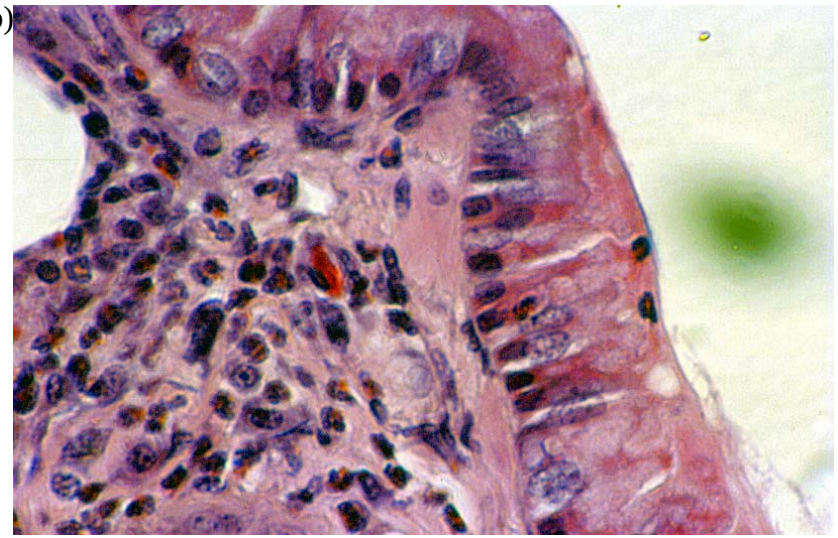


Figure 6.6. Histological analysis of lung sections from OVA sensitised and challenged mice adoptively transferred with OT-II CD4+ T lymphocytes. (a) Histological section (20 x objective) typical of OVA sensitised and challenged mice. Note the pronounced peribronchial and perivascular infiltrate surrounding the airways and blood vessels, and occluded airway typical of goblet cell hyperplasia. (b) Histological section (40 x objective) typical of OVA sensitised and challenged mouse adoptively transferred with OT-II $CD4^+$ T lymphocytes. In all sections examined, OT-II recipients were not readily distinguished from control mice. Lung tissue was stained with Lendrum's carbolchromotrope stain.

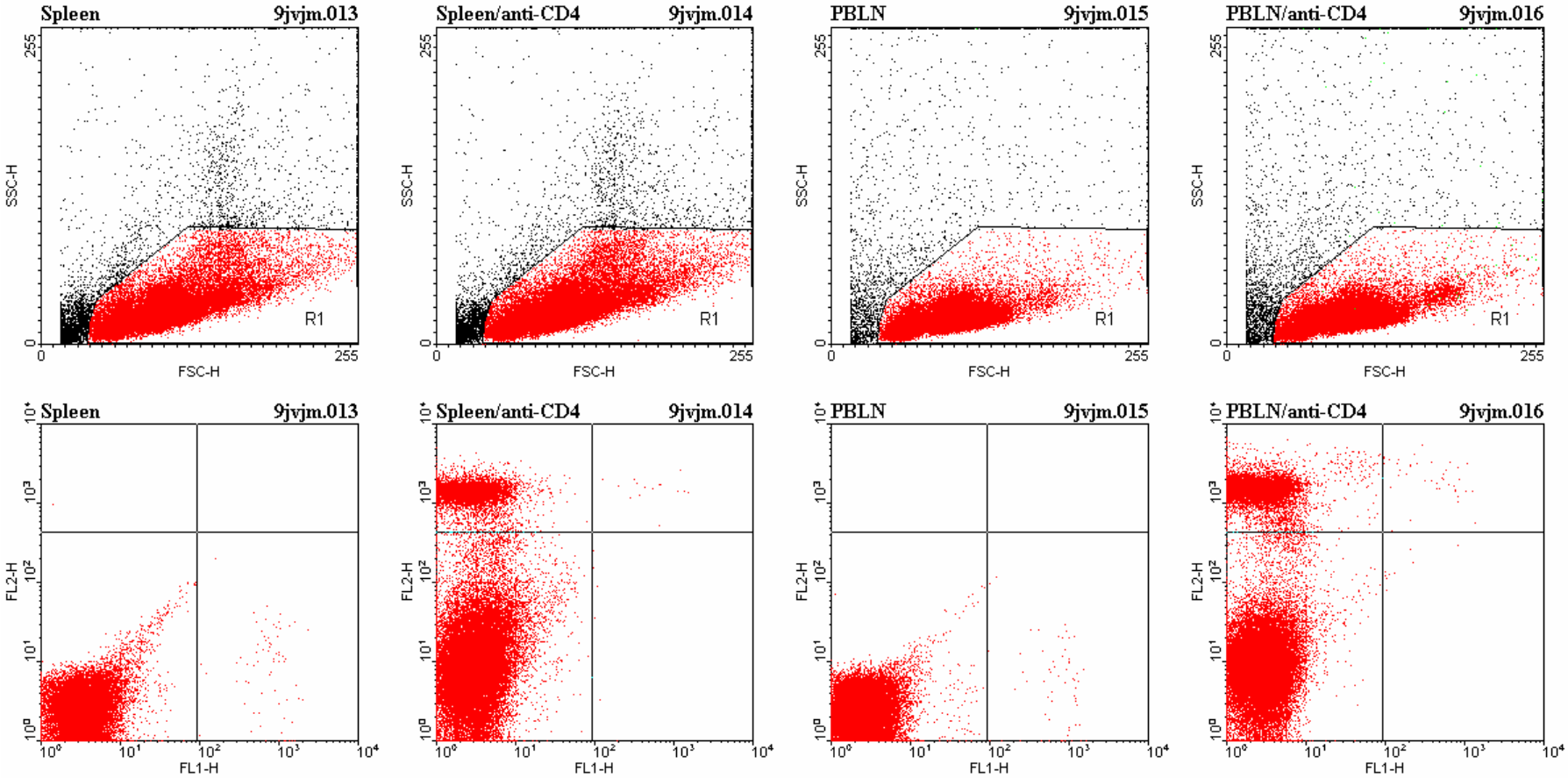


Figure 6.7. OT-II transgenic $CD4^+$ T lymphocytes migrate to the spleen and peribronchial lymph nodes when transferred to antigen sensitised and challenged wild type hosts. Lymphocytes were gated on the basis of forwards and side scatter profiles (R1, upper plots). CFSE (FL1-H channel) and $CD4^+$ (FL2-H channel) double positive cells (upper right quadrants) are adoptively transferred OT-II $CD4^+$ T lymphocytes, and represent 0.03 and 0.09% of gated cells in spleen and PBLN tissue respectively.

6.4 DISCUSSION

The OT-II transgenic mouse was initially obtained to study putative antigen-dependent interactions between eosinophils and T lymphocytes, in the same manner as T cell hybridomas have been used by others (Del Pozo *et al.*, 1992). Early experiments with eosinophils, OT-II splenocytes, and relevant peptide were disappointing, because observed proliferative responses did not correlate with detectable Th2 cytokine release (see Chapter 4). Therefore, OT-II transgenic mice were characterised in an allergic airways disease model to gain some insight into the way in which these animals responded to relevant antigen *in vivo*. OT-II mice failed to develop the characteristic peripheral blood eosinophilia normally seen in wild type mice following sensitisation and subsequent airways challenge with OVA (Figure 6.1a). Analysis of BALF revealed appreciable lymphocyte infiltration of the airways in both saline and OVA sensitised OT-II mice, but attenuated levels of eosinophils in BALF when compared to wild type sensitised and challenged controls (Figure 6.1b), and both IL-13 and IL-5 secretion from whole splenocytes was significantly reduced (Figure 6.2a and b). Serum OVA-specific IgG_1 was also notably absent in OT-II mice (Figure 6.2c). Collectively these data suggested that OT-II transgenic mice were refractory to allergic disease induction and progression when using this model of allergic airways disease.

The OT-II transgenic T cell is $CD4^+$, and bears the Vα2/Vβ5-TCR which enables it to respond to the $OVA_{323-339}$ peptide (Barnden *et al.*, 1998; Robertson *et al.*, 2000). Investigation of the cytokines secreted by OT-II $CD4^+$ T lymphocytes in response to stimulation with peptide-pulsed APCs revealed an appreciable secretion of IFN-γ, but not IL-13 nor IL-5 (Figure 6.3b). To the author's best knowledge, this is the first reported instance of appreciable IFN-γ secretion from OT-II transgenic cells obtained from naïve mice.

IFN-γ can attenuate antigen-induced eosinophil recruitment to the airways (Iwamoto *et al.*, 1993; Kung *et al.*, 1995), and has been implicated in the regulation of eosinophil migration from lung tissue to the airways lumen in IL-4 deficient mice (Cohn *et al.*, 2001). The ability of IFN-γ to influence Th2 mediated allergic disease could be linked to its ability to suppress IL-4 induced commitment of Th cells to the Th2 phenotype (Pene *et al.*, 1988), and also its ability to suppress both IL-4 and IL-5 production by

$CD4^+$ Th2-type lymphocytes (Demeure *et al.*, 1994). Therefore, the ability of IFN-γ secreting OT-II $CD4^+$ T lymphocytes to counterbalance Th2 mediated allergic airways disease was investigated.

OT-II $CD4^+$ T lymphocytes were administered to naïve hosts concomitant with i.p. sensitisation, and mice were subsequently exposed to a mild regime of airways exposure to OVA. On investigation, OT-II $CD4^+$ T cell recipient mice were found to have significantly reduced numbers of eosinophils in BALF. Peripheral blood eosinophil levels were similar between allergic mice and OT-II recipients, although it was noted that OT-II recipients had significantly increased levels of eosinophils in blood on day 19, which may have represented a "backing up" of eosinophils in the blood compartment due to altered recruitment to the allergic lung. Eosinophils are known to express functional IFN-γ receptors (Ochiai *et al.*, 1999), and in this Chapter, transferred OT-II $CD4^+$ T cells were observed to reside in the pulmonary draining lymph nodes and to a lessor extent in the lung tissue. Therefore, OT-II cells secreting IFN-γ in response to presentation of OVA by endogenous APCs within these host tissues may have modulated eosinophil function and infiltration into the allergic lung, such that eosinophil infiltration of the airways lumen was inhibited. Analysis of histological sections revealed that inflammatory cell infiltrate in the lungs of OT-II recipients were virtually indistinguishable from allergic controls, suggesting that although BALF eosinophils were reduced, eosinophil infiltration of lung tissue was not impaired. In addition, the airway response to methacholine challenge, and serum OVA-specific IgG_1 responses in OT-II recipients remained intact, despite the reported ability of IFN-γ to suppress IL-4 mediated IgG_1 production (Snapper and Paul, 1987; Finkelman *et al.*, 1988a), and for Th1 cells to reduce AHR in the rat (Huang *et al.*, 2001). Collectively, these data suggest that transferred IFN-γ secreting OT-II T cells can influence eosinophil infiltration into the airways lumen, but are not able to counterbalance allergic airways disease induction and progression in this animal model. IFN-γ has been shown to inhibit eotaxin synthesis by dermal fibroblasts *in vitro* (Miyamasu *et al.*, 1999), and therefore the release of IFN-γ from adoptively transferred OT-II $CD4^+$ T cells in response to inhaled OVA may have reduced eotaxin synthesis within the airways epithelium.

It is possible that significantly increasing the numbers of adoptively transferred OT-II T cells during sensitisation could attenuate other characteristic markers of AAD. In this way, substantially greater amounts of IFN-γ production during disease induction (sensitisation) could influence the adaptive immune response such that the developing Th2 phenotype is either greatly suppressed, or even absent. The therapeutic value of such a strategy in human asthma may be limited however, as allergic asthma is usually diagnosed after the fact – that is following significant development of a Th2 type response to a normally innocuous environmental antigen. In this regard, attempts to counterbalance the disease using Th1 type T cells may not be ideal, particularly when levels of eosinophils in the lung parenchyma remains refractory to such treatment (Huang *et al.*, 2001 and this Chapter). Instead, interventional therapies based simply on IFN-γ or its regulation within the lung may be beneficial. Indeed, the delivery of IL-12, a potent regulator of IFN-γ to the airways mucosa can suppress measurable parameters including parenchymal eosinophils in allergic mice (Hogan *et al.*, 1998b; Sur *et al.*, 2000a; Sur *et al.*, 2000b). However, the systemic administration of recombinant human IL-12 does not alter airways hyperreactivity nor the late asthmatic reaction in mild asthmatic patients, despite significant reduction in sputum and peripheral blood eosinophils (Bryan *et al.*, 2000).

In summary, the OT-II mouse has been partially characterised in a mouse model of asthma, and is refractory to allergic airways disease induction and progression. When stimulated *in vitro*, OT-II transgenic T cells have been observed to secrete appreciable amounts of IFN-γ, and are able to attenuate eosinophil infiltration of the airways, but not AHR or lung inflammation when transferred to allergic hosts. The mechanism whereby adoptively transferred OT-II $CD4^+$ cells may attenuate pulmonary eosinophilia may be related to the ability of large amounts of IFN-γ to bias an existing response towards Th1 cytokine production, and through the inhibition of established Th2 responses and eosinophil recruitment. However, it must be noted that transgenic TCR mice are not always suitable to study disease or disease models, given the specificity of the TCR to a single T cell epitope, and the potential for commonly used antigens to contain more than one antigenic peptide.

Chapter 7

Neonatal Antigen Delivery Induces Th-2 Mediated Allergic Airways Disease In The OT-II Transgenic Mouse.

7.1 INTRODUCTION

The adaptive immune system has evolved to mount responses to foreign antigen, whilst at the same time remaining unresponsive to self. Billingham and colleagues first demonstrated acquired tolerance in 1953, wherein the administration of allogeneic cells to neonatal mice enabled them to accept allogeneic skin grafts as adults (Billingham *et al.*, 1953). Neonatal immunity has previously been attributed to clonal deletion and/or anergy (Nossal, 1983; Gammon *et al.*, 1986; Qin *et al.*, 1989), but more recent evidence suggests that immune deviation is the basis for neonatal tolerance. The lymph nodes that drain immunogen bearing tolerant grafts have since been shown to produce a 10-100 fold higher ratio of IL-4 to IFN-γ (Chen and Field, 1995), and the administration of IL-12 (Donckier *et al.*, 1998), IFN-γ (Chen *et al.*, 1996), or antibodies to IL-4 (Gao *et al.*, 1996) during tolerance induction can prevent transplantation tolerance in mice, suggesting that immune-deviation from a Th1 to a protective Th2 is the basis for tolerance in these models. However, Th1 responses to non-self are not totally inhibited. A/J tolerant BALB/c mice show diminished Th1 $CD4^+$ and $CD8^+$ cell immunity (IFN-γ production) to A/J *in vitro*, whilst displaying enhanced $CD4^+$ Th2 responses (IL-4 production). When $CD4^+$ cells are removed from splenic cultures, IL-2 can restore the ability of $CD8^+$ cells to proliferate and secrete IFN-γ in response to A/J antigen, which suggests that tolerance relies, in part, on the inhibition of cell immunity by regulatory $CD4^+$ Th2 cells (Field and Gao, 1998).

Neonatal responses to environmental antigens are of particular interest when trying to understand the predisposition towards allergic disease in humans. For instance, reduced IFN-γ production by antigen stimulated cord blood mononuclear cells is a risk factor for the development of allergic disorders in later life (Kondo *et al.*, 1998), and it has been shown that stimulation of cord blood cells with common environmental allergens results in predominantly Th2 type cytokine secretion, suggesting that a predisposition towards allergy may reside in an inability to deviate such responses from Th2 to Th1 (Prescott *et al.*, 1998; Prescott *et al.*, 2003).

T cell maturation in the thymus has been characterised as a very destructive process, in which only about 2% of $CD4^+CD8^+$ double positive (DP) cells will mature into $CD4^+$and $CD8^+$ single positive (SP) cells (Robey and Fowlkes, 1994; Fink and Bevan,

1995; Jameson and Bevan, 1998). The process involves the "positive selection" of those DP cells whose TCRs have a mild to low level recognition of self peptide/MHC complexes on cortical epithelial cells, effectively rescuing them from "death by neglect" (Fink and Bevan, 1995; Jameson and Bevan, 1998). These cells may then progress from the cortex to the medulla, which is populated with bone marrow derived APCs (Sprent, 1995), and permeable to soluble antigens in the blood (Surh and Sprent, 1994). Contact with high affinity peptides within either the medulla or the cortex leads to strong TCR signaling, and rapid apoptosis of the developing DP/SP cells. This is suggested to be one of the central tenets of "negative selection", and avoidance of autoimmune reactions in the continually evolving adaptive immune system.

Recently, a mouse model of allergic airways disease was used to determine the effects of neonatal antigen delivery in mice (Hogan *et al.*, 1998a). A medium dose of insoluble OVA (100 μg) in incomplete Freund's adjuvant (IFA) administered s.c. could prime for Th2-mediated AAD in adult mice, whilst soluble OVA (100 μg in PBS) could not (although these mice still developed AAD following sensitisation and challenge with OVA). Interestingly, very low doses of soluble OVA (10 μg) could also prime for Th2-mediated AAD, without the requirement for OVA sensitisation of adult mice. Conversely, a high dose of soluble antigen (1 mg OVA) during neonatal development was shown to anergise both Th1 and Th2 adult responses to OVA, regardless of sensitisation. This high dose of soluble OVA totally negated proliferative spleen cell responses to OVA, and may have been indicative of clonal deletion of OVA-reactive cells. In their article, Hogan *et al.* introduced the concept of effective dose, and postulated that IFA may have sequestered antigen such that a lower effective dose was administered, and led to Th2 responses in the same way as did very low doses of soluble antigen (Hogan *et al.*, 1998a). As such, low effective dose was shown to predispose adult mice to Th2 mediated AAD, whilst high soluble dose led to anergy in both Th1 and Th2 compartments.

In Chapter 6, naïve OT-II transgenic T cells having an easily detectable TCR were shown to secrete IFN-γ when stimulated with OVA. Using this knowledge of the adult phenotype, OT-II neonates were given high doses of soluble OVA, with a view to determining the effects of high affinity antigen administration upon the phenotype and fate of TCR transgenic cells.

7.2 MATERIALS AND METHODS

7.2.1 Animals.

C57BL/6 (males, 4 wk old) and OT-II mice (timed pregnant females, N10 C57BL/6, 7-8 wk old) (Barnden *et al.*, 1998), were obtained from the SPF unit of the ANU and housed in an approved containment facility. Mice were treated according to ANU animal welfare guidelines.

7.2.2 Antigen treatment of mice.

Within hours of birth on day 1, OT-II neonates were administered 1 x s.c. injection of 1 mg OVA in 50 μL saline (OVAN mice). As a control, age matched OT-II litters also received 50 μL of saline vehicle (SALN mice). All mice were then sex-determined and weaned on day 30. It should be restated that all male OT-II mice carry the transgene, which appears to be linked to the Y chromosome.

7.2.3 Detection of $CD4^+/V\beta5$ double positive cells using flow cytometry.

OT-II transgenic T cells are $CD4^+$, and express the Vα2/Vβ5 TCR which is specific for $OVA_{323-339}$ (Barnden *et al.*, 1998; Robertson *et al.*, 2000), and may be detected in the thymus and the spleen, using antibodies to either Vα2 or Vβ5 in conjunction with anti-CD4 (Bill *et al.*, 1990; Barnden *et al.*, 1998).

A subset of mice from OVAN and SALN groups (n=3) were sacrificed on day 30, and spleens excised and harvested into ice cold HBSS and physically disaggregated using sterile wire gauze (0.5mm). The isolated cells were centrifuged at 500 g at 4^oC for 10 minutes and the cell pellet resuspended in 500 μl RBC lysis solution and incubated at 37^oC for 5 minutes, before the addition of 1 ml ice cold HBSS. 1×10^5 freshly isolated splenocytes were then incubated with biotinylated mouse anti-mouse Vβ5 (Bill *et al.*, 1990) (Clone MR9.4) in 50 μl HBSS for 30 minutes on ice. 1 ml HBSS was then added, and cells FCS underlaid before being centrifuged at 500 × g for 5 min at 4° C. The cell pellet was then incubated with streptavidin-FITC and phycoerythrin (PE)-conjugated anti-mouse CD4 (clone L3T4) for a further 30 minutes on ice. Cells were again FCS underlaid, centrifuged at 500 × g for 5 min at 4° C, and the cell pellet resuspended in ice-cold HBSS before analysis by flow cytometry. Analysis was performed on a Becton and Dickinson FACScan flow cytometer using Cellquest (Becton and Dickinson, San

Jose, CA) and WinMDi software packages (kindly provided by J. Trotter, Scripps Research Institute, La Jolla, CA). Approximately 10,000 cells were analyzed for each sample.

7.2.4 Sensitisation and challenge with OVA.

Both OVAN and SALN treated OT-II mice (males) were sensitized by i.p. injection with 50 μg OVA/1 mg Alhydrogel (Commonwealth Serum Laboratories (CSL), Parkville, Australia) in 0.9% sterile saline on days 30 and 42. For comparison, age-matched wild type C57BL/6 mice were also i.p. sensitised with OVA (WT OVA i.p.). On days 54, 56, 58 and 60, all mice were aeroallergen challenged as in Section 3.2.5. Peripheral blood smears were collected on days 53, 55, 57, 59, and 61, and analysed as in Section 5.2.5. All mice were sacrificed on day 61, with BALF collected and analysed as in Section 2.2.4, serum collected for OVA specific IgG_1 analysis as in Section 2.2.8, lung sections taken for histological analysis as in Section 5.2.6, and spleens excised for stimulation with OVA and cytokine analysis.

7.2.5 Stimulation of OVAN and SALN treated mouse splenocytes with OVA.

Spleens from i.p. sensitised and challenged OVAN and SALN mice were separately disaggregated using sterile wire gauze (0.5mm) and suspended in ice cold PBS. Erythrocytes were then lysed, and the washed splenocytes were resuspended at 10^7 cells/ml in MLC medium containing 10% heat-inactivated FCS, 2 mM L-glutamine, and 50 mg/L neomycin sulfate. Splenocytes from each group were then plated out at a density of 2 x 10^6 cells/well in round-bottom tissue culture plates, both with and without addition of exogenous OVA (100 μg/well). Following 72 hours of culture, cell-free supernatants were removed and stored at –70^{o}C for later cytokine analysis.

7.2.6 Cytokine analysis.

IL-13 and IL-5 concentrations in stimulated splenocyte supernatants were determined as in Section 5.2.8. IFN-γ was detected as in Section 6.2.7.

7.3 RESULTS

7.3.1 Ovalbumin administration to OT-II neonates does not alter CD4$^+$/Vβ5 double positive T cell population.

The administration of OVA to OT-II neonates could potentially alter the proportion of OVA-transgenic CD4$^+$/Vβ5 double positive cells in the adult periphery. To explore this possibility, antibodies to both CD4 and Vα2 were used to verify the presence of transgenic CD4$^+$/Vβ5 double positive cells in the adult spleen. On investigation, it was noted that both OVAN and SALN mice retained significant numbers of transgenic CD4$^+$/Vβ5 double positive cells within the spleen (Figure 7.1), suggesting that neonatal OVA administration did not deplete transgenic CD4$^+$ T cells in OT-II mice.

7.3.2 Ovalbumin administration to OT-II neonates promotes Th2-mediated allergic airways disease in adult mice.

Following sensitisation and airways challenge with OVA, peripheral blood eosinophil levels were similar between OT-II OVAN and comparative WT mice following repeated airways exposure to OVA (Figure 7.2a). Analysis of BALF revealed a prominent airways eosinophilia in OT-II OVAN mice which was not significantly different from that of sensitised and challenged WT mice (Figure 7.2b). Of particular note was the restoration of serum OVA-specific IgG_1 in OT-II OVAN mice (Figure 7.2 c), a feature which is noticeably absent in both OT-II SALN mice and OT-II mice in general (see Chapter 6). Analysis of histological sections revealed that OT-II SALN treated mice had notable inflammatory infiltrate surrounding the airways and blood vessels, with some eosinophil infiltration of this tissue (Figure 7.3). In OT-II OVAN mice, there was a heavy inflammatory infiltrate throughout the lung, including the parenchyma and particularly surrounding the airways and blood vessels (Figure 7.4). Numerous eosinophils were also seen throughout OT-II OVAN lung sections, and also what appeared to be bronchoalveolar lymphoid tissue structures associated with the small airways. Ovalbumin stimulation of whole spleen cell populations from i.p. sensitised and challenged OT-II OVAN mice produced appreciable IL-13 and IL-5 secretion, but not IFN-γ (Figure 7.5a-c). Only splenocytes from OT-II SALN mice retained the capacity to secrete appreciable amounts of IFN-γ in response to OVA stimulation (Figure 7.5c).

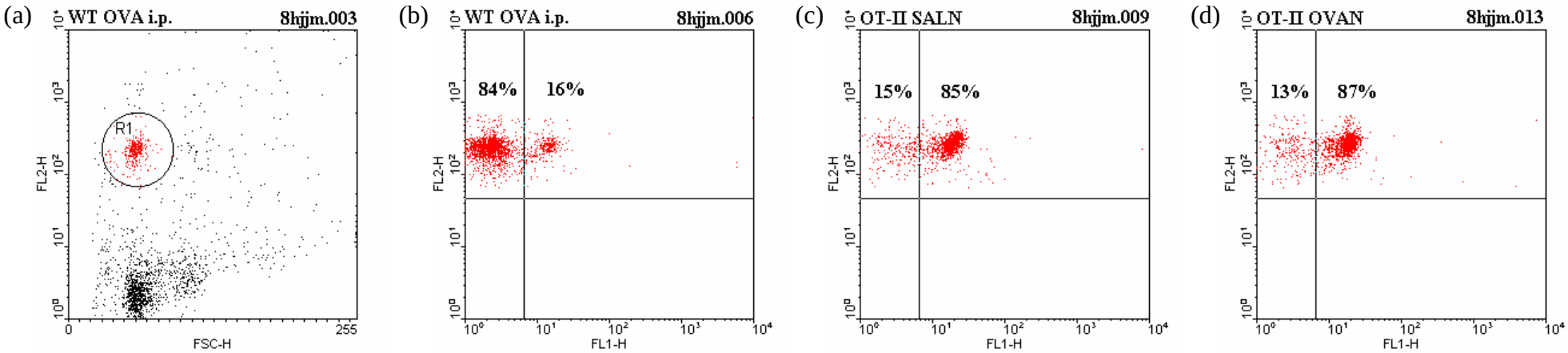


Figure 7.1. Neonatal exposure to ovalbumin does not deplete transgenic $CD4^+$ T cells in OT-II mice. (a) $CD4^+$ splenocytes from OVA sensitised and challenged wild type mice (WT OVA i.p.) were gated (R1) on the basis of size (forward scatter, FSC-H) and staining with antibody to CD4 (FL2-H). (b) WT OVA i.p. splenocytes stained with anti-CD4 antibody and mouse anti-mouse Vα2 (clone MR9.4, FL1-H channel). Single-CD4 positive cells reside in the upper left quadrant, while low numbers of naturally occurring Vα2/CD4 double positive cells reside in the upper right quadrant. Percentages denote the proportion of gated cells (from R1) in each quadrant. (c) Splenocytes from OT-II mice treated with saline as neonates (OT-II SALN), contain normal numbers of Vα2/CD4 double positive cells (upper right quadrant). (d) OT-II mice treated with soluble OVA as neonates (OT-II OVAN) have normal numbers of Vα2/CD4 double positive cells in the spleen when compared to OT-II SALN controls. Data is representative of 3 animals per group.

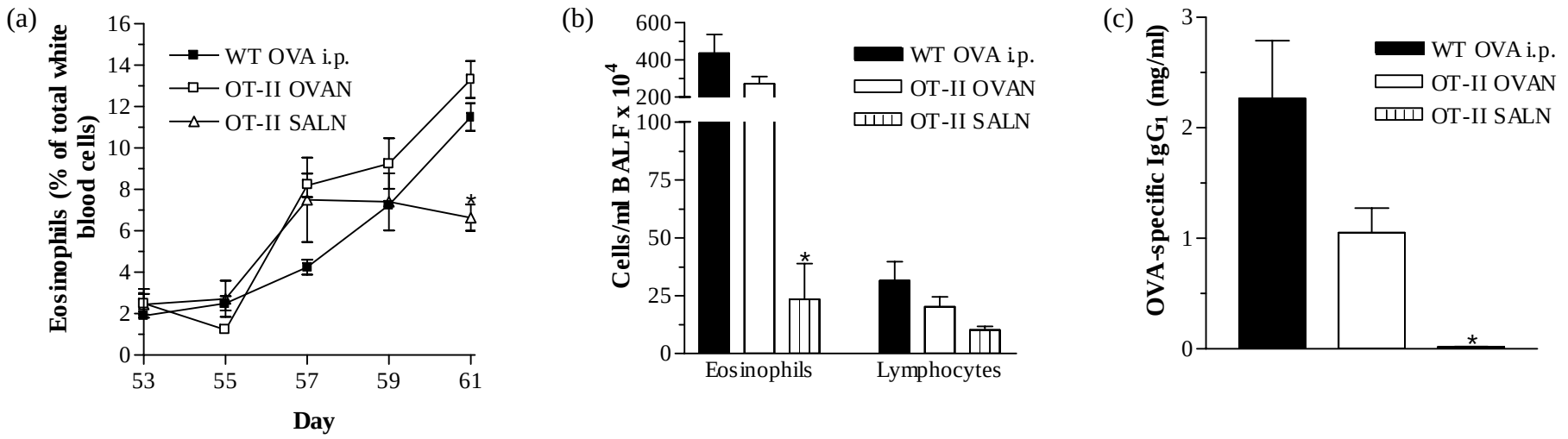


Figure 7.2. Neonatal exposure to ovalbumin promotes allergic airways disease in OT-II mice. (a) Peripheral blood eosinophilia in OVA sensitised and challenged OT-II transgenic mice, exposed to soluble OVA (OT-II OVAN) or saline vehicle (OT-II SALN) as neonates. Data for OVA sensitised and challenged wild type mice (WT OVA i.p.) are also shown for comparison. (b) Eosinophil and lymphocyte numbers in BALF. (c) Serum OVA-specific IgG_1 levels. Data represents mean ± SEM for groups of 6-7 mice. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$). * $P < 0.05$ when compared to OT-II OVAN and WT OVA i.p. mice.

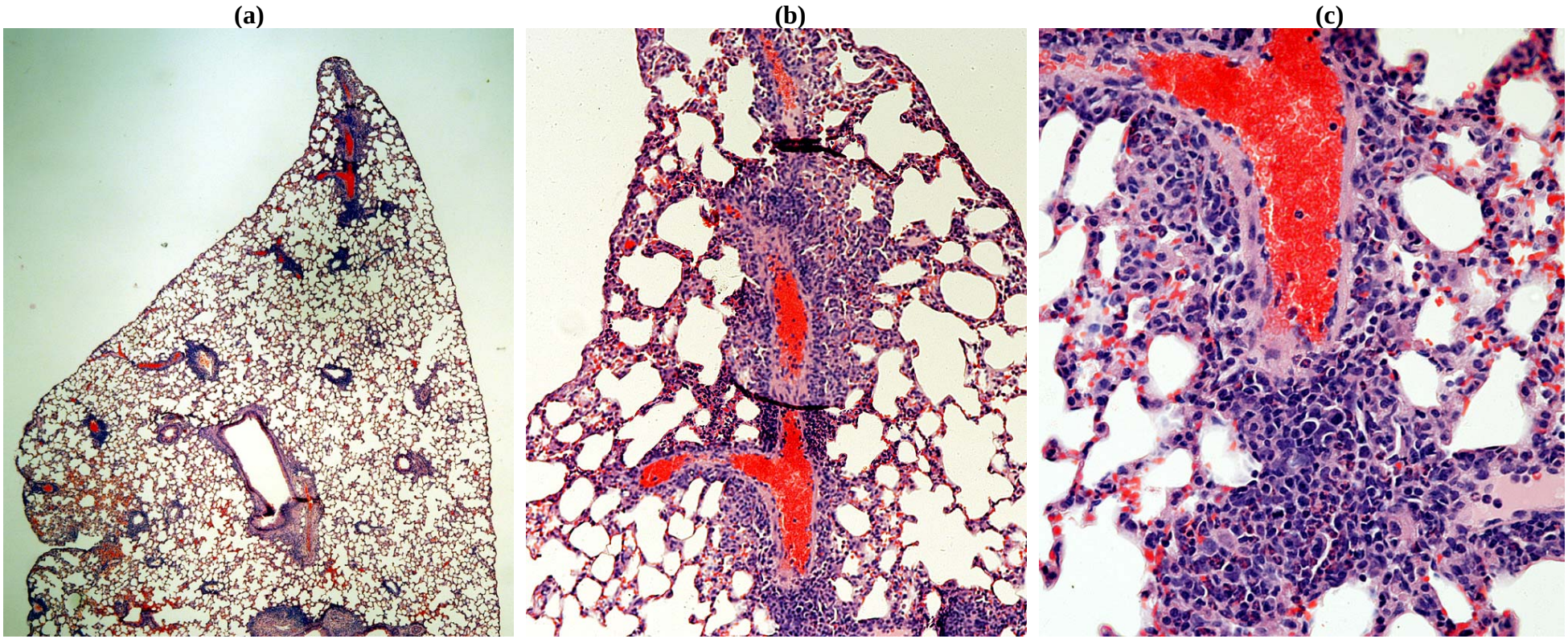


Figure 7.3. Histological analysis of lung sections from OT-II mice treated with saline as neonates. (a) Histological section (4 x objective). Note the peribronchial and perivascular infiltrate surrounding the airways and blood vessels, whilst the parenchyma is relatively intact. (b) There is a large inflammatory infiltrate surrounding the blood vessels (20 x objective). (c) Most of the infiltrating cells appear to be lymphocytes, although a significant number of eosinophils may be seen in association with inflammatory cells surrounding the airways and blood vessels (40 x objective). Lung tissue was stained with Lendrum's carbolchromotrope stain.

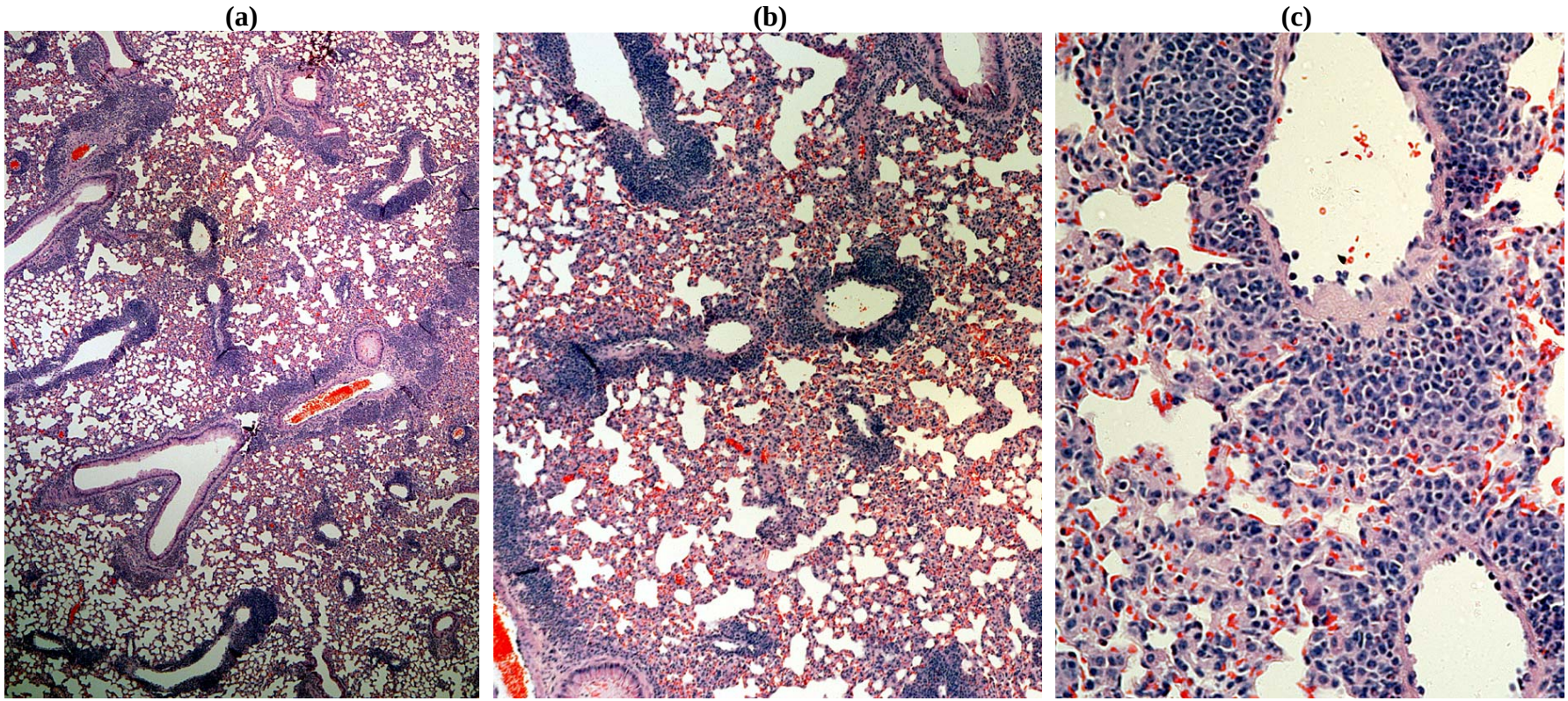


Figure 7.4. Histological analysis of lung sections from OT-II mice treated with soluble ovalbumin as neonates. (a) Histological section (4 x objective). Note the very pronounced peribronchial and perivascular infiltrate surrounding the airways and blood vessels, and spreading throughout the parenchyma. (b) There is a large inflammatory infiltrate surrounding the blood vessels (10 x objective). (c) Most of the infiltrating cells appear to be lymphocytes, and a large number of eosinophils may be seen (20 x objective). What appears to be bronchoalveolar lymphoid tissue may be seen in top left. Lung tissue was stained with Lendrum's carbolchromotrope stain.

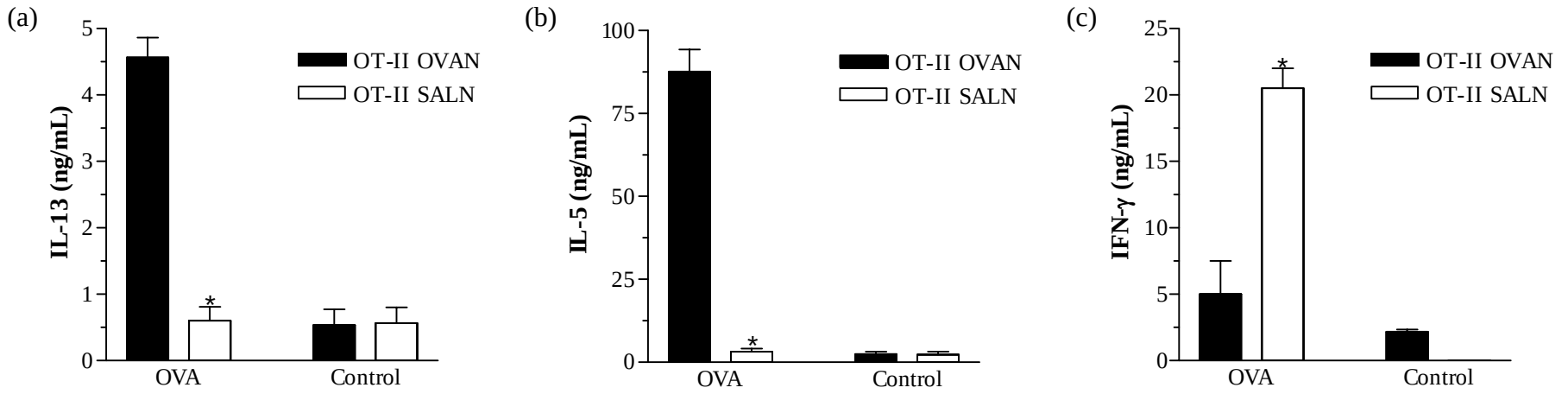


Figure 7.5. Neonatal exposure to ovalbumin promotes Th-2 cytokine release from ovalbumin stimulated OT-II spleen cells. (a) IL-13 release from spleen cells in response to ovalbumin (OVA) stimulation. Spleen cells were sourced from i.p. sensitised and challenged OT-II transgenic mice exposed to ovalbumin (OT-II OVAN) or saline vehicle as neonates (OT-II SALN). Cytokine levels from cells incubated without added exogenous OVA also shown (Control). (b) IL-5 release from OT-II OVAN and OT-II SALN spleen cells in response to stimulation with OVA. (c) IFN-γ release from OT-II OVAN and OT-II SALN spleen cells in response to stimulation with OVA. Data represents mean ± SEM for triplicate wells. Significant differences in means were determined by Student's unpaired *t* test ($P < 0.05$). * $P < 0.05$ when compared to OT-II OVAN mice.

7.4 DISCUSSION

In Chapter 6, the OT-II mouse was shown to be refractory to AAD induction and progression. This may be attributed to significant IFN-γ production by transgenic $CD4^+$ cells in response to OVA during sensitisation, and is an interesting finding given that $OVA_{323-339}$ peptide is as an immunodominant T cell epitope that preferentially skews naïve T cell responses towards Th2 cytokine secretion (Janssen *et al.*, 1999; Janssen *et al.*, 2000). High doses of soluble OVA administration to wild type neonatal mice has previously been shown to induce anergy in both Th1 and Th2 compartments (Hogan *et al.*, 1998a), and may have been indicative of clonal deletion of antigen reactive T cells. To test if administration of high doses of antigen during neonatal development led to anergy or clonal deletion, OT-II neonates were given high doses of OVA, and subsequently characterised as adults. Upon investigation, OVA administration to OT-II neonates was shown to have no effect on splenic $CD4^+/V\beta5^+$ T cell populations in comparison to mice injected with vehicle (Figure 7.1). This result prompted a further analysis of the effects of neonatal antigen administration upon AAD induction and progression in the OT-II mouse, with a view to understanding the phenotypic fate of transgenic T cells. Surprisingly, soluble OVA administration to OT-II neonates promoted AAD induction and progression in i.p. sensitised and airways challenged adults, which was accompanied by increased IL-13 and IL-5, and decreased IFN-γ production from OVA stimulated splenocytes (Figures 2-5). Restoration of serum OVA specific IgG_1, was also a surprising result, and implied that IL-4 secretion was also a possible feature of this immune deviation (Snapper and Paul, 1987; Finkelman *et al.*, 1988a; Finkelman *et al.*, 1990).

These observations suggest two possibilities (at least) for immune deviation in the OT-II mouse. (1) The development and emergence of an OVA-specific Th2 subset that can both promote AAD and suppress the IFN-γ producing transgenic T cells, or (2) total reprogramming of the transgenic T cell response to OVA. In the first instance, the transgenic T cell represents almost 90 % of the $CD4^+$ population and remains within the T cell repertoire of OT-II OVAN mice (Figure 7.1). Their relative abundance and IFN-γ secretion in response to OVA (Chapter 6) does not support the hypothetical emergence of a separate Th2 T cell population having the capacity to suppress this abundant population of OVA transgenic T cells.

It is proposed that the OT-II TCR transgenic T cell may have been reprogrammed towards the Th2 phenotype in response to soluble OVA administration during ontogeny. Medullary thymic epithelial cells can present soluble OVA to T cells in the thymus (Mizuochi *et al.*, 1992), and it is possible that TCR transgenic cells that were developing in the thymus would have been negatively selected following the administration of OVA bolus to OT-II neonates, as the interaction between presented OVA peptides and developing TCR transgenic cells would be a high affinity interaction. Therefore, those cells that were developing in the thymus during and soon after OVA administration in OT-II OVAN mice may not have contributed to the observed phenotype in adult mice. Instead, the observed result may have been due to the reprogramming of the already mature TCR transgenic T cells that were circulating through the periphery at that time. Wild type neonates have a reduced capacity to produce IL-12 and IFN-γ (Chen *et al.*, 1996; Donckier *et al.*, 1998), and develop Th2 type cytokine responses to exogenous antigens (Gao *et al.*, 1996). The OT-II neonate may be similar in this respect, as antigen encounter by TCR transgenic T cells in the periphery during ontogeny appears to have led to peripheral reprogramming of these cells towards the Th2 phenotype, which then promoted the development of allergic airways inflammation in adult mice.

In summary, neonatal antigen administration in the OT-II mouse suggests that there is no intrinsic defect in the transgenic T cell's ability to produce IL-5 and IL-13. In the unmanipulated adult, significant IFN-γ production is a pre-programmed response that leaves the animal refractory to AAD induction and progression. This may be overcome by neonatal administration of soluble OVA, which is proposed to reprogram those T cells circulating in the periphery to become Th2 cells. Further experiments may validate this proposed mechanism.

Chapter 8

General Discussion

This thesis has explored the ability for eosinophils to respond to antigen deposition in the naïve lung and to modulate $CD4^+$ T cell function. In particular, the spatial and temporal distribution of mouse eosinophils within the allergic lung and lymphoid tissues was examined, concomitant with analysis of eosinophil function as antigen presenting cells both *in vitro* and *in vivo*.

8.1 INNATE IMMUNITY OF THE NAÏVE LUNG

Allergic responses to innocuous environmental antigens may represent a misdirected activation of the effector arm of the immune system (Th2), which that has evolved to combat parasite infections. Culley *et al.* recently hypothesised that the mechanism of eosinophil recruitment during parasite infection may be related to those seen during allergic inflammation (Culley *et al.*, 2002). In their study, the appearance of parasite larvae in lung tissue following primary infection with *Necator americanus* induced eotaxin and macrophage inflammatory protein 1α (MIP-1α) production. Importantly, the levels of these eosinophil chemoattractants were no different between naïve and immune mice, and both developed a pronounced pulmonary eosinophilia that was dependent on IL-5 (Culley *et al.*, 2002). Eosinophils have previously been identified as the primary source of IL-4 in response to the i.p. administration of parasite eggs (Sabin and Pearce, 1995; Sabin *et al.*, 1996). Furthermore, eosinophils recruited to the lung during *N. brasiliensis* infection are a major source of IL-4, and their appearance coincides with the induction of a Th2 response to this parasite (Shinkai *et al.*, 2002). Thus, the naïve lung secretes selective chemoattractants during primary responses to parasites, which promote the recruitment of IL-4 producing eosinophils that can participate in the generation of protective immune responses.

In Chapter 2, substantial evidence is provided for a similar mechanism of eosinophil recruitment to the naïve mouse lung, which operates in response to antigen (OVA) deposition. Eosinophil recruitment was only observed in PMBS treated mice that had a substantial pool of eosinophils located distally within the peritoneal cavity, suggesting that significant IL-5 generation in response to antigen was not a feature of the IES. The IES in naïve mice exposed to aerosolised OVA was significantly attenuated in eotaxin deficient mice, implicating this selective chemoattractant in what is suggested to be an innate response to antigen deposition in the naïve mouse lung. Notably, eotaxin plays a

role in the homeostatic regulation of eosinophils and thus has the potential to function as a rapid response signal to foreign antigen. Ordinarily, this mechanism would go unnoticed in naïve mice lacking a distal pool of eosinophils, although detailed analysis of histological sections of the lungs of naïve wild type and eotaxin deficient mice may further illuminate the operation of the IES at baseline.

It has recently been recognised that commercial OVA preparations contain bacterial endotoxin (Watanabe *et al.*, 2003), and LPS (a component of endotoxin), is known to induce granulocyte recruitment via eotaxin and RANTES expression in the mouse lung (Larsson *et al.*, 2000). However, while LPS administration induces both eotaxin and RANTES expression, antibodies to either of these molecules do not inhibit eosinophil recruitment (Penido *et al.*, 2001). In Chapter 2, the IES was significantly attenuated in PMBS treated mice that were deficient for eotaxin, and therefore the mechanism of recruitment cannot readily be attributed to endotoxin contamination of the OVA preparations that were used.

It would be interesting to further characterise the downstream effects of the IES described in Chapter 2. GM-CSF is an eosinopoietic cytokine (Arm and Lee, 1992), implicated in eosinophil survival *in vivo* (Corrigan *et al.*, 1995; Park *et al.*, 1998). GM-CSF transgene expression in the airways induces a transient pulmonary eosinophilia, and concurrent exposure to aerosolised OVA promotes allergic sensitisation via the airways in naïve mice (Stampfli *et al.*, 1998). Thus, one question that could be answered is this: were PMBS-treated mice subsequently sensitised following repeated exposures to OVA antigen? There is some evidence for this in Figure 2.10, wherein PMBS treated mice at the completion of the experiment had detectable OVA specific IgG_1. It was found that repeated PMBS injections were not tolerated in BALB/c mice, and at the time this study was conducted, IL-4 deficient mice were not available in the C57BL/6 background. Therefore, it would be very interesting to repeat these experiments in PMBS treated wild type and IL-4 deficient animals, with a view to determining the contribution of the IES, IL-4 and eosinophils to allergic sensitisation via the airways. Detailed examination of histological sections from both lung and lymph node tissue before, during, and after antigen exposure would also be very informative, when combined with immunohistochemistry and detection of IL-4 relative to eosinophils.

In summary, it seems that the naïve lung can generate eotaxin and possibly other factors, in response to antigen deposition, which can recruit eosinophils to pulmonary tissues. Under these conditions, there is no IL-5 available to promote the maturation and release of eosinophils from bone marrow, and the phenomena goes virtually unnoticed at baseline. In PMBS treated mice however, there are abundant eosinophils located in the peritoneal cavity that can rapidly respond to this signal. Further experiments are necessary to better define the IES and its role in immune surveillance and in priming of the adaptive response.

8.2 ANTIGEN PRESENTATION BY EOSINOPHILS

The ability of eosinophils to quickly respond to antigen deposition in the naïve airways prompted a detailed analysis of the temporal and spatial distribution of eosinophils in the lung and draining lymph nodes of allergic mice. Eotaxin is constitutively expressed in the lung (Rothenberg *et al.*, 1995b; Humbles *et al.*, 1997), and in Chapter 3, low levels of eosinophils were found to reside in BALF and lymph node tissues of sensitised mice prior to airways challenge. Using a model of allergen challenge, eosinophil numbers in the draining lymph nodes of sensitised mice were significantly elevated 3 hours after the first exposure to allergen, which actually preceded observed elevations in BALF and blood eosinophil levels (Figure 3.2).

Further investigation revealed that a sustained antigen challenge regime promoted a significant eosinophil infiltration of the lung and draining lymph node tissues, which persisted well after the cessation of airways challenge. Programmed cell death or apoptosis mediated by Fas is currently thought to be the process by which tissue eosinophilia is resolved (Woolley *et al.*, 1996), and both macrophage and small airways epithelial cells may ingest apoptotic eosinophils (Stern *et al.*, 1992; Walsh *et al.*, 1999). Interestingly, deficiency in Fas has been shown to extend tissue eosinophilia and AHR in a mouse model of allergic airways inflammation (Duez *et al.*, 2001), and defects in Fas signaling may underlie the chronic eosinophilic inflammation seen in asthmatic individuals (Tsuyuki *et al.*, 1995). In sensitised mice, IL-5 is expressed in the draining lymph nodes soon after allergen challenge (Gajewska *et al.*, 2001), and may contribute to prolonged eosinophil persistence in this tissue.

Notably, electron microscopy revealed that eosinophils residing in lymphoid tissue exhibited a low level of activation, with the loss of crystalloid granule cores and matrix from eosinophilic granules (Figure 3.4b). Accordingly, it was hypothesised that eosinophils residing in the lung prior to-, or those that were recruited following allergen provocation may actively sample the extracellular environment before translocating to the draining lymph nodes via the afferent lymphatics.

Analysis of eosinophil surface molecule expression showed that eosinophils derived from the allergic lung possess the molecular machinery (MHC-II, CD80, and CD86), necessary for effective antigen presentation to T lymphocytes (Chapter 4). Furthermore, these cells were able to internalise and process complex antigen both *in vitro* and *in vivo* within the allergic lung (Figure 4.3 and 4.4). Importantly, eosinophils co-incubated with *in vitro* derived Th2 cells promoted the release of IL-4, IL-5 and IL-13 (Figure 4.8), which are cytokines that have been implicated in the development of Th2 mediated allergic inflammation (Snapper and Paul, 1987; Swain *et al.*, 1990), and eosinophil recruitment to tissues (Ying *et al.*, 1997b; Webb *et al.*, 2000; Woltmann *et al.*, 2000; Kumar *et al.*, 2002). Therefore, eosinophils which have previously been shown to populate the lung and draining lymph node tissues following allergen challenge can interact with memory T cells via presentation of relevant antigen in the context of MHC-II, concurrent with the delivery of appropriate costimulatory signals.

In the future, these *in vitro* experiments may be extended by analysis of eosinophil ability to prime for Th2 and even Th1 responses. For instance, the methodology of Section 4.2.9 may be suitably adapted to incorporate eosinophils as the antigen presenting cells that drive Th T cell production. In the presence of appropriate antibodies, either Th1 or Th2 $CD4^+$ T cells may be generated from naïve T cells *in vitro*, and would further our knowledge of eosinophil function during immune surveillance. In these future experiments, it would be very interesting to use separate Th1-like and Th2-like eosinophil populations that have been identified previously (Lamkhioued *et al.*, 1996), which may further exhibit preferential Th1 or Th2 priming capacities *in vitro*. These studies may further link innate (eosinophils) and adaptive (T cell) responses.

Alternatively, the EliCell assay developed by Bandeira-Melo *et al.*, may be used to further define the antigen presenting capacity of eosinophils (Bandeira-Melo *et al.*,

2003). This recently developed microscopic assay has been used to identify cytokine release by individual eosinophils in response to environmental stimuli, when placed in a semi-solid growth medium. The assay may be suitably adapted to study the cognate interactions between antigen loaded eosinophils and naïve or antigen-specific T cells, and the cellular source of relevant cytokines, particularly IL-4, may be delineated. Furthermore, the semi-solid growth medium may be adapted to contain antibodies to cell surface molecules, including MHC-II, CD80/CD86, and ICAM-1, to further characterise the contribution of these molecules to antigen presentation by eosinophils. In addition, the semi-solid medium may be supplemented with proteins and other cell types (e.g. endothelial cells) that mimic *in vivo* allergic tissue conditions. For example, fibronectin has previously been shown to promote eosinophil survival *in vitro* (Anwar *et al.*, 1993), and the addition of this molecule to the semi-solid growth medium seems logical when attempting to study eosinophil function *ex vivo*.

In Chapter 5, eosinophils loaded with OVA antigen *ex vivo* promoted the development of Th2 mediated allergic airways inflammation when administered to naïve mice. Eosinophil recipient mice exhibited significant peripheral blood and pulmonary eosinophilia in response to airways challenge with relevant antigen, and stimulation of lymph nodes cells revealed significant Th2 cytokine production (IL-5 and IL-13). Importantly, OVA specific IgG_1 was not detected in the serum of eosinophil recipients at the end of the experiment, and indicated a lack of free OVA available for stimulation of naïve B cells.

A significant component of this thesis has been submitted for peer review, and subsequently published in an internationally recognised journal (MacKenzie *et al.*, 2001). Recently, Van Rijt *et al.*, have refuted the ability for eosinophils to present antigen to naïve T cells, and maintain that dendritic cells are superior in activating T cells within the draining lymphoid tissues of the lung (Van Rijt *et al.*, 2003). In their study, Van Rijt *et al.*, demonstrate that unlike dendritic cells, eosinophils instilled into the naïve airways are unable to stimulate the proliferation of OVA TCR transgenic T cells residing in the draining lymph nodes. This lack of function may however, be an artifact of their stringent purification procedure which, despite the best intentions, still only produced an eosinophil population of greater than >96% purity. The FACS sorted eosinophils used by Van Rijt *et al.*, were enriched from the BALF of allergic BALB/c mice on the basis of size, granularity, and $CCR3^{hi}/CD11c^{intermediate}$ surface molecule

expression. Whilst these two surface markers would certainly contribute to attaining a highly enriched eosinophil population, it should be noted that incubating eosinophils with antibodies to these surface molecules – particularly CCR3, may alter the activation status of these cells by non-specific receptor activation. For instance, eotaxin induces aggregation of guinea pig eosinophils *in vitro* (Griffiths-Johnson *et al.*, 1993), is an activator of the respiratory burst (Elsner *et al.*, 1996), and mobilises preformed IL-4 from the eosinophilic granules of human eosinophils (Bandeira-Melo *et al.*, 2001). Importantly, antibody-mediated ligation of surface molecules expressed by human eosinophils (CD28) induces cytokine release (Woerly *et al.*, 2002), and may alter any subsequent eosinophil responses to the microenvironment. Therefore, the purification procedure used by Van Rijt *et al.*, may have been detrimental to eosinophil function, and notably, they neglect to demonstrate translocation of their enriched eosinophils to the draining lymphoid tissues. In contrast, the freshly isolated eosinophils used in the experiments described in this thesis were FACS sorted on the basis of size (forwards scatter), granularity (side scatter), and the ability to polarize light, which reliably produced cell populations of >98% purity. These cells were duly capable of regulating $CD4^+$ T cell function *in vitro*, and *in vivo*.

In summary, intimate communication between eosinophils and T lymphocytes in allergic tissue may exacerbate allergic inflammation *in vivo*, through the production of Th2 cytokines (Figure 8.1). It is shown that eosinophils recruited to the allergic lung may take up and process antigen following repeated exposure to aeroallergen, and either present antigenic peptides to antigen–specific T cells within lung tissue, or traffic to the draining lymph node and present antigenic peptides to resident T cells. Ultimately, IL-4, IL-5 and IL-13 may be released in response to eosinophil immunomodulatory events, which may act distally (e.g. IL-5) to promote further eosinophilpoiesis, or locally (e.g. IL-4, IL-5 and IL-13), to exacerbate eosinophilic inflammation and airways hyperresponsiveness.

Alternatively, cognate interactions between antigen presenting eosinophils and antigen-specific T cells may serve to limit antigen-specific T cell function. Previously, eosinophils in the lungs of mice with allergy-independent pulmonary eosinophilia were demonstrated to release MBP following antigen deposition in the lung, and significantly, the depletion of $CD4^+$ T cells attenuated this MBP release (Mould *et al.*, 2000). More recently, antigen-activated $CD4^+$ memory T cells were shown to mediate

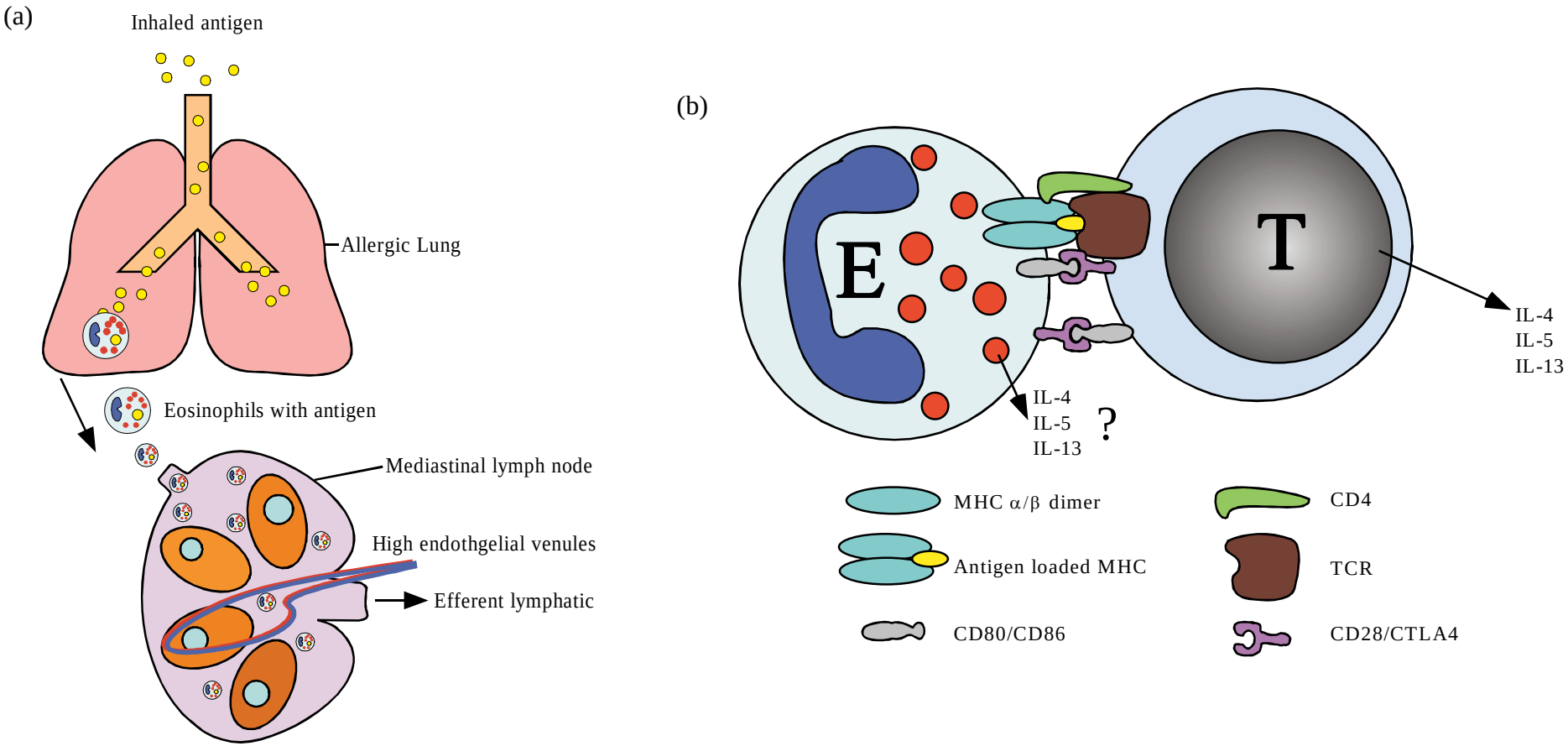


Figure 8.1. Proposed mechanism for eosinophil acquisition of aeroallergen and presentation to antigen specific T cells. (a) Eosinophils encounter antigen in the allergic lung, which is then processed in situ. They may either remain in the allergic lung, or traffic via the afferent lymphatic system to the draining lymph nodes. (b) Eosinophils (E) located within lung or draining lymphoid tissue present peptide antigens to $CD4^+$ T cells (T) in the context of MHC class II. Such immunomodulatory events may induce IL-4, IL-5 and IL-13 Th2 cytokine secretion and promote disease exacerbation. In addition, CD28 ligation on the eosinophil cell surface by T cell expressed B7 molecules may also promote the release of preformed cytokines from eosinophils.

eosinophil degranulation within the lungs of *N. brasiliensis* infected mice, but only in the context of relevant antigen (Shinkai *et al.*, 2002). Thus, antigen-activated $CD4^+$ T cells may mediate eosinophil degranulation in the lung, possibly as a result of bidirectional signaling events. The release of cytotoxic granule proteins during cognate interactions may then limit the activity of T cells, and also result in localised tissue damage. Again, the recently developed EliCell assay may be used to illuminate T cell mediated degranulation by eosinophils during cognate interactions. In particular, differing concentrations of antigen may be added to the semi-solid growth media in order to demonstrate antigen induced eosinophil degranulation mediated by $CD4^+$ T cells.

In conclusion, the work presented in this thesis explores the ability of eosinophils to participate as antigen presenting cells during allergic inflammation. The experiments and results described herein suggest that eosinophils recruited to pulmonary tissues may collectively act to modulate $CD4^+$ T cell function via antigen presentation and the induction of Th2 cytokine release. This has particular relevance for human atopic diseases, including asthma. It has already been recognised by others that eosinophils obtained from the lungs of allergic asthmatics display increased HLA-DR expression (Sedgwick *et al.*, 1992), and that human eosinophils are clearly capable of presenting complex antigens to memory T cells (Weller *et al.*, 1993, Weller *et al.*, 1997). Therefore, the eosinophil may provide a bridge between the innate and adaptive immune systems. Thus this cell may play a role in immune surveillance, normally populates the gastrointestinal mucosa, and is responsive to helminth parasite infections. Eosinophils may also play a small yet functional role in the education/priming of naïve T cells, and may transiently amplify memory T cell responses to allergen, and thereby exacerbate allergic disease.

8.3 THE OT-II MOUSE AND POSSIBLE FUTURE EXPERIMENTS.

Towards the end of this study, the OT-II mouse was procured with a view to investigating interactions between antigen presenting eosinophils and antigen-specific T cells. Initial experiments suggested that eosinophils could present antigen to OT-II T cells, however no detectable Th2 cytokine production could be measured despite specificity of the transgenic TCR for the Th2 skewing $OVA_{323-339}$ peptide (Janssen *et al.*, 1999; Janssen *et al.*, 2000). The OT-II mouse was subsequently characterised using

a mouse model of allergic airways inflammation, and was found to be refractory to disease induction, which was attributed to significant IFN-γ release from T cells in response to stimulation with OVA (Chapter 6).

T cell therapies have been effectively used to restore anti viral responses in immunocompromised patients (Heslop *et al.*, 1996; Rooney *et al.*, 1998), and the infusion of opposing Th1 cells in order to attenuate Th2 mediated allergic disorders is an exciting prospect. For instance, the adoptive transfer of antigen-specific Th1 cells that secrete IFN-γ has previously been shown to attenuate characteristics of allergic airways disease in animal models, including reduced pulmonary eosinophilia and airways hyperresponsiveness (Cohn *et al.*, 2001; Huang *et al.*, 2001). Subsequent attempts at using adoptively transferred IFN-γ secreting OT-II $CD4^+$ T cells to reduce allergic airways disease in C57BL/6 mice gave mixed results. While the recruitment of eosinophils to the airways lumen was certainly inhibited, recruitment to lung tissue, airways hyperresponsiveness and OVA specific immunoglobulin were left intact. This suggests that such immunotherapies based on homogenous populations of T cells with opposing cytokine responses to allergen may be of little therapeutic value, although it is acknowledged that using cells derived from transgenic animals that only recognise a single T cell epitope may not be an ideal foundation on which to predict future results.

The availability of antibodies directed against the OT-II TCR Vβ5 element enabled a brief and interesting investigation into the effects of neonatal antigen administration, which has previously been shown to inhibit both Th1 and Th2 responses (Hogan *et al.*, 1998a). Soluble OVA administration to OT-II neonates was shown to modulate adult responses to sensitisation and challenge with OVA, and led to the restoration of pulmonary eosinophilia, Th2 cytokine production, and OVA-specific IgG_1 to levels equivalent to those seen in OVA sensitised and challenged wild type mice. In Chapter 7, it was suggested that antigen delivery to OT-II neonates reprogrammed circulating T cells in the periphery during ontogeny, and that these cells were responsible for later development of allergic airways disease in OVA sensitised and challenged adults. It is reasonable to think that subsequent *de novo* production of TCR transgenic T cells in OVAN treated OT-II adults may still lead to mature T cells exiting from the thymus with the observed phenotype (IFN-γ secretion) seen in unmanipulated OT-II mice. Therefore, it is quite possible that in OVAN treated OT-II mice, subsequent i.p.

sensitisation and challenge with OVA led to the suppression and/or immunomodulation of these recent *de novo* produced thymic emigrants, by the Th2 type TCR transgenic cells (and their descendents) that were reprogrammed in the neonatal periphery. Future experiments using elispot assays and individual peripheral $CD4^+/V\beta5^+$ DP T cells may confirm the presence of two distinct populations of TCR transgenic T cells in the unsensitised OT-II OVAN mouse: one population that may secrete IFN-γ in response to OVA and which is representative of recent thymic emigrants, and an older population comprising those peripheral T cells and their descendents that were reprogrammed in the neonatal periphery, and which secrete IL-5 and IL-13 in response to OVA.

This question remains: why did a large soluble dose of OVA delivered to OT-II neonates not induce the previously observed anergy seen in wild type mice (Hogan *et al.*, 1998a)? In the wild type mouse, maturing thymocytes specific for OVA peptides may only represent a small fraction of all developing thymocytes. Administration of soluble OVA may have led to the negative selection of those cells that were developing in the thymus at that time (Liblau *et al.*, 1996), and the retention of OVA peptides by thymic medullary APCs may have prolonged the negative selection of the subsequently developing OVA-specific T cells. The (few) mature OVA-specific T cells in the wild type neonatal periphery may have been overwhelmed by such a large dose of soluble antigen and been subject to clonal exhaustion and apoptosis (Boehme and Lenardo, 1993; Radvanyi *et al.*, 1993; Nagata and Golstein, 1995; Iezzi *et al.*, 1998). In the OT-II neonate, a disproportionately large number of OVA-specific TCR transgenic T cells may be expected to reside in the periphery, and these cells may have been impervious to such an overwhelming dose of soluble antigen by virtue of their sheer numbers, and possibly synchronised responses afforded by the possession of a common TCR. In Figure 8.2, a mechanism is proposed wherein OT-II $CD4^+$ T cells may be reprogrammed during ontogeny. Future experiments may reveal that OVA administration to OT-II neonates can promote the induction of allergic inflammation of the lung (when subsequently exposed to inhaled allergen) without the requirement for sensitisation to OVA.

In summary, the availability of the OT-II mouse has provided a brief but unique opportunity to study neonatal responses to exogenous antigen during neonatal ontogeny.

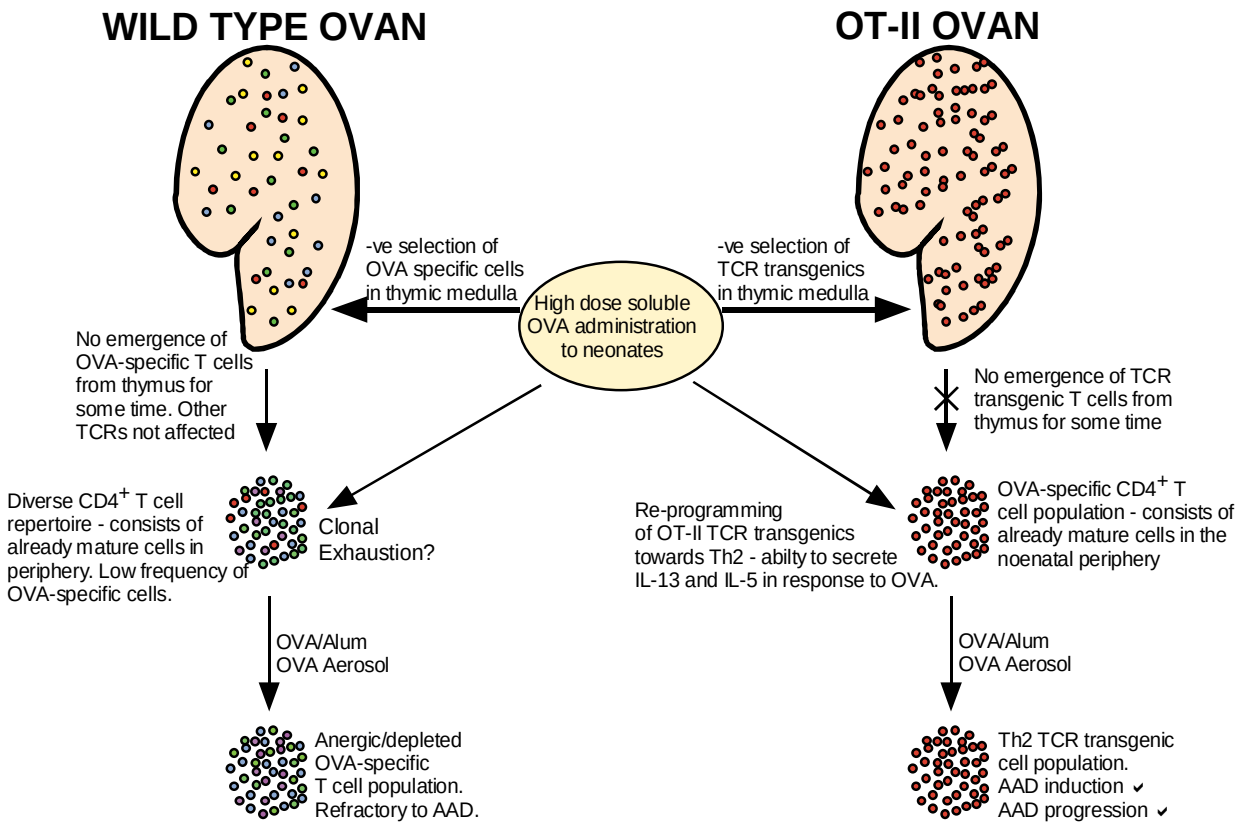


Figure 8.2. Proposed mechanism for reprogramming in the OT-II neonate exposed to a high dose of soluble OVA. In the wild type neonate, a high dose of soluble OVA (WILD TYPE OVAN) may promote the negative selection of OVA-specific thymocytes, and this process may continue into adulthood due to the retention of OVA peptides by medullary APCs. In the wild type periphery, the few recent thymic emigrants that are OVA-specific are overwhelmed by the large amount of soluble antigen, and either die or become anergic through clonal exhaustion. The wild type OVAN mouse is therefore refractory to allergic airways disease (AAD) induction and progression, and Th1 responses are also attenuated. In the OT-II neonate, negative selection in the thymus may also occur, and may also continue well into adulthood. There are many OVA-specific TCR transgenic T cells in the periphery, which are not overwhelmed by large amounts of soluble antigen. Instead, they may be collectively reprogrammed towards the Th2 phenotype. This promotes AAD induction and progression in OVAN treated OT-II mice.

Future experiments may illuminate the mechanism for reprogramming of the transgenic T cell during the neonatal period, and further our understanding of neonatal immunity.

Chapter 9

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RESPONSE TO EXAMINERS WRITTEN REPORTS, APRIL 2004.

EXAMINER 1:

Comment on Chapter 2. The experimental design and its interpretation imply that eosinophils detected in the lung fluid have arrived there via trafficking through the peritoneum. Do you know this is really the case?

Response: The data provided in Figure 2.1 show the difference in recoverable eosinophils from the peritoneal cavity both prior to (day 0) and following (day 8) exposure to aerosolised OVA on previous days 1, 3, 5, and 7. Looking to Figure 2.2, only WT PMBS treated mice developed a significant peripheral blood eosinophilia following exposure of the naïve airways to OVA, suggesting that a humoral factor may act to draw eosinophils from the distal peritoneal pool into the bloodstream. Analysis of BALF in Figure 2.3 shows that both WT and EKO PMBS treated mice had significant numbers of eosinophils in BALF, although this response was greatly amplified in WT PMBS treated mice.

In this model, it is the repeated injections of PBMS that induce the peritoneal eosinophilia in naïve mice, and which may be attributed to mast cell degranulation (Pincus, 1978). On discontinuation of the PMBS injections, I think that the eosinophils located within the peritoneum may endure alternative fates including: (1) non-specific egress via the blood and subsequent removal via apoptosis in the periphery, or (2), remain in the peritoneal cavity prior to subsequent removal via apoptosis in that compartment. Some eosinophils may invade the surrounding tissues, and histochemical analysis of surrounding tissues may be illuminating. The data presented in Figures 2.2 and 2.3 would suggest that antigen deposition in the naïve airway can elicit a humoral factor, possibly eotaxin, that can attract eosinophils from the peritoneal compartment to the lung, via blood. The alternative is that eosinophils may be recruited from other tissues or even from the bone marrow, however this is unlikely and the WT Control data in Figures 2.1-2.3 do not support any notion that antigen deposition in the lung may drive eosinophil accumulation in the absence of this distal and responsive pool. Nonetheless, adoptive transfer of fluorescently labelled eosinophils to the peritoneum of WT PMBS and WT Control mice may be an informative exercise in future experiments, and confirm egress from the peritoneal cavity via blood and subsequent recruitment to the naïve lung.

Comment on Chapters 3&4. While you have presented evidence suggesting that eosinophils can function as antigen presenting cells *in vivo*, how effective are they when put up against the classic antigen presenting cells (i.e…dendritic cells, etc)? Do you think eosinophils fill a specific niche in time? space (i.e…nature of organ)? nature of antigen?

Response: Eosinophils are poor antigen presenting cells in comparison to classic APCs (Del Pozo *et al.*, 1992; Mawhorter *et al.*, 1994), and yet I still believe that the evidence presented in this thesis shows that large numbers of antigen loaded eosinophils may serially trigger T cell receptors (Valitutti and Lanzavecchia, 1997), and exacerbate inflammation type responses e.g. following repeated antigen deposition within the allergic airways mucosa. This exacerbation may occur locally within lung tissue, as well as distally in draining lymphoid tissues (see for example figures 3.3 and 3.4). Ultimately, modulation of T cell function by this inefficient yet highly prevalent

granulocyte having APC function is considered to have effects on eosinophil activation status in the lung, and also on subsequent eosinophilpoiesis and recruitment, which is mediated by such factors as T cell (and possibly eosinophil) derived IL-4, IL-5 and IL-13.

Do I think eosinophils fill a specific niche in time? Yes, but only in that they may (following initial recruitment), reside in inflamed tissues for the duration of their short lives. If, during the period in which they reside, that tissue is exposed to further antigen, then large numbers of eosinophils with antigen presenting capacity may continue to modulate T cell function en masse, be they memory or naïve T cells.

As to the nature of the antigen, the experiments in this thesis dealt solely with a soluble antigen (OVA), which has no intrinsic enzymatic property. Many of the known allergens are soluble proteins having intrinsic enzymatic properties. For instance Fel d 1 antigen derived from cat saliva is a soluble antigen having associated fibronectin-degrading ability (Ring *et al.*, 2000), and may be carried by airborne house dust particles (Ormstad *et al.*, 1998). Similarly, Der p 1 antigen from house dust mites has some associated proteolytic activity, and may also modulate the balance between IL-4 and IFN-γ (Comoy *et al.*, 1998). It would be interesting to study the ability of eosinophils to present soluble antigens with such associated intrinsic activities.

Comment on Chapters 6&7. These chapters are a bit short on experimental data, and while your chapter discussions and overall discussions not "future experiments", it might be useful, just as a post-thesis exercise, to outline precisely what those future experiments might be.

Response: I agree with the examiners sentiments. This suggested exercise may be undertaken in collaboration with Prof. Foster at some time in the future. However, I would prefer not to publish any planned future experimental procedures here in any detail, with a view to protecting future research efforts/directions.

EXAMINER 2

Comment on Chapter 1. In Chapter 1, page 8, 2nd paragraph. It was unclear how a lack of protective immunity in IL-5 KO mice can be concluded from studies showing no difference in recovery rates between non-immunized controls and IL-5 KO mice?

Response: Le Goff et al., (Le Goff *et al.*, 2000), demonstrated that "Mice genetically deficient in IL-5 were unable to generate a protective immune response when vaccinated with irradiated larvae, whereas C57BL/6 mice were fully protected from challenge infection" (excerpt from the abstract). My intention was to draw the readers attention to the fact that eosinophils may play a role in the development of protective immunity – aside to their purported roles in innate immunity and parasite clearance (Weller, 1994; Ovington and Behm, 1997), which are actively being challenged (Dent *et al.*, 1997).

Comment on Chapter 1. 3rd last line, page 24. A square bracket appears in the line.

Response: I propose to remove said square bracket.

Comment on Chapter 1. 1st line page 26. The sentence begins by referring to studies in the 1990's, yet a paper from 1981 is cited?

Response: I propose to change the paragraph to "In the late 1900's…"

Comment on Chapter 1. It may have been helpful to give the reader an indication of the relative efficiency of different APC's including eosinophils with respect of antigen presentation. Particularly in the context of presentation to naïve and memory T cells?

Response: The readers attention is drawn to the response to comments of Chapters 3 and 4, raised by Examiner 1 (*supra*). In addition to these comments, it appears that eosinophils may be a potential source of early IL-4 in naïve responses to parasite eggs (Sabin and Pearce, 1995; Sabin *et al.*, 1996), and may dictate the early development of a Th2 type response in parasite models.

Comment on Chapter 2. In Chapter 2, the candidate uses polymyxin B to raise the levels of eosinophils in the peritoneal cavity. As a consequence, ovalbumin exposure to the lung results in eosinophil recruitment to this site. There is no rationale for the use of polymyxin B nor how it raised eosinophil levels in the peritoneal cavity. The study of Pincus refers to studies in guinea-pig and this agent induces mast cell degranulation. Is this the proposed mechanism in these studies? This rationale should have been given in the introduction.

Response: Polymyxin B has previously been used routinely in Prof. Fosters laboratory to induce eosinophils in the peritoneal cavity, providing an easily obtainable source of these cells for *in vitro* and *in vivo* experiments (Mould *et al.*, 1997). Polymyxin B is thought to induce mast cell degranulation, the downstream effects of which lead to eosinophil accumulation (Pincus, 1978). I am not aware if anybody has since elucidated the exact mechanism of polymyxin B-generated eosinophilia, other than identifying IL-5 as being necessary (studies in IL-5 deficient mice, Dr. Klaus Matthei - personal communication). It is interesting to note that polymyxin B is known to bind endotoxin (James *et al.*, 1996), although LPS contamination of polymyxin B for injections is unlikely.

Comment on Chapter 2. Is the effect of ovalbumin on eosinophil recruitment observed if another agent is used to augment in the peritoneal cavity (eg IL-5 or PAF)?

Response: Unknown. I think it might be important to use a stimulus that does not generate a large peripheral blood eosinophilia (bolus IL-5 administered i.p. may do this), and to use a stimulus which gives reproducible eosinophilia in large numbers of naïve mice. At the time, polymyxin B injections provided the means with which to do this.

Comment on Chapter 2. Is it possible that polymyxin B is inducing the inflammatory mediators systemically (eg eotaxin, IL-5), which then facilitates eosinophil recruitment to the lung?

Response: Yes of course, although it should be remembered that despite eotaxin deficient animals having similar numbers of eosinophils in the peritoneal cavity, pulmonary eosinophilia was significantly attenuated in comparison to wild type mice following exposure of the naïve airways to ovalbumin aerosol (see Figures 2.1(a) and 2.3(a)). Only polymyxin B treated mice had significant pulmonary eosinophilia

following exposure of the naïve airways to ovalbumin aerosol, as wild type controls (WT Control) mice (not exposed to polymyxin B) did not.

Comment on Chapter 2. It is unclear from the figures whether WT controls refer to animals not treated with polymyxin but exposed to ovalbumin? If animals were not exposed to ovalbumin, then please indicate the levels of lung eosinophils enumerated in BAL fluid.

Response: The mice were not treated with polymyxin but exposed to ovalbumin. There were no eosinophils recovered in BAL for these mice.

General comment. Non paired t test was frequently used to determine statistical significance between groups. In a number of situations more than two treatment groups were present and therefore, an analysis of variance followed by an appropriate multiple comparison test (eg Bonferroni) should have been used. While the conclusions drawn will not change, the data analysis would be more appropriate.

Response: From GraphPad Prism (Ver 2.01) helpfile: “The only advantage of the Bonferroni method is that it is well known and easy to understand. Its disadvantage is that it is too conservative, leading to P values that are too high and confidence intervals that are too wide.” Whilst the examiner favors this test, there was no special inducement to use it. As the examiner has indicated, the conclusions drawn would not change.

Comment on Chapter 5. Page 83. The candidate considers the possibility that contamination by T cells could not account for the effects observed when eosinophils from antigen-sensitized mice were transferred into recipients. Would in not have been possible to undertake control experiments with anti-CD4 during purification to eliminate contamination by T cells?

Response: Yes it was possible with hindsight, and the suggested controls would have adequately answered the question as to T cell contamination.

Comment on Chapter 8. Page 110. ‘microscopical’ should be replaced with ‘microscopic’.

Response: I propose to change said word to "microscopic".

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