CO-OPERATION BETWEEN HUMORAL AND CELLULAR IMMUNITY IN PULMONARY LUNG INFLAMMATION

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ABSTRACT

Asthma is a respiratory disease characterised by reversible airway obstruction, elevated levels of immunoglobulin E (IgE) in serum, chronic eosinophilic airway inflammation and airway hyperresponsiveness (AHR) to bronchospasmogenic stimuli. Many studies have been performed to dissect the role of T lymphocytes in asthma but not many studies specifically address the role of IgE in asthma. *In vitro* studies have shown enhanced activation of allergen specific T cells when they were cultured with allergen and allergen-specific IgE, suggesting that the role of IgE is more than just a mast cell activator but rather it plays a part in up-regulating the effects of CD4+ T cells in asthma. Hence the aim of the current study was to elucidate the interaction between allergen-specific IgE and allergen-specific Th2 CD4+ T cells *in vivo*. Mice that were immunised by intraperitoneal (i.p.) injection of ovalbumin (OVA) followed by intranasal (i.n.) challenge with OVA had a significantly higher percentage of eosinophils in bronchoalveolar lavage (BAL) compared to the control group animals. Moreover, levels of OVA-specific IgE were a 1000-fold higher in experimental animals than in control animals. To study the role of IgE in airway inflammation, a passive sensitisation model was developed. Mice were intravenously (i.v.) given OVA-specific IgE before they were i.n. challenged with OVA and responses of these mice were analysed by BAL. No eosinophilic inflammation of the airways was observed regardless of the relatively high doses of mouse anti-OVA IgE that were used. To study the role of CD4+ T cells in airway inflammation, Th2-polarised antigen-specific CD4+ T cells were intravenously transferred into naïve animals before they were intranasally challenged with OVA. Massive numbers of eosinophils was recruited into the BAL with the adoptive transfer model mice. Once these two models were independently established, the role of IgE aiding in the airway

inflammation induced by antigen-specific CD4+ T cells was studied by combining the two models. The mice were passively challenged with IgE and given sub-optimal numbers of Th2 cells a day before they were intranasally challenged with OVA. Mice that had received just the Th2 cells had a higher level of eosinophils in the BAL when compared to animals that were passively sensitised and given Th2 cells. However mice that had received IgE had a higher percentage of T-cells and almost twice the amount of transgenic T-cells recruited into the lungs thus suggesting that IgE might play a role in the recruitment of T-cells but not in the enhancement of T-cell mediated responses.

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ABBREVIATIONS

AHR	Airway hyperresponsiveness
APC	Antigen presenting cells
BAL	Bronchial alveolar lavage
CD	Cluster of differentiation
CO_2	Carbon dioxide
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
IFN	Interferon
IL	Interleukin
Ig	Immunoglobulin
i.n	intranasal
i.p	Intraperitoneally
i.v	Intravenously
mAb	Monoclonal antibody
MHC	Major histocompatability complex
NK	Natural killer
OVA	Ovalbumin
PB	Pacific Blue
PBS	Phosphate buffered saline
PE	Phycoerytherin
PMA	Phorbol myristate acetate
TCR	T-cell receptor
Th	T helper
Tc	T cytotoxic
Treg	T regulatory
APC	Allophycocyanin

CHAPTER 1: INTRODUCTION

1.1 Immunology of the respiratory tract

The main function of the respiratory tract is to allow efficient gas exchange between the pulmonary circulation and the thin epithelial lining of the alveoli during breathing. Successful host defence comprises of an effective barrier function and an immune system that deals with potentially dangerous invaders efficiently, while avoiding an over-reaction to harmless airborne particles [1]. Inevitably, the exposed surface and conducting airways have to be defended against airborne irritants and infectious agents in ways different from other externally exposed areas such as the skin [1]. The primary defence against foreign particles consists of a thin layer of mucus secreted by globlet cells and the mucous glands found in the conducting airways. If these passive barriers are insufficient in clearing the foreign agents, the airways are then defended by a combination of non-specific phagocytosis by alveolar and tissue macrophages and the specific immune responses such as antibodies and cell-mediated immunity [1].

1.2 Innate versus adaptive immunity

Immunological defences in vertebrates consist of two distinct arms — innate and adaptive immunity. The innate immune system of defence consists of both cellular and non-cellular components. The cellular components of the innate immune system include dendritic cells, monocytes, macrophages, granulocytes, and natural killer T cells, as well as the skin, pulmonary, and gut epithelial cells that form the interface between an organism and its environment. The non-cellular aspects of the innate system are diverse and range, from the simple barrier function of the stratum corneum, skin and etc to complex pathways such as the complement cascade. These

non-cellular elements seek to prevent the entry of pathogens through physical blockade, or once invaded, to destroy pathogens directly or call them to the attention of phagocytes. The cellular aspects of the innate immune system respond by recognising conserved motifs in pathogens known as pathogen-associated molecular patterns (PAMP) as well as a number of other indicators of cell stress or death. The innate immune system recognises PAMP using pathogen-recognition receptors (PRR), which are a group of germline-coded, evolutionary conserved proteins. PRR do not only comprise of cell-surface pathogen receptors, present on innate immune cells, but also secreted and locally produced molecules that mediate many steps in inflammation including directed phagocytosis, activation of inflammatory signalling pathways, induction of cell death, and activation of the complement or coagulation cascades [2]. One of the most studied PRR is the Toll-like Receptors (TLRs).

The key elements of the adaptive immune system are T and B cells. Flexibility and memory are the hallmarks of the adaptive immune response. Flexibility is provided by the unique antigen receptors expressed on T and B cells enabling them to recognise virtually any antigen. T and B cells that have previously encountered antigen persist over the long term within an organism and provide rapid and specific responses to re-infection, a concept known as immunologic memory. The adaptive immune response might be slower but is more flexible and is more efficient at combating infections that have managed evade the rapid and blunt responses of the innate system. However without the innate immune system to instruct the cells of the adaptive immune system, they may never have the chance to respond [2].

1.3 Humoral immunity

Antibodies, which mediate the humoral arm of the immune system, are produced by B cells and are grouped into different isotypes based on their heavy chains. There are five different antibody isotypes, each performing different roles (Table 1). Antibodies can exist in two forms; a soluble form that is secreted into the blood and tissue fluids, and a membrane-bound form attached to the surface of a B cell and is known as the B cell receptor (BCR). The BCR allows a B cell to detect when a specific antigen is present in the body and the antigen:BCR complex triggers B cell activation. Activated B cells differentiate either into plasma cells that secrete soluble antibody, or into memory cells that survive and remain dormant in the body for years. B cells need two signals to initiate activation. Most antigens are T cell-dependent, requiring T cell activation for antibody production. The first activation signal comes from antigen cross-linking BCRs and the second activation signal is from the T cell and this occurs when the T-dependent antigens are presented by Class II major histocompatability complex (MHC) molecules present on the surface of B cells to T cells, which then provide co-stimulation to trigger B cell proliferation and differentiation into plasma cells. Antibody isotype switching to IgG, IgA, and IgE and memory cell generation occur in responses to T-dependent antigens under the control of specific cytokines. However there are some antigens that are T-independent and these antigens deliver both signals to the B cell. For example there are bacteria that have repeating carbohydrate epitopes that stimulate B cells to respond with IgM synthesis in the absence of T cell help. Fine tuning of antigen specificity of T-dependent antibody is accomplished by affinity maturation, a process that involves hyper-mutation of antibody genes and selection of high affinity antibody expressing cells that are better

able to solicit T cell help [2], sending survival signals to B cells through anti-apoptotic receptors such as Bcl-2.

Isotype	% of total Ig (adult serum)	Biological half-life (days)	Biological Functions
IgA_1	11-14	5.9	Pathogen neutralisation in mucosal secretions
IgA_2	1-4	4.5	
IgD	0.2	2-8	Membrane BCR
IgE	0.004	1-5	Mast cell histamine release
IgG_1	45-53	21-24	Pathogen neutralisation in tissues Classical complement activation Opsonisation Natural Killer (NK) cell antibody-dependent cell-mediated cytotoxicity (ADCC) Transplacental transfer
IgG_2	11-15	21-24	Pathogen neutralisation in tissues
IgG_3	0.03-0.06	7-8	Pathogen neutralisation in tissues Classical complement activation Opsonisation NK cell ADCC Transplacental transfer
IgG_4	0.015-0.045	21-24	Pathogen neutralisation in tissues Transplacental transfer
IgM	10	5-10	Classical complement activation Membrane BCR (monomer)

Table 1: Properties of human antibody isotypes. (Source: Leffell, 1997) [3]

1.4 T cell mediated immunity

T cells play a central role in cell-mediated immunity. They enter the bloodstream and are carried by the lymphatic and blood circulation once they have completed their development in the thymus. T cells leave the bloodstream once they have reached a peripheral lymphoid organ only to enter the circulation if they have not encountered their specific antigen via an antigen presenting cell (APC) and this cycle occurs until they do. To participate in an adaptive immune response, a naive T cell must first encounter the appropriate antigen, and then be induced to proliferate and differentiate into cells capable of contributing to the removal of the antigen and such cells are known as effector T cells because they act very rapidly when they encounter their specific antigens. Effector T cells fall into two functional classes that detect peptide antigens derived from different types of pathogen. Peptides from intracellular pathogens that multiply within the cytoplasm of cells are carried to the cell surface by MHC class I molecules and presented to CD8+ T cells which then differentiate into cytotoxic T (Tc) cells that directly kill infected target cells. Peptide antigens from pathogens multiplying in intracellular vesicles, and those derived from ingested extra cellular bacteria and toxins, are carried to the cell surface by MHC class II molecules and presented to CD4+ T cells [2].

Just like B cells that have a surface receptor, the T cell receptor (TCR) is structurally similar to the BCR. However, unlike the BCR, which has the ability to recognise native antigen, T-cell receptors recognise a composite ligand, consisting of the foreign peptide bound to a (self) MHC molecule and each TCR is specific for a particular combination of foreign peptide-MHC complex. TCR recognition is, however insufficient for activation. Activation of T/B cells requires the simultaneous delivery

of a co-stimulatory signal by the antigen-presenting cell. The most potent activators of naive T cells are mature dendritic cells and these are thought to initiate most T-cell responses *in vivo*. Immature dendritic cells take up antigen at sites of infection and consequently travel to local lymphoid tissue where they mature into cells that express high levels both co-stimulatory and adhesion molecules. These expressed molecules mediate the interactions between the mature dendritic cells and the naive T cells that are continually recirculating the lymphoid tissues.

1.4.1 CD4+ T cells

CD4+ T cells also known as helper T (Th) cells can be grouped into two subsets based on the effector cytokine expression. Th1 cells secrete interferon-gamma (IFN-?) and Th2 cells secrete interleukin-4 (IL-4) [4]. Recently, it has been recognised that there are other subsets of CD4+ T cells namely Tr1 (IL-10-secreting), Th3 (transforming growth factor [TGF] ß-producing), ThFH (follicular helper cells), peripherally-induced T regulatory (Treg; FoxP3-positive) and Th17 (IL-17A-producing). The differentiation of naïve T cells to Th1 cells is regulated by transcription factors such as T-bet, Signal Transducers and Activator of Transcription-1 **(**Stat1) and Stat4, as well as cytokines such as IL-12, IL-18 and type 1 Interferons and IFN-? [5]. On the other hand, Th2 differentiation is controlled by transcription factors such as Stat6, GATA-3, c-Maf, Nuclear factor of activated T-cells (NFATs) and the cytokine IL-4 [5]. Th1 effector cells produce IFN-? and promote cellular immunity, which is critical to the control of intracellular pathogens such as *Mycobaterium tuberculosis*. IFN-? activates macrophages, enhancing their ability to phagocytose and destroy microbes. Th2 effector cells produce IL-4, IL-5 and IL-13 and promote humoral immunity and resistance to helminthic infections. IL-4 induces isotypic switching in B-cells to IgE

and IL-5 plays a key role in the eosinophilic activation. Th1 cells have also been implicated in organ-specific autoimmunity and similarly Th2 cells with allergy and asthma.

1.4.2 CD8+ T cells

Naive CD8+ T cells differentiate into Tc cells and upon activation are effective in killing cells infected with viruses or other intracellular pathogens. However, their ability to produce various cytokines suggests additional immune functions. CD8+ T cells can be classified as being either Tc1 (IFN-? producing) or Tc2 (IL-4 producing) subtype. Naive CD8+ T cells show a stronger preference to differentiate into Tc1 cells [6]. IFN-? and IL-12 promote differentiation to Tc1 cells and substantial amounts of IL-4 together with anti-IL-12 and anti-IFN-? blocking antibody is required for Tc2 differentiation [7, 8]. Despite the differences in cytokine expression, both Tc1 and Tc2 have similar cytotoxic ability regardless of whether killing is mediated by the perforin pathway or Fas pathway [7, 9, 10]. Perforin deficient Tc2 cells, are able to provide some help for IgM production but it cannot be compared to that provided by Th2 cells because Tc2 subtypes are unable to induce strong antibody responses compared to Th2 cells [7]. Although Tc2 might differ from Th2 in terms of their inflammatory responses, cytokines secreted by Tc2 could provide bystander help for Th2 mediated responses because during a Tc2 mediated delayed-type hypersensitivity (DTH) a higher number of eosinophils are recruited compared to Tc1 mediated DTH [11].

1.5 Hypersensitivity

Gell and Coombs (1963) devised the classification of allergic reactions; types I - IV. Type I reactions occur rapidly and are mediated by IgE antibodies (to the allergen) which bind strongly to the surface of mast cells. The synthesis of IgE antibodies is triggered by Th2 cells which produce a number of inflammatory cytokines in the process. The most important cytokine in these type I responses is IL-4. Cross linking of bound-IgE with its appropriate antigen results in mast-cell degranulation and the consequent release of histamine (causing an immediate reaction), leukotrienes (resulting in the more delayed symptoms) and other mediators. Type-II reactions are antibody-mediated. They are caused by cytotoxic antibodies directed against cell surface antigens, which are primarily IgM or IgG. Cell damage results from two main mechanisms. The first mechanism is the direct action of macrophages, neutrophils and eosinophils that are linked to Ig-coated target cells via their Fc receptors. The second mechanism induces the antibody-mediated activation of the complement pathway that results in cell lysis. Type III hypersensitivity reactions occur when antibody reactions occur in the blood or tissues, resulting in the formation of antigen–antibody complexes, which are deposited in the glomerular and/or pulmonary basement membranes. Here, the presence of these complexes, in addition to the polymorphonuclear cells (PMNs) attracted by complement activation, results in tissue injury and compromised function. Type IV hypersensitivity reactions are mediated by T cells, and tissue damage is caused by macrophages and Tc cells. Contact dermatitis is a clinical example of a type IV hypersensitivity reaction.

1.6 Asthma

Asthma is one of the most common disorders encountered in clinical medicine in both children and adults. Affecting approximately 5-10% of the adult population, its reported incidence is increasing dramatically in developed nations. It is characterised by three major features: (1) intermittent and reversible airway obstruction leading to recurrent episodes of wheezing, breathlessness, chest tightness, and cough; (2) AHR, which is defined as an increased sensitivity to bronchoconstrictors such as histamine or cholinergic agonists; and (3) airway inflammation. There is an established strong correlation between the presence of eosinophils and the presence of Th2 cells in asthmatic airways. Th2 cell-derived cytokines, namely IL-4, IL-5, IL-9 and IL-13, play a critical role in orchestrating and amplifying allergic inflammation in asthma [12].

1.6.1 Mast cells and IgE

The classical type 1 hypersensitivity reaction in acute asthma and the early response to allergen challenge results from IgE cross-linking by allergen leading to Fc epsilon Receptor I (FceRI) signalling. Cross-linking of IgE bound to mast cells triggers the release of stored preformed mediators such as histamine and also initiates the synthesis of prostaglandins and leukotrienes which have roles in bronchoconstriction, edema, and recruitment of inflammatory cells. Numerous human studies on asthma have demonstrated an increase in mast cell numbers in the airways and have detected histamine, Prostaglandin D_2 **(**PGD_2), and tryptase in BAL fluid both in symptomatic asthma and after allergen inhalation challenge, suggesting mast cell degranulation [13-15].

Results from a mouse model of asthma showed that AHR and tissue eosinophilia could still be evoked in mast cell-deficient mice sensitised with intraperitoneal OVA with aluminium hydroxide adjuvant (which favours a Th2 response), but not if sensitisation was without adjuvant [16]. Although mast cells can cause AHR, it can also be elicited without these cells. Work done using animal models had dissected the early and late asthmatic response and from these studies, there is wide agreement that the early asthmatic reaction (EAR) is IgE-dependent and mast cells play a pivotal role [17] the late asthmatic reaction (LAR) can be IgE-dependent or IgE-independent, as seen by isolated LAR induced by intradermal injection or inhalation of allergen-derived peptide fragments that activate T cells but not IgE. However there was no evidence of mast cell activation in such reactions [18-20]. Passive sensitisation of athymic mice with anti-OVA IgE induced immediate allergic responses and mast cell degranulation but upon challenge with OVA after passive sensitisation, athymic mice didn't develop AHR [21] which corroborates the data from reports that T cells are required for the full development of asthma.

1.6.2 Eosinophils

Eosinophilia is a characteristic feature of asthma and eosinophil cell numbers and activation state broadly correlate with disease severity. Activated eosinophils are thought to contribute to airway inflammation and AHR by the direct release of basic granules, leukotrienes and other mediators and also indirectly by their interactions with numerous cell types. Eosinophils are also a rich source of cysteinyl leukotrienes, which directly contribute to bronchoconstriction and also increase vascular permeability and contribute to inflammatory cell recruitment. In addition, eosinophils indirectly contribute to the development of AHR by the induction of mast cell and

basophil degranulation, leading to the production of prostaglandins, leukotrienes and histamine, all of which can induce AHR. Studies have also shown that interactions of eosinophils with T cells may also contribute to the development of AHR. The transfer of eosinophils to OVA-sensitised IL-5 knockout(KO) mice resulted in the development of eosinophilia, Th2 cytokine production and the development of AHR similar to WT mice but treatment of IL-5 KO mice with anti-CD4+ antibody diminished the effect of adoptive transfer of eosinophils on AHR [22].

Although eosinophils secrete a range of mediators that can contribute to airway remodelling, it was recently shown that an association exists between airway remodelling and eosinophils. Eosinophils were genetically ablated in mice by the deletion in the high affinity GATA-binding site in the GATA-1 promoter. After a prolonged allergen challenge using a well established model the wild type had more prominent features of airway remodelling [23].

1.6.3 CD4+ T cells

The "Th2 hypothesis for asthma" was first suggested by Mosmann in 1989, who had earlier discovered the presence of two distinct subtypes of helper T cells in mice, namely, Th1 and Th2 [4]. The Th2 hypothesis for asthma stated that asthma was caused by a relative increase in Th2 cellular response in combination with a decrease in Th1 response. The consequent alteration in cytokine levels in the lung with excess Th2 cytokines (i.e. IL-4, IL-5, and IL-13) in concert with decreased Th1 cytokine levels (i.e. IFN-? and IL-12), drove the development of asthma. Evidence of such a shift in the Th1/Th2 balance arose from studies of human studies that profiled the cytokine production from cells collected from BAL [24]. mRNA expression study of

the cells from asthmatic patients showed an increase in Th2-type cytokine mRNA levels. Furthermore, using adoptive transfer models, investigators were able to show that antigen-specific Th2 clones were able to induce eosinophilia and bronchial hyperresponsiveness in naïve mice following antigenic challenge. Co-transfer of antigen-specific Th1 cells dose-dependently reversed bronchial hyperresponsiveness (BHR) and BAL but not mucosal eosinophilia [25].

1.6.3.1 Th2 cytokines

Two essential biological activities of IL-4 lead to the development of allergic inflammation. IL-4 drives differentiation of naive Th0 cells into Th2 cells, which secrete IL-4, IL-5, IL-9 and IL-13 but not IFN-? and IL-4 induces B cell isotypic switching to IgE. Studies using IL-4-deficient mice clearly showed that IL-4 was required for the development of allergic inflammation, as antigen-induced allergic inflammation was significantly decreased in IL-4-deficient mice as compared with wild-type mice [26]. Coyle *et al* also demonstrated that the administration of neutralising anti-IL-4 antibody prior to immunisation prevented the development of antigen-induced airway inflammation, whereas the administration of the same antibody after immunisation but prior to antigen inhalation was not effective for preventing antigen-induced airway inflammation [27]. These studies show that even though IL-4 is essential for the initial differentiation and expansion of antigen-specific Th2 cells, it may not be important for the induction of allergic airway inflammation at a later stage. Other studies have shown that IL-4 may have a broader action. The importance of IL-4 in promoting allergic inflammation at an effector phase by inducing the recruitment of Th2 cells was shown by Cohn *et al* [28]. OVA-specific Th2 cells from IL-4-deficient mice were not recruited to the lung but this defect in

homing was overcome by administration of TNF-alpha. Thus it is possible to conclude that IL-4 is required by Th2 cells to home to the lung and this function of IL-4 is distinct from its effects on Th2 cell development.

IL-5 has been recognised as the major maturation and differentiation factor for eosinophils in animal models of asthma[29]. Using neutralising anti-IL-5 monoclonal antibody (mAb) Nakajima *et al* showed that IL-5 was important in antigen-induced AHR and eosinophil infiltration in the airways of mice [30]. The lack of BHR and eosinophilia in the lungs of antigen-sensitised and antigen-challenged IL-5-deficient mice further demonstrated the importance of IL-5 in allergic airway inflammation [31]. In a clinical study using humanised anti-IL-5 mAb to treat mild allergic patients, the effects of treatment on the levels of blood and airway eosinophils (measured in induced sputum) were examined, as were the effects on the responses to an inhaled allergen challenge administered 1 week and 4 weeks after the treatment [32]. Results of this study confirmed the importance of IL-5 in eosinophilic inflammation in human. However, anti-IL-5 antibody was not able to reduce asthmatic symptoms and airway reactivity, suggesting that IL-5 independent mechanisms contribute to asthma.

Based on similarities in structure and common receptor components with IL-4, it was hypothesised that IL-13 may play a role in the development of allergic airway responses. The importance of IL-13 was demonstrated by the findings of 2 groups [33, 34] who showed that neutralisation of endogenously released IL-13 with a soluble form of IL-13Ra2 (which binds IL-13 specifically but not IL-4) during antigen exposure inhibited the characteristics of asthma in murine models. Its importance as an effector molecule in asthma was further evidenced by the finding of

Walter DM *et al*, showed that antigenic challenge of IL-13-deficient mice failed to elicit AHR, airway inflammation and mucus production although IL-4 and IL-5 were present [35]. Using an over-expression transgenic approach to characterise the *in vivo* effector functions of IL-13 [36], lung-specific expression of IL-13 resulted in the development of the characteristic features of asthma. These results suggest that IL-13, independent from other Th2 cytokines, is necessary and sufficient to induce key features of allergic inflammation at an effector phase.

Genetic studies have revealed a possible role for IL-9 in asthma as it was found to be localised within a region of chromosome 5 that has been identified to carry a major gene for asthma [37]. IL-9 was shown to have a significant association with serum total IgE but not with histamine induced BHR. It was further demonstrated that expression of IL-9 was increased in bronchial biopsy samples of asthmatics when compared with non-asthmatic controls [38] . Over-expression of IL-9 in the mouse lung, was shown to induce AHR in addition to morphological changes that bear similarities to asthma [39]. Treatment of sensitised mice with anti-IL-9 antibody prior to challenge resulted in a significant increase in AHR, lung inflammatory cells, and BALF IL-4, IL-5, and IL-13 in BALF. Treatment with anti-IL-9 antibody significantly prevented airway hyperreactivity in response to methacholine inhalation as well [40]. On the other hand, in IL-9-deficient mice eosinophilia and granuloma formation were not affected. Also IL-9 was not required for T cell development or differentiation and generation of naive or antigen-driven antibody responses but was required for the generation of pulmonary goblet cell hyperplasia and mastocytosis in response to lung challenge.

1.6.4 CD8+ T cells

Although CD4+ T cells are the predominant effector population in asthma, other types of T lymphocyte (e.g., CD8+, and natural killer T [NKT] cells) also possess the capacity to respond to allergen and modulate asthma. Naïve CD8+ T cells just like their CD4+ counterparts can differentiate into at least two subsets of cytolytic effector cells with distinct cytokine patterns: Tc1 cells that secrete IFN-? and Tc2 cells that produce IL-4 IL-5 and IL-13. Investigators have shown that CD8+ T cells do have a role in the up-regulation of AHR and airway inflammation. Gelfand *et al* using a mouse model of CD8+-deficient mice showed that CD8+ T cells are important in the full development AHR and IL-13 from these cells appear to be the key element [41, 42].

Depletion of CD8+ T cells increases airway inflammation in animal models of asthma [43, 44]. Kemeny *et al* showed that CD8+ cells regardless of their cytokine profile, inhibit IgE by inducing IL-12 production from dendritic cells and IL-12 is required for the development of Th1 cells [45]. The group have also shown that IFN-? from CD8+ T cells is not necessary for the regulation of the IgE response. More recently Noble *et al* showed that CD8+ cells specific for inhaled allergens suppress allergic airway inflammation through induction of IL-12 in the lung during interaction with respiratory dendritic cells [46].

1.7 Aim of project

A patient who suffers from allergic airway disease often demonstrates increased IgE, Th2- type cytokines, and eosinophilic inflammation thus making it difficult to evaluate the inter-relationship or importance of IgE in the induction of airway inflammation and AHR. Therefore the mouse provides an excellent model to investigate the contribution of individual components to the development of AHR. Adoptive transfer of both CD4+ and CD8+ T cells were used to dissect the roles of T cells and the different cytokines secreted by them in the development of AHR and IgE responses [45, 47, 48]. In contrast to the recognised importance of T cells, Th2- type cytokines, the roles of eosinophils, IgE in persistent airway inflammation and AHR is unclear. Previous studies by two different groups [49, 50] reported that IgE levels in serum or BAL fluid in patients suffering from bronchial asthma are often increased and may correlate with the incidence or severity of the disease. Hamelmann *et al* using different modes of sensitisation showed that systemic sensitisation induces the strongest AHR followed by mice that were passively sensitised and airway sensitised [51]. Using both normal and athymic BALBc mice, Hamelmann *et al* showed independence of IgE-mediated immediate reactions from T cells by showing that passively sensitised athymic mice are fully capable of generating immediate cutaneous hypersensitivity reactions [21]. However the combination of passive sensitisation with local airway challenge with allergen triggered the development of AHR only in normal, but not in athymic mice and restoration of T cells by adoptive transfer from normal to nude mice before passive sensitisation with IgE followed by airway challenge re- established the capacity for development of AHR in athymic mice. If these athymic mice were passively sensitised and were treated with IL-5 before being challenged, they were capable of developing AHR. However if the

athymic animals were just treated with IL-5 before being challenged, they did not develop AHR but only eosinophilia in the lungs. Therefore the study shows that IgE is one of the key elements in inducing AHR in this particular model.

The role of IgE in persistent airway inflammation and AHR is not well defined compared to the role of T cells. Mehlhop *et al* used an IgE deficient mice (on a 129/SVEV background) to show that airway inflammation and AHR is not dependent on IgE production [52] but these responses could be easily elicited from naïve mice that were adoptively transferred with OVA-specific Th2 cells before being challenged with OVA intranasally [28]. It was also previously shown by Oshiba A *et al* that co-culturing sensitised T cells with allergen and allergen- specific IgE *in vitro* enhanced the activation of T cells [53]. Therefore this project aims to determine/define the co-operation that exists between humoral immunity and cell-mediated immunity in airway inflammation *in vivo.* This study begins with establishment of a normal immunisation/sensitisation murine model of asthma, followed by the use of polarised Th2 cells to induce asthma in naïve animals. Thereafter the responses mediated by antigen-specific Th2 cells were compared with responses that were mediated by antigen-specific IgE antibody and eventually to establish whether there is co-operation between humoral and cell-mediated immunity in asthma.

CHAPTER 2: MATERIALS AND METHODS

Reagents

Please refer to Appendix for reagents used and the preparation of buffers.

2.1 Animal Protocols

During the *in vivo* experiments, the various mouse strains were maintained in separate isolators, unless involved in an experimental protocol, during which time mice were housed in filter-top boxes. All mice were fed pelleted mouse diet and water *ad libitum.* Animals used were between the ages of 4 to 8 weeks old. All inter group and inter experiment groups were age, sex and weight matched. The mice used in this study were commercially available from the university breeding centre (CARE). All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC).

2.1.1 Immunisation protocols

2.1.1.1 Intraperitoneal (i.p.) immunisation

The animal was anaesthetised by placing it in a chamber into which oxygen and isoflourane were introduced. The animal was manually restrained by holding the scruff of the neck and its abdomen exposed by tilting the head downward. The abdomen was swabbed with 70% ethanol before it was injected. Using a 1ml syringe with a 25-G needle 100µl of inoculum was injected into the lower right quadrant of the abdomen (known as the peritoneal cavity), with moderate pressure and speed.

2.1.1.2 Intranasal (i.n.) immunisation

The animal was anaesthetised to a sufficient level such that it remained unconscious for 30 seconds by placing it in a chamber into which oxygen and isoflourane were introduced. It was manually restrained by gripping the skin over the back of the neck and holding it in a vertical position. Using a micropipette 10µl of inoculum was introduced drop wise into the nasal passage of the animal and was maintained in that position for 20 seconds. If the animal had sneezed out the inoculum, it was put back into the anaesthesia chamber and i.n. was attempted again. It was important that the animal was sufficiently anaesthetised as judged by respiration rate.

2.1.1.3 Intravenous (i.v.) immunisation

The animal was restrained with a commercial restraint and the position of the animal adjusted till the tail vein was visible. The animal was warmed up using a heat lamp. The injection site was disinfected and, using a 1ml syringe with a 26-G needle, innoculum was slowly injected into the vein at a slight angle. Clearing of the lumen at the vein was observed if successful. If not, a slight bulge will result in the tail. When this occurred, needle was removed and process was repeated proximal to previous site. Upon completion the needle was removed and pressure was applied to injection site. A new needle was used for each animal.

2.1.2 Blood collection

Blood is most frequently sampled for evaluation of serum antibodies. This section describes blood collection methodology for small rodents. Blood collection is the most common interventional procedure conducted with laboratory animals and is an essential requirement for many studies. The protocol offered in this section describes

collection of blood from the cheek pouch of the mouse. With the appropriate technique, small amounts of blood can be obtained with little ill effect on the animal. Bleeding procedures that should be performed on the anaesthetised animal include collection from the mouse by cardiac puncture, which is also known as a terminal bleed and should only be done when blood samples are no longer needed from animals.

2.1.2.1 Blood collection by cardiac puncture of mouse

The animal was sacrificed using a $C0_2$ chamber (should take about 2 minutes for the animal to die) and death was ensured by pinching one of its footpads. If there wasn't any reflex reaction, it was placed on its back on a clean, dry absorbent paper. A 1ml syringe with a 25-G needle was inserted just below and slightly to the left of the xiphoid cartilage at the base of the sternum at a 15 to 30° angle. The needle was advanced slowly and a very slight negative pressure was applied on the barrel of the syringe. If the tip of the needed had entered one of the chambers of the heart, blood will flow into the hub of the needle. Gently aspirate until the blood flow cease. Approximately, 0.5 to 0.8 ml of blood can be collected. The blood was allowed to clot at room temperature, left overnight at 4°C for the clot to retract and next day, centrifuged for 10 minutes at 400g. Serum was collected and stored at -20°C until assessment.

2.1.2.2 Blood collection by submandibular pouch (cheek) puncture of mouse

The mouse was restrained by gripping the skin over the back of the neck and held upright to provide a good view of the cheek pouch. The area was swabbed with alcohol and a lancet was inserted quickly into the bundle of vessels located at the back

of the cheek pouch and then quickly withdrawn. Once the blood started to flow, up to 200µl was collected in a 0.6ml microfuge tube. Once sufficient blood was collected, pressure was applied to the site of puncture for at least 20 seconds with a clean gauze pad to stop the blood flow. If desired, serial blood samples can be obtained at weekly intervals by alternating cheeks. Blood was allowed to clot at room temperature and left overnight at 4°C for the clot to retract and the next day centrifuged for 10 minutes at 400g. Serum was collected and stored at -20°C until assessment.

2.1.3 Removal of lymphoid organs

The removal of lymphoid organs for isolation and culture of cell populations, provide cells for proliferation assays and for isolation of CD4+ and/or CD8+ T cells for culture or reconstitution of other animals. This section covers the identification and removal of mouse lymphoid organs.

The animal was sacrificed in a CO_2 chamber as mentioned earlier. It was placed on its back on clean, dry absorbent paper in a BSL-2 cabinet. The fur was swabbed with 70% ethanol to reduce the possibility of contamination. Scissors and forceps were sterilised with 70% ethanol and a midline incision made with the scissors. The skin below the head and above the thighs was retracted by pulling it with gloved fingers. The animal was turned to its left side for spleen removal. Skin was lifted and a 1-inch incision at the left of the peritoneal wall was made with surgical scissors. The spleen was grasped and gently pulled free from peritoneum, tearing the connective tissue behind to release the spleen. The organ was then placed in a 20 ml universal tube containing cold sterile PBS supplemented with 1% FCS. This was kept on ice until the organ was processed.

2.1.4 Bronchoalveolar Lavage (BAL)

The animal was sacrificed by i.p. injection of pentobarbitol; 0.1ml/mouse. The trachea was cannulated and the lungs flushed with three 1ml aliquots of ice cold PBS/2% FCS. BAL was spun down at 300g/4°C/10 minutes and supernatant (BAL fluid) was saved for analysis of cytokine levels by ELISA. RBC lysis was carried out by resuspending the cells in 0.1ml of ammonium chloride. This was done at room temperature for 1 minute. After 1 minute, ammonium chloride was quenched by adding 2.0ml of cold PBS. BAL was spun down and washed twice in cold PBS. A cell count was done after the 2^{nd} wash and concentration of cells were adjusted to 1×10^5 cells/ml. 200ul (containing approximately 10^4 cells) of cell suspension was added to cytospin funnels and centrifuged at 800rpm/5mins/medium acceleration. The slides were air dried, fixed and stained as described in section 2.7.

2.1.5 Isolation of total lung cells

Animals were sacrificed as described earlier. The chest of the mouse was swabbed with 70% ethanol and opened with anatomical scissors. The lung were removed from the mouse with anatomical scissors and tweezers and transferred into a 15 ml tube containing medium and immediately stored on ice. Lung was transferred into a 15 ml tube containing 2 ml of liberase digestion solution (0.5mg/ml) and was cut into very small pieces (approximately 1–2 mm^2) before it was digested at 37 °C for 1 hour under constant agitation. A 40µm sieve was placed on a 50 ml tube and the lung digest was transferred to the 40µm sieve. With the help of a syringe plunger, the remaining lung pieces were pushed through the sieve. The sieve was washed with 5–10 ml of medium and the lung digest centrifuged at 350*g*/4°C for 5 minutes. The supernatant was discarded and the cell pellet re-suspended in 10 ml of red blood cell

lysis buffer. The cell suspension was incubated at room temperature (18–25 °C) for 5 minutes. After 5 minutes, medium was added till tube was full. Cells were spun down at 350*g*/4 °C for 5 minutes. Washing was repeated for another 3 times with PBS.

2.2 Immunisation of mice

2.2.1 Preparation of antigen-alum precipitate

During the course of this study, the immune responses of mice were investigated. In order to evoke these responses, OVA with an aluminium-based adjuvant was administered intraperitoneally (i.p.). An adjuvant is an agent that enhances the immunogenicity of an antigen and many experiments have shown that aluminium hydroxide and aluminium phosphate possess adjuvant activity.

The protein antigen solution was made up to 10mg/ml in sterile PBS. 4.5ml of 1M $NaHCO_3$ in sterile distilled water was added to 10ml of the antigen stock solution at room temperature and gently mixed. 10ml of 0.2M KAl $(SO_4)_2.12H_20$ in sterile distilled water (preferably freshly prepared) was added drop-wise to the mixture while stirring. The mixture was maintained at 25°C for 20 minutes and then centrifuged at 3000g for 10 minutes. The precipitate was washed three times in sterile PBS. After the last wash, the supernatant was discarded and the cell pellet re-suspended in 10ml of sterile PBS. Alum-antigen mixture was stored at 4°C for up to 24 hours.

2.3 Preparation of mononuclear cell Suspension from spleen and lymph nodes

Mouse spleens provide a convenient source of large numbers of T cells. Cell suspensions from homogenised spleens and lymph nodes contain polymorph nuclear leukocytes, red blood cells and non-viable cells as well as cells of the lymphoid and

monocyte lineages. Mononuclear cells and platelets collect on top of the Ficoll-Hypaque layer because they have lower density than red blood cells and granulocytes, which collect at the bottom of the Ficoll-Hypaque layer.

Mice were sacrificed and spleens obtained as described in section 2.1.3. The parathymic, posterior mediastinal, cervical, inguinal, axillary and mesenteric lymph nodes (LN) was also obtained when required. Working in a BSL-2 cabinet, freshly removed spleen and LN were placed in a pre-wet 70um nylon mesh filter that was placed over the mouth of a 50ml tube. With a scissors, the organ was cut into several pieces and using a circular motion, the pieces were pressed against the mesh with the plunger of a 5 ml syringe until mostly fibrous tissue remained. Occasionally, chilled PBS/1% FCS was added to dislodge cells from the filter. Cell suspension was centrifuged for 10 minutes at 300g/4°C. Supernatant was discarded and cells resuspended in 3ml of room temperature PBS. Cell suspension was layered onto 5ml of room temperature Ficoll-Hypaque. Density gradient centrifugation was performed at 800g for 20 minutes at 20°C with maximum acceleration and minimum deceleration (no brake). Mononuclear cells were isolated with a 3ml sterile Pasteur pipette from the interface between the PBS and Ficoll-Hypaque. Cells were resuspended in a total volume of 50ml of sterile PBS and centrifuged at 600g for 10 minutes. Cells were washed twice with complete medium and spun at 300g for 7 minutes. Final cell pellet was resuspended in 10ml of complete medium. 10ul of cell suspension was removed and mixed with an equal volume of trypan blue dye and counted on a haemocytometer to estimate the total viable white cell numbers. From a 6-week-old mouse, recoveries of live lymphocytes is generally around 5-15 $\times$ 10^7 from the spleen and about 2 $\times$ 10^7 from lymph nodes.

2.4 Isolation of mouse CD4+ T cells using magnetic particles

Magnetic cell separation (*MACS*) is based on the labelling of cell surface antigens with specific monoclonal antibodies coupled to magnetic beads. The labelled cells are then placed over a separation column in the presence of a magnet. Unlabelled cells pass through the column and can be collected as the negative subset while labelled cells (the positive fraction) are retained on the column and are eluted after the column is removed from the magnet. One of two magnet systems is used to isolate the cells.

Mouse mononuclear cells were prepared as described in section 2.3. Cells were resuspended in MACS buffer to a concentration of approximately 1 x 10^8 cells/ml and were incubated with anti-CD4+ micro beads (Miltenyi) at a concentration of 4ul / 1x10^7 total cells for 30 minutes at 4°C. Cells were resuspended and centrifuged for 10 minutes at 200g/4°C. During centrifugation, Midi MACS Separation Unit was placed on the MACS Multi-stand, an LS column in the magnet, and a sterile 15-ml tube under the column. The column was washed with 5ml cold, degassed MACS buffer and the flow-through was discarded and a clean sterile 15-ml tube was placed under the column. Cells were collected from the centrifuge and the supernatant discarded and the pellet was resuspended thoroughly in *MACS* buffer to a concentration of 1 × 10^8 cells/ml. Cells were passed through a 40-µm preseparation filter which was placed over the mouth of the LS column and filter was washed with an additional 0.1 to 0.4 ml *MACS* buffer. Filter unit was removed and the column was washed three times with 3 ml of *MACS* buffer, loading the first 3 ml slowly to avoid disturbing the cells in the column. Flow through was saved if desired. Column was removed from the magnet and placed over a fresh, sterile, 15-ml tube. *MACS* buffer was added to the column to the full capacity and the positive fraction was eluted by using the plunger

provided. Cell suspension was centrifuged for 10 minutes at 200g/4°C. Supernatant was discarded and cell pellet was washed 2 more times in complete medium before it was resuspended in 5ml of complete medium and viable cell count was performed.

2.5 Cell culture

2.5.1 Proliferation assay

In several cases, antigen specific reactivity of the cells had to be assessed, by determining the levels of cell division. In these assays, radio labelled thymidine, [^{3}H]-thymidine was used to assess cell division. The [^{3}H]-thymidine provided an alternative nucleotide that could be incorporated into DNA. As cells grew and divided, DNA was synthesised and the amount of incorporation was proportional to the level of cell growth.

The test lymphocyte suspension was prepared from spleen or LN in complete medium as described in section 2.3 and 2.4. The cell suspension was centrifuged for 10 minutes at 200g/4°C. The supernatant was discarded and cell pellet resuspended in 15-ml of complete medium. The responder cell concentration was adjusted to $1x10^6$ cells/ml with complete medium. Working solutions of activating agents were prepared in 15-ml conical tubes at room temperature as follows. For mAb, toxin, or lectin, a series of dilutions from 1 mg/ml stock solutions—e.g., 30, 10, 3, 1, 0.3 and 0.1 µg/ml were prepared in complete medium. 20 µl of each dilution of activating reagent (mAb, enter toxin or lectin) was added to each of three wells of a 96-well flat -bottom microtiter plate. Control wells with 20 µl of complete medium only were included. To the wells, 2×10^5 cells in 0.2 ml were added. Plate was placed in a humidified 37°C, 5% CO_2 incubator for 2 days. After 2 days, 0.5uCi of [^{3}H] thymidine was added to

each well and the plate was returned to the CO_2 incubator for another 18 to 24 hr. The cells were harvested using a semi automated sample harvester and measured cpm in ß scintillation counter.

2.5.2 Production of CD4+ Th1 and Th2 cell lines using non-antigenic stimulation

Spleen and lymph nodes from wild-type mouse were removed and a single-cell suspension prepared, CD4+ cells were purified using positive-selection immunomagnetic bead purification. Viable cell numbers were determined by trypan blue exclusion and cell concentration adjusted to 4×10^6 cells/ml in complete medium. For Th1 cultures: A Th1 working solution containing complete medium supplemented with anti-IL-4 (20µg/ml), recombinant mouse IL-12 (20ng/ml) and anti-CD28 (4µg/ml) was prepared. 1 ml of CD4+ T cell suspension and 1ml of the Th1 working solution were added to each well of a 6-well plate that was precoated with 1ug/ml anti-CD3 in sterile PBS. For Th2 cultures: Th2 working solution containing complete medium supplemented with recombinant mouse IL-4 (20ng/ml), anti-IFN-? (20µg/ml), anti-IL-12 (20µg/ml), PMA (20ng/ml) and anti-CD28 (6µg/ml) was prepared. 1ml of CD4+ T cell suspension and 1ml of the Th2 working solution were added to each well of a 6-well plate that was precoated with 1ug/ml anti-CD3. On day 2, fresh medium containing recombinant IL-2 (final concentration 20U/ml) was added to the culture. Cell growth was monitored and if culture medium was being used up (medium turns yellow), cells were split using fresh medium containing IL-2 and either Th1 or Th2 polarisation cocktail. On day 7, cells were harvested into 15- or 50-ml tubes and centrifuged for 10 minutes at 300g/4°C. The cell pellet was resuspended in PBS and centrifuged as before. A cell count was performed and cells

were adjusted to desired concentration for adoptive transfer or for FACS analysis of intracellular cytokines.

2.5.3 Production of CD4+ Th2 cell lines using antigenic stimulation

Spleen and lymph nodes were excised from transgenic mouse (OT-II/DO11.10), a single-cell suspension prepared and CD8+ cells removed from the cell suspension using positive-selection immunomagnetic bead purification. A cell count was performed to determine the numbers of viable cells by trypan blue exclusion and cell concentration was adjusted to 4×10^6 cells/ml in complete medium. For Th2 cultures: Th2 polarising solution containing medium supplemented with recombinant mouse IL-4 (20ng/ml), recombinant mouse IL-2 (10U/ml), anti-IFN-? (20µg/ml), anti-IL-12 (20µg/ml) and $OVA_{323-339}$ (20µg/ml) was prepared. 1ml of cell suspension and 1ml of the Th2 polarising solution was added to each well of a 6-well plate. Cell growth was monitored and whenever culture medium was rapidly used up, cells were split using fresh medium containing the polarisation cocktail. On day 7, cells were harvested into 15- or 50-ml tubes, topped up with complete medium and centrifuged for 10 minutes at 300g/20°C. The cell pellet was resuspended and washed twice in PBS before a density gradient centrifugation was performed to remove all the dead cells. Viable cells were counted, resuspended in complete medium and restimulated under Th2 polarising conditions together with mitomycin C treated CD90-depleted cells from C57BL6 mice (acting as feeder cells, see protocol 2.5d) and steps 3-4 were repeated for another 2 weeks. At the end of 3 weeks, cells were harvested and a density gradient centrifugation was done and viable cells were adjusted to desired concentration for adoptive transfer.

2.5.4 Preparation of mitomycin C treated feeder cells

Spleen from wild-type mouse was excised; single-cell suspension prepared and CD90-positive cells were removed from the cell suspension using positive-selection immunomagnetic bead purification. Numbers of viable cells were determined by trypan blue exclusion and cell concentration adjusted to a concentration of 5×10^7 cells/ml in PBS. Mitomycin C was added to cell suspension to a final concentration of 50µg/ml, the tube was wrapped in aluminium foil and cells were incubated for 20 minutes at 37°C. An excess of complete medium was added and suspension was centrifuged for 10 minutes at 300g. Supernatant was discarded and washing procedure was repeated two more times. Cell pellet was resuspended in complete medium, a cell count was done and cell concentration was adjusted to 8×10^6 cells/ml. Feeder cells were used at a ratio of 2:1 i.e for every one T cell, there were 2 feeder cells.

2.6 Preparation of cells for flow cytometry

Flow cytometry is widely used for analysing the expression of cell surface and intracellular molecules (on a per *cell* basis), characterising and defining different cell types in heterogeneous populations, assessing the purity of isolated subpopulations, and analysing cell size and volume. This technique is predominantly used to measure fluorescence intensity produced by fluorescent-labelled antibodies or ligands that bind to specific cell-associated molecules.

2.6.1 Cytofluorographic analysis of cell surface markers

Cells were harvested into 15ml tubes, and centrifuged for 10 minutes at 300g/4°C. The supernatant was discarded and cell pellet resuspended in 10ml of staining buffer, 4°C. A viable cell count was determined by trypan blue exclusion and the cell

concentration adjusted to 1 × 10^6 cells/ml in staining buffer, 4°C. 1ml of cell suspension (10^6 cells) was added to round-bottom FACS tubes and was spun down for 10 minutes at 300g/4°C, and supernatant discarded. 0.2ul of one or more labelled mAb was added to cell pellet and was incubated at 4°C for 30 minutes in the dark. Excess antibodies were removed by washing the cells with 2ml of cold staining buffer. The cell suspension was centrifuged for 6 minutes at 300g/4°C and the supernatant discarded by rapid inversion of the tubes taking care not to loose cells. Wash steps were repeated two more times before stained cell pellets were resuspended in 500µl of 4°C 1% PFA. Tubes were covered with foil and kept on ice until the cells were analysed by flow cytometry.

2.6.2 Intracellular staining

On several occasions, cytokine productions by cultured cells had to be investigated. In order to simultaneously detect two or more cytokines within a single cell, thereby permitting true Th1 versus Th2 determination, cells were intracellularly stained for specific cytokines and subsequently analysed by flow cytometry.

Cultured cells were harvested into a 15ml tube and were spun down for 7 minutes at 350g at room temperature. The supernatant was saved for cytokine analysis by ELISA and cell pellet was resuspended in prewarmed complete medium. A viable cell count was determined by trypan blue exclusion and cell concentration adjusted to 2x10^6 cells/ml with prewarmed complete medium. Activation medium containing 20ng/ml PMA, 800ng/ml ionomycin and 6uM monesin was prepared. 1 ml of cell culture was added to an equal volume of activation medium in a well of a 12-well plate. After 6 hours, cells were harvested and centrifuged for 7 minutes at 350g/4°C. The cell pellet

was resuspended in FACS buffer and cell viability was determined. Cell concentration was adjusted to $1x10^6$ cells/ml and 1 ml of cell suspension added to each FACS tube. Cells were spun down and the supernatant discarded. 0.2ul of anti-CD4+ mAbs was added and cells were incubated for 20 minutes at 4°C, in the dark. 2ml of ice-cold PBS was added to each tube and gently vortexed and cell suspension was spun down at 350g/4°C for 5 minutes. Washing was performed twice. After the third wash, the cell pellet was loosened and 0.5ml of 4% PFA (prewarmed to 37°C) to each tube was added and incubated for 5 minutes at room temperature in the dark. Cells were vortexed periodically. Cells were washed with 2ml of FACS buffer and centrifuged for 5 minutes at 350g/4°C. 1ml of Perm buffer was added to each tube and incubated for 30 minutes at room temperature in the dark. Cells were spun down and cell pellet loosened up, before 1 µl of PE or FITC-labelled anti-IL-4 or IL-5 or IFN-? mAbs were added. Cells were incubated for 45 at 4°C, in the dark. The cells were washed with Perm buffer twice and resuspended in 1% PFA. Tubes were kept in the dark at 4°C till analysis by flow cytometry.

2.7 Haematoxylin and eosin (H&E) staining

Slides with the BAL cytospin were prepared as described in section 2.1.4. Haematoxylin stains negatively charged nuclei acids (nuclei and ribosomes) blue. Eosin stains cytoplasm pink.

The 3 different Diff-Quik reagents were poured into 3 different coplin jars The slides were first dipped into the coplin jar that contained the fixative 10 times. Then the slides were dipped into the second jar containing the 2nd component of the Diff-Quik (eosin) for another 10 times. Finally the slides were dipped into the jar containing the

last component of Diff-Quik (haematoxylin) 5 times. The slides were washed thoroughly until water ran clear. The slides were dried and read by microscopy.

2.8 Enzyme-linked immunosorbent assay (ELISA)

Over the course of this study the enzyme-linked immunosorbent (ELISA) was used to detect the cytokine content of culture supernatants and BAL samples. It was also used to determine the levels of OVA-specific IgG1, OVA-specific IgE and total IgE in serum. ELISA is a well-established method for measuring cytokine levels *in vitro*. Cytokines were measured using a sandwich ELISA, the plate is coated with a capture antibody (an antibody that binds to the cytokine of interest). Culture supernatant is added followed by the detection antibody, which only binds to the cytokine of interest and do not cross react with the capture antibody. Substrate is added and colour development occurs if cytokine of interest is present. The amount of cytokine measured is proportional to the antibody bound and this in turn can be correlated to biologic activity when suitable cytokine standards of known biologic potency are used to calibrate the immunoassay.

2.8.1 ELISA for IL-2, IL-4, IL-5 IL-13, IFN-? and total IgE

Wells of the ELISA plate were coated with 50µl of capture antibody at the recommended working concentration. The plate was sealed and incubated overnight at 4°C. Each well was aspirated and washed with wash buffer, repeating the process two more times for a total of three washes. After the last wash, any remaining wash buffer was removed by inverting the plate and blotted by banging the plate against clean paper towels. Wells were blocked by adding 300µl of block buffer to each well and the plate was incubated at room temperature for an hour. During the blocking

step, samples and standards are prepared. Aspiration/wash was repeated as in step 2. 50µl of sample or standards in reagent diluent were added to each per well and the plate was covered with foil and incubated for 2 hours at room temperature. Aspiration/wash was repeated as in step 2. 50µl of the detection antibody, diluted in reagent diluent, was added to each well. The plate was covered and incubated for 2 hours at room temperature. Aspiration/wash was repeated as in step 2 and 50µl of the working dilution (1/200) of streptavidin-HRP was added to each well. The plate was covered and incubated for 15 minutes at room temperature in the dark. Aspiration/wash was repeated as in step 2 and 50ul of substrate solution (TMB) was added to each well. The plate was incubated for 20 minutes at room temperature in the dark. 25ul of stop solution (2N $H2SO_4$) was added to each well. The optical density of each well was determined immediately, using micro plate reader set to 450nm.

2.8.2 ELISA for OVA-specific IgG1

The wells of the microtitre plate were coated with 10ug/ml OVA and incubated overnight at 4°C. Standards were prepared using OVA-14 (mouse anti-OVA IgG1) and 1000-fold dilutions of serum samples were prepared in assay diluent. The plate was washed five times and after the last wash, any remaining wash buffer was removed by inverting the plate and blotting against clean paper towels as before. Diluted serum samples or standards were added to each well and incubated at RT for an hour. The plate was washed five times and 50µl 1ug/ml of biotin conjugated rat anti-mouse IgG1 antibody was added to each well. The plate was incubated at RT for an hour, was washed five times and 50ul of 1000-fold diluted avidin-alkaline phosphatase added to each well and incubated at RT for 1 hour in the dark. Plate was washed five times and 50ul of pNpp substrate was added to each well and plate

incubated in the dark for 45 minutes. Enzymatic reaction was stopped by adding 50ul of 3M NaOH to each well. Optical density of each well was determined immediately, using micro plate reader set to 405nm. If dual wavelength correction is available, set it to 655nm.

2.8.3 ELISA for OVA-specific IgE

The microtitre plate was coated with 3 µg/ml of anti-IgE mAb (LO-ME-3) in coating buffer at 50µl per well and was incubated at 4°C overnight. The plate was washed 5 times before wells were blocked with 1% BSA in PBS at RT for an hour. Dilutions of anti-OVA IgE mAb (MCA2259) standards and samples were prepared in assay diluent. The plate was washed 5 times before 50µl of each diluted standards or samples were added into per well in triplicates. The plate was incubated at RT for 2hours. The plate was washed 5 times before adding biotinylated OVA (diluted 1:500) at 50µl per well and was incubated at RT for 2hours. The plate was washed 5 times before avidin conjugated alkaline phosphatase (diluted 1:1000) was added at 50µl per well and incubated for an hour at RT. The plate was washed 5 times before adding 50µl of pNpp per well. Plate was incubated at RT for 30 - 45 minutes. Enzyme reaction was stopped by the addition of 50µl of 3M NaOH per well. Absorbance was measured at 405nm (correction wavelength if available is 655nm).

2.9 Statistical analysis

Results are representative of at least three independent experiments. Data were analyzed by standard statistical packages for one way analysis of variance (ANOVA) followed by Student's *t*-test for unpaired values. A value of $p<0.05$ was considered significant. Results are expressed as means ± SEM.

CHAPTER 3: RESULTS

3.1 Optimisation of immunisation protocol

The first objective of this project was to establish an immunisation protocol for C57BL6 mice. The mice were intraperitoneally immunised with various doses of OVA ranging from 10µg to 3000µg in alum at 100µl/mouse. Control mice were given 100µl of PBS. The immune response of the animals was determined by ELISA, measuring the amount of OVA-specific IgG1 and the total IgE in the serum collected 14 days after immunisation. There was a dose-dependent IgG1 response (Figure 1a) at the lower end of the range. However the response plateaued at doses >1000µg. Total IgE production responded in a similar dose-dependent manner (at 30-3000µg OVA). However the control and 10µg OVA groups had higher than expected total IgE production at 900ng/ml and 200ng/ml, respectively. There are two key possibilities for the divergence of observation from this experiment. Either an error occurred during preparation of the OVA/alum or failure to illicit an immunological response from the animals.

To discriminate between the possibilities, a similar experiment was repeated with a different set of animals (Figure 2). The results obtained did not corroborate with previous observations. OVA-specific IgG1 levels did not show a dose response. Similarly, total IgE levels were dose-independent and 2- to 3-fold higher compared to the previous experiment. Furthermore, the control group showed a 2-fold increase in IgE levels. The similarity of the control group in both experiments suggested that the mice had an inherent problem that prevented them from responding to an antigenic challenge.

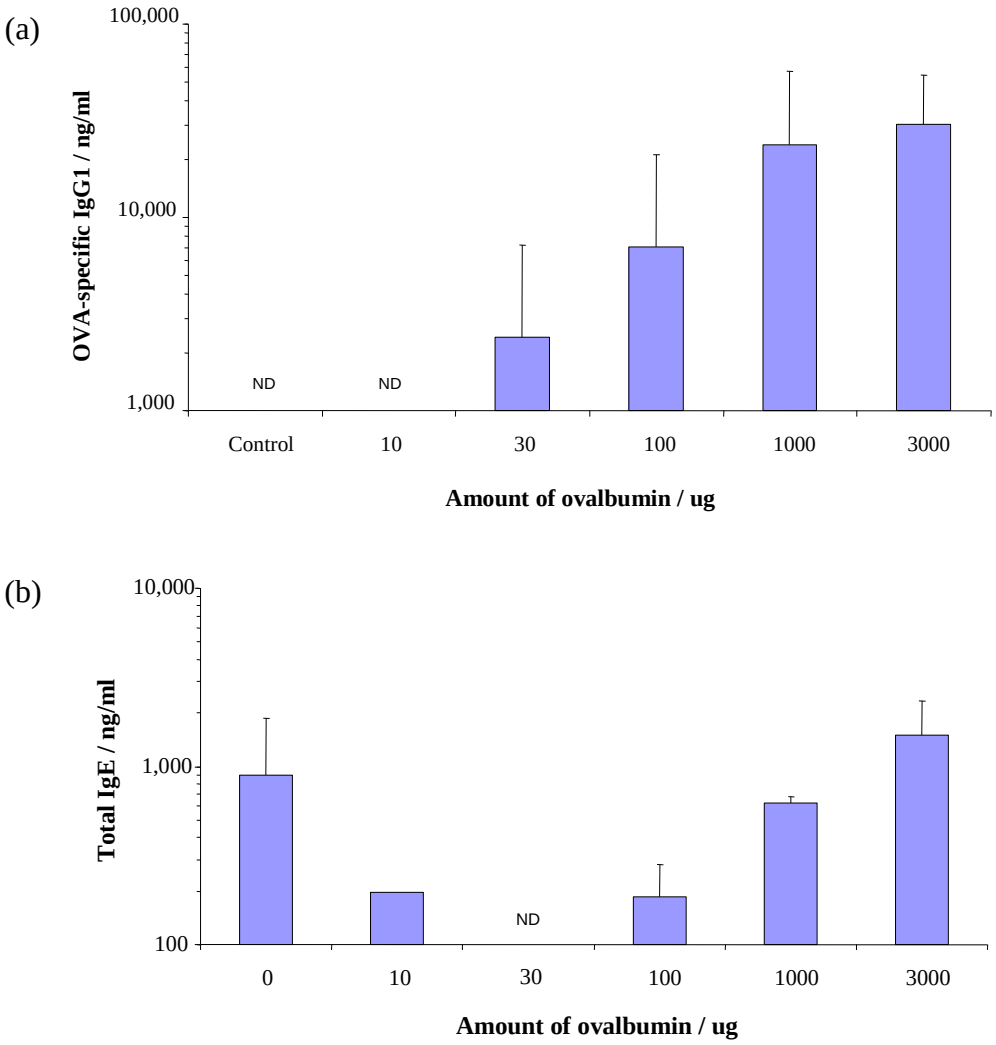


Figure 1: Optimisation of ovalbumin (OVA) dosage for immunisation. C57BL6 mice (n=4), were immunised with varying doses of OVA, ranging from 10µg/mouse to 3000µg/mouse. Mice in control group were given 100µl of PBS. Blood was collected on Day 14 by cardiac puncture and sera were tested for OVA-specific IgG1 (a) and total IgE (b) by ELISA. Results are expressed as ng/ml by reference to a standard curve prepared by a monoclonal antibody specific for OVA. ND = not detectable

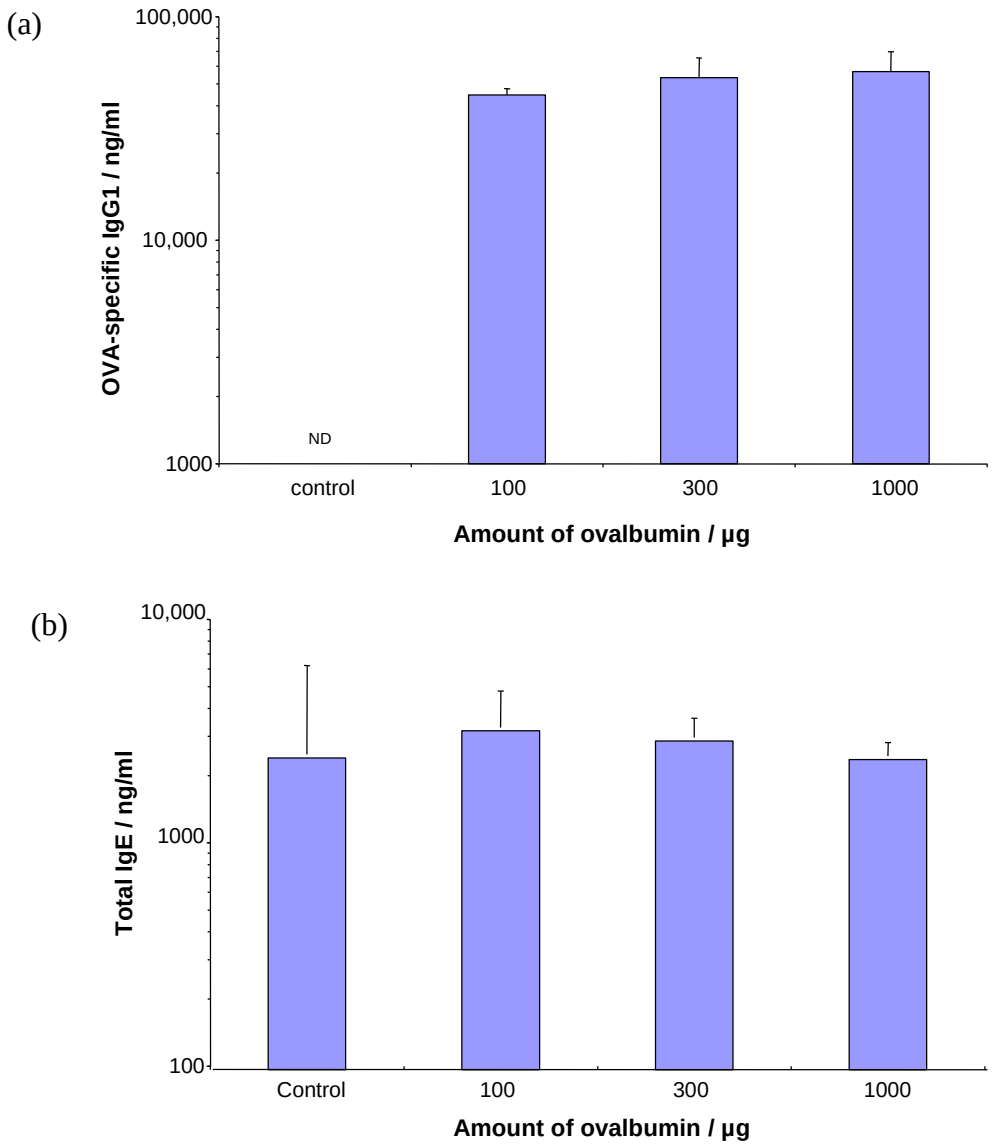


Figure 2: Optimisation of OVA dosage for immunisation. C57BL6 mice (n=4) were immunised with varying doses of OVA, ranging from 100µg/mouse to 3000µg/mouse. Mice in control group were given 100µl of PBS. Blood was collected on Day 14 by cardiac puncture and sera were tested for OVA-specific IgG1 (a) and total IgE (b) by ELISA. ND = not detectable

Despite using high OVA doses (up to 3000µg OVA), animals did not elicit the expected IgE immune response. It was reasoned that if the animals were repeatedly immunised with OVA/alum the expected IgE response would be obtained. Therefore animals were challenged once, twice or three times with OVA/alum (Figure 3). There was no significant increase in OVA-specific IgG1 and total IgE in animals that received multiple immunisations when compared to mice that only received a single immunisation. When compared to earlier experiments, levels of OVA-IgG1 were 4-fold higher and the levels of total IgE were comparable. A possible contributing factor to the observed variations in the immune response was the self-made alum. Therefore, alum-preps from commercial sources were used for comparison.

Adjuvants from a panel of sources were tested (Figure 4). There were no observed significant differences in the OVA-IgG1 and total IgE responses obtained between the panel of adjuvants used. Although levels of OVA-IgG1 were comparable to the first 2 experiments (Figures 1 and 2), levels of total IgE were up by 10-fold in the current experiment. It was deduced that the animals were not making a typical IgE response because they were suffering from infections. Hence, new breeder pairs were brought in and a new colony of specific pathogen free animals was set-up.

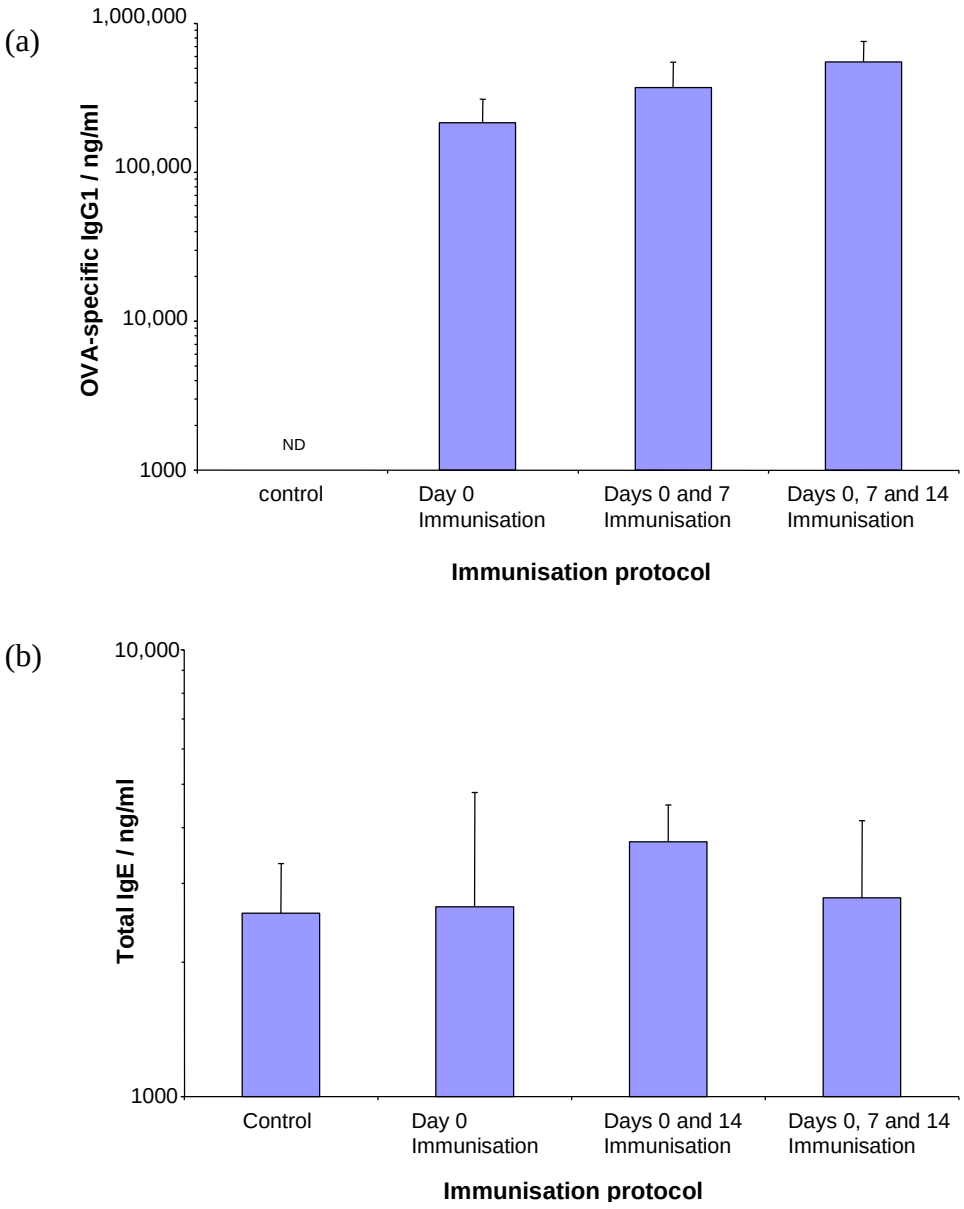
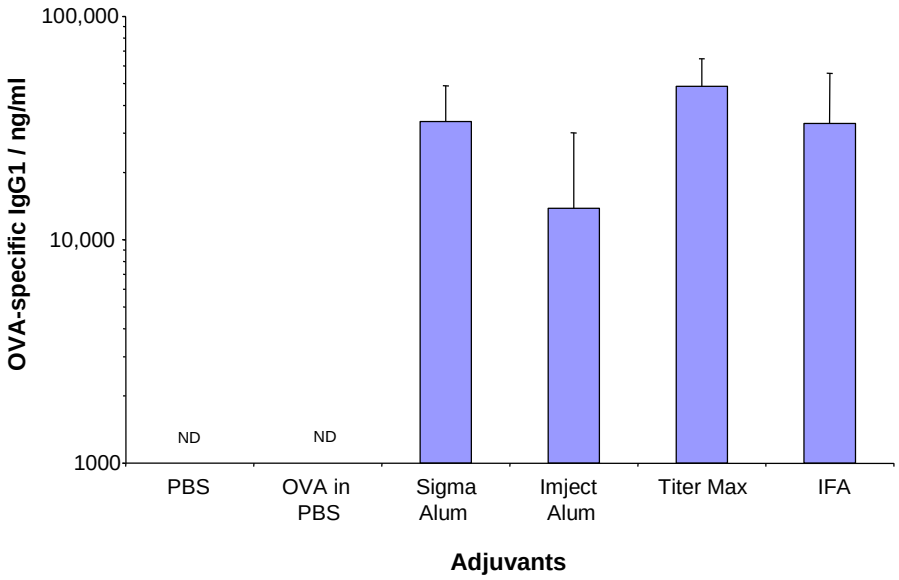


Figure 3: Optimisation of booster injections. C57BL6 mice (n=4), were immunised with OVA at 100µg/mouse. Mice were immunised once, twice or thrice, as indicated. Animals in control group were given 100µl of PBS. Blood was collected on Day 21 by cardiac puncture and sera were tested for (a) OVA-specific IgG1 and (b) total IgE by ELISA. ND = not detectable

(a)



(b)

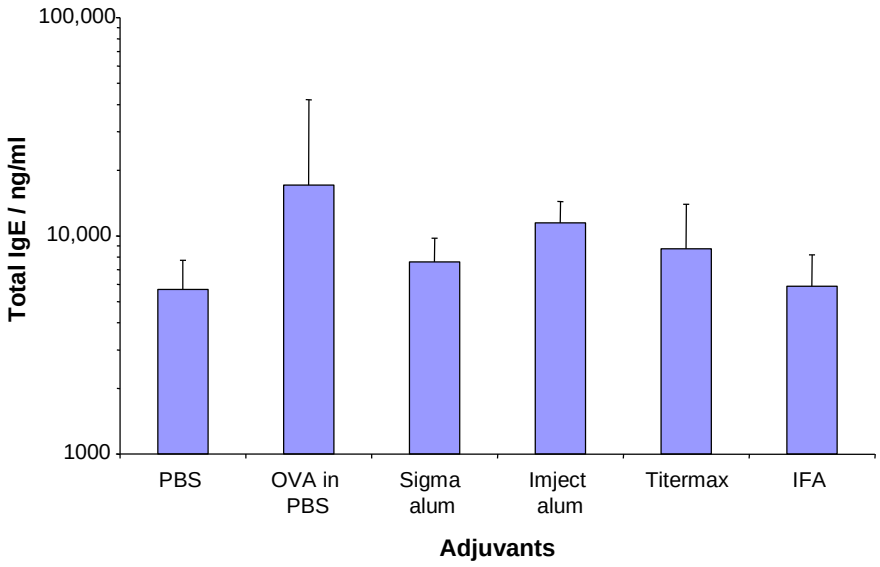


Figure 4: Determination of the best adjuvant for immunisation. C57BL6 mice (n=4), were immunised with a single dose of OVA at 300 µg/mouse but with different adjuvants. Mice in control group were given 100µl of PBS. Blood was collected on Day 15 by cardiac puncture and sera were tested for (a) OVA-specific IgG1 and (b) total IgE by ELISA. ND = not detectable

Using animals from the new colony, optimal dose of OVA/alum was determined as mentioned earlier (Figure 5). BALBc was included as a positive control as it is the most commonly used animal model for asthma. Among the C57BL6 mice, a slight dose-dependent response was observed in both OVA-specific IgG1 and total IgE levels but in BALBc mice, there was no dose-dependent trend in the IgG1 response but there was a detectable dose response in the IgE levels. Levels of IgG1 and IgE in C57BL6 were comparable to previous experiments.

To identify the peak of the immune responses, C57BL6 and BALBc mice were immunised i.p. with various doses of OVA (1µg to 1000µg OVA/mouse) (Figures 6 and 7). They were bled on days 7, 14 and 21 and sera were tested for OVA-IgG1 and total IgE. OVA-IgG1 responses from both strains of mice did not have distinguishable peaks but generally increased over time regardless of the OVA dose used. However total IgE response peaked on Day 14 for both strains and declined to levels lower than those of Day 7. As observed in the previous experiment, dose-dependent response was seen in both IgG1 and IgE.

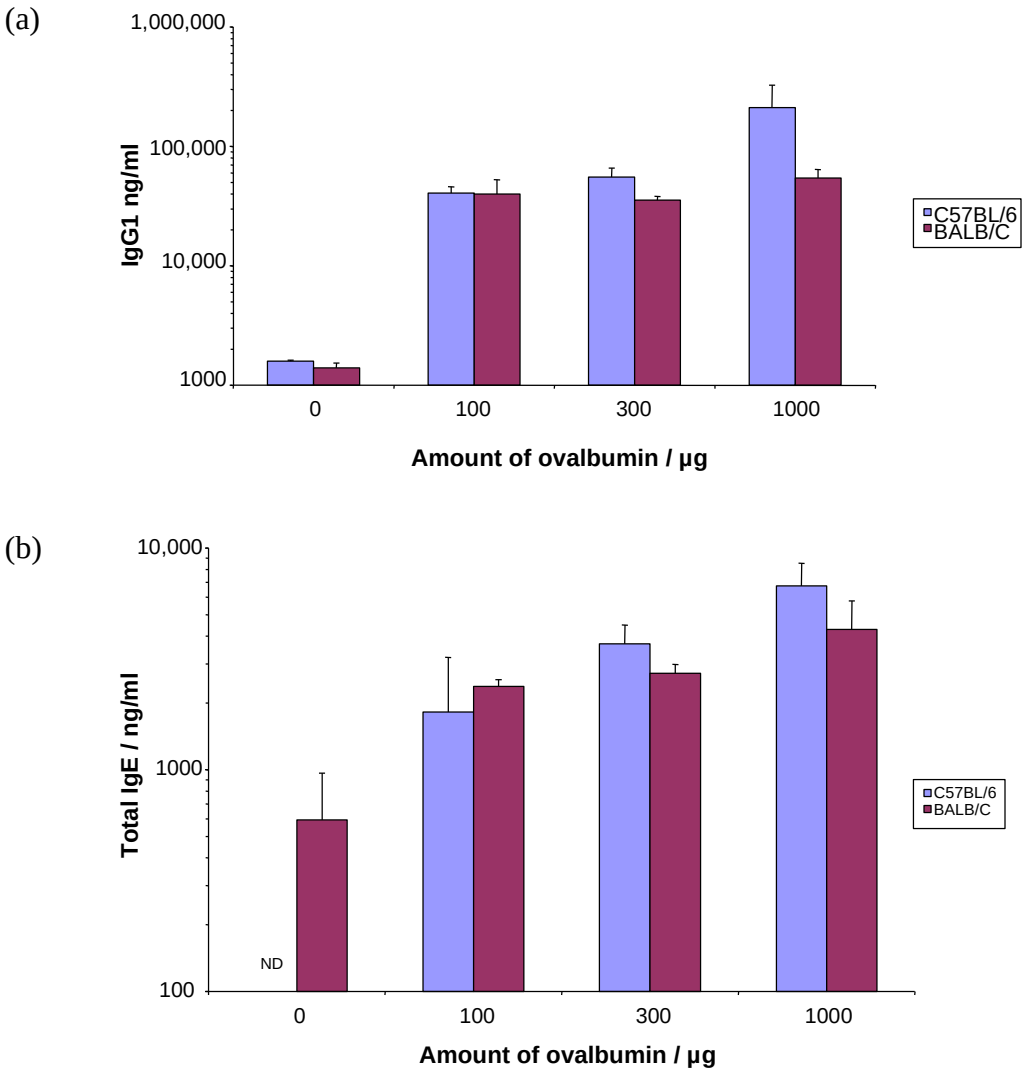


Figure 5: Comparison of immune responses between 2 different murine strains after immunisation. C57BL6 and BALBc mice (n=4) from Harlan-Olac, UK, were immunised with varying doses of OVA, ranging from 100µg/mouse to 1000µg/mouse. Mice in control group were given 100µl of PBS. Blood was collected on Day 21 by cardiac puncture and sera were tested for (a) OVA-specific IgG1 and (b) total IgE by ELISA. ND = not detectable

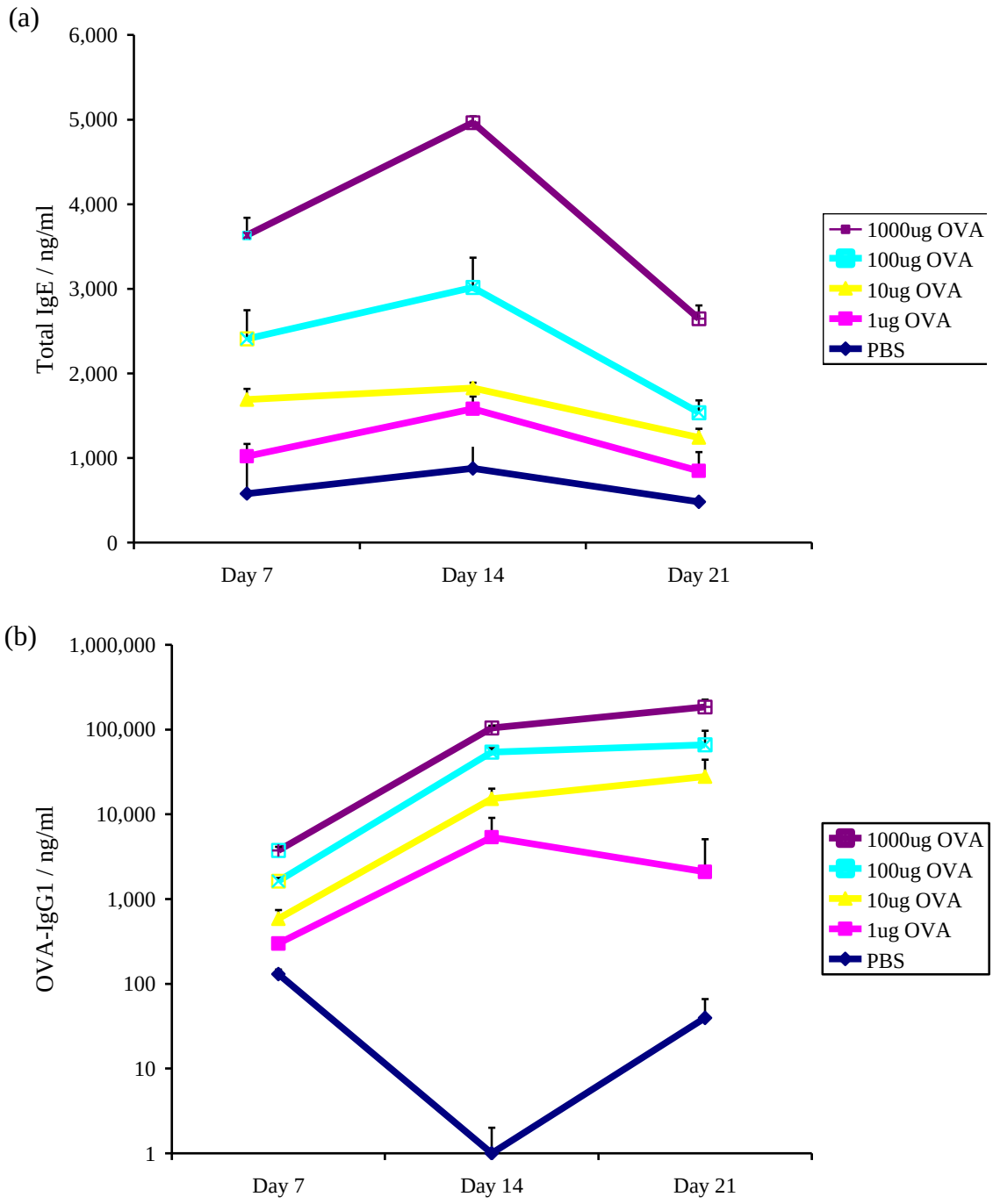


Figure 6: Optimisation of OVA dose for immunisation protocol. BALBc mice (n=4) from Harlan-Olac, UK, were immunised with varying doses of OVA, ranging from 1µg/mouse to 1000µg/mouse. Mice in control group were given 100µl of PBS. Blood was collected on Days 7, 14 and 21 by cardiac puncture and sera were tested for (a) OVA-specific IgG1 and (b) total IgE by ELISA.

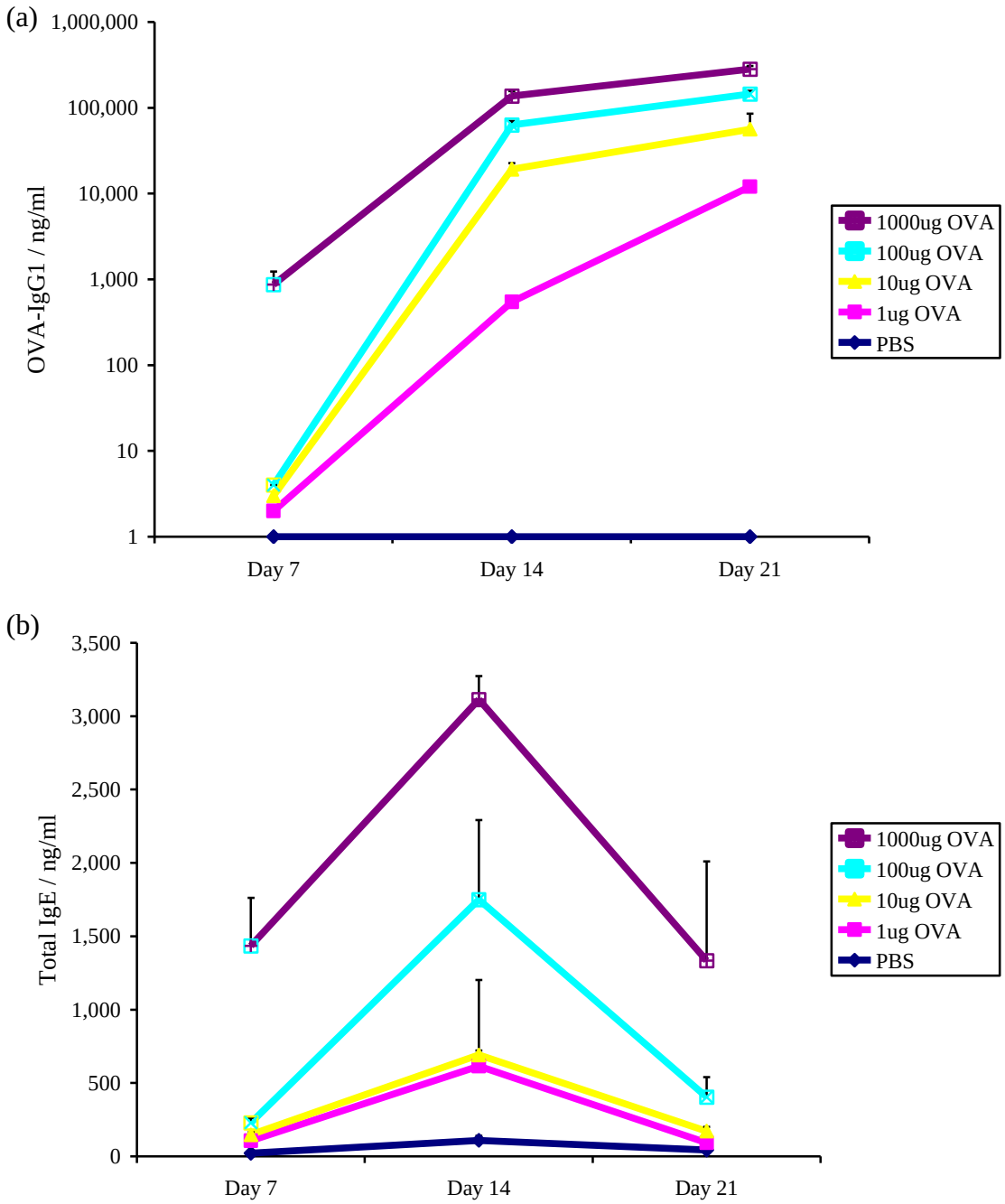


Figure 7: Optimisation of OVA dose for immunisation protocol. C57BL6 mice (n=4) from Harlan-Olac, UK, were immunised with varying doses of OVA, ranging from 1µg/mouse to 1000µg/mouse. Mice in control group were given 100µl of PBS. Blood was collected on Days 7, 14 and 21 by cardiac puncture and sera were tested for (a) OVA-specific IgG1 and (b) total IgE by ELISA.

To ascertain that the adjuvant that was used for the previous experiments (Figures 5 - 7) is comparable to the commercially available ones, the immune response of C57BL6 and BALBc mice that were immunised with fresh alum ppt were compared with mice that were immunised with either of the 2 commercially available adjuvants (TMG: Titermax Gold, IFA: Incomplete Freund's Adjuvant) (Figure 8). OVA-specific IgG1 and IgE levels were generally similar among the different adjuvants in C57BL6 mice. However C57BL6 mice that were immunised with fresh alum ppt had total IgE levels that were 2-5 fold higher than the animals from the other 2 groups. In BALBc, OVA-IgG1 levels were similar across the 3 groups but the group that had Titermax Gold as the adjuvant had slightly higher total and specific IgE responses.

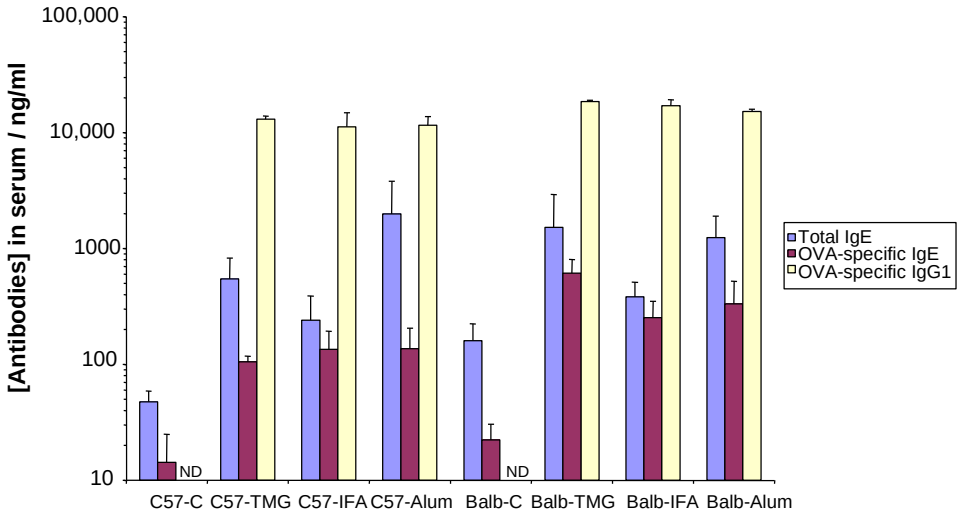
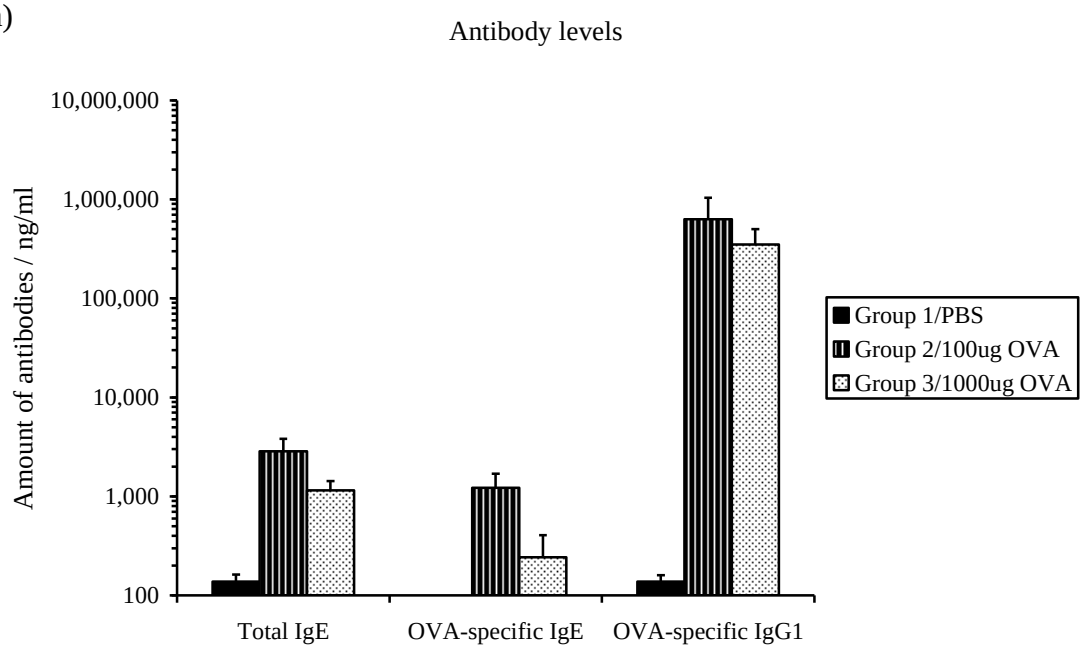


Figure 8: Determination of the best adjuvant for immunisation protocol. C57BL6 and BALBc mice (n=4) from Harlan-Olac, UK, were immunised with a single dose of OVA, of 100µg/mouse. However different adjuvants were used (TMG: TiterMax Gold, IFA: Incomplete Freund's adjuvant and Alum). Mice in control group were given 100µl of PBS. Blood was collected on Day 21 by cardiac puncture and sera were tested for total IgE, OVA-specific-IgE and IgG1 by ELISA.

Once the various conditions for the optimum response of C57BL6 after immunisation were determined, the immune responses after immunisation and challenge were studied. C57BL6 mice were immunised i.p. twice on days 0 and 14 with either 100µg OVA (Group 2) or 1000µg OVA (Group 3) in fresh alum ppt. Control mice received 100µl PBS(Group 1). All 3 groups were challenged i.n. with 100µg OVA from days 21-23. 24 hours after the last challenge, mice were scarificed, blood and BAL collected. Serum samples were checked for antibody response and BAL was analysed by differential cell counts and ELISA for cytokine levels (Figure 9). Antibody levels were generally higher in Group 2 animals and cytokines levels in BAL fluid were similar across all 3 groups. Eosinophilic inflammation of the airways was only observed in animals that were immunised with OVA (Groups 2 and 3). Eosinophil recruitment was higher at 100µg than 1000µg of OVA.

(a)



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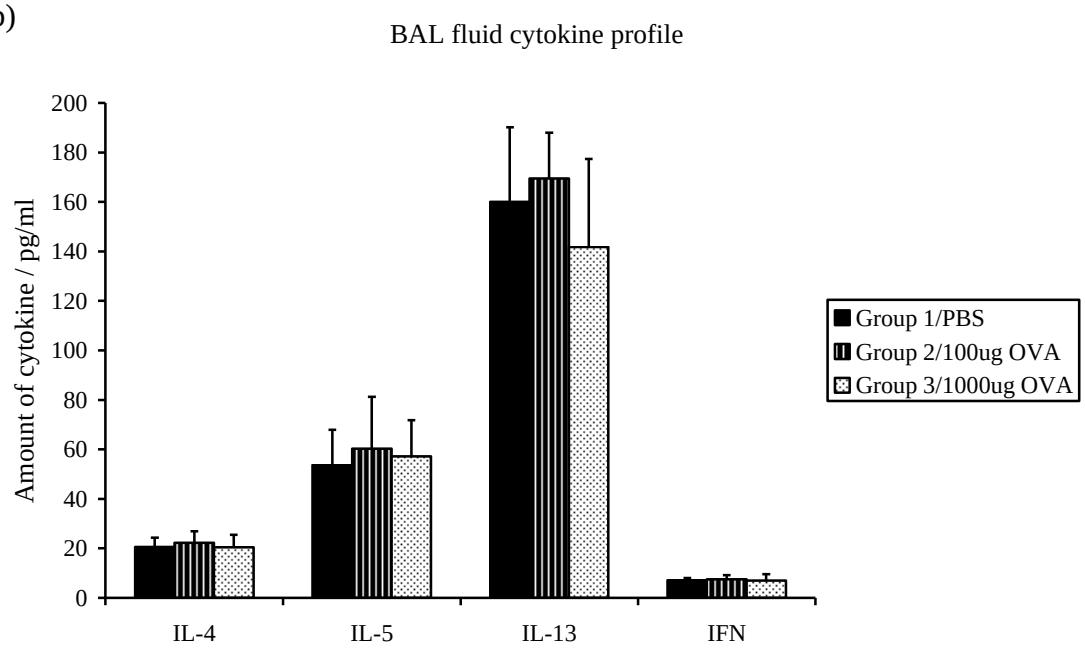

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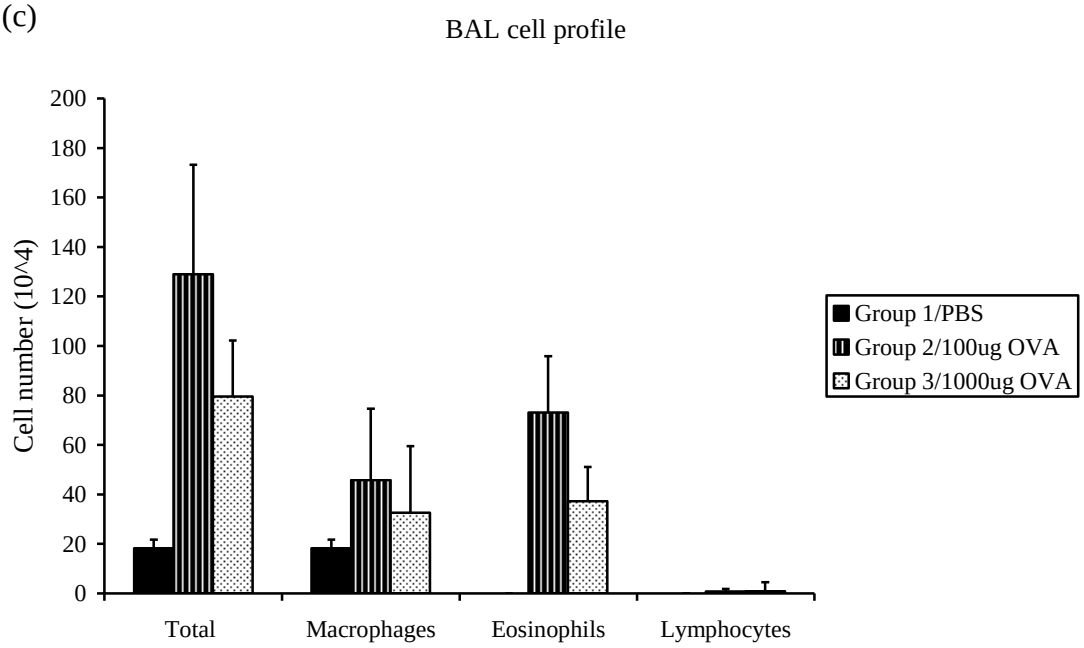


Figure 9: Determination of responses after an immunisation and challenge protocol. C57BL6 mice (n=4) from Harlan-Olac, UK, were immunised with either 100µg of OVA/mouse (Group 2) or 1000µg of OVA/mouse (Group 3) in fresh alum ppt on Days 0 and 14. Mice in control group were given 100µl of PBS (Group 1). All three groups were challenged with 100 µg OVA intra-nasally from days 21 to 23. Blood was collected on Day 24 by cardiac puncture and sera were tested for total IgE, OVA-specific-IgE and IgG1 by ELISA (a). BAL fluid was tested for cytokines by ELISA (b) and cells differentiated by H&E staining (c).

3.2 Optimisation of cell proliferation assay

A number of agents can specifically or non-specifically induce T cell activation and ultimately proliferation of the activated T cells. T cell proliferation assay conditions were optimised using a variety of known T cell stimulants such as PHA and anti-CD3 and anti-CD28. These agents are capable of activating unprimed T lymphocytes in culture either by direct cross-linking of the T cell receptor (TCR) on a large percentage of responder cells (anti-CD3 and anti-CD28 monoclonal antibodies), or by cross-linking other surface ligands such as CD2 [54].

Total splenocytes or purified CD4+ T cells were stimulated with various doses of PHA (0.1µg to 10µg/ml) and proliferation levels were determined by quantitating the amount of IL-2 in the culture supernatant by ELISA (Figure 10). Splenocytes that were stimulated with less than 10µg/ml of PHA had no detectable amounts of IL-2 in the culture supernatant. CD4+ T cells had detectable amount of IL-2 when they were cultured with PHA at concentrations of 5µg/ml and above.

The next stimulus tested was the pair of anti-CD3 and anti-CD28 monoclonal antibodies. Total splenocytes and CD4+ T cells were stimulated with various amounts of plate-bound anti-CD3 and anti-CD28 in solution. After 48 hours of culture, supernatant was tested by ELISA for IL-2 levels (Figure 11). A dose-dependent response was observed for both total splenocytes and CD4+ T cells with anti-CD3 but there was no dose response with anti-CD28 for either culture. At the same anti-CD3 and anti-CD28 concentrations, IL-2 levels were much higher in the CD4+ T cell culture than in total splenocytes culture. A similar experiment as mentioned previously was carried out but using anti-CD3 and anti-CD28 in solution (Figure 12).

Both total splenocytes and CD4+ T cells failed to show clear dose-dependent response with either anti-CD3 or anti-CD28. Moreover levels of IL-2 detected were much lower than 200pg/ml regardless of the conditions the cells were cultured under.

Once it was determined that bound anti-CD3 and anti-CD28 at 1µg/ml each was the optimal culture condition for proliferation of CD4+ T cells, optimisation of polarisation conditions was carried out. Generation of polyclonal Th1 and Th2 cell lines from naive CD4+ T cells were carried out using anti-CD3 and anti-CD28 stimulation of purified CD4+ T cells from either BALBc or C57BL6. The two subsets of Th cells exhibit helper function in different ways and can be distinguished by the patterns of cytokines they synthesise. When naive CD4+ T cells are primed with appropriate antigen, they undergo a process of differentiation and division. These cells may polarise into Th1 cells, which produce IFN-? and or into Th2 cells, which produce IL-4, IL-5 and IL-13. Although factors such as strength of antigen signal, antigen dose, co stimulators, and genetic polymorphism play a role in determining the differentiation to Th1 or Th2, the cytokine environment encountered by the naive CD4+ T cell plays a dominant role. IL-12/IFN-? and IL-4 present early in naive T helper cell activation are the crucial cytokines in determining the Th1 and Th2 phenotype, respectively.

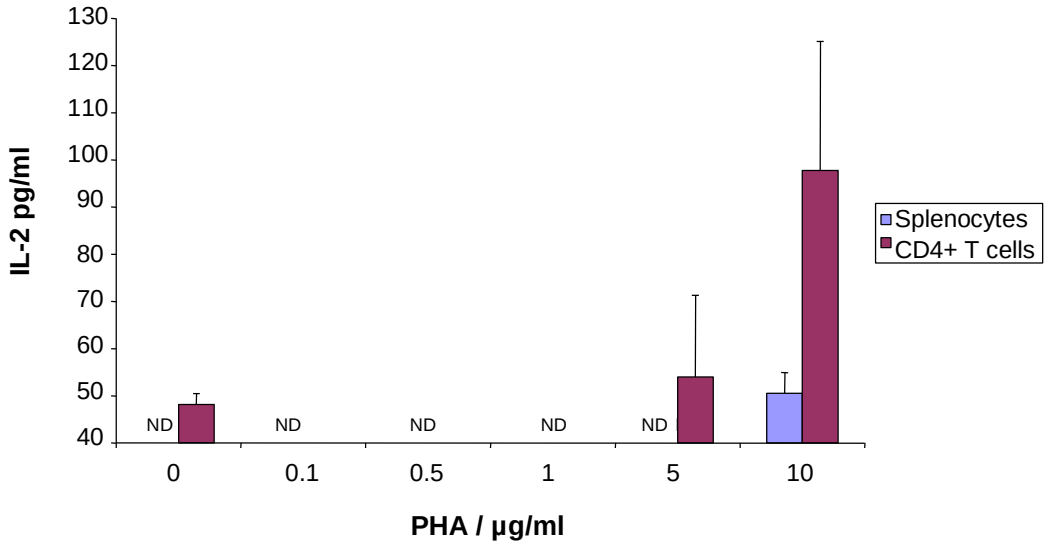


Figure 10: Optimisation of PHA dose for proliferation assay. Purified CD4+ cells or total splenocytes from spleens of C57BL6 mice were cultured for 3 days with varying doses of PHA (0µg/ml to 10µg/ml). Supernatants were tested by ELISA for IL-2 levels after 48 hours of culture. ND = not detectable

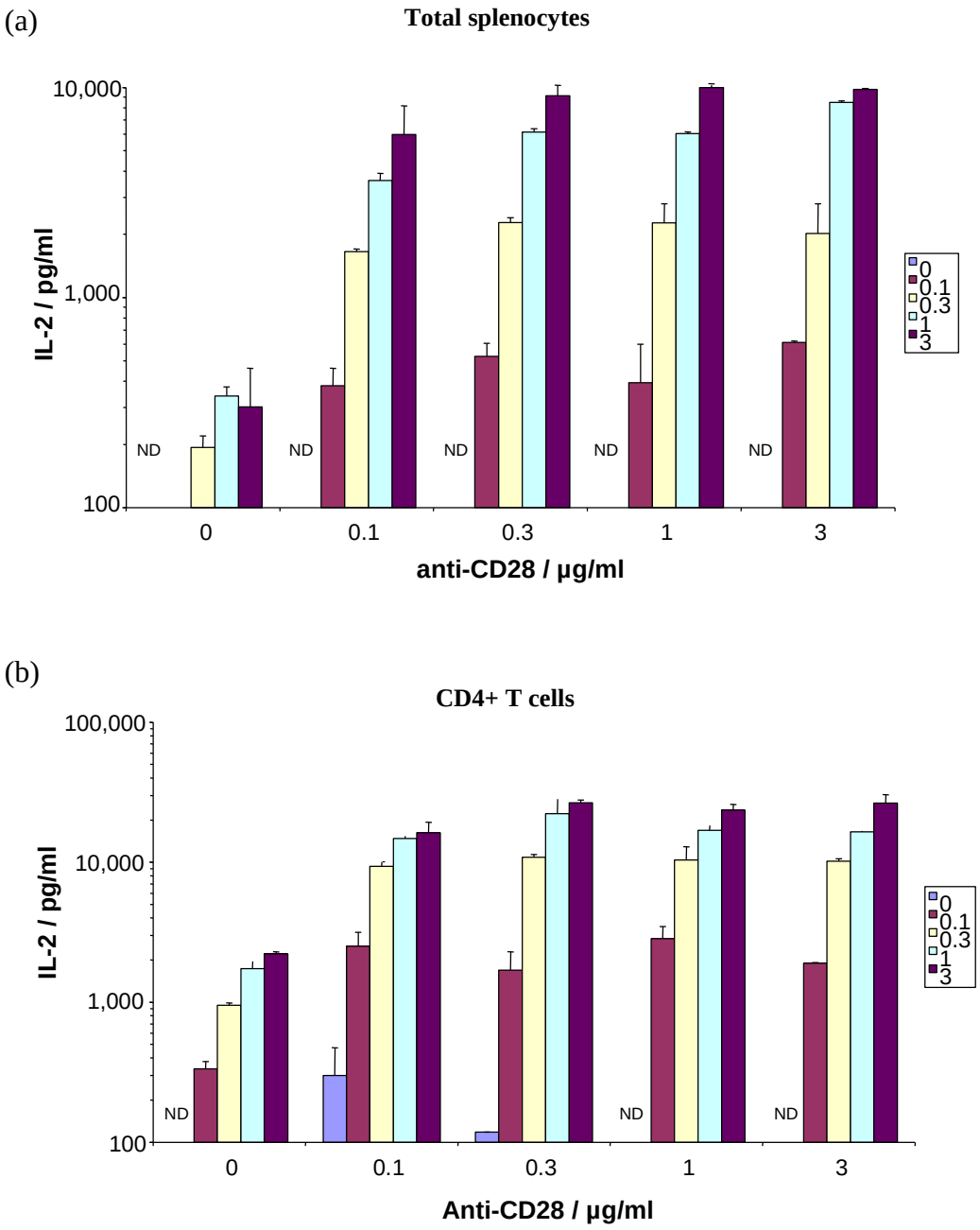


Figure 11: Optimisation of anti-CD3e and anti-CD28 conditions for cell proliferation assay. Total splenocytes (a) or CD4+ T cells (b) from C57BL6 mice were cultured with varying amounts of anti-CD28 (0.1µg/ml to 3.0µg/ml) and plate-bound anti-CD3*e* (0.1µg/ml to 3.0µg/ml). Supernatants were tested by ELISA for IL-2 levels after 48 hours of culture. ND = not detectable

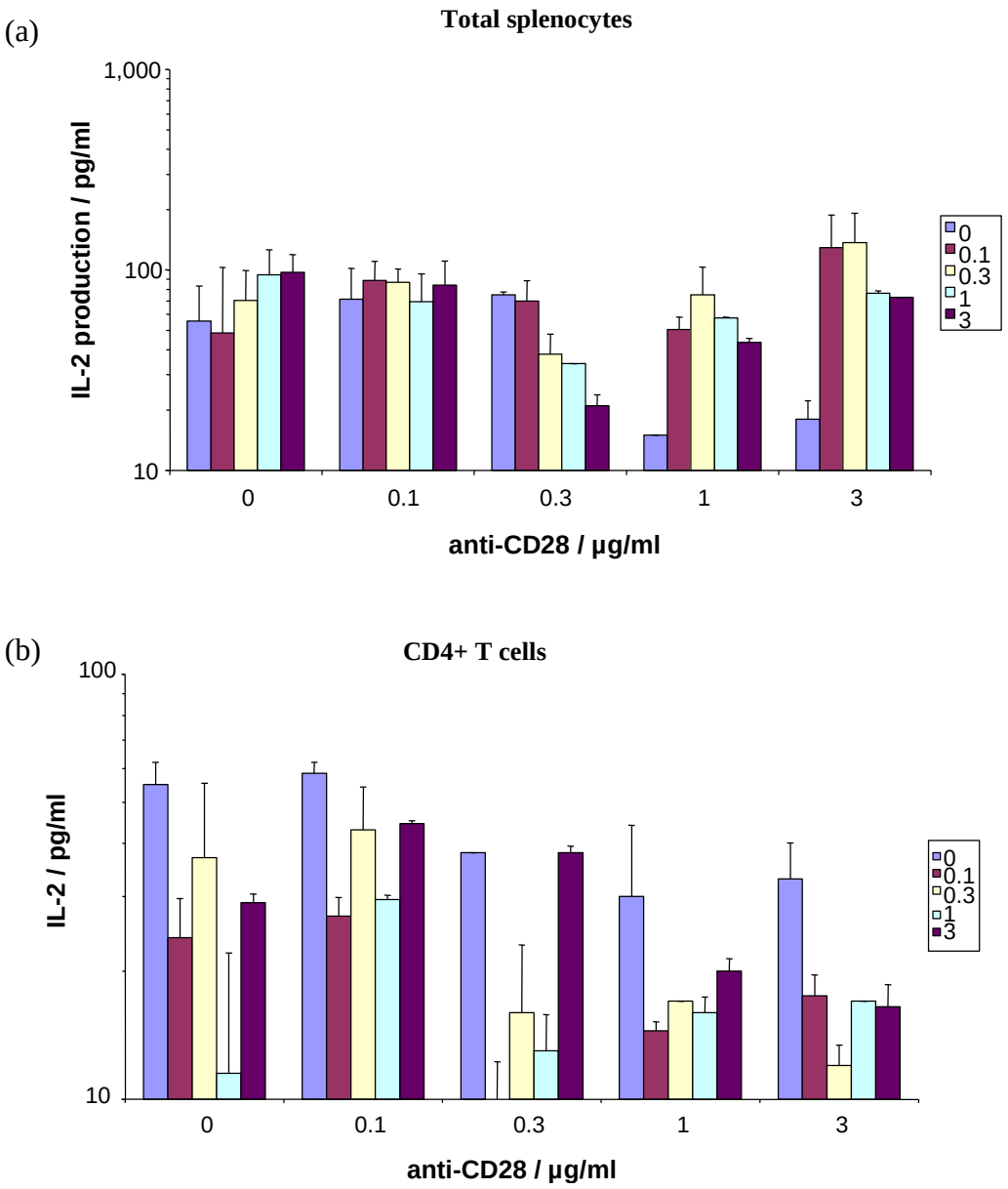


Figure 12: Optimisation of anti-CD3e and anti-CD28 conditions for cell proliferation assay. Total splenocytes (a) or CD4+ T cells (b) from C57BL6 mice were cultured with varying amounts of anti-CD28 (0.1µg/ml to 3.0µg/ml) and unbound anti-CD3*e* (0.1µg/ml to 3.0µg/ml). Supernatants were tested by ELISA for IL-2 levels after 48 hours of culture.

3.3 Polarisation studies

Splenic CD4+ T cells from C57BL6 mice were used to generate Th1, Th2 and Th0 subpopulations. Cells were cultured with plate bound anti-CD3 and anti-CD28 in solution and polarising cytokines. Recombinant murine IL-12 (10ng/ml) and anti-IL-4 (10µg/ml) neutralising antibodies were used to generate Th1 cells; recombinant IL-4 (10ng/ml), anti-IL-12 (10µg/ml) and anti-IFN-? (10µg/ml) neutralising antibodies were used to generate Th2 cells and to generate Th0 cells, cells were cultured with just anti-CD3 and anti-CD28. IL-2 was added on day 3 of culture at 10U/ml. After 1 week of culture, cells were washed and were either restimulated for 6 hours with PMA, ionomycin and monensin and tested for intracellular cytokine using FACS staining or were recultured under the various polarising conditions for another 1 week. Attempts to generate Th1 cells *in vitro* were successful with about 38% of IFN-? and less than 2% of IL-4 positive cells as determined by FACS (Figure 13a) Generation of Th2 cells was also successful as there were about 34% of IL-4 and less than 1% of IFN-? positive cells (Figure 13c). However attempts to generate Th0 cells were not as successful as about 41% of the cells were positive for IL-4 (Figure 13e). Th1 cells that were cultured under polarising conditions for 2 weeks yielded similar results with about 38% of cells being IFN-? positive (Figure 14a). However, in the Th2 culture there was a 2-fold drop in the number of cells that were IL-4 positive, with only 13% of cells being IL-4 positive (Figure 14c). A similar trend was observed in the Th0 cultures, with a significant drop in the number of IL-4 positive cells (Figure 14e).

A similar experiment was done using splenic CD4+ T cells from BALBc mice. About 32% of cells cultured under Th1 conditions for a week were IFN-? positive (Figure

15a), 26% of cells cultured under Th2 conditions were IL-4 positive (Figure 15c). 26% of cells cultured under Th0 conditons were IL-4 positive as well (Figure 15e). After 2 weeks of culture the levels of polarised cells dropped as observed in the previous experiment. The biggest drop was observed in the Th2 conditions with a 2-fold drop in the number of cells expressing IL-4 (Figure 16c).

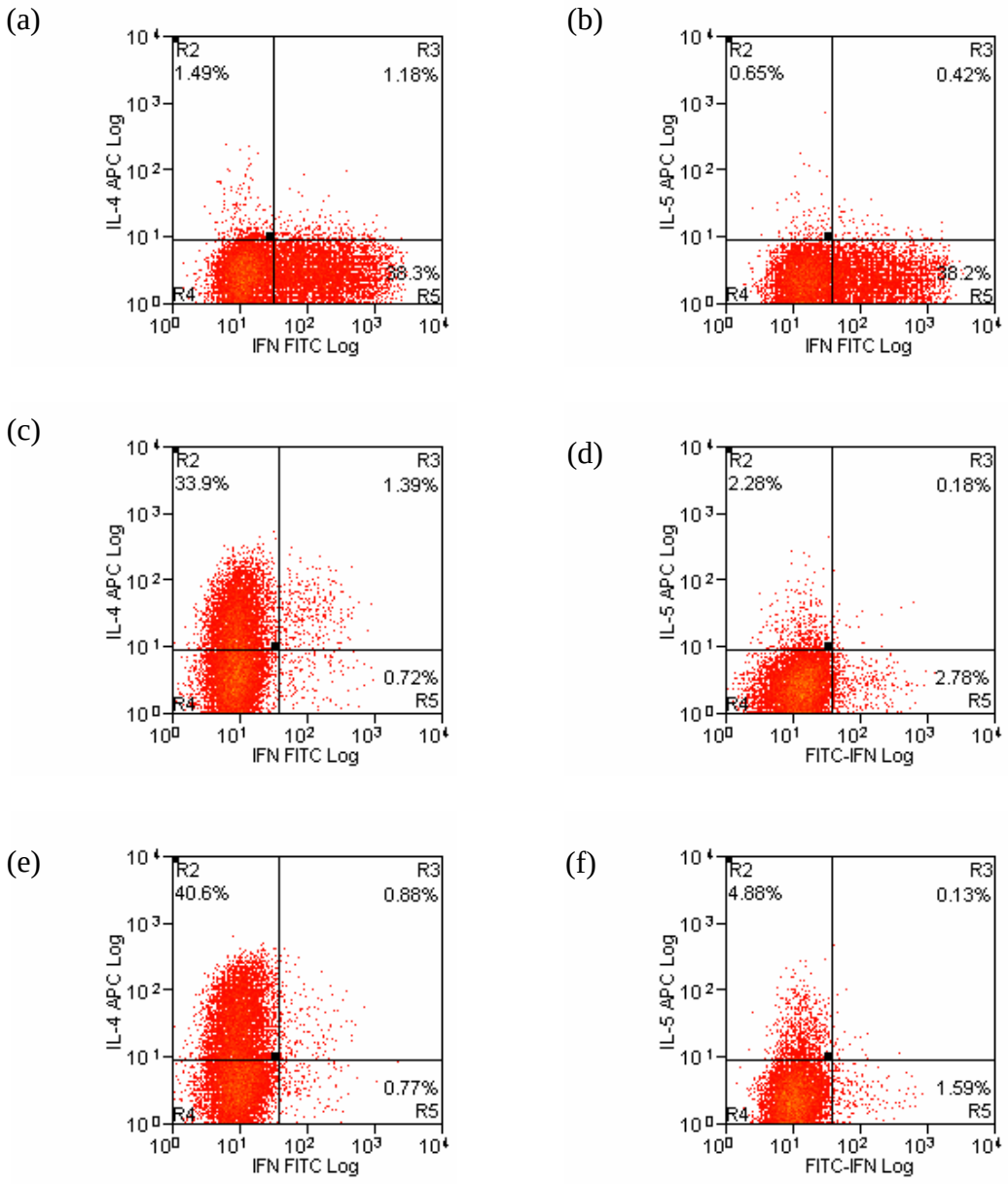


Figure 13: Non-antigenic polarisation of CD4+ T cells from C57BL6 mice for one week. Purified CD4+ T cells from C57BL6 mice were cultured under Th1 (a-b), Th2 (c-d) or Th0 (e-f) polarising conditions. After a week, cells were either restimulated under similar polarising conditions as mentioned above or they were stained for intracellular cytokines; IL-4, IL-5 and IFN-?.

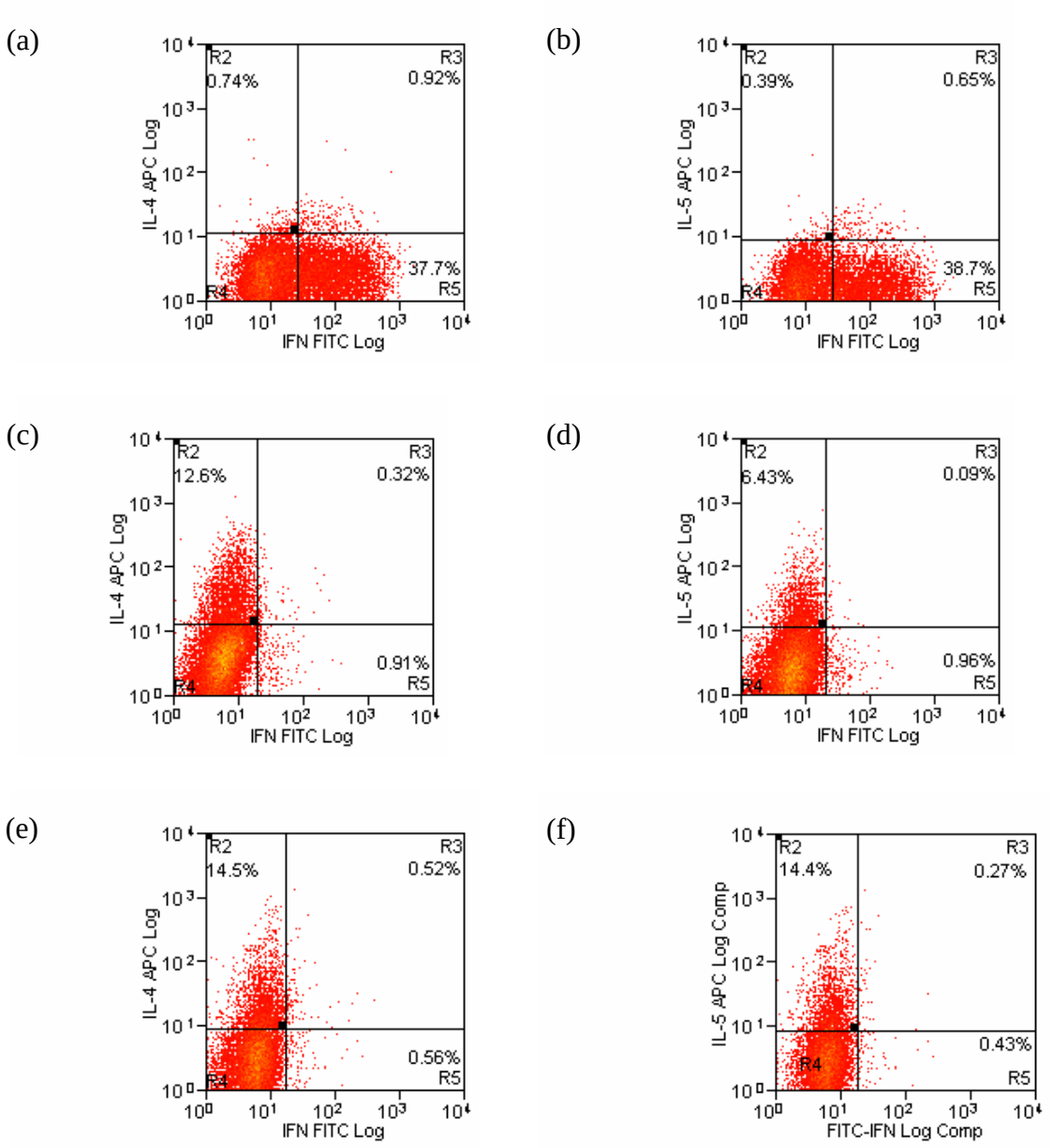


Figure 14: Non-antigenic polarisation of CD4+ T cells from C57BL6 mice for two weeks. Purified CD4+ T cells from C57BL6 mice were cultured under Th1 (a-b), Th2 (c-d) or Th0 (e-f) polarising conditions. After 2 weeks, cells were stained for intracellular cytokines; IL-4, IL-5 and IFN-?.

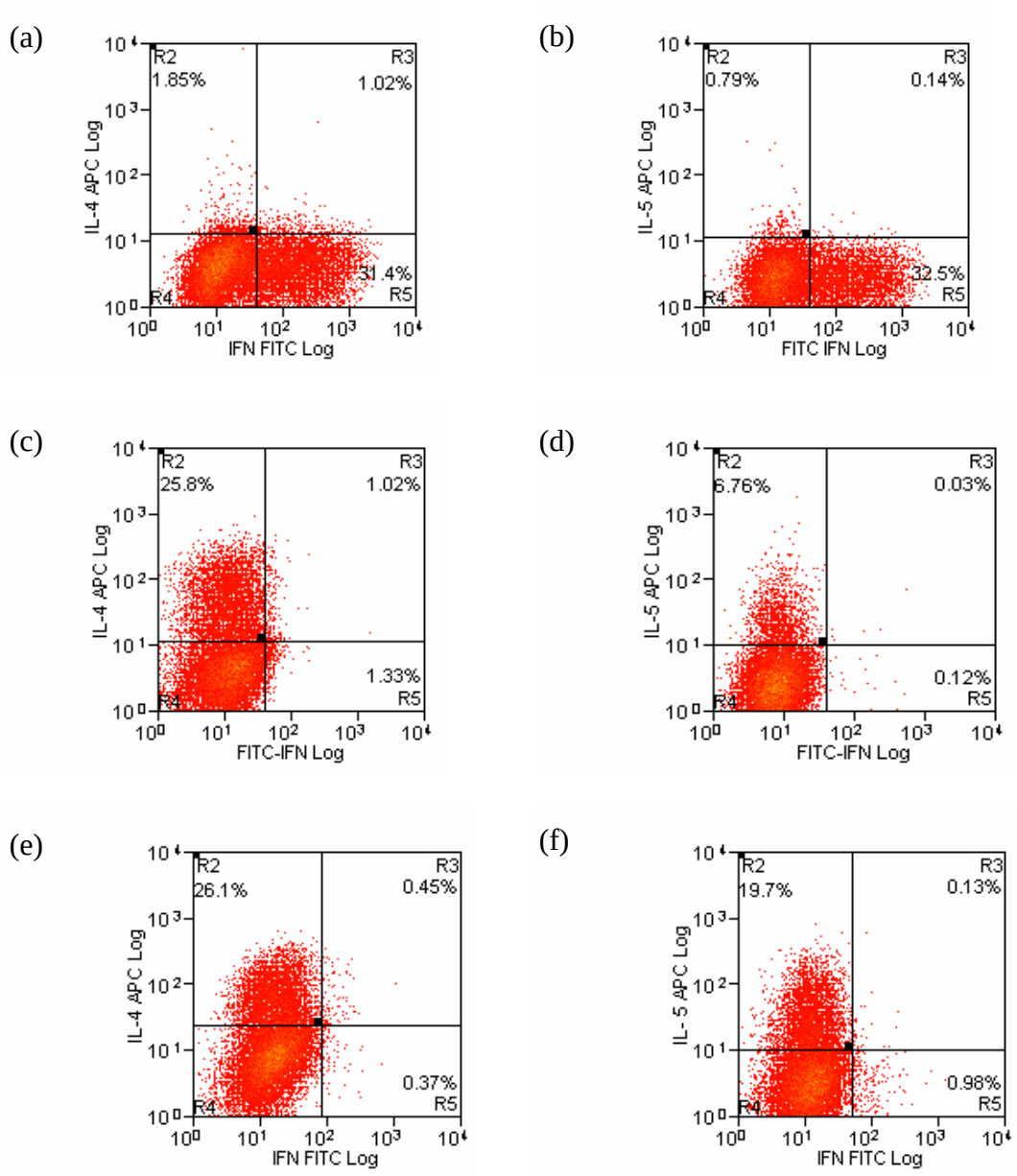


Figure 15: Non-antigenic polarisation of CD4+ T cells from BALBc mice for one week. Purified CD4+ T cells from BALBc mice were cultured under Th1 (a-b), Th2 (c-d) or Th0 (e-f) polarising conditions. After a week, cells were either restimulated under similar polarising conditions as mentioned above or they were stained for intracellular cytokines; IL-4, IL-5 and IFN-?.

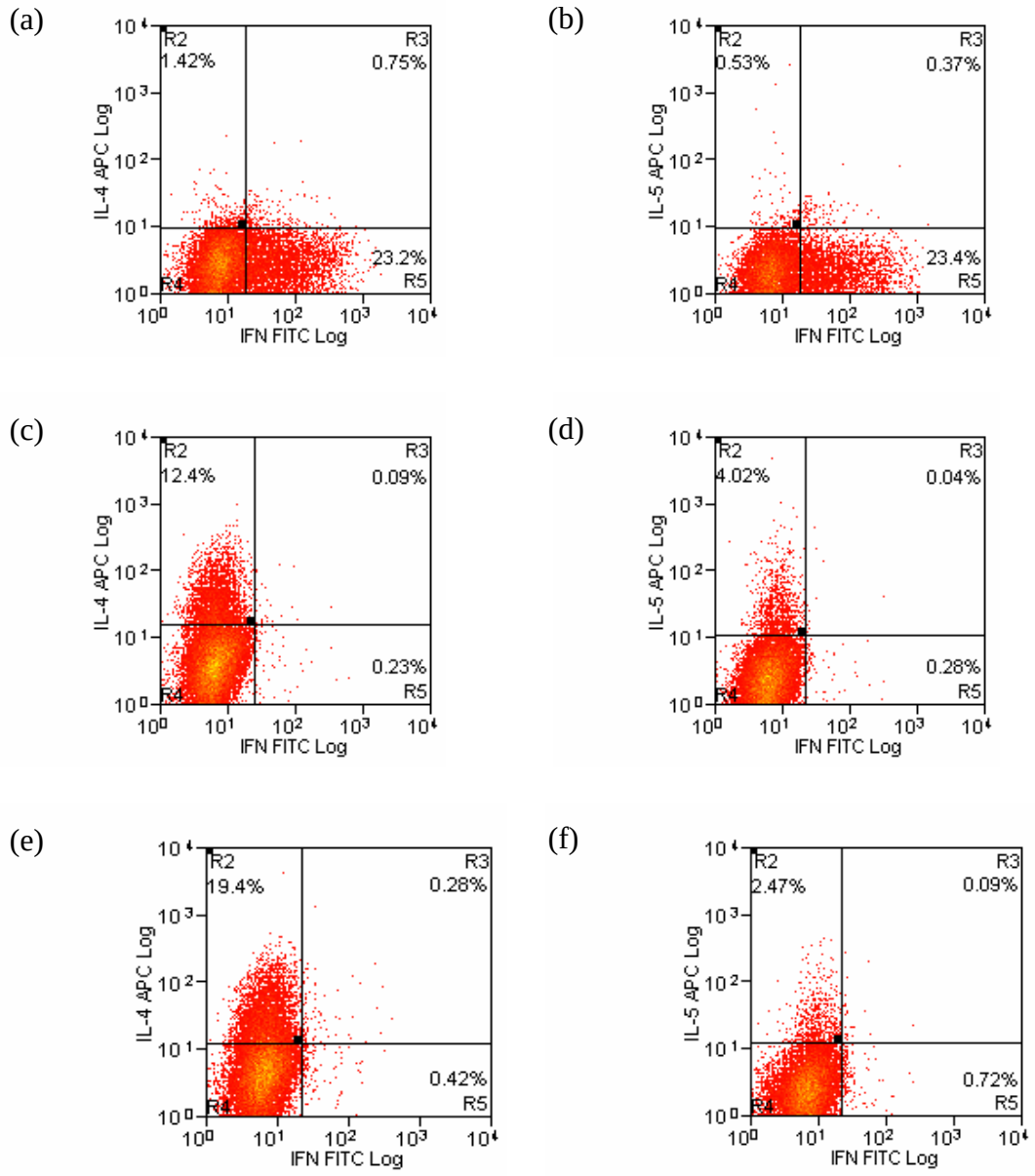


Figure 16: Non-antigenic polarisation of CD4+ T cells from BALBc mice for two weeks. Purified CD4+ T cells from BALBc mice were cultured under Th1 (a-b), Th2 (c-d) or Th0 (e-f) polarising conditions. After 2 weeks, cells were stained for intracellular cytokines; IL-4, IL-5 and IFN-?.

However, Th0 cells under these conditions were expressing high levels IL-4 only when they should be expressing both IL-4 and IFN-? equally. Hence a second protocol was developed. In the 2nd approach, generation of Th2 cells from CD4+ T cells from OT-II mice was studied. CD8+ depleted splenocytes were cultured with $OVA_{323-339}$ (10µg/ml), IL-4 (10ng/ml), anti-IL-12 (10µg/ml), anti-IFN-? (10µg/ml) and IL-2 (5U/ml) for one week. After a week, cells were washed and were either restimulated for 6 hours with PMA, ionomycin and monensin or tested for intracellular cytokine using FACS staining or were recultured under Th2 polarising conditions for another week. This was repeated for over a period of 3 weeks (Figure 17). In the first week, about 22% of cells were IL-4 positive, with less than 22% being IFN-? positive (Figure 17a). By the end of the 2nd week, the percentage pf cells positive for IL-4 had more than doubled to about 54% with less than 1% IFN-? positive cells (Figure 17c). However by the end of the 3rd week, the percentage of IL-4 positive cells dropped to levels similar to that seen in the first week. On the other hand, levels of IL-5 positive cells were very low at the end of the first week but had increased to about 24% by the end of 2nd week and had dropped only slightly to about 19% at the end of the experiment (Figures 17b, d and e).

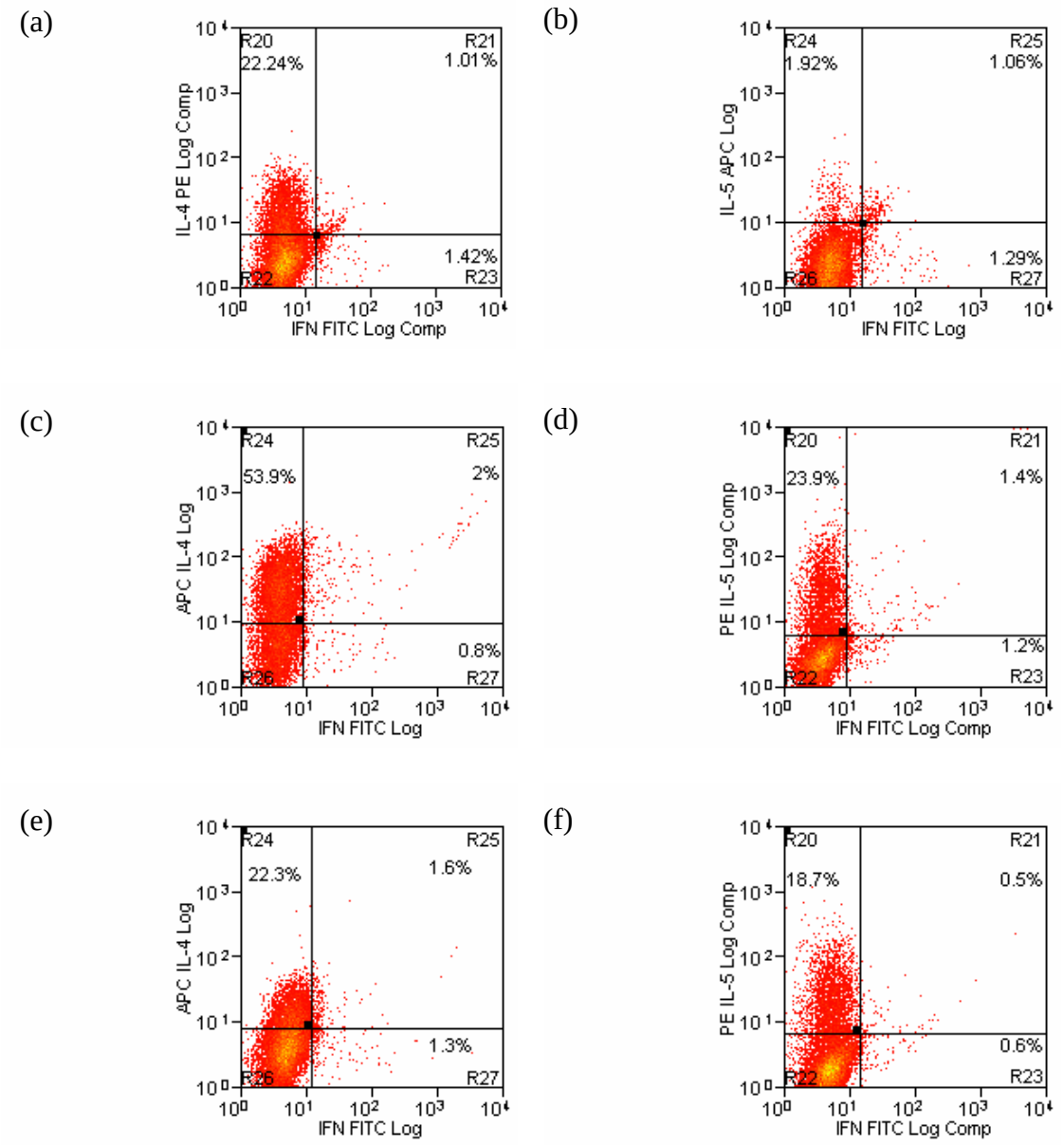


Figure 17: Antigenic stimulation of cells. Splenocytes from OT-II mice which were depleted of CD8+ T cells were cultured with IL-4 (10ng/ml), anti-IL-12 (10µg/ml), anti-IFN-? (10µg/ml), IL-2 (5U/ml), and $OVA_{323-339}$ (10µg/ml) in complete medium. After a week, cells were either restimulated as mentioned above or they were stained for intracellular cytokines; IL-4, IL-5 and IFN-?. Top panel (a-b): cells were stimulated for one week, second panel (c-d): cells were stimulated for two weeks and bottom panel (e-f): cells were stimulated for three weeks.

Non-antigenic based polarisation of C57BL6 CD4+ T cells			
Polarisation condition	Th1 (IFN-?)	Th2 (IL-4)	Th0 (IFN-? + IL-4)
Week 1	38.3%	33.9%	0.88%
Week 2	37.7%	12.6%	0.52%

Non-antigenic based polarisation of BALBc CD4+ T cells			
Polarisation condition	Th1 (IFN-?)	Th2 (IL-4)	Th0 (IFN-? + IL-4)
Week 1	32.5%	25.8%	0.45%
Week 2	37.7%	12.4%	0.28%

Antigenic based polarisation of OT-II cells	
Polarisation condition	Th2 (IL-4)
Week 1	22.2%
Week 2	53.9%
Week 3	22.3%

Table 2: Summary of FACS data from polarisation studies. Cells were polarised under the different conditions and the percentage of cells positive for IFN-? (Th1 condition) or IL-4 (Th2 condition) or IFN-? and IL-4 (Th0) were analysed by FACS after the cells were stained for the various intracellular cytokine.

3.4 Adoptive transfer and passive sensitisation studies

Th2 cells were used to establish the animal model of Th2 mediated lung inflammation. Naïve C57BL6 mice were intravenously injected with 5×10^6 Th2 cells and control mice received 100µl PBS. Animals were intranasally challenged with 100µg OVA 24hrs after the cell transfer on 3 consecutive days. 24 hours after the last antigen challenge, BAL was collected and analysed using differential cell counts and cytokine levels were measured by ELISA (Figure 18). Mice that had received Th2 cells had a high number of eosinophils recruited into the airways but the control mice had none (Figure 18a) but cytokine levels in BAL fluid of both groups did not yield any significant differences (Figure 18b).

In the next experiment, responses of mice that were actively sensitised and challenged with OVA (Group 2) were compared with mice that had received Th2 cells before being challenged with OVA (Group 1). In addition, mice that were actively sensitised with OVA and received Th2 cells one day before being challenged with OVA was also included (Group 3). BAL was collected and analysed using differential cell counts and fluid tested by ELISA for cytokine levels. Blood collected was tested for serum levels of OVA-IgG1, IgE and total IgE (Figure 19). Eosinophil numbers in Groups 1 and 3 were comparable with each other but were much higher when compared to group 2. However cytokine levels between the 3 groups were similar. Antibody levels were only detected in groups that were actively sensitised (Groups 2 and 3) and levels between these 2 groups were similar.

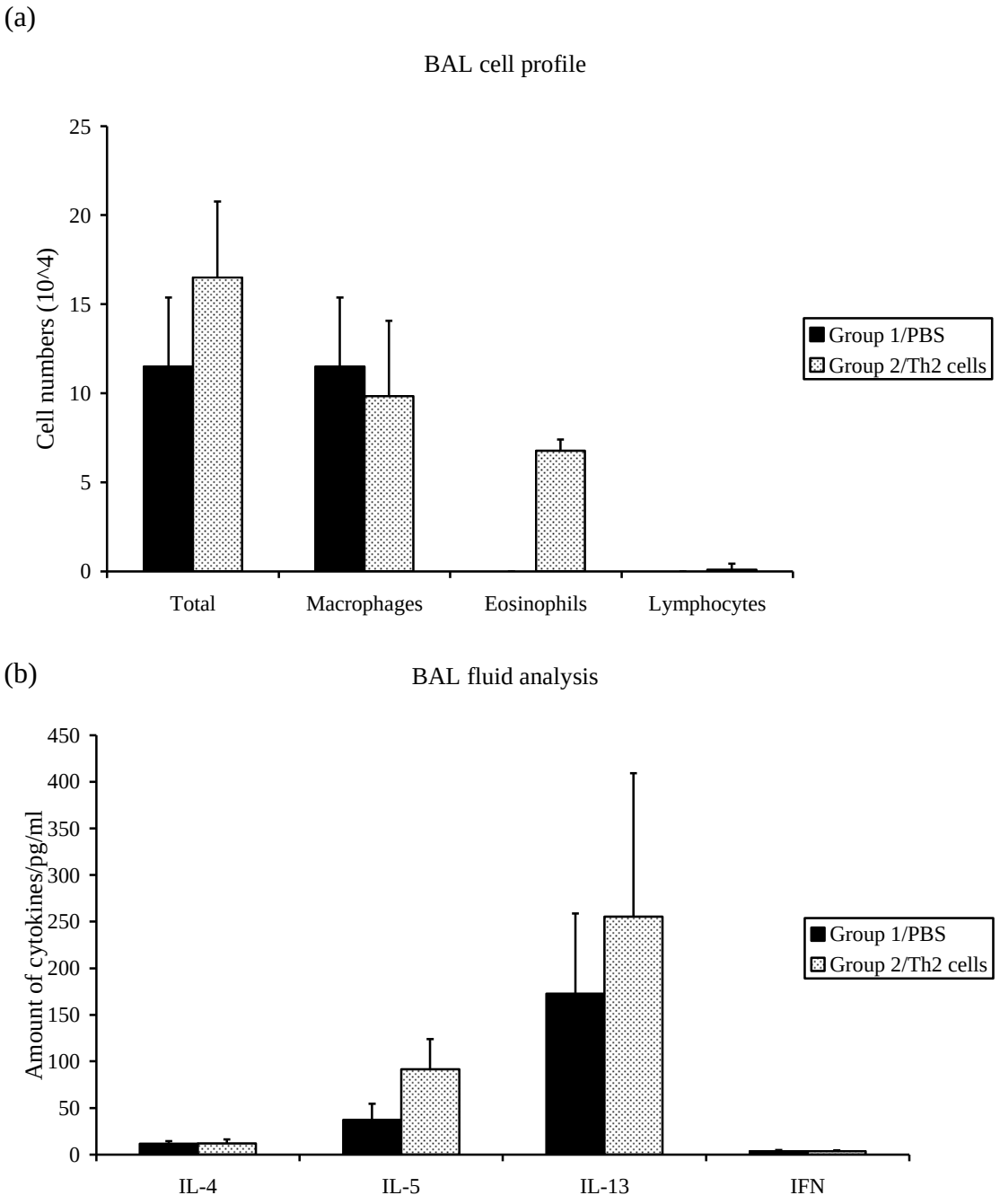
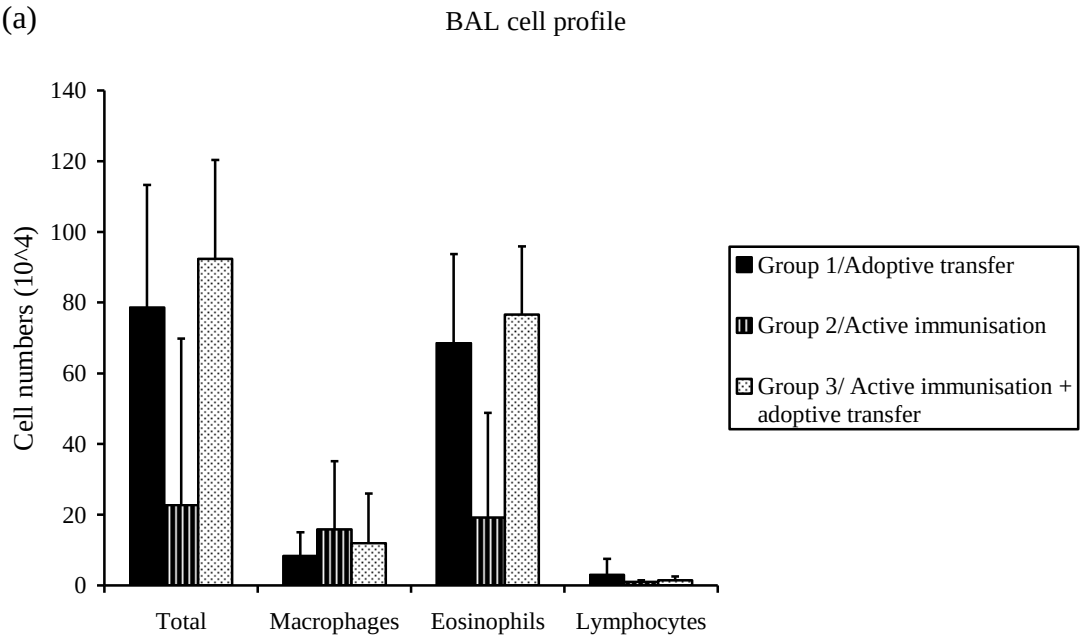


Figure 18: Effect of adoptive transfer. Naïve C57BL6 were intra-nasally (i.n.) challenged with 100µg OVA on Day 0. On Day 1, animals were either given 100µl PBS (Group 1) or 5 $\times 10^6$ Th2 polarised CD4+ OT-II cells (Group 2) intravenously. They were then challenged with 100µg OVA on Days 3 and 4 i.n. and were sacrificed on Day 6. Bronchoalveolar lavage was done. BAL cells were differentiated according to morphology and supernatant was tested for IL-4, IL-5, IL-13 and IFN-? by ELISA.

(a)



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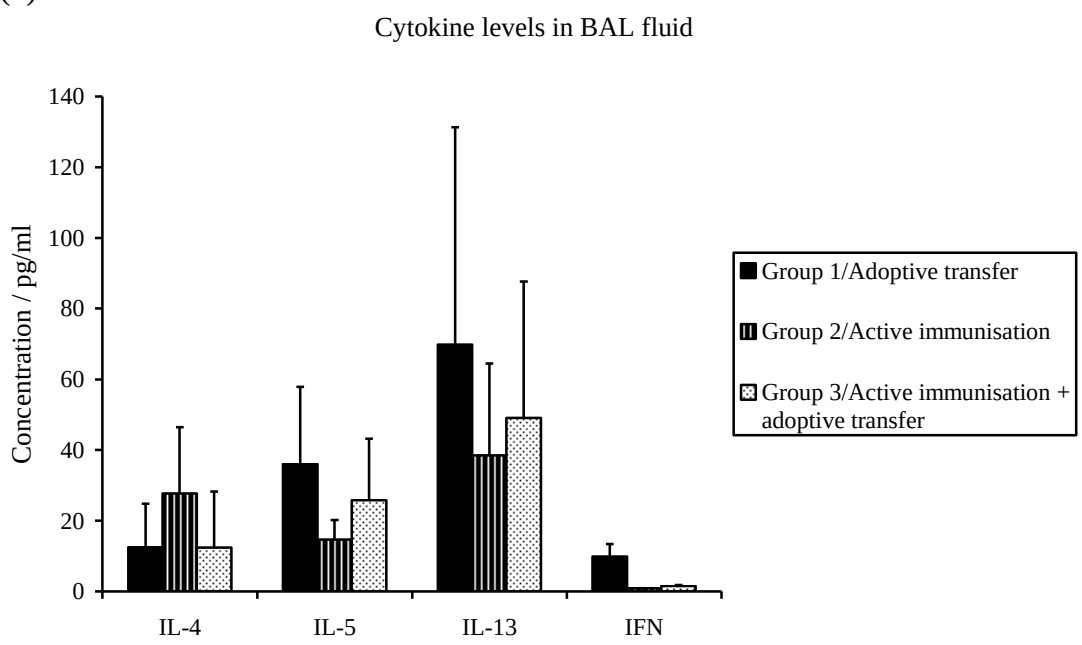


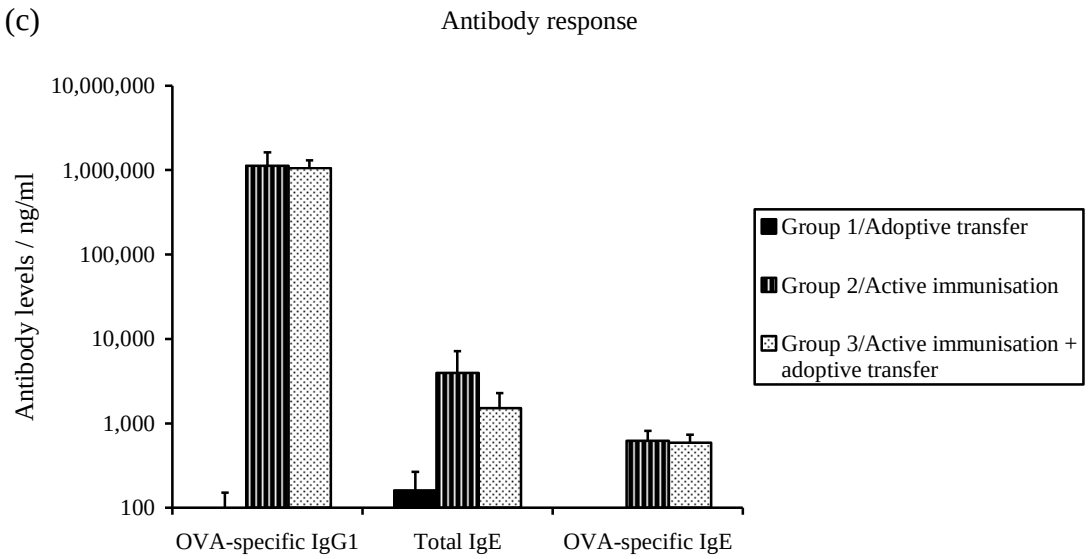


Figure 19: Effect of adoptive transfer on active immunisation. Naïve C57BL6 were immunised with 100µg OVA on Days 0 and 14 (Groups 2 and 3). On Day 21, animals in all 3 groups were challenged with 100µg OVA intranasally. Animals were given 5 $\times 10^6$ Th2 polarised CD4+ OT-II cells in 100 µl PBS (Group 1 and 3) or 100 µl PBS intravenously. They were then challenged with 100µg OVA on Days 23 and 24 i.n. and were sacrificed on Day 26. Bronchoalveolar lavage was done. BAL cells were differentiated according to morphology (a) and supernatant was tested for IL-4, IL-5, IL-13 and IFN-? by ELISA (b). Sera collected were tested for OVA-IgG1, OVA-IgE and Total IgE (c).

One of the objectives of the project was to study the immune responses of passively sensitised mice after they were intranasally challenged with OVA. Mice in the following experiment were passively sensitised by intravenous injections with a commercially available mouse anti-OVA IgE. 24 hours later, they were intranasally challenged with OVA and after another 24 hours, they were sacrificed and BAL obtained and analysed by differential cell count and ELISA for cytokine levels (Figure 20). Based on differential counts, no other cells except for macrophages were detected in all of the groups that received anti-OVA IgE. Cytokine levels were barely detectable in all 4 groups (data not shown).

Once it was shown that passive sensitisation with anti-OVA IgE do not induce eosinophilic airway inflammation, the next stage was to study whether passive sensitisation of animals will affect the immune responses of mice that had received Th2 cells. In the next experiment (Figure 21), mice were given suboptimal levels of Th2 cells (3×10^6) on Day 0. At the same time, Group 1 received intravenously, 10µg anti-OVA IgE and Group 2 received 100µl PBS as control. Mice were challenged intranasally with OVA for 2 days and 24 hours after the last antigenic challenge were sacrificed. BAL obtained was analysed for differential cell counts and cytokine levels. Lungs were also harvested to compare the recruitment of the adoptively transferred T cells between the 2 groups. There was no significant difference between the 2 groups in the differential cell counts (Figure 21a). Cytokine levels in the fluid were also similar between the 2 groups. However FACS data showed that there was a 2-fold increase in the level of transgenic T cells that were recruited into the lungs in group 1 but the low numbers might suggest that the difference between the two groups might not significant (Figure 21c).

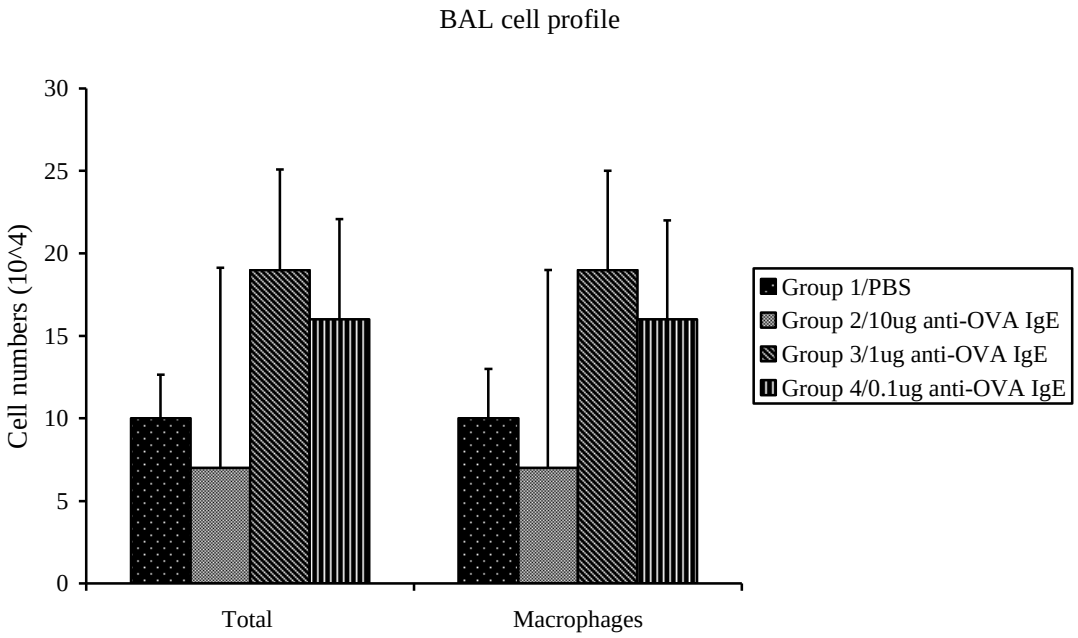
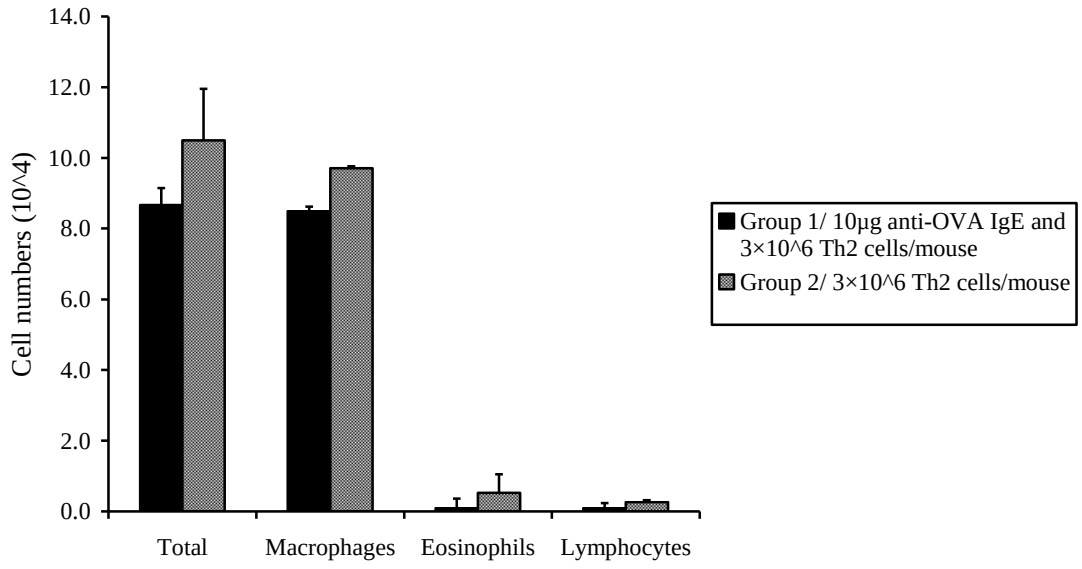
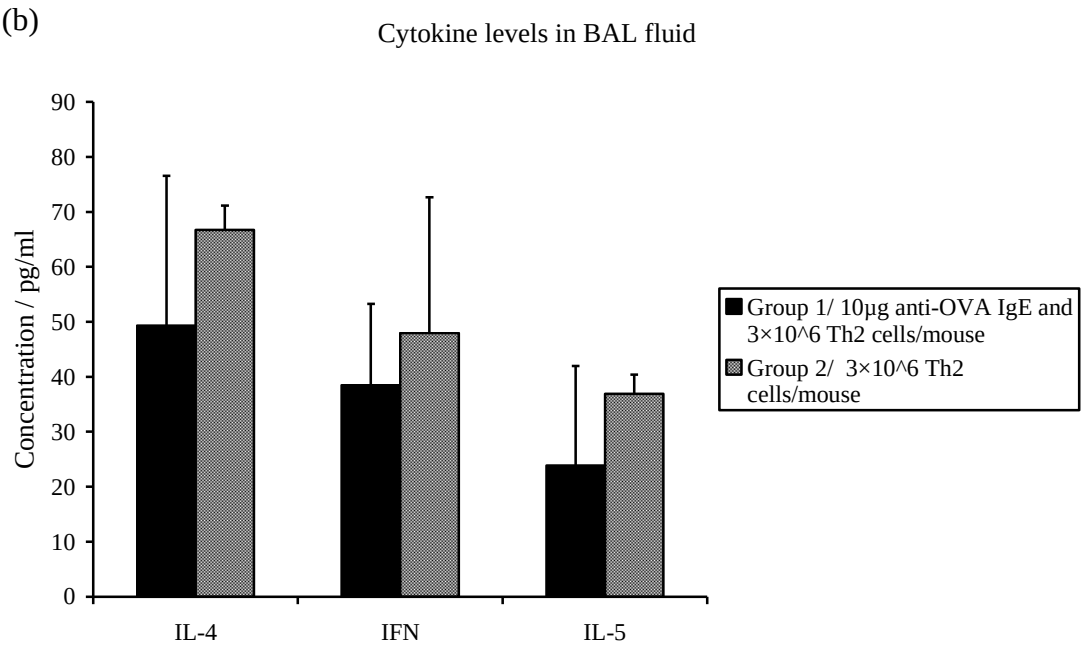


Figure 20: Effect of passive sensitisation of animals with mouse anti-OVA IgE. C57BL6 (n=4) were intravenously given varying amounts of anti-OVA IgE; 10µg anti-OVA-IgE/ mouse (Group 2), 1µg anti-OVA-IgE/ mouse (Group 3) and 0.1µg anti-OVA-IgE/ mouse (Group 4) on Day 0. Control animals were given 100µl PBS IV. On Day 1, all the animals were intra-nasally challenged with 100µg OVA. 24 hours later, they were sacrificed and Bronchoalveolar lavage was performed. Experiments were done twice and error bars are standard deviations.

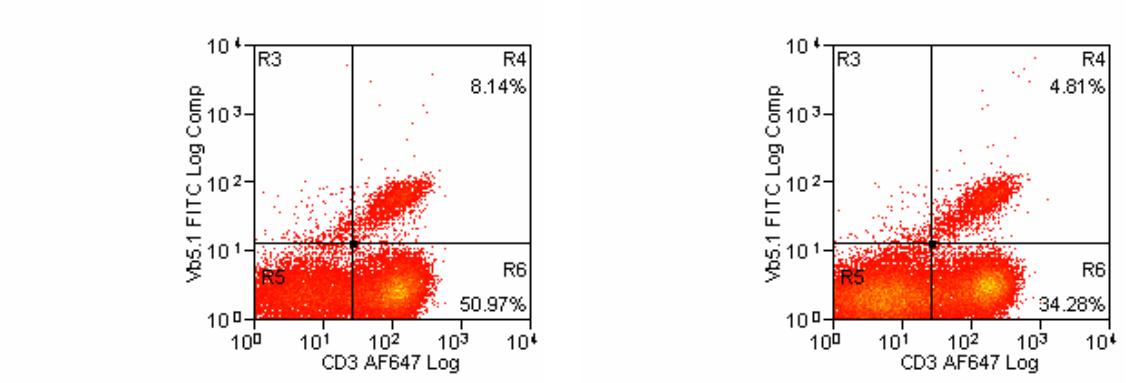
(a)



(b)



(c)



Group 1 Group 2

Figure 21: Effect of passive sensitisation on adoptive transfer. C57BL6 mice were either intravenously given 10µg anti-OVA IgE in 100µl PBS (Group 1) or PBS alone (Group 2) on day 0. At the same time, both groups of mice intravenously received 3×10^6 cells/mouse. On days 1 and 2, both groups were given 100µg OVA i.n. and 24 hours later were sacrificed. After BAL was done, lung tissues were processed and cells were stained to identify the transgenic T cells and population was analysed by FACS.

CHAPER 4: DISCUSSIONS

4.1 Optimisation of immunisation protocol

One of the hallmarks of asthma is the high production of serum IgE. An asthmatic response after an allergen exposure is associated with IgE-mediated mast cell activation, which induces the accumulation of Th2 lymphocytes and eosinophils in the airways [55, 56]. Hence it was crucial to establish an animal model of asthma that produced high amounts of IgE when sensitised with an allergen as this study's aim was to shed some light on the role of IgE in an acute model of asthma. In the first experiment of this study (Figure 1), OVA-specific IgG1 response was quickly established in the animals, but the animals were not making the appropriate IgE responses. Control animals in the experiment were making an IgE response that was as high as, if not higher than IgE responses of animals that were immunised with the allergen (OVA). To rule out the possibility that a human error could have contributed to the unexpected IgE response, a second experiment was done using a different group of animals and with a smaller dose range of OVA. A similar IgE response was obtained which indicated that the first experiment's unexpected result was not due to human error. The next two experiments were done to study the effects of repeated dosing and adjuvants on IgE responses. It was reasoned that perhaps with repeated dosing, animals would make a more pronounced IgE response when compared to the control groups because a survey of literature indicated that most groups immunised animals more than once. But the results obtained showed that repeated dosing does not increase the levels of IgE significantly when compared to the control group. Selected adjuvants, which are known to be good inducers of Th2 immune responses, were tested out. However in this experiment IgE responses of control animals were comparable with those from the experimental groups. It was also observed that in all 4

experiments, control animals made IgE responses that were above the reported background values in literature which is about 15ng/ml [57]. In fact, values for IgE of OVA immunised and challenged animals were reported in literature to be about 2000-3000ng/ml [58]. Since control animals in our experiments had IgE levels that were as high as 5000ng/ml, this suggested that these mice had no inherent problems that prevented them from making an IgE response but rather they were making non-specific IgE responses. It was postulated that the animals could be suffering from a parasitic infection, as a parasitic infection induces a typical Th2 response which includes production of non-specific IgE [59]. A study done by Wohlleben *et al* showed that helminth infection does not affect the levels of OVA-specific serum IgE and IgG1 [60] thus we tried to determine the levels of serum OVA-IgE in the animals. A working ELISA protocol for the detection of OVA-IgE was only established later in the project for easier detection of OVA-IgE but for these experiments OVA-specific IgE was measured by passive cutaneous anaphylaxis. Since it was identified that the main cause for the lack of proper IgE responses could be the mice, tests were done to verify if the animals were suffering from an infection. The tests did confirm what was suspected and steps were taken to establish a new colony of mice. Using these animals, experiments were repeated to optimise the conditions for the immunisation protocol. 100µg of OVA and the self-made alum were chosen as the optimal conditions to evoke OVA-IgE and IgG1 responses that were comparable with those found in literature. An immunisation and challenge protocol was set-up to study the immune responses of mice. Responses were measured by analysing the cells recruited into the airways and also the cytokines of the BAL fluid. Analysis of BAL cells demonstrated a typical Th2 inflammation i.e recruitment of eosinophils into the airways. However, levels of the different cytokines were similar amongst the groups.

This was an unusual observation because whenever high eosinophilia was reported, IL-4 and especially IL-5 levels in BAL fluid were reported as being high as well [61] when compared to control groups. Levels of IL-4 and IL-5 were reported to be as high as 150pg/ml but in this study levels of IL-4 and IL-5 were about 50pg/ml. This could perhaps be explained by the low levels of lymphocytes that were detected in the BAL. Lymphocytes are the main producers of IL-4 and IL-5 and if there were low numbers of lymphocytes, it is reasonable to expect low amount of IL-4 and IL-5 in the BAL fluid. Using these results, the roles of antigen specific-IgE and CD4+ T cells were studied in isolation and in combination and this will be discussed further in the sections below.

4.2 Optimisation of polarisation protocol

The first step in getting a good polarisation data is to find out the best T cell stimulus for non-transgenic T cells. Two of the most commonly used stimuli to stimulate T cells *in vitro*, PHA and anti-CD3 and anti-CD28, were tested. Initially [^{3}H] thymidine incorporation assay was the only assay used to check proliferation levels however, the levels of proliferation in the control groups did not tally with what was reported (Maria's thesis). Our earlier results from immunisation, suggested that there might be a parasitic infection in the mice. Wohlleben *et al* has shown that modulation of airway inflammation of OVA-sensitised, parasite infected mice was due to IL-10 and the group had postulated that the source of IL-10 could be Tregs hence our conclusion that in our mice there might had been significantly higher proportion of activated Tregs [60]. We believed that the cultures might have been contaminated with a significantly high numbers of Tregs because even though we might have purified for CD4+ T cells, Tregs which are also CD4+CD25+ might have also been isolated

together with the cells. Therefore to rule out the possibility that our cultures were contaminated with Tregs, proliferation levels were checked by quantitating IL-2 levels in the supernatant by ELISA in the later experiments. This because with the proper stimulus for T cells, activation of naïve CD4+ T cells will induce the secretion of IL-2 whereas activation of Tregs will result in consumption of IL-2 therefore accurately measuring the rate of proliferation of naïve CD4+ T cells. We had also compared proliferation between total splenocytes and purified CD4+ T cells because we wanted to show that the stimuli used were specific for the activation and proliferation of CD4+ T cells and not the other cell types that could be found in splenocytes.

As mentioned in the result section, bound anti-CD3 and unbound anti-CD28 at 1µg/ml each was found to be the best stimulus for T cell growth. Nguyen *et al* reported that T cells stimulated with anti-CD3 alone can induce activation and proliferation which was also observed in our proliferation experiments and in the presence of anti-CD3 and anti-CD28, proliferation rates hit a maximum which suggests that both signals are crucial to induce an optimum cell proliferation [62]. The next stage was to optimise the conditions for polarisation. Results shown are the optimised conditions. However under the non-polarising conditions, activated T cells were making a Th2 polarised response, which was unexpected for we had expected these Th0 cells to be producing both IFN-? and IL-4 [63]. This was a slightly unexpected result however, there is a study that have shown that naïve CD4++ T cells that were stimulated with anti-TCR, anti-CD28 and IL-2 had significantly increased production of IL-4 [64] compared to non-stimulated cells and they attributed this phenomenon to the presence of IL-2 in the culture. However these authors reported

that BALBc CD4+ T cells produced higher levels of IL-4 and IFN-? compared to CD4+ T cells from C57BL6 mice and this was probably due to the genetic makeup of the mice, BALBc mice being more prone to produce a Th2 response than C57BL6 mice which are more predisposed to producing a Th1 response. However in our studies, it was consistently shown that C57BL6 mice without the skewing medium produced a better Th2 response than cells from BALBc mice. This phenomenon is rather hard to explain as there are no studies that have shown C57BL6 mice capable of producing a better Th2 response than BALBc mice when stimulated with the above-mentioned skewing medium. However there could be the possibility that BALBc mice that we had been using in our studies were not as clean as the C57BL6 that we had used.

Cells from both BALBc and C57BL6 mice that were just stimulated with anti-CD3, anti-CD28 and IL-2, had a cytokine profile similar to cells that were stimulated with anti-CD3, anti-CD28, IL-4, IL-2, anti-IL-12 and anti-IFN. One likely possibility is that the mice were infected as mentioned earlier and hence the reason why the cells from these mice were skewed towards a typical Th2 response although they have not been exposed to any skewing conditions. Neither can we explain why cells not stimulated with skewing medium had a higher percentage of IL-4 producing cells compared to cells that were stimulated with Th2 skewing medium.

Adoptive transfer of these Th2 polarised cells did not yield a typical Th2 response in naïve mice after an antigen challenge as others have reported (data not shown) [25] hence we switched to using OT-II mice which express TCRs specific for $OVA_{323-339}$ and polarising them using a slightly modified protocol [61] that yields well polarised

Th2 cells (Figure 17). This we felt would be a better model which mimics what happens during OVA sensitisation and challenge, where antigen specific Th2 cells are the main culprit that induces the typical airway inflammation after an antigen challenge. In the earlier polarisation model, we were basically polarising T cells that were not necessarily specific for the OVA and hence the reason why we were not able to get the typical Th2 response in mice that were adoptively transferred with these polarised cells after the challenge.

4.3 Adoptive transfer model and passive sensitisation model

With the adoptive transfer protocol established, we looked at our passive sensitisation model. Gelfand *et al*, passively immunised BALBc mice with antigen-specific IgE which were later aerosolly challenged with OVA. Not only did the mice have increased AHR but also increased numbers of eosinophils in BAL, in cells extracted from the lungs, and in the peribronchial areas [51]. In our experiments, the mice did not respond in a similar manner. There are several reasons that could have contributed to the differences in the results observed. One of the reasons could be due to the strain of mouse we had used. In their experiments, they had used BALBc and BALBc could be more susceptible to passive sensitisation [65] as they are a strain known to be more susceptible to Th2-mediated responses. In our study we had used C57BL6 mice and they have been shown previously to be less susceptible to passive sensitisation [66]. The second reason could be that they had used an in-house produced mouse anti-OVA IgE, whereas we had used a commercially available mouse anti-OVA IgE. This monoclonal antibody had worked very well as a standard for the OVA-specific IgE ELISA that we had developed for this study however when we had tried to use it as a

positive control in our PCA assays, it did not function as we had expected it to. We have now come to conclude that the binding affinity of this mAb is very low *in vivo*.

As mentioned in the introduction, this study was done to test the hypothesis that IgE might have a role to play in acute airway inflammation together with the help T cells and thus our experiment of passively sensitising the animals as well as transferring antigen-specific Th2 cells a day before they were challenged. From our FACS data, we have shown that IgE does play a role in airway inflammation as the group of animals that were passively sensitised had a almost twice number of T cells recruited into the lungs than in the unsensitized animals.

4.4 Summary and future directions

Even though there were difficulties establishing a proper IgE response in immunised mice, we were finally able to evoke a good IgE response when we had brought in mice from Harlan-Olac and bred them in our own facility. These mice not only had low background IgE levels but they were also making appropriate OVA-specific IgG1 responses. IgE responses in animals are highly variable as shown by Holmes, BJ (PhD thesis, 1998) but we were able to induce an immune response that was consistent throughout this study. During the course of this study, we have also shown that the best stimulus to induce proliferation of T cells is the anti-CD3 and anti-CD28. We have also developed protocols for non-antigenic and antigenic-polarisation of CD4+ T cells and finally we have shown that adoptive transfer of Th2 cells into naïve animals can induce Th2-like responses after an antigenic challenge.

In this study, we have shown some evidence that antigen-specific IgE may play a role in acute asthma. However there are a few areas of this project that needs to be addressed before we can come to any firm conclusions. One of these, would be the establishment of antigen-specific Th2 clones. During the course of this study, isolating a highly purified population of naïve CD4+ T cells was a difficult task. Hence the reason why we had used CD8+ depleted cell population for our antigenic stimulation experiments. It was only recently, when we were able to isolate lymph nodes from animals were we able to get a better purity levels. Moreover, in our polarising medium we did not add anti-IL-10 as a safeguard against Tregs and this is one area that needs to be looked at because a study done by Cousins DJ *et al* showed that IL-10 is prevalent during the early stages of Th2 differentiation and IL-10 is now being recognised as a Treg promoting cytokine [67]. Hence to prevent the Tregs from outgrowing the Th2 cells, it would be a good idea to have some anti-IL-10 in the medium.

The other area that needs to be addressed would be that of passive sensitisation with the commercially available anti-OVA IgE. To ensure that a monoclonal antibody was functioning *in vivo* and that we were using an optimal dose, we need to develop an assay whereby we are able to check for immediate cutaneous hypersensitivity reactions after passively sensitising the mice with anti-OVA IgE. Besides looking for eosinophil infiltration of the airways, we could also look at AHR to methacholine because AHR has been shown to develop without the help of antigen specific CD4+ T cells [51]. Lastly, this study focused on an animal strain that is known to be a low Th2 responder. We could also look at the role of IgE in an acute asthma model of a high Th2 responder like BALBc. Finally, if IgE does not play a role in airway

inflammation perhaps IgG might. Katz *et al* has shown that cultured mast cells can be triggered to degranulate by IgG hence it is possible that OVA-specific IgG could play some role in airway inflammation [68].

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APPENDIX

1-, 5-ml syringes	BD
2-mercaptoethanol	Sigma
25-, 26- and 27-G needles	BD
70µm nylon filters	BD
Allophycocyanin (APC)-conjugated rat IgG1, k isotype control immunoglobulin (554686)	BD
Aluminium hydroxide gel (A8222)	Sigma
Ammonium chloride (A382345)	Sigma
Anti-CD4+ (L3T4) micro beads	Miltenyi Biotec
Anti-CD8+ (Ly-2) micro beads	Miltenyi Biotec
Anti-CD90 (Thy1.2) micro beads	Miltenyi Biotec
Anti-mouse IgE (LO-ME-3)	Serotec
APC-conjugated rat anti-mouse IL-4 monoclonal antibody (554436)	BD
APC-conjugated rat anti-mouse IL-5 monoclonal antibody (554396)	BD
Avidin-alkaline phosphatase (A7294)	Sigma
Diethanolamine (D8885)	Sigma
Diff-Quik Reagents	Dade Behring
EDTA (E6758)	Sigma
Evan’s blue dye	Sigma
Ficoll-Hypaque	GE-Healthcare
FITC-conjugated rat anti-mouse IFN-? monoclonal antibody (554411)	BD
FITC-conjugated rat IgG1 isotype control immunoglobulin (554684)	BD
FITC-conjugated rat IgG2b, k monoclonal immunoglobulin isotype control (556923)	BD
Fluorescein Isothiocyanate (FITC)-conjugated rat anti-mouse CD3 molecular complex monoclonal antibody (555274)	BD
Foetal calf serum (FCS)	Hyclone
IgE mouse ELISA kit (555248)	BD

Imject alum (77161)	Pierce
Incomplete Freund's adjuvant (F5506)	Sigma
Ionomycin calcium salt (I0634)	Sigma
$KAl(SO_4)_2.12H_2O$ (A7210)	Sigma
$KHCO_3$	Sigma
LS+, MS+ ferrous columns	Miltenyi Biotec
MACS Multi-stand	Miltenyi Biotec
$MgCl_2.5H_2O$	Sigma
Micro titre plates (Maxisorb)	Nunc
Midi MACS Separation Unit	Miltenyi Biotec
Monensin	Sigma
Monoclonal anti-chicken egg albumin clone OVA-14 (A6075)	BD
Mouse anti-OVA IgE (MCA2259)	Serotec
Mouse IFN-? ELISA kit (DY485)	RnD Systems
Mouse IL-13 ELISA kit (DY413)	RnD Systems
Mouse IL-2 ELISA kit (DY402)	RnD Systems
Mouse IL-4 ELISA kit (DY404)	RnD Systems
Mouse IL-5 ELISA kit (DY405)	RnD Systems
$NaHCO_3$	Sigma
NH_4Cl	Sigma
NaN_3	Sigma
Non-essential amino acids	Sigma
$OVA_{323-339}$	Anaspec
p-Nitrophenyl phosphate (pNpp) tablets (N2765)	Sigma
Pacific Blue (PB)-conjugated rat anti-mouse CD4+ (L3T4) (558107)	BD
Paraformaldehyde (P6148)	Sigma
PB-conjugated rat anti-mouse CD8+a (Ly-2) (558106)	BD
PB-conjugated rat IgG2a, k monoclonal immunoglobulin isotype control (558109)	BD
PBS (10x)	1^{st} Base
Penicillin-Streptomycin	Sigma
Phorbol 12-myristate 13-acetate (PMA) (P1585)	Sigma

Purified bovine serum albumin (grade V)	Sigma
Purified hamster anti-mouse CD28 monoclonal antibody (553294)	BD
Purified hamster anti-mouse CD3e monoclonal antibody (553057)	BD
Purified ovalbumin (grade V) (A550)	Sigma
Purified rat anti-mouse IFN-? monoclonal antibody (554408)	BD
Purified rat anti-mouse IL-12 (p40/p70) monoclonal antibody (554475)	BD
Purified rat anti-mouse IL-4 monoclonal antibody (554432)	BD
R-PE-conjugated rat anti-mouse IL-4 monoclonal antibody (554389)	BD
R-PE-conjugated rat anti-mouse IL-5 monoclonal antibody (554395)	BD
R-PE-conjugated rat IgG2a, k monoclonal immunoglobulin isotype control (553930)	BD
R-PE-conjugated rat IgG2b, k isotype control immunoglobulin (556925)	BD
R-Phycoerythrin (R-PE)–conjugated rat anti-mouse CD4+ (L3T4) monoclonal antibody (557308)	BD
R-Phycoerythrin (R-PE)–conjugated rat anti-mouse CD8+a (Ly-2) monoclonal antibody (553032)	BD
Rat anti-mouse IgG1Heavy chain: Biotin (LO-MG1-2)	Serotec
Recombinant mouse IL-12p70 (554592)	BD
Recombinant mouse IL-2 (550069)	BD
Recombinant mouse IL-4 (550067)	BD
RPMI 1640	Gibco
Saponin (47036)	Sigma
Sodium pyruvate	Sigma
TMB Substrate Reagent Set	BD
TMB Substrate reagent set (555214)	BD
Trypan blue dye	Sigma
Tween 20	Sigma

1x PBS

Dilute 100ml of 10x PBS in distilled water. Adjust pH to 7.2 with 1M HCl or 1M NaOH and top it up to 1L. Sterile filter or autoclave the solution if it is to be used for sterile purposes. Store buffer at room temperature.

ELISA wash buffer

Dilute 100ml of 10x PBS in distilled water. Add 0.5ml of Tween 20. Adjust pH to 7.4 with 1M NaOH or 1M HCl. Top it up to 1L and store solution at 4°C.

Blocking buffer

1% BSA in 1x PBS.

Reagent diluent

0.1% BSA, 0.05% Tween 20 in 1x PBS. Adjust pH to 7.4.

Staining/FACS buffer

1% FCS and 5mM EDTA in 1x PBS. Adjust pH to 7.4

Perm buffer

1% FCS, 5mM EDTA and 0.1% saponin in 1x PBS.

4% PFA.

Dilute 4g of Paraformaldehyde (PFA) in 90ml of 1x PBS. Adjust pH to 7.2 and top up to 100ml with PBS.

1% PFA

Dilute 1g of Paraformaldehyde (PFA) in 90ml of 1x PBS. Adjust pH to 7.2 and top up to 100ml with PBS.

Diethanolamine buffer, pH 9.8, 0.05M

101 mg of $MgCl_2.5H_2O$ was dissolved in 800ml of distilled water and 97ml of diethanolamine was added and mixed thoroughly. pH was adjusted with concentrated HCl to 9.8 and mixture was made up to 1L. NaN_3 was added and solution was stored at 4°C in the dark.

Culture medium

RPMI-1640 medium or DMEM

Heat inactivated FCS	10% (v/v)
Non-essential amino acids	1% (v/v)
2-Mercaptoethanol	5µM
Streptomycin	100µg/ml
Penicillin	100 IU/ml

RBC lysis buffer

Dissolve 8.26 g ammonium chloride (NH_4Cl), 1 g potassium bicarbonate ($KHCO_3$) and 0.037 g EDTA in 1 liter ddH2O. Mix well and autoclave. Store up to 6 months at 4°C. RBC lysis buffer should always be used at room temperature.