THE ROLE OF VIRAL AND BACTERIAL INFECTIONS IN ASTHMA EXACERBATIONS AND CORTICOSTEROID RESISTANCE

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DOCTOR OF PHILOSOPHY

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SUMMARY

Asthma is a chronic inflammatory disease of the airways characterised by early and late asthmatic responses (EAR & LAR) to allergen, airways hyperresponsiveness (AHR) to inhaled spasmogens, airway inflammation and airway oedema. Viral infections and lipopolysaccharide (LPS) from bacteria and environmental sources contribute to exacerbations of asthma and the development of insensitivity to corticosteroids. Complete insensitivity to oral corticosteroids is rare and most patients lie on a continuum of steroid responsiveness. This thesis aimed to examine the effect of viral infection and LPS in a guinea-pig model of asthma and determine the sensitivity to inhaled and systemic corticosteroids.

Sensitised guinea-pigs challenged with ovalbumin displayed EAR, LAR, AHR to histamine, airways inflammation and airway oedema. Inoculation of guinea-pigs with parainfluenza-3 virus alone induced AHR to histamine and airway inflammation. However this response was not consistent. Inhaled LPS alone induced an immediate bronchoconstriction, AHR, airway inflammation and oedema and goblet cell hyperplasia. LPS co-administered with ovalbumin exacerbated the allergen response by lengthening the EAR, prolonging the bronchoconstrictor response to histamine, increasing airway inflammation and oedema and goblet cell hyperplasia.

In guinea-pigs challenged with ovalbumin alone, treatment with inhaled fluticasone propionate (FP) and inhaled and systemic dexamethasone decreased the LAR, abolished AHR, airway inflammation and oedema. Responses to LPS alone were not reduced by inhaled dexamethasone or FP but partially reduced by systemic dexamethasone. Ovalbumin and LPS combined responses were insensitive to inhaled corticosteroids, except lavage fluid protein. These responses were partially sensitive to systemic dexamethasone, with the prolonged EAR, inflammation and airway oedema all reduced.

The data in this thesis suggests that LPS inhalation exacerbates ovalbumin-induced functional and inflammatory responses rendering them insensitive to inhaled corticosteroids but partially sensitive to systemic corticosteroids. Thus, the experimental combination of ovalbumin with LPS might represent a useful preclinical model of corticosteroid-insensitive airway inflammation.

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CHAPTER 1 General Introduction

Chapter 1

1.1 Definition

The current operational definition of asthma by the National Asthma Education and Prevention Program Expert Panel Report 3 (2007) is

"Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role: in particular, mast cells, eosinophils, neutrophils (especially in sudden onset, fatal exacerbations, occupational asthma, and patients who smoke), T lymphocytes, macrophages, and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of coughing (particularly at night or early in the morning), wheezing, breathlessness, and chest tightness. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment" from Busse, (2007)

Maintaining a precise definition that includes all aspects of the disease remains problematic. One particular problem in asthma's definition stems from the overlap between symptoms of other respiratory conditions such as COPD. In particular, airways hyperresponsiveness is one of the key characteristics of asthma but is absent in some patients, despite clear evidence of other asthma symptoms. It is also present in some individuals without other significant respiratory symptoms (Pattemore *et al*, 1990). There are also wide differences in the reversibility of airway obstruction, especially in asthmatics that smoke or work in polluted environments (Barnes *et al*, 1998). Accordingly, diagnosis is not always clear cut and the re-assessment of symptom histories can lead to re-classification of diagnosis from asthma.

1.2 Prevalence

Asthma has seen a growing diagnosis/prevalence over the last 50 years, especially in the western world, with an estimated 300 million people worldwide now suffering from the affliction (ISAAC, 1998). Approximately 180,000 deaths across the world annually are asthma related. In the UK alone an estimated 5.1 million people have asthma costing

the NHS £2.5 billion (Holgate *et al*, 2008). The exact reasons for increased incidence of asthma are unknown but factors such as genetic background, early life infection and the adoption of a western lifestyle are all implicated. More recently, disturbances in the bacterial community of the lungs have been implicated (Hilty *et al*, 2009).

1.3 ASTHMA SUBTYPES

Both the environment and genetic factors are likely to lead to the creation of a phenotypically heterogeneous disease with numerous aetiologies (Barnes *et al*, 1998). Many forms of asthma such as childhood asthma, aspirin sensitive asthma, chronic respiratory infection related asthma, occupational asthma and severe asthma have been described in the past but often overlap in definition and do not consider more than one symptom. More recent attempts to classify asthma subtypes have used multiple factors to designate subgroups (Moore *et al*, 2008).

The first distinction between asthma subtypes can be made based on the presence of atopy. 70% of asthmatics are atopic and demonstrate increased circulating levels of IgE antibodies to specific allergens; although these allergens may not be the driving force behind the condition in all cases. The other 30% of asthmatics are non-atopic, with an onset commonly in the forties and an association with intolerance to non-steroidal anti-inflammatory drugs (Botturi *et al*, 2011). Further distinctions can be made on the basis of independent factors including clinical markers (Body mass index and age of onset), lung function (Forced expiratory volume in 1 second, FEV_1), inflammatory markers (eosinophils and neutrophils) and treatment use (frequency and type) (Rosi *et al*, 1999; Moore *et al*, 2008). Severe asthmatics are one such subgroup and demonstrate high numbers of eosinophils, more FEV_1 variability and a higher risk of tracheal intubation during exacerbation. The presence of high neutrophil counts and increased resistance to steroid therapy is also observed in some individuals (Leung & Bloom, 2003).

The term ‘asthma’ in the context of this thesis will refer to atopic, IgE-related asthma. Eosinophil dominant inflammation is also a key characteristic but not an exclusive term as the presence of neutrophils is often observed in severe asthma, particularly at times of exacerbation (in't Veen *et al*, 1999). IgE-mediated asthma is characterised by hypersensitivity to allergens such as pollen and house dust mites, and in experimental animals to ovalbumin from egg whites. Hypersensitivity to an allergen results from initial sensitisation and subsequent allergen re-encounter which triggers the effector phase.

1.4 Asthma pathology

1.4.1 Allergen sensitisation

In asthma, the sensitisation process begins with the inhalation of allergen and its active uptake by antigen presenting cells (APCs) such as macrophages and dendritic cells. The antigen is internalised, then proteolysed into peptide for incorporation into major histocompatibility complex (MHC) class II at the cell membrane. Following this, APCs migrate to local lymph nodes or lymph mucosa and present antigen to T and B lymphocytes (Lambrecht & Hammad, 2011). Depending on the presence of certain co-stimulatory molecules such as OX40L, these cells then become active or undergo apoptosis. T lymphocyte differentiation requires IL-2. Whether lymphocytes become the Th1 or Th2 type is dependent on the presence of IL-12 or IL-4 respectively. In allergen sensitisation Th2 cells predominate (Galli *et al*, 2008). These cells secrete IL-4 and IL-13, which drive immunoglobulin class switching to IgE (Geha *et al*, 2003). IL-4 is also able to increase the expression of the high affinity IgE receptor (FcεRI) on mast cells and basophils. Monovalent binding of IgE to the high affinity IgE receptor (FcεRI) results in receptor priming for degranulation upon further exposure to the same allergen (Gould & Sutton, 2008).

1.4.1.1 ENHANCEMENT OF SENSITISATION

Several factors contribute to an enhancement of allergic sensitisation. The first is the breakdown of the airway epithelial barrier which in asthmatics has leaky tight junctions which facilitate penetration of allergens (Xiao *et al*, 2011). This problem is further augmented by intrinsic properties of allergens such as house dust mite and cockroach extract which can disrupt the epithelial barrier and trigger the release of danger signals. Pollutants, viruses, ozone, LPS and tobacco smoke can also augment allergen sensitisation. The second factor is the enhancement of dendritic cell maturation and activation. Pattern recognition receptors on dendritic cells recognise pathogen associated molecular patterns (PAMPs) such as those on LPS and single stranded (ss)RNA. Danger associated molecular patterns (DAMPs) such as those on ATP are also recognized. These enhance dendritic cell maturation, allergen sampling and the production of co-stimulatory molecules via mediators such as TSLP, IL-33 and IL-25, which are secreted by airway epithelial cells in response to pathogens (Holgate, 2012).

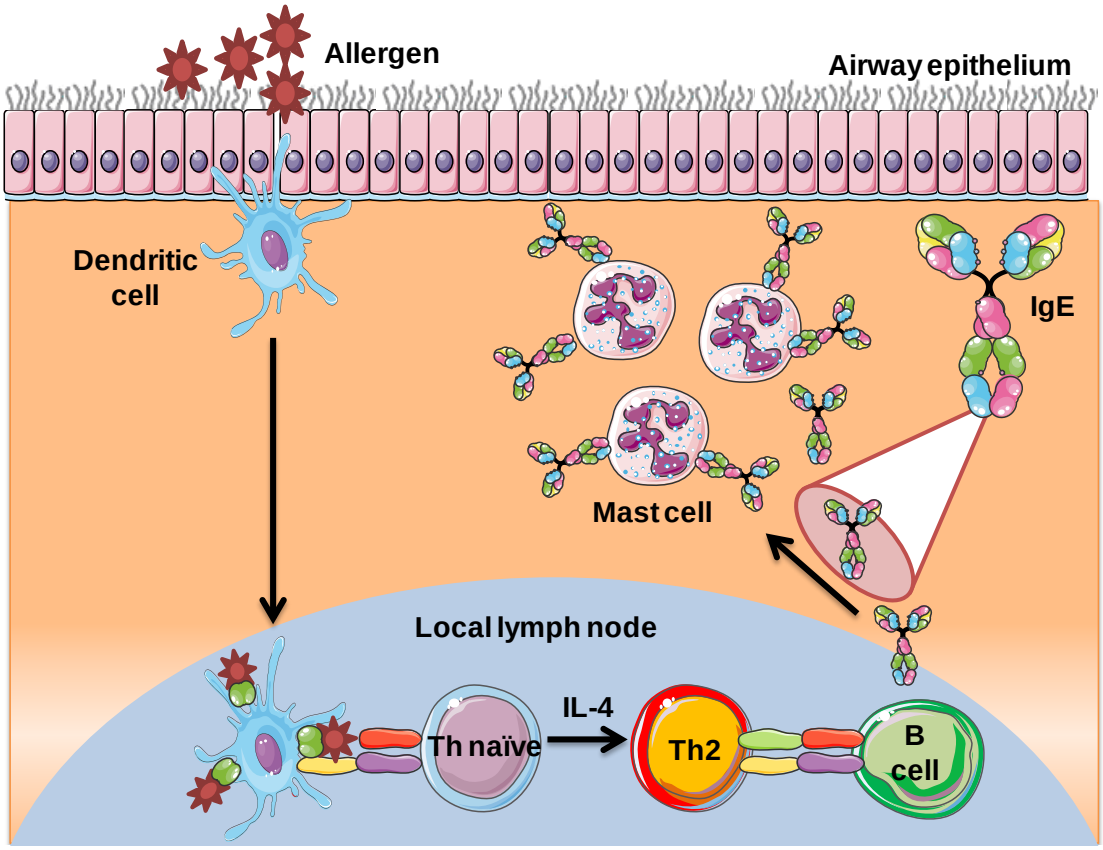


Figure 1: Simplified diagram of allergic sensitisation in the airways. Antigen presenting cells such as dendritic cells capture allergen, internalise it and process it to the cell surface. This along with co-stimulatory molecules promotes the differentiation of T helper (Th) cells into Th2 cells which stimulate the production of immonoglubulin E (IgE) from B lymphocytes. IgE binds to the surface of mast cells in the airways. Figure adapted and redrawn from Galli *et al*, 2008.

1.4.2 Effector phase

The effector phase results from re-exposure to an allergen to which an individual is sensitised. This phase can be divided into 2 distinct categories; that of the early asthmatic response (EAR) and the late asthmatic response (LAR). The EAR occurs within minutes of allergen exposure and is mediated by IgE crosslinking on the surface of mast cells (Gould & Sutton, 2008). Crosslinking triggers a complex intracellular cascade of signalling molecules leading to mast cell degranulation and the secretion of three classes of active products. The first set of mast cell products are those stored in cytoplasmic granules prior to degranulation and include biogenic amines (e.g.

histamine), serglycin proteoglycans (e.g. heparin and chondroitin sulphate), serine proteases (e.g. chymases, trypases and carboxypeptidases) and other granule associated products like TNF-α and vascular endothelial growth factor A (VEGFA). The second class of mast cell product are lipid mediators and include prostaglandins, leukotrienes, cysteinyl leukotrienes and platelet activating factor (PAF). The third class of mast cell products is cytokines, chemokines and the growth factors (Bradding *et al*, 2006). The 1st two classes of product act within the duration of the EAR and are responsible for physiological changes including vasodilation, increased vascular permeability (causing tissue swelling), contraction of bronchial smooth muscle (causing airflow obstruction) and increased mucus secretion (contributing to airflow obstruction in the lower airways). Sneezing, coughing and itching can also be evoked by stimulation of nociceptors on sensory nerves. These effects are shown in Figure 2.

The LAR develops 6-9 hours after allergen exposure. This phase is characterised by the recruitment, activation and migration of eosinophils, lymphocytes, neutrophils and macrophages into the lamina propria, epithelium and the airway lumen (Galli *et al*, 2008). The cytokines, chemokines and growth factors produced during the EAR drive this process and allow for further release of mediators from these cells and further bronchoconstriction.

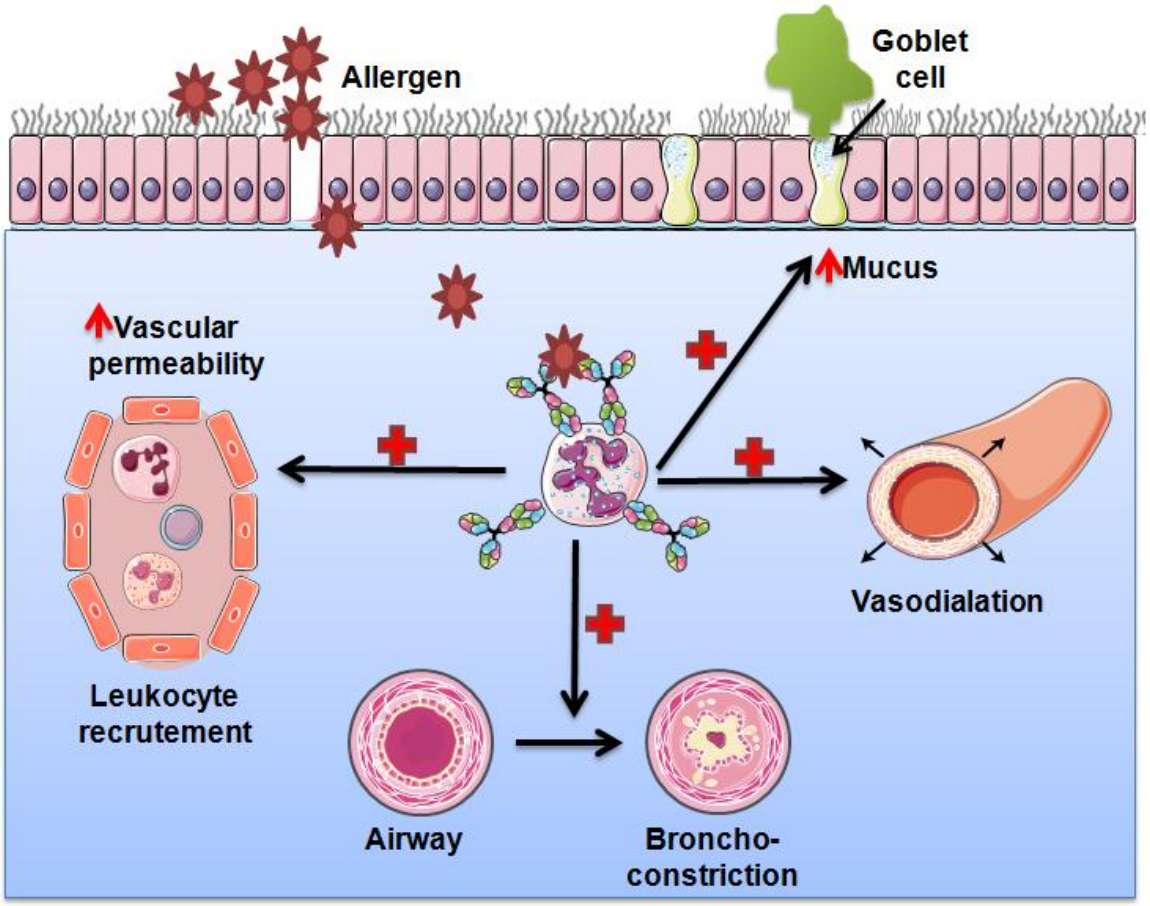


Figure 2: Diagram of the main processes during the early asthmatic response (EAR) evoked by an allergen. Allergen enters the airway and binds with high affinity IgE receptors on mast cells resulting in degranulation. Mast cell degranulation products increased leukocyte recruitment, increased vascular permeability, vasodilatation, bronchoconstriction and increased mucus production Based on Galli *et al*, 2008.

1.4.3 Inflammation

Inflammation of the lungs is one of the key hallmarks of asthma and is observed across the entire spectrum of disease severity (Laitinen *et al*, 1996). The extent of inflammation has also been shown to correlate with disease severity, suggesting inflammation may be important in the pathogenesis of the disease (Barnes, 1996). The relationship between inflammation and the clinical symptoms of asthma is complex, with inflammation likely contributing to them both directly and indirectly. The inflammatory infiltrate consists of

numerous cell types both from the innate and adaptive branches of the immune system. Discussed below are the aspects of the various cell types.

1.4.3.1 EOSINOPHILS

Eosinophils are the predominant cell type during the late asthmatic response to allergens, with their numbers peaking 24 hours after allergen challenge (Smith & Broadley, 2007; Toward & Broadley, 2004). Eosinophils are found in the lungs of those even with mild asthma. An association between eosinophil activation and asthma severity and bronchial responsiveness exists (Bousquet *et al*, 1990; Bradley *et al*, 1991). Eosinophils release many biological products including eicosanoids, oxygen free radicals, Th2 cytokines, growth factors and toxic granule products which contribute to contraction of smooth muscle, airways hyperresponsiveness (AHR) and increased vascular permeability (Bousquet *et al*, 2000). Granule products such as major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN) contribute directly to the development of AHR and epithelial shredding (Gleich *et al*, 1993). Other products such as growth factors, metalloproteases and elastase are involved in fibrosis and tissue remodelling (Bousquet *et al*, 2000).

1.4.3.2 MAST CELLS

Mast cells play a key role in both sensitisation to an allergen and also in the effector phase of asthma. Mast cell numbers are 2-6 times higher in the bronchial lavage fluid of asthmatics compared to non-asthmatics (Hamid *et al*, 2003). Mast cells are commonly found in their degranulated state in both stable asthma and following allergen exposure (Bousquet *et al*, 2000). Their cytoplasmic granules contain products including tryptase, prostaglandin (PG)-D2, histamine, cysteinyl leukotrienes (Cys-LT), which when released can elicit bronchoconstriction, mucus secretion, and oedema (Broide *et al*, 1991). Other products such as chymase may contribute to increased collagen deposition (Kofford *et al*, 1997). Mast cells also produce heparin, which has biological activities including modulation of wound healing and cell proliferation and differentiation (Bousquet *et al*,

2000). Heparin is also thought to have an anti-inflammatory role by binding and inhibiting chemokines; in addition to inhibiting the effects of several eosinophil-specific granule proteins such as major basic protein (MBP) (Diamant and Page, 2000).

1.4.3.3 MACROPHAGES

Macrophages are derived from monocytes and are the most commonly found inflammatory cell type in asthma (Hamid *et al*, 2003). Macrophages seem to have a dual role in asthma in both increasing and decreasing inflammation. Classical activation of macrophages by cytokines such as interferon-γ (IFN-γ), IL-1β, IL-6, IL-12 and tumour necrosis factor (TNF)-α lead to differentiation into M1 macrophages, which are efficient at phagocytosis and pathogen clearance. They also release cytokines, growth factors and chemoattractants directly and indirectly via epithelial cells and fibroblasts. Factors such as CCL2 (MCP-1) and CCL5 (RANTES) attract eosinophils and further macrophages (Barnes, 1996). In the presence of Th2 cytokines such as IL-4, macrophages can differentiate into M2 macrophages with anti-inflammatory actions such as IL-10 production. Macrophages can also suppress inflammation by decreasing lymphocyte activity. However, this effect may be impaired following allergen exposure (Hamid *et al*, 2003). M2 macrophages can also become a source of IL-13 and worsen asthma increasing AHR and mucus production (Byers & Holtzman, 2011).

1.4.3.4 NEUTROPHILS

Neutrophils are important in severe cases of asthma along with asthma exacerbations where their numbers are significantly elevated compared to milder forms of the disease. (Taha *et al*, 2001; Douwes *et al*, 2002). Neutrophils are also implicated in the pathology of steroid insensitive asthma (Monteseirín, 2009). Neutrophils may also play a role in milder forms of asthma, having been implicated in the early asthmatic response. In patients that died within 2 hours of an asthma attack, neutrophil numbers were found to be significantly elevated as compared to individuals that died from slow-onset fatal asthma (Sur *et al*, 1993). Neutrophil numbers are also found raised in animal models,

peaking only 2 hours after allergen challenge (Toward & Broadley, 2004). Neutrophils are able to produce a wide range of substances including metalloproteinases, elastase, lactoferrin, myeloperoxidase and reactive oxygen species (ROS). These have the capability to increase inflammation and increase the extent of tissue damage (Monteseirín, 2009).

1.4.3.5 LYMPHOCYTES

Lymphocytes play an indispensable role in the asthmatic inflammatory response. B lymphocyte's main role is in the secretion of IgE antibodies. T lymphocytes can be broadly divided on the presence of the cell surface marker CD4 or CD8. CD8+ cells are generally cytotoxic (T_C) cells and are more rarely found in asthmatics. CD4+ cells are normally T helper (Th) cells and the predominant T lymphocyte type observed in asthma (Corrigan *et al*, 1995). The activation of CD4+ lymphocytes has been shown to correlate with eosinophil number and bronchial responsiveness (Robinson *et al*, 1993). Several subtypes of Th cells exist including Th1, Th2, Th17, and Th9. These cells differ in their cell surface markers, cytokine secretion pattern and role in the disease. Classically Th2 cells, secreting IL-4, IL-5 and IL-13 have been identified as the main Th cell type in the mediation of allergic airway inflammation (Galli *et al*, 2008). T regulatory (Treg) cells may be important in suppressing the persistent inflammation. Other innate-like T cells such as the natural killer and γδ cells may also have roles. A detailed analysis of specific lymphocyte sub-populations is beyond the scope of this thesis. Table 1 contains an overview of the main roles of T lymphocytes in asthma and their cytokine mediators.

Cell Type	Cytokines secreted	Role in Asthma
CD8+ T cell	IFN-γ, IL-4, IL-13	AHR, eosinophilia, viral exacerbation?
Th1 cell	IFN-γ	Exacerbations?
Th2 cell	IL-4, IL-5, IL-13	IgE production, eosinophilia, AHR, mucus
Th17 cell	IL-17	Neutrophilia, steroid insensitivity?
Th9 cell	IL-9	IgE production, mucus, mast cell recruitment
T-reg cell	IL-10, TGF-β	Suppression of inflammatory response
Natural killer cell	IFN-γ, IL-13	eosinophilia, AHR, IgE production,
γδ T cell	IL-4, IL-17	Subtypes respectively suppress and promote AHR

Table 1: An overview of the different T lymphocyte sub-populations, their role in asthma and cytokine mediators secreted.

1.4.3.6 Inflammatory cytokines

Cytokines, chemokines and other inflammatory mediators play a central role in coordinating the actions of inflammatory cells and promoting the functional changes seen in asthma. Table 2 shows some of the main mediators involved in asthma. Of particular importance are the Th2 cytokines, IL-4, IL-5 and IL-13, cytokines triggered by toll like receptor signaling such as TNF-α and IL-8. Th17 cytokines such as IL-17 are also important and have been implicated in steroid insensitivity.

Cytokine	Effect in asthma	Mechanism
T helper cell cytokines (Th2, Th9, Th1, Th17)		
IL-4	↑ disease	↑ IgE, Th2 cells
IL-5	↑ disease	↑ eosinophils
IL-9	↑ disease	↑ mast cells
IL-13	↑ disease	↑ IgE, AHR
IL-12	↓ disease	↑ Th1 cells
IL-17	↑ disease	↑ neutrophils
IL-18	↑ disease	↑ IFN-γ release
IFN-γ	↓ disease	↓ Th2 cells
Pro-inflammatory and innate cytokines		
IL-1β, IL-6	↑ disease	↑ inflammation
TNF-α	↑ severe disease	↑ inflammation
TSLP, IL-25, IL-33	↑ disease	↑ Th2 cells, dendritic cell maturation
Growth factors and anti-inflammatory		
EGF	↑ disease	↑ mucus secretion
GM-CSF	↑ disease	↑ eosinophils, neutrophils
TGF-β	↑ severe disease	↑ fibrosis, ↓ inflammation
IL-10	↓ disease	↓ inflammation

Table 2: Table showing the some of the main inflammatory cytokines involved in asthma. IL = Interleukin; IFN = Interferon; EGF = TNF = Tumour necrosis factor; TSLP = Thymic stromal lumphopoietin; Epidermal growth factor; GM-CSF = Granulocyte-macrophage colony stimulating factor; TGF = Transforming growth factor. Adapted from Barnes, 2008

1.4.4 AIRWAY HYPERRESPONSIVENESS

Airway hyperresponsiveness (AHR) is an increased response to a bronchoconstrictor stimulus of natural or pharmacological origin. AHR is an essential component of asthma and is a requirement for diagnosis. However, the presence alone of AHR is not an indicator of asthma as it can also be present in asymptomatic people and sufferers of other respiratory diseases like COPD (Woolcock *et al*, 1987). The AHR seen in asthmatics can be episodic, persistent or both (Cockcroft & Davis, 2006). On this basis it has been suggested that AHR can be divided into 2 subtypes, persistent and variable. Persistent AHR which is thought to be associated with airway remodelling is often seen in chronic asthma. Variable AHR is more commonly associated with acute inflammation.

Bronchoconstrictive agents can be divided into those that act directly or indirectly on airway smooth muscle. Direct stimuli include histamine and methacholine which act on H_1 and muscarnic receptors respectively and are used clinically for diagnosis. Other direct acting stimuli not used clinically, include cysteinyl leukotrienes, thromboxanes, and prostaglandins (O'Byrne *et al*, 2009). Indirect stimuli include adenosine monophosphate (AMP) and allergens, but also stimuli that alter the physical environment including cold air, exercise, hypertonic saline, mannitol and voluntary hyperventilation. These agents cause the release of bronchoconstrictor mediators from inflammatory cells, which then act on the smooth muscle (O'Byrne *et al*, 2009). Clinically, AHR is measured using bronchial provocation tests such as FEV_1 following the inhalation of a direct stimulus like methacholine. Both asthmatics and non-asthmatics are able to respond to these stimuli, although the dose required in asthmatics is far lower. AHR is expressed as provocative dose (PD) of the stimuli causing a 20% reduction in FEV_1.

AHR constitutes two distinct components: airway hypersensitivity and airway hyperreactivity and increased maximum response (Figure 3). Airway hypersensitivity is a decrease in the threshold dose of the bronchoconstrictor stimulus required to elicit

bronchoconstriction of the airways. Graphically, this is represented as a leftward shift in the dose-response curve to bronchoconstrictor stimulus from one in non-asthmatics. Airway hyperreactivity is a greater degree of closure in the airways in response to bronchoconstrictor stimuli. Graphically it is represented by an increased gradient in the slope of a dose-response curve (O'Connor *et al*, 1999). An increase in the maximum response is graphically represented as a greater degree of bronchoconstriction before the response plateaus. These changes are more pronounced in severe asthmatics compared to milder forms of the disease. The mechanisms behind these components of AHR may be different. Airway hypersensitivity has been linked to epithelial disruption and changes in the neuronal regulation of the airways. Airway hyperreactivity has been linked to changes in the structure in the lung including smooth muscle hyperplasia (Sterk & Bel, 1989.)

The causes of AHR are still unclear. Variable AHR demonstrates a link between airways inflammation and the LAR (Meijer *et al*, 1999). Many inflammatory cells and mediators have been suggested as being important in the development of AHR. In particular, eosinophils have been implicated. However, treatment with an anti-IL-5 antibody does not reduce AHR despite reducing eosinophilia, suggesting eosinophils are not essential in this process (Lekie *et al*, 2000). Other inflammatory cells such as CD4+ and CD8+ T cells and the cytokine IL-13 have also been investigated for a potential role but a definitive answer as to what in the inflammatory milieu is responsible for AHR is still unknown (Cockcroft & Davis, 2006). On the other hand persistent AHR seems to be more clearly related to the consequences of airway remodelling including increased airway smooth muscle mass (Rennard, 1996).

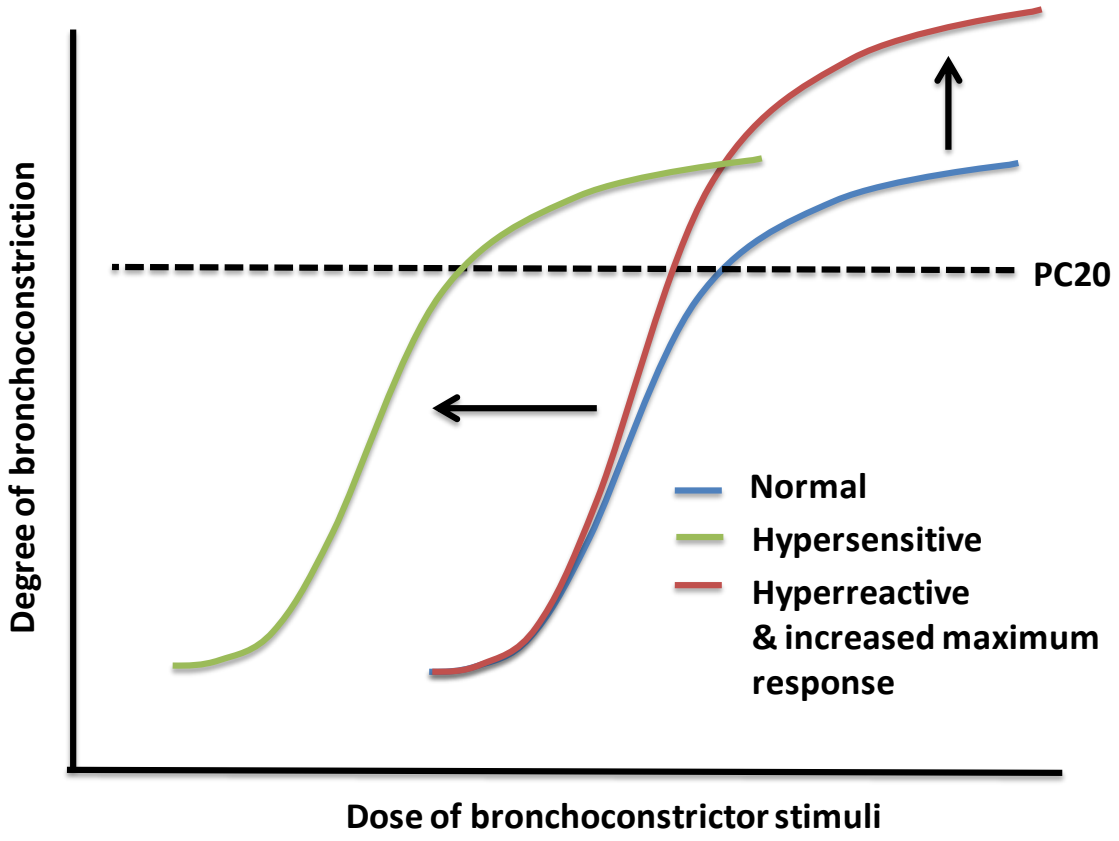


Figure 3: Graphically demonstrates the three components of airways hyperresponsiveness (AHR) to a bronchoconstrictor stimuli. Hyperreactivity (red), hypersensitivity (green), increased maximum response (orange) and normal response (blue). PC20 expressed as the provocative concentration of the bronchoconstrictive agent required to cause a 20% reduction in forced expiratory volume in 1 second (FEV_1). Redrawn from Lotvall *et al*, 1998 and Sterk & Bel, 1989.

1.4.5 Airway remodelling

Airway remodeling is typically seen in chronic cases of asthma and is related to the severity of the disease (Rennard, 1996). More recently, it has been suggested that airway remodelling can occur in parallel and/or may be required for persistent inflammation (Galli *et al*, 2008). Several structural changes are observed in the disease. Increased vascularity and detachment of the epithelium, the latter leads to exposure of epithelial nerves and increase their exposure to irritant stimuli (Sumi & Hamid, 2007; Laitinen *et al*, 1987). Subepithelial fibrosis is also observed as a result of increased collagen and fibronectin deposition in the lamina reticularis (Roche *et al*, 1989). Elastic

fibres, which give the lungs their elastic recoil, are often found fragmented and tangled, although the number of fibres appears unchanged (Bousquet *et al*, 1996). These two changes make the lungs less compliant and can reduce the maximum improvement in lung function with therapy. An increase in smooth muscle mass is also observed in asthmatic airways and can be as much as 2-4 times that seen in normal subjects. This increase is thought to result from both hyperplasia and hypertrophy (Bousquet *et al*, 2000).

Mucus in the lungs is secreted by mucus glands and goblet cells distributed throughout the airways. An increased mucus production is one of the hallmarks of asthma and is attributable to increased goblet cell number, along with hyperplasia of the submucosal glands (Aikawa *et al*, 1992; Bousquet *et al*, 2000). The excessive mucus in asthma can lead to mucus plugging in both the central and peripheral airways and is a frequent contributor to fatal asthma (Andoh *et al*, 1992). A decrease in mucociliary clearance may also contribute to this effect (Pavia *et al*, 1985). The increase in mucus production has been atrributed to cytokines such as IL-4, IL-9 and IL-11, which can increase the expression of the mucus genes, MUC2 and MUC5AC (Temann *et al,* 1997).

1.5 Asthma Exacerbations

Asthma exacerbations are acute or sub-acute episodes of worsening symptoms such as wheezing, chest tightness, cough and shortness of breath. Underlying this is an increase in airflow obstruction due to smooth muscle contraction, mucus obstruction of the airway lumen and oedema (Hogg, 1997). Mucus in particular seems to be prominent, as emphasised by its finding in fatal cases of asthma. Cases of near fatal asthma are often associated with segmental lung collapse due to mucus plugging (Kuyper *et al*, 2003). Clinically, exacerbations can be measured as a further decrease in expiratory airflow by spirometry and peak flow from pre-exacerbation values (Busse, 2007).

Asthma exacerbations can be divided into two types. The first are severe exacerbations, which require urgent action on the part of the patient and physician to prevent a life threatening outcome. The second are moderate exacerbations which are troublesome to the patient, require prompt change in treatment but are not life threatening (Reddel *et al*, 2009). It is the former that lead to hospitalisations and represent a significant economic burden in the developed world. The number of asthma sufferers that have an exacerbation requiring hospital treatment is a relatively small percent of the total population of asthmatics (20%) but accounts for about 80% of the total direct costs (Rodrigo *et al*, 2004). The incidence of asthma exacerbations is similar across all age groups and races, when socioeconomic factors are taken into consideration. The occurrence of exacerbations increases in obese individuals and the presence of female sex hormones (Dougherty & Fahy, 2009).

Asthma exacerbations can become self reinforcing, further increasing the risk of recurrent future exacerbations, independent of socioeconomic factors. (Miller *et al*, 2007). A particular group of patients which have an ‘exacerbation prone’ phenotype has been identified. They are typically characterised by irreversible airflow limitation, psychological dysfunction, chronic sinusitis and intolerance to non-steroidal anti-inflammatory medications (Koga *et al*, 2006). Asthma exacerbations may also have long

term effects on the lung. The exacerbation prone phenotype is associated with an accelerated loss of lung function, over asthmatics with a relatively stable form of the disease (Bai *et al*, 2007). This may be due to the increased inflammatory burden during an exacerbation, in addition to the activation of alternative inflammatory pathways (Holgate, 2007).

1.6.1 CAUSES OF EXACERBATION

Factors that contribute to the exacerbation of asthma include occupational exposures (cleaning products, grains, flours), hormones, drugs (NSAIDs, β-blockers), stress, exercise, air pollutants (diesel particulates) and bacterial infections (chlamydia pneumonia) and high doses of allergens (pollen, dust mites, animal dander). However, the most common cause is viral infection (Nicholson *et al*, 1993). Lipopolysaccharide (LPS) from gram-negative bacteria, which is also found ubiquitously in the environment, is also recognised as an important contributor to asthma exacerbations (Douwes *et al*, 2006).

1.6.1.1 VIRAL EXACERBATIONS OF ASTHMA

Viruses are main cause of asthma exacerbations in both adults and children (Busse *et al*, 2010). The importance of viruses in asthma exacerbations is well demonstrated by the higher rates of asthma mortality during the winter, coinciding with higher rates of influenza at that time of year (McCoy *et al*, 2005). Varying levels of virus detection have been made during asthma exacerbations. Using PCR techniques, viral detection rates in asthma patients have been recorded to be as low as 57%, to as high as 85% (Nicholson *et al*, 1993; Johnston *et al*, 1995). Common viral infections found during exacerbation include rhinovirus (RV), coronaviruses, influenza, parainfluenza and respiratory syncytial virus (RSV) (Atmar *et al*, 1998). The most commonly detected virus is RV; being reported in 65% of cases (Nicholson *et al*, 1993). Parainfluenza viruses (PIV) are also commonly found and thus represent a relevant human pathogen in asthma exacerbations. Viral exacerbations of asthma are described in more detail in chapter 4.

1.6.1.2 LIPOPOLYSACCHARIDE-INDUCED EXACERBATIONS OF ASTHMA

LPS is found in the cell wall of gram-negative bacteria and also ubiquitously in the environment. A range of studies in humans and animal models have demonstrated that LPS also contributes to asthma exacerbations. Correlations between wheezing episodes and LPS exposure in humans have been demonstrated (Douwes *et al*, 2006). Additionally, correlations between the concentration of LPS in the domestic environment and severity rating of asthma exist (Michel *et al*, 1996). In animal models an increase in allergen-induced inflammation and decreased lung function with LPS has been demonstrated (Delayre-Orthez *et al*, 2004; Murakami *et al,* 2006). LPS-induced exacerbations of asthma are described in more detail in chapter 5.

1.6 TREATMENTS

Treatments for asthma can be broadly divided in to those that provide symptomatic relief and those that target the underlying inflammation of the disease. The former include bronchodilators such as β_2 adrenoreceptor agonists, muscarinic receptor antagonists and phosphodiesterase 4 (PDE4) inhibitors which relax airway smooth muscle and reduce bronchoconstriction. Anti-inflammatory therapies indicated in the treatment of asthma include leukotriene antagonists, PDE4 inhibitors, anti-IgE monoclonal antibodies and corticosteroids. The Global strategy for asthma management and prevention (GINA) outlines in its 2008 executive summary the 5 step treatment program indicated in the treatment of asthma across a range of disease severity (Bateman *et al*, 2008) (see Figure 4). The general principles of treatment are the use of a short acting β_2 agonist as a reliever in all patients and a gradual introduction of longer acting β_2 agonists and inhaled corticosteroids if symptomatic control is not achieved.

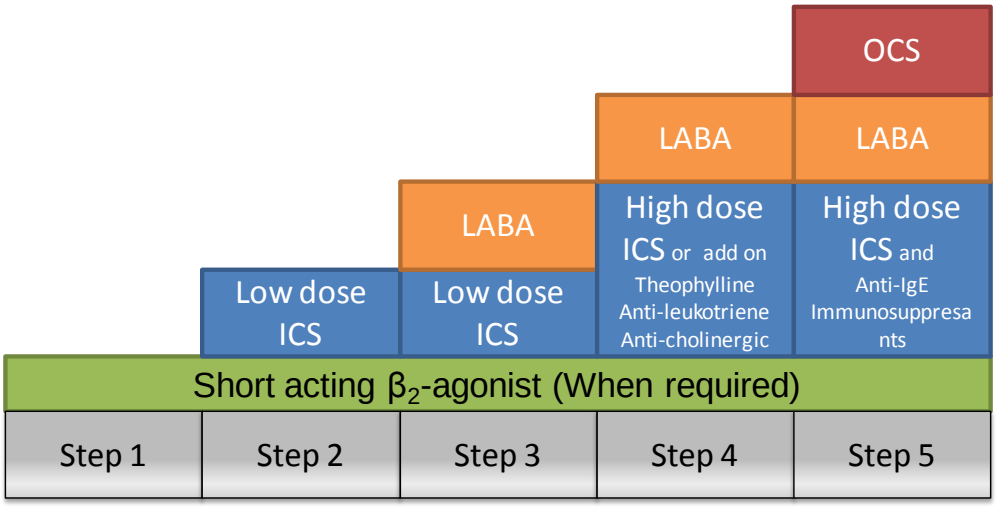


Figure 4: A schematic of the treatment steps taken in the management of asthma. Treatment is taken to the next step if symptomatic control is not achieved. Step 1 introduces short acting β_2 agonists. Step 2 adds a low dose inhaled corticosteroids (ICS). Step 4 adds long acting β_2 agonist (LABA). Step 3 either increases the dose of ICS or adds an additional therapy such as theophylline, anti-leukotriene or anti-cholinergic. Step 5 trials anti-IgE therapy or immunosuppresants and periodic oral corticosteroid (OCS) use. Adapted and redrawn from Bateman *et al*, 2008

1.6.1 B_2 ADRENORECEPTOR AGONISTS

β_2 adrenoreceptor agonists are the frontline bronchodilator used in the treatment of asthma. Both short and long acting β_2 agonists (LABA) are available and differ in their use according to their pharmacological properties. Short acting β_2 agonists such as salbutamol have a short duration of action but work rapidly so are used for symptomatic control when required, especially during exacerbations. Longer acting β_2 agonists such as salmeterol have a longer duration of action and are used in long term control of symptoms. Additionally, several ultra-LABA are currently in development and indicated for once daily dosing. Controversy still exists regarding the safety of long acting β_2 agonists despite recent systematic reviews (Cates *et al*, 2012). Both classes of drug work on the β_2 adrenergic receptor, which is the predominant subtype in the lungs. Binding of the β_2 agonist to its receptor activates intra-cellular adenylate cyclase, which increases the production of cyclic adenosine monophosphate (cAMP). cAMP then acts via a variety of mechanisms to promote relaxation of smooth muscle. In addition, they may be able to prevent the inflammatory cell mediator release, inhibit cholinergic transmission, reduce vascular permeability and increase mucocilliary clearance (Barnes *et al*, 1998).

1.6.2 ANTI-CHOLINERGIC DRUGS

Anti-cholinergic drugs such as atropine have been established as bronchodilators for several centuries but have a wide range of side effects. They work by blocking the actions of the parasympathetic nervous system on the contraction of smooth muscle and mucus production in the airways (Barnes, 1989). More recently drugs which are selective for the muscarinic receptor subtypes of the parasympathetic nervous system have been developed. Ipratropium bromide is a non-selective muscarinic receptor antagonist, blocking the M_1, M_2 and M_3 receptors (Barnes *et al*, 1998). However, the blocking of the M_2 receptor on pre-synaptic cholinergic terminals is undesirable as it would enhance transmitter release and oppose the benefits from blocking the post-

junctional M_3 receptors on airway smooth muscle. M_3 receptor specific antagonists would be of clinical benefit but have been hard to develop (Maesen *et al*, 1993).

1.6.3 Phosphodiesterease (PDE) inhibitors and Theophylline

Phosphodiesterease (PDE) inhibitors such as theophylline have been used to treat asthma for over a century. The PDE4 isoform of the enzyme is found predominantly in inflammatory cells and airway smooth muscle. It is involved in the breakdown of cAMP to the inactive substance adenosine monophosphate (AMP). Inhibition of PDE will cause cAMP levels to increase and the inhibition of inflammatory cells (Boswell-Smith *et al*, 2006).

Clinically, theophylline is able to significantly improve asthmatic symptoms (Evans *et al*, 1997). However, the extent of PDE4 inhibition at therapeutically relevant concentrations is low suggesting an alternative mechanism of action (Polson *et al*, 1978). One alternative mechanism suggested is antagonism of adenosine receptors. Adenosine causes bronchoconstriction in asthmatics by promoting the release of leukotrienes and histamine (Björck *et al*, 1992). These effects are prevented by therapeutic concentrations of theophylline (Cushley *et al*, 1984). Theophylline also seems to have some anti-inflammatory effects such as inhibition of the LAR and the activation of HDAC-2 which decreases pro-inflammatory gene transcription (Pauwels *et al*, 1985; Jaffar *et al*, 1996). The drug may also be able to attenuate inflammatory cell mediator release and lower the oxidative burden of the lung, potentially being useful in treating exacerbations (Nielson *et al*, 1988). The negative side effect profile of theophylline compared to β_2 agonists has given the drug limited usage. More selective PDE4 inhibitors including roflumilast and cilomilast have been approved for use in severe exacerbations of COPD. Clinical studies in asthma have suggested that these drugs may also be beneficial in the treatment of asthma (Boswell-Smith *et al*, 2006).

1.6.4 Cysteinyl leukotrienes

Cysteinyl leukotrienes (Cys-LTs) are arachidonic acid derived inflammatory mediators. Their effects include bronchoconstriction, inflammatory cell recruitment and mucus hypersecretion (Riccioni *et al*, 2007). Montelukast, an anti-cysteinyl leukotriene drug has both bronchodilator and anti-inflammatory effects and works by antagonising the receptor of leukotriene D_4: the Cysteinyl Leukotriene 1 receptor. These receptors are found on mast cells and can prevent release of histamine and development of the EAR (Taylor *et al*, 1991). Montelukast is also able to attenuate symptoms of the late phase such as mucus hypersecretion and eosinophil recruitment (Barnes *et al*, 1998). These drugs have not proved to be as successful as inhaled corticosteroids in corticosteroid-sensitive asthma in controlling symptoms and consequently are used as 3rd line therapy.

1.6.5 Corticosteroids

Corticosteroids continue to be the mainstay treatment in long term control of asthma. They are mainly given as an inhaled preparation due to their decreased systemic side effect profile compared to oral preparations. Oral preparations are reserved for treating severe asthma exacerbations and patients with inhaled corticosteroid insensitivity. They also have wide ranging effects on both functional and inflammatory components of asthma. Corticosteroids are able to suppress numerous inflammatory cell types including eosinophils, lymphocytes and mediators (Underwood *et al*, 1997, Krouwels *et al*, 1996; Barnes *et al*, 1998A). Corticosteroids act via the glucocorticoid receptor (GR), which translocates to the nucleus and both suppresses pro-inflammatory and increases anti-inflammatory gene transcription (Marwick *et al*, 2007). A more detailed overview of inhaled and systemic corticosteroids including their mechanisms can be found in chapter 6 and 7 respectively.

1.6.6 New and Future therapies

The most recent anti-asthma drug to be approved is the anti-IgE monoclonal antibody, omalizumab. Currently, issues such as certain patients demonstrating lack of symptom improvement and the cost of the therapy have prevented wider adoption of the treatment. Other treatments based on blocking key cytokines in the allergic response such as IL-4, IL-5, IL-9 and IL-13 are at various stages of clinical and pre-clinical development (Barnes, 2010). So far these have shown mixed results and may indicate the need to better understand the various mechanisms that underlie different forms of asthma.

1.6.7 Treatment of asthma exacerbations

Corticosteroids have been shown to be the most protective in the reduction of exacerbations, decreasing their occurrence by 55% when compared with short acting β2 agonist or placebo (Sin *et al*, 2004). They also seem to be able to reduce AHR, which is likely to contribute to better exacerbation control (Sont *et al*, 1999). Despite this, asthma exacerbations continued to be under treated. One problem in the treatment of asthma exacerbations is the poor compliance with inhaled drug treatment within the asthmatic population. Due to this, treatment is often only begun when the symptoms of an asthma exacerbation have emerged and treatment is less effective. The effectiveness of corticosteroids during exacerbations remains under debate. In particular, the effectiveness of corticosteroids in reducing viral-induced exacerbation remains controversial, despite an extensive Cochrane review that found that inhaled corticosteroids are an effective strategy in dealing with respiratory viral infection (Zhang *et al*, 2007; McKean & Ducharme, 2000). The mechanism behind this may be an enhancement of innate immunity and epithelial defense, and also reduction in Th2 pro-inflammatory cytokine production, which is weakly anti-viral (Zhang *et al*, 2007). In animal models, viral exacerbations have been shown to be refractory to inhaled corticosteroids (Singam *et al*, 2006) LPS-induced exacerbations of asthma have also

been shown to be partially refractory to even systemic corticosteroids (Komlosi *et al*, 2006). Alternative strategies to treat asthma exacerbations are also under investigation. Exogenous interferon-β has been shown to have both anti-viral and anti-inflammatory properties *in vitro* on primary bronchial epithelia cells from asthmatics (Cakebread *et al*, 2011). Thus this may represent an alternative and/or complimentary strategy to corticosteroids for treating viral specific asthma exacerbations.

1.7 Steroid Insensitivity

Approximately 5% of asthmatics have been found to be unresponsive to inhaled corticosteroid treatment and show decreased responsiveness to oral corticosteroid treatment. This is termed steroid insensitivity. Relatively few people are completely resistant to oral steroids (Ito *et al*, 2006). Steroids are a front line treatment for controlling asthma and few alternative anti-inflammatory therapies exist. Therefore, patients that are unresponsive to steroid treatment are more likely to have poorly controlled asthma and present considerable management and economic problems. The underlying mechanisms of steroid insensitivity in asthma are largely unknown, although several pieces of evidence have suggested possible causes including a decrease in the activity of a group of enzymes called the histone deacetylases, which suppress pro-inflammatory gene transcription (Ito *et al*, 2006). New animal models that reflect human steroid insensitivity in asthma are required. These will enable the development of novel therapeutics to directly treat patients or to restore normal steroid response, allowing for better symptom control. A more detailed discussion of steroid insensitivity can be found in chapter 6 and 7.

1.8 Animal Models

Animals are used to model diseases and test the efficacy of new drugs. However, since asthma is a complex disease, no one animal model can represent the heterogeneity of the human condition. Animals do not develop asthma spontaneously but some

demonstrate diseases akin to human asthma including cats, dogs, sheep and horses although their pathogenesis may be vastly different (Kurucz & Szelenyi, 2006). As a result, the majority of models of asthma are created through allergen sensitisation and subsequent allergen challenge. A variety of different allergens have been used in these models including ovalbumin (Ova), cockroach extract, ragweed, Aspergillus fumigatus and house dust mite (Smith & Broadley, 2007; Canning & Chou, 2008).

A discussion of Ova allergen challenge models can be found in chapter 3. A discussion of viral and LPS induced asthma exacerbation models can be found in chapter 4 and 5 respectively. A discussion of inhaled and systemic corticosteroid insensitivity models can be found in chapters 6 and 7 respectively. Many different animals have been used as models for asthma including monkeys, dogs, mice and guinea-pigs. Guinea-pigs in particular have been the most commonly used small animal species in preclinical testing for asthma (Canning & Chou, 2008). Although guinea-pigs have offered vital insight into processes such as immediate hypersensitivity reactions and the actions of histamine, their value as an animal model must be carefully considered.

1.8.1 Guinea-pig models

Guinea-pigs offer several advantages over larger species such as sheep in modelling asthma. They are relatively cheap; so that using them is fairly cost effective. Like other rodents they are small and therefore easy to handle. Unlike other rodents many aspects of the guinea-pig lung physiology and anatomy is similar to humans (Ressmeyer *et al*, 2006). As in humans, guinea-pigs have a pseudo-stratified epithelium lining the trachea, a similar bronchi structure, vagal innervation of the epithelial and sub epithelial spaces, goblet cell and mucus glands that are both neuronally and locally regulated and smooth muscle of similar anatomical location and functional properties (Canning & Chou, 2008). Importantly, the pharmacology of receptors including responses to airway smooth muscle relaxants and contractile agonists are nearly identical to humans, with a few exceptions (Muccitelli *et al*, 1987). In addition, in both humans and guinea-pigs the EAR

is mediated by histamine and leukotrienes from mast cells, whereas in species such as mice and rats, mast cells contain 5-HT (Lamm *et al*, 1984; Roquet *et al*, 1997). Finally, the docile nature of guinea-pigs makes the use of anaesthetic unnecessary for accurate measurements of airway function.

The use of guinea-pigs does have several disadvantages. There exists in guinea-pigs an axonal reflex that results in the local release of tachykinins and the induction of bronchospasm, mucus secretion, inflammatory cell recruitment, vascular engorgement and plasma extravasation. It appears that this reflex does not exist in humans and thus serves as a drawback of the guinea-pig model (Barnes, 2001). It should also be noted that in humans the main antibody involved in type 1 hypersensitivity reactions is IgE whereas in guinea-pigs it is IgG (Regal, 1987). Guinea-pigs have seen decreased use as models for asthma due to a lack of available biochemical and molecular biological reagents. Guinea-pigs also have a more uncharacterised genome as compared to mice and thus genetic manipulation is more difficult (Canning & Chou, 2008). However, the advantages of guinea-pigs as models of asthma outweigh the disadvantages and they continue to be a useful tool in drug discovery and modelling.

Ultimately, no animal model can seek to represent the full human asthmatic condition due to its heterogeneous nature, its complex causes and underlying mechanisms. Criticism that animal models fail to replicate the full human condition represent a misunderstanding of the use of animal models (Holmes *et al*, 2011). Animal models are best applied to recreating certain aspects of asthma and having their mechanisms shown as relevant in humans.

1.8.2 Use of whole body plethysmography

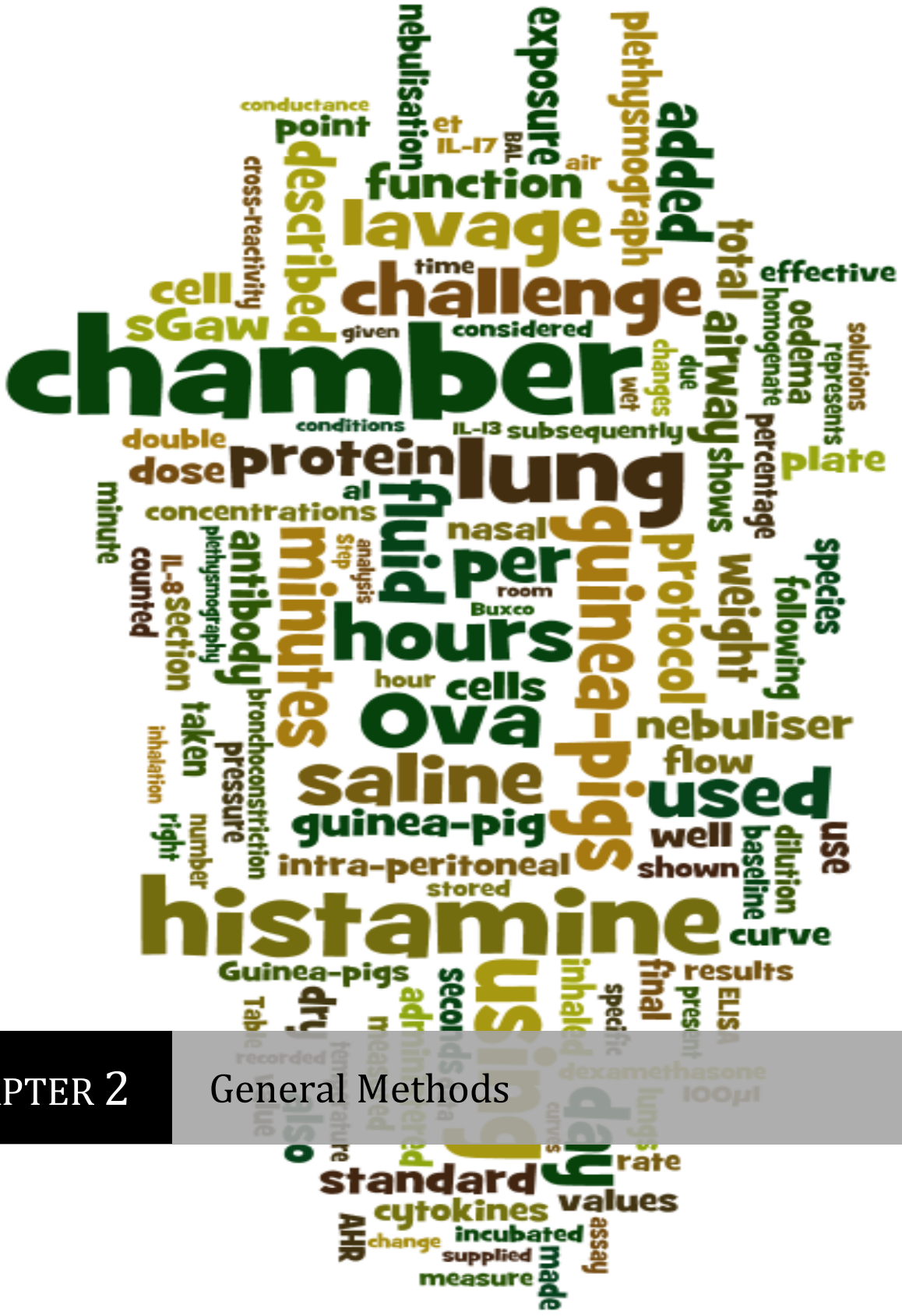
Whole body plethysmography allows for conscious measurement of lung function over a prolonged period. This enables measurement of both the early and late asthmatic responses in the same animal, along with measurement of other asthma characteristics such as AHR and cellular inflammation at a separate time point. This technique has been

successfully employed to measure early and late asthmatic responses in a range of studies (Johnson & Broadley, 1999; Underwood *et al*, 1995, Evan *et al*, 2011). The disadvantage of this method is that it does not allow for direct measurement of bronchoconstriction in specific airways, along with a direct measurement of airway resistance and compliance. Whole body plethysmography does overcome the issues with using anaesthetic, as required for invasive measurement of lung function. Anaesthetic can interfere with the vagal reflex in the lung which can be important in bronchoconstrictor responses (Toward & Broadley, 2004).

1.9 AIMS

The overall aims of this thesis are:

- Establish an acute allergen challenge model of asthma demonstrating an early and late asthmatic responses, airways hyperresponsiveness and airway inflammation.
- Establish a model of asthma exacerbation demonstrating a worsening of the parameters observed above using live virus, viral mimetic or lipopolysaccharide. Also to establish the effect of these agents alone.
- Establish the inhaled and systemic corticosteroid sensitivity of experimental asthma, asthma exacerbation models and virus/LPS alone.



CHAPTER 2 General Methods

A complete list of the materials, equipment and solutions used in this thesis and their suppliers is available in Appendix 1. Figures in this thesis were produced using "Servier Medical Art" (www.servier.com**).**

2.1 *IN VIVO* METHODS

2.1.1 ANIMAL HUSBANDRY

Male Dunkin-Hartley guinea-pigs, 200-300g were purchased from Harlan Ltd, UK or Charles River, Germany. On arrival guinea-pigs were given one week to acclimatise with their new surroundings before commencement of experiments. Guinea-pigs were housed in pathogen free conditions; with environmental enrichment in the form of cardboard tubes and hay. The housing room conditions were: twelve hour light/dark cycles, at 50% humidity and room temperature of 20°C±2°C. All guinea-pigs were given food and water *ad-libitum*. All procedures were carried out in accordance with Home office licence conditions and legislation covering animal husbandry and severity limits.

2.1.2 MEASUREMENT OF LUNG FUNCTION

Guinea-pig airway function was measured using non-invasive double chamber plethysmography supplied by Buxco systems Ltd. During respiration, an exchange of air between the airways of the animal and the surrounding environment (plethysmography chamber) takes place. This exchange is induced by changes in the volume of the small airways and alveoli of the lungs. During inspiration the volume of the lungs increases, resulting in a decrease in the airway pressure. Air then travels down its pressure gradient resulting in reduction in the volume of air of the surrounding environment. The opposite process takes place during expiration. The plethysmograph allows the difference between the rate of change of thoracic displacement volume and the nasal flow to be measured when the chamber is kept at a constant temperature.

The chamber is split into both a nasal and thoracic sections, separated by a neck seal and monitored by separate transducers. This allows thoracic and nasal flows to be compared. The difference in pressure between the alveoli and the nose or mouth, when divided by the airflow between them produces a measure of airway resistance (Raw). The reciprocal of Raw, airway conductance (Gaw), is considered a better measurement of airway function as it accounts for change in lung tissue tension and transpulmonary pressure. Gaw is influenced by changes in the thoracic gas volume (TGV), as occur in the alveoli and therefore a further correction is required. The value produced is termed specific airway conductance (sG_{aw}) (Griffiths-Johnson *et al*, 1988) (Figure 1). The greater the resistance in airway, the greater the time delay between the nasal and thoracic flows, which consequently alters sG_{aw}. A fuller description of the measurement of lung function can be found in Appendix 2.

Gaw = 1/ Raw

sGaw = Gaw/TGV

Figure 1: Equation used to calculate specific airway conductance (sGaw) from airway resistance (Raw). Airway conductance (Gaw), Thoracic gas volume (TGV).

Guinea-pigs were placed in a double chamber plethysmograph and prevented from moving their bodies by use of a neck restraint (Figure 2). The restraint also serves the function of separating the nasal and thoracic compartments of the chamber and makes them both airtight. Air temperature and gas percentages were kept constant by use of a bias flow supply unit. Box pressure changes in both compartments were measured by pressure transducers. The pre-amplified output is converted to waveforms by Finepoint software (Buxco system Ltd). An example of the waveform is given in Figure 3. The software is also able to derive sGaw based on the inputs it receives. Readings are taken every 2 seconds and at least 20 breaths are recorded during any time point measurement. For calculation of sGaw at a particular time point, 15 values are taken at random and the average is calculated.

Prior to the commencement of lung function measurements, all guinea-pigs were acclimatised to being restrained in plethysmograph chambers for at least 20 minutes on two separate occasions. This reduces movement related signal 'noise' during measurements. It also reduces animal stress during subsequent restraint, reducing the interference of stress-related hormones such as cortisol and adrenaline on allergen responses.

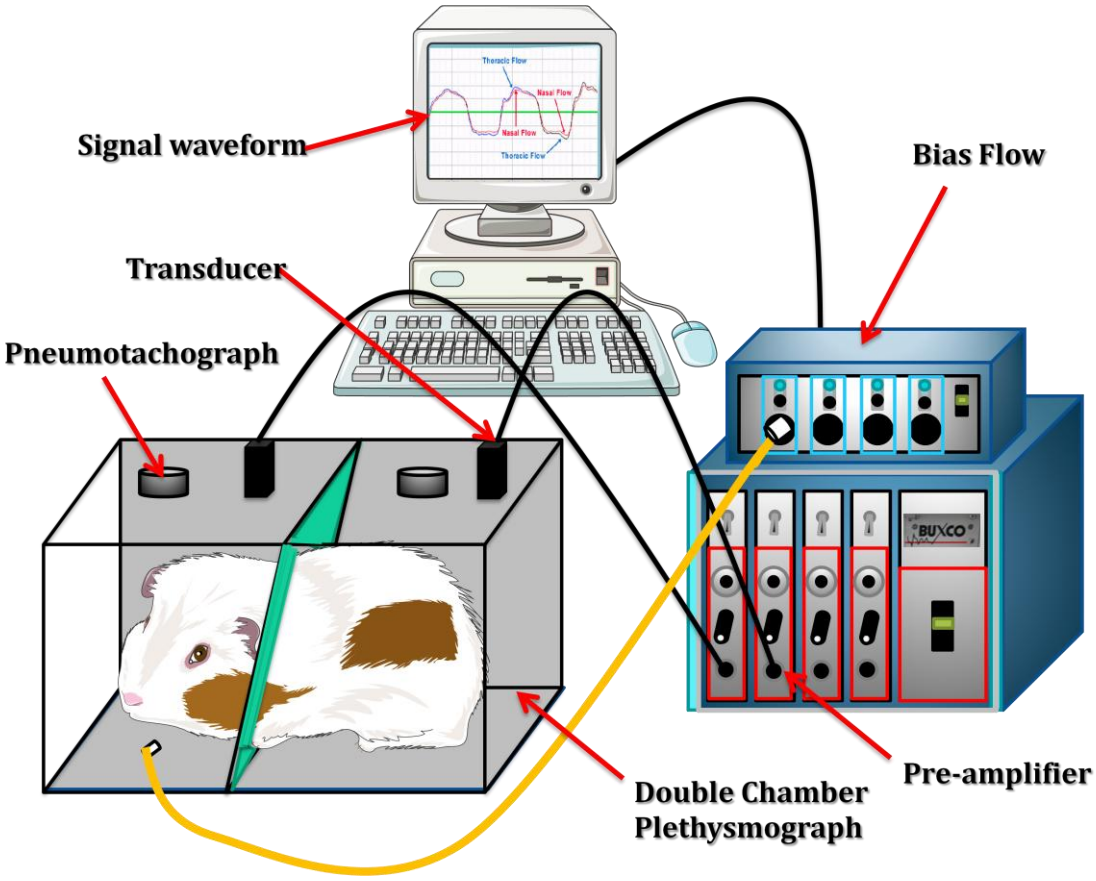


Figure 2: Diagram of double chamber plethysmograph used for measurement of specific airway conductance (sGaw) in conscious, restrained guinea-pigs. Airflow is measured by aid of pnuemotachograph and pressure changes by transducer. The difference in these values is captured using the pre-amplifiers and specific airway conductance (sGaw) calculated using Finepoint software.

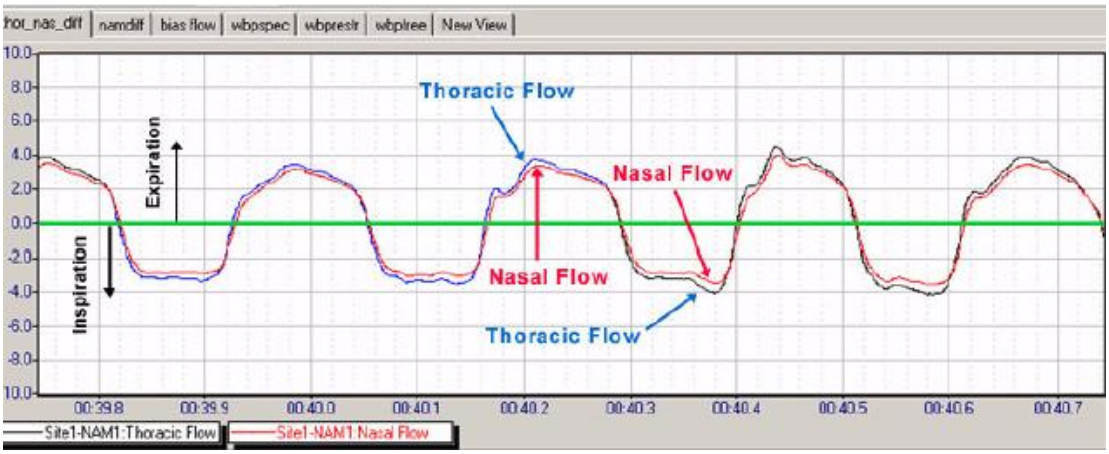


Figure 3: A trace of the waveform data produced from double chamber plethysmography. Thoracic flow (blue) and nasal flow (red) are both represented as near overlapping waveforms. Taken from (Lomask, 2005).

2.1.3 Ovalbumin and combined ovalbumin and LPS protocols

2.1.3.1 Sensitisation

Guinea-pigs were sensitised by 3 bilateral intra-peritoneal (I.p) injections (day 1, 4 and 7) of ovalbumin (Ova, 150ug) and aluminium hydroxide (100mg) in 1ml of normal saline, unless otherwise stated. All further procedures commenced on day 15. This method of sensitisation is modified from Smith and Broadley, 2007 and is described in more detail in chapter 3.

2.1.3.2 Acute ovalbumin challenge protocol

Modifications were made to this protocol and are described in chapter 3. This description represents the final protocol developed and used in all subsequent experiments. Airways hyper-responsiveness (AHR) was determined by histamine inhalation, both pre- and post-saline or Ova challenge on day 15 and 22 respectively (described in more detail in section 2.1.6). Guinea-pigs were exposed to inhaled ovalbumin (0.03% w/v) or saline challenge on day 21. Exposure was performed in a Perspex exposure chamber (15x30x15cm) using a DeVilbiss nebuliser, delivered at a rate of 0.3ml/min and at an air pressure of 20 ib p.s.i. Guinea-pigs were exposed for an hour or until they appeared distressed, in which case exposure was considered

complete. Lung function was subsequently measured over 12 hours (described in more detail in section 2.1.5). Further to histamine inhalation on day 22, guinea-pigs were sacrificed and bronchoalveolar lavage (BAL) performed (described in more detail in section 2.2.1). Using the fluid returned from the lungs, total and differential cell counts were performed. Figure 4 shows the acute protocol.

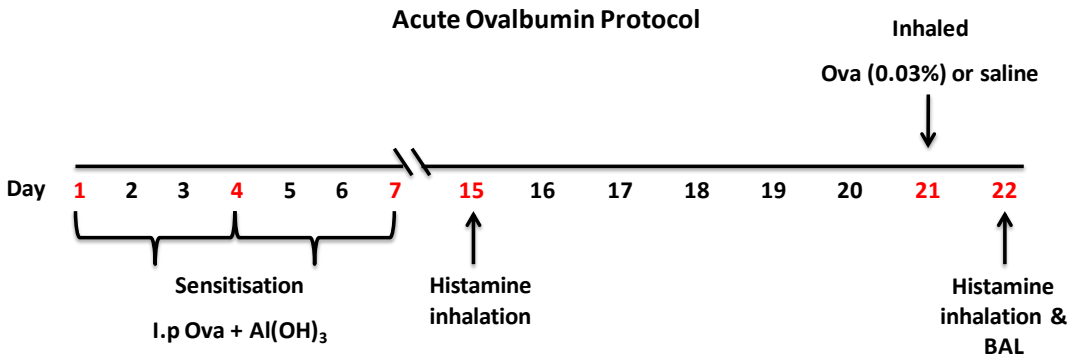


Figure 4: A diagram of the acute ovalbumin protocol. Ova: Ovalbumin; BAL: bronchoalveolar lavage

2.1.3.3 OVALBUMIN AND LPS CO-ADMINISTRATION PROTOCOL

Variations of this protocol were used in chapter 5 and 6. This description refers to the protocol using 2 LPS exposures used in chapter 5-8. Airways hyperresponsiveness (AHR) was determined by histamine inhalation both pre- and post-saline or Ova challenge on day 15 and 22 respectively (described in more detail in section 2.1.6). Guinea-pigs were exposed to inhaled LPS (30µg/ml) 48 hours before Ova challenge and both LPS (30µg/ml) and Ova (0.03% w/v) co-administered on day 21. Exposures were performed for an hour using the same equipment mentioned in section 2.1.3.2. Lung function was subsequently measured over 12 hours (described in more detail in section 2.1.5). Following the second histamine inhalation on day 22, guinea-pigs were sacrificed and bronchoalveolar lavage performed (described in more detail in section 2.2.1). Using the fluid returned from the lungs, total and differential cell counts were performed. Figure 5 shows a diagram of this protocol.

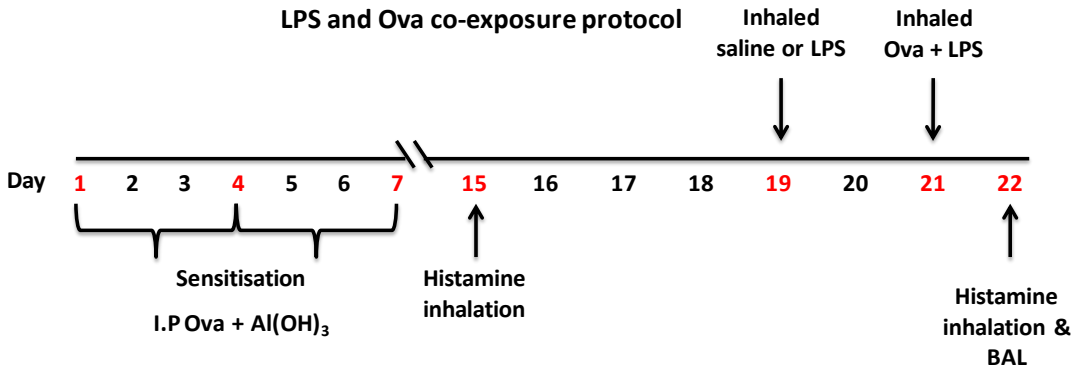


Figure 5: Diagram of LPS and Ova co-exposure protocol. Guinea-pigs were exposed to saline or LPS (30µg/ml) on day 19 and both Ova and LPS co-administered on day 21.

2.1.4 LPS EXPOSURE PROTOCOL

Guinea-pigs were exposed to inhaled histamine both pre and post- LPS exposure on days 1 and 8 respectively of the protocol to determine the presence of AHR (described in more detail in section 2.1.6). Guinea-pigs were exposed to saline or LPS (30µg/ml) on day 5 and 7 of the protocol. Exposures were performed for an hour using the same equipment mentioned in section 2.1.3.2. Lung function was determined for 12 hours after the 2nd LPS or saline exposure to determine the presence of a bronchoconstriction as described in section 2.1.5. Bronchoalveolar lavage was performed on day 8, following histamine inhalation and sacrifice. Total and differential cell counts were performed to determine airways inflammation.

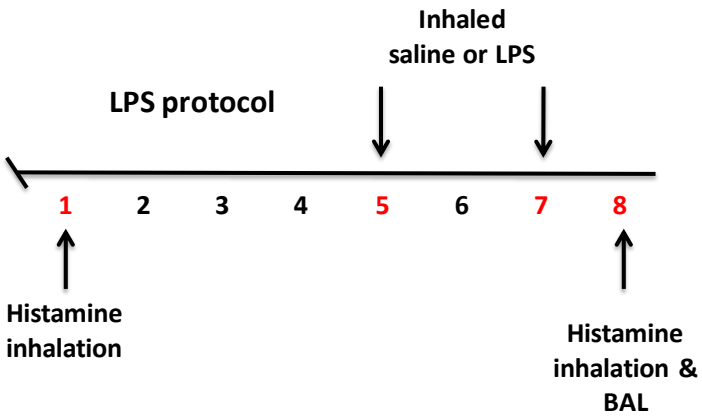


Figure 6: Diagram of the LPS alone exposure protocol. Guinea-pigs are exposed to 30µg/ml of LPS on day 5 and 7.

2.1.5 Recording Lung function

Lung function was measured by the use of sGaw immediately following saline, Ova or LPS challenge. Values for sGaw were taken hourly for 12 hours and every 15 minutes during the first hour of measurements. A final measurement was also taken 24 hours post-challenge. sGaw was expressed as a percentage of the baseline reading, taken before saline, Ova or 2nd LPS challenge. A negative percentage change in baseline value represents a bronchoconstriction, whereas a positive change in percentage represents a bronchorelaxation. In ovalbumin challenged animals early and late asthmatic responses (EAR and LAR, respectively) which are characterised by distinct bronchoconstrictions, were determined. The EAR occurs within the first hour but can take as long as 6 hours to fully return to baseline. Therefore, to account for any individual differences between guinea-pigs the peak value between 0-6 hours is considered to represent the EAR. The LAR starts from about 6 hours onwards and shows considerable temporal variation between animals. Therefore the peak value between 6-12 hours is considered to represent a LAR.

2.1.6 Measurement of Airways Hyperresponsiveness

The development of airway hyperresponsiveness (AHR) following Ova, LPS or saline challenge was determined by measuring the response to the bronchoconstrictor agent histamine using plethysmography. Histamine was delivered to the guinea-pigs by the use of a Buxco nebuliser chamber, which directly feeds into the nasal chamber of the plethysmograph (Figure 7). The rate of gas flow into this chamber is dependent on the total of the bias flow units suction vs the rate of the nebuliser control units extraction. The nebuliser control unit was set to extract histamine from the nebulisation chamber at a rate of 2 litres per minute (LPM), per chamber, which when subtracted from the bias flow extraction rate of 2.5LPM per chamber results in a flow of histamine from the nebuliser chamber to the nasal chamber of the plethysmograph of 0.5 LPM per chamber. Nebulisation of histamine took place over the first two minutes, with a 10% duty setting per chamber i.e. 1.2 seconds out of

every 6 seconds spent nebulising. The third minute included a 1 minute drying period which removes excess aerosolised histamine from the nebulisation chamber.

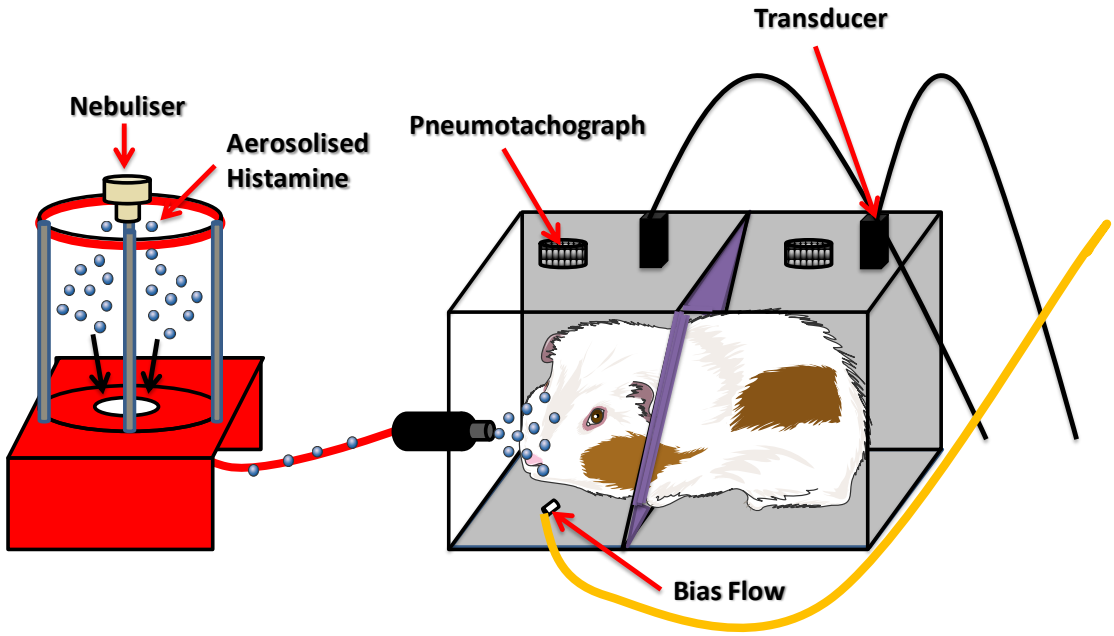


Figure 7: A diagram of double chamber plethysmograph with nebuliser unit for performing assessment of the bronchoconstrictor response to aerosolised histamine. Solutions are added to the nebuliser, aerosolised into the chamber below and sucked into the nasal portion of the plethysmograph chamber where the guinea-pig inhales it. Aerosolised histamine is removed from the chamber by the bias flow unit. Specific airway conductance (sGaw) values are derived an from both the nasal and the thoracic portions of the plethysmograph chamber by the transducers finepoint software.

A concentration of histamine that evokes minimal bronchoconstriction in naïve guinea-pigs was determined by dose-response curve (Figure 8). The effect of different nebulisation regimes on histamine response was also determined (Figure 9). From these studies a histamine concentration of 0.3mM, 2 minutes nebulisation + 1 minute drying, 10% duty cycle per chamber was chosen. This protocol produces a small bronchoconstriction in naïve guinea-pigs which ensures that a failure to see AHR to histamine is not due to the dose of histamine being too low.

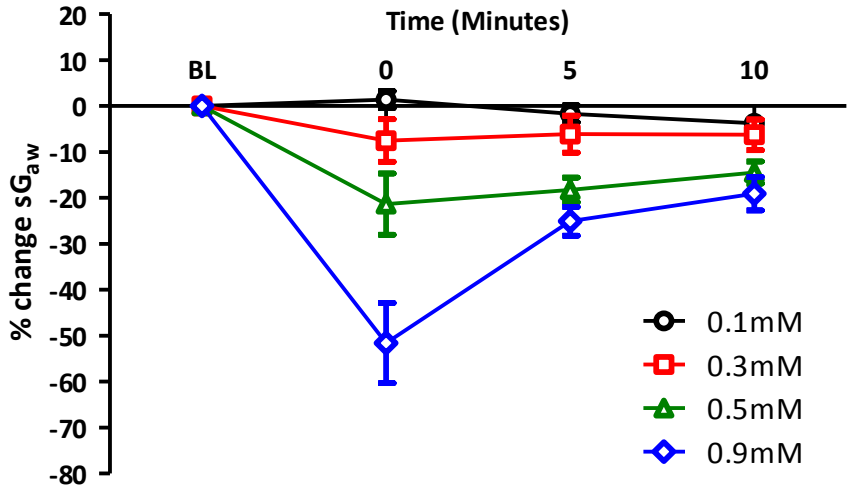


Figure 8: A dose-response curve to histamine performed in naïve guinea-pigs. Settings used were 1 minute nebulisation, 1 minute drying. Net flow to each chamber was 0.5LPM (2.5LPM from bias flow against 2LPM extraction from neubliser control unit), 20% duty per chamber.

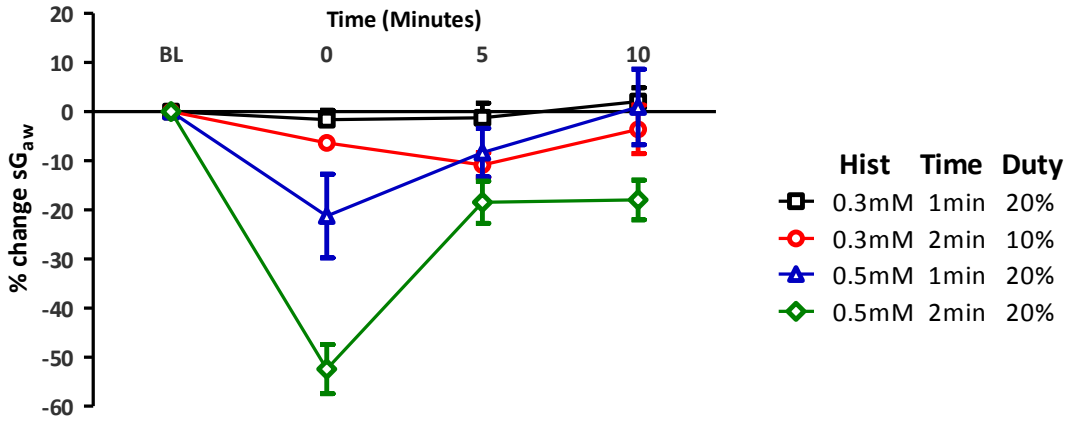


Figure 9: The effect of altering the nebulisation period and duty cycle on the bronchoconstrictor response to 2 different doses of histamine. Neb: nebulisation time, Duty: duty cycle for duration of nebulisation, per chamber.

To determine the bronchoconstrictor response to histamine, a 1 minute baseline value of lung function is recorded using sGaw and all other values recorded are taken as a percentage of this. Further sGaw values are recorded at time points 0, 5 and 10 minutes post histamine exposure. A decrease in sGaw following histamine exposure

represents a bronchoconstriction, suggestive of AHR. In each protocol, histamine challenges were performed both pre- and 24 hours post-Ova or LPS challenge. Pre-Ova or saline histamine exposures were used to determine individual guinea-pigs sensitivity to histamine prior to Ova challenge. A direct comparison between the pre- and post-Ova, LPS or saline responses accommodates for group to group variation in responsiveness to histamine.

2.1.7 Drug administration

The effect of inhaled (fluticasone propionate and dexamethasone) and systemic corticosteroid (dexamethasone) were used on various acute asthma models of asthma throughout this thesis. Drugs were all administered for 6 consecutive days before lavage, to reflect the clinical situation where corticosteroids treatment is underway before asthma exacerbation. On days of Ova or LPS challenge, corticosteroid treatment was administered 30 minutes prior to subsequent challenge. The dosing regimen used for each drug is described in Table 1.

Drug	Administration	Daily Doses	Vehicle	Frequency
Fluticasone propionate	Inhaled (nebulised) 15 minutes	0.05, 0.1, 0.5 and 1mg/ml	30% ethanol 30% DMSO 40% saline	Split into 2x daily dose, 6 hours apart
Dexamethasone 21-phosphate disodium salt	Inhaled (nebulised) 15 minutes	4 & 20mg/kg	25% DMSO 75% Saline	Once daily
Dexamethasone 21-phosphate disodium salt	Intra-peritoneal	5, 10 & 20mg/kg	25% DMSO 75% Saline	Once daily

Table 1: A list of the compounds used throughout this thesis.

The corticosteroid vehicle selected was chosen to minimise side effects whilst fully dissolving the drug. Previous work with dexamethasone used 50% DMSO and 50% saline as a vehicle (Toward & Broadley, 2004). This was reduced to 25% DMSO to reduce local irritation at the site of injection and potential anti-inflammatory actions. This fully solubilised dexamethasone at even the highest concentrations used for both inhaled and intra-peritoneal routes. The vehicle for fluticasone propionate included ethanol due to issues with solubility in DMSO and saline alone. This mix has been shown to have minimal effects on Ova and histamine responses (Nevin & Broadley, 2004).

Drugs administered by inhalation were delivered for 15 minutes in a Perspex exposure chamber (15x30x15cm) using a DeVilbiss nebuliser delivered at a rate of 0.3ml/min and at an air pressure of 20 ib p.s.i. Fluticasone propionate was administered twice daily to reflect its clinical administration and according to a protocol already shown to be effective on Ova-induced allergic and functional responses (unpublished data). Dexamethasone was administered once daily due to its long biological half life of 36-54 hours, in spite of its short plasma half life (3.5-4.5 hours) in humans (Sparrow & Geelhoed, 2006).

Drug doses selected were based on previous work and dose-response relationships performed in this thesis. Previous work with inhaled fluticasone propionate has found that 0.5mg/ml is a dose effective at reducing Ova induced LAR, AHR and airway inflammation (Evans *et al*, 2012). Similarly, an intra-peritoneal injection of 20mg/kg dexamethasone has been shown effective at reducing the same parameters (Toward & Broadley, 2004). Dexamethasone is not commonly given by the inhaled route and consequently there are no studies using it in guinea-pigs on which to estimate an effective dose. The dose used was extrapolated from one effective in mice (Jungsuwadee *et al*, 2004), relative to intra-peritoneal mouse doses reported in the literature (Komlósi *et al*, 2006; Korideck & Peterson, 2009). From this a ratio (5:1) between effective intra-peritoneal and inhaled doses was determined and used to calculate an inhaled dose for guinea-pigs.

2.2 EX VIVO METHODS

2.2.1 MEASURING AIRWAY INFLAMMATION

At 24 hours post-Ova exposure and following final histamine challenge, guinea-pigs were sacrificed by an intra-peritoneal overdose of sodium pentobarbitone (euthatal 400mg/kg). Death was confirmed by the absence of neural reflexes and heartbeat. Guinea-pigs were then bled via severance of the carotid artery. An incision into the neck was made and subsequently an intravenous 7-9FG (trachea size-dependent) cannula was inserted into the trachea. Dissection was then performed to excise the lungs. Subsequently, the right lung, consisting of 4 lobes was clamped off. Bronchoaleveolar lavage (BAL) was performed on the non-clamped left lung using normal saline (0.5ml per 100g of guinea-pig weight) instilled through the cannula for 3 minutes. This process was then repeated and the 2 volumes of lavage return fluid combined. The largest of the right lobes was stored in 10% buffered formaldehyde for later histology. The smallest lobe had its wet weight measured and was subsequently dried overnight in an oven at 40^{o}C. The remaining two lobes were stored at -80^{o}c for future protein or RNA analysis.

To measure cellular influx, both total and differential cell counts were performed on BAL fluid. The total number of cells (per ml of lavage fluid) was counted using a Neubauer haemocytometer under a light microscope at 40x magnification. Cell counts were performed using undiluted BAL fluid. Lavage fluid returns were consistent across experiments. 100µl of lavage solution was pipetted under a coverslip placed on top of the haemocytometer, evenly distributed by the aid of capillary action. 2 grids of 25 squares are present on the haemocytometer, with the number of cells in 5 of these squares being counted each time (always the top left, top right, bottom, right, bottom, left and central square). The formula used to calculate total cell numbers per ml of BALF is shown in (Figure 10). The resulting figure was multiplied by 5 and averaged with the total from the other grid of 25 cells. As each of the squares measure 0.04 mm^2 and 25 were counted, a figure of number

of cells per 1 mm^2 was established. However, the depth of the chamber is 0.1 mm, therefore giving a number of cells per 0.1 mm^3. The cell count figure was multiplied by 10^4 to provide a total cell count per ml ($1cm^3$)

$$Cells/1mm^3 = [Average\ (GC_1 x5\ \&\ GC_2 x5)]\ x10^4$$

GC_1= Grid count 1

GC_2= Grid count 2

Figure 10: Formula used to calculate total cell numbers/ml of bronchoalveolar lavage fluid.

Differential cell counts were the second count performed. 100µl of undiluted, 1:5 diluted or 1:10 diluted BAL fluid stock was centrifuged for 7 minutes at 1000rpm onto a glass slide using a cytospin. Slides were subsequently air dried to aid cell adhere to the slide. Slides were then stained with 1.5% Leishmans solution in 100% methanol for 6 minutes and rinsed in dH_20 twice. Slides were air dried overnight. Once slides were dry, cells were counted at x100 magnification to determine the subpopulations of leukocytes present. The subpopulations of leukocytes counted included eosinophils, macrophages, lymphocytes and neutrophils. No mast cells were observed. Cell types were differentiated on the basis of their granularity, cell size, nucleus shape and stain colouration (Figure 11).

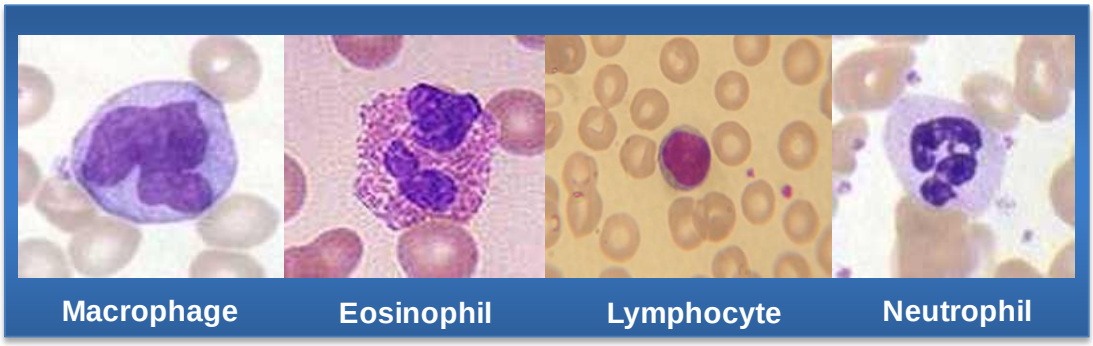


Figure 11: Photographs of the four subtypes of leukocytes, stained with Leishman's solution, counted in a differential count. Adapted from (Gude *et al.*, 1982).

Macrophages are the predominant inflammatory cell in BAL fluid. They are also the largest ranging from 15-20µm. Eosinophils are most easily identified on the basis of their bi-lobed nucleus, red/pink staining of granules in the cytoplasm and their smaller size (10-14 µm), compared to macrophages. Lymphocytes are mononuclear and are the smallest cell type of the 4 (<10 µm). Identification is reliant on the presence of a large spherical, darkly stained nucleus, taking up 70-80% of the total cell volume. Neutrophils are similar in size to eosinophils but easily distinguished from them by the presence of a multi-lobed, blue stained nucleus. The cytoplasm also stains a light blue further differentiating the 2 cell types (Gude *et al*, 1982).

2.2.2 Determination of airways oedema

Initially airway oedema was determined by assessment of the wet vs dry weight of the lungs (described in section 2.2.2.1). This technique was used in chapter 4. Further studies used total protein content in lavage fluid as an indicator of airway oedema. This measure correlates with the wet/dry weight measure and allows for clearer determination of differences in oedema.

2.2.2.1 Wet vs dry weight of lung

Wet lung oedema was determined by comparison of lung wet weight with dry weight. This was performed as follows. The 3rd largest lobe of the right lung was weighed following excision from the guinea-pig and then dried overnight in an oven

at 40ºC. The lobe was again weighed. The difference between the wet and dry weight of the lung allows determination of lung water content and consequently oedema. However, percentage changes are small and do not allow for differences in oedema to be easily seen. Therefore the difference was expressed as a percentage of the dry weight according to the following equation: [Airway oedema= ((wet weight-dry weight)/dry weight x 100)].

2.2.2.2 BICINCHONINIC ACID (BCA) PROTEIN ASSAY

Total protein content was determined in lavage fluid supernatant using a BCA protein assay as per the manufacturer's instructions (Pierce protein biology). This was performed as follows. 10µl of a 1:3 dilution of lavage fluid, PBS or protein standard (range 2- 0.025mg/ml) was added to 96 well plate. 200µl of BCA working reagent (See Appendix A1.3) was added to each well, covered and put on a plate shaker for 30 seconds. The plate was then incubated at 37^{o}C for 30mins. The plate was then cooled and read at 540nm on a plate reader. Protein content in lavage fluid was determined using an 8 point protein standard curve.

2.2.3 QUANTIFICATION OF CYTOKINE LEVELS USING ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

To quantify the changes in cytokine levels ELISAs were performed on lung homogenate and lavage fluid. An initial list of cytokines of interest was determined and included the Th2 cytokines IL-4, IL-5 and IL-13, the Th1 cytokine IL-12p70 and IFN-γ, TNF-α, IL-10, IL-17 and the chemoattractant IL-8. However, due to the limited availability of guinea-pig specific kits an attempt at cross-reacting readily available mouse and human antibodies was made. The guinea-pig genome has not been fully sequenced yet and so antibodies for other species are not routinely screened for guinea-pig protein cross-reactivity. Therefore, cross-reactivity between guinea-pigs and mouse and human antibodies is unknown

2.2.3.1 Bioinformatic assessment of potential species cross reactivity

To maximise the chances of successful cross-reactivity between non-guinea-pig antibodies and guinea-pig proteins, an assessment of the protein sequence homology between species was made. Using Pubmed, the protein sequences for guinea-pig cytokines of interest were obtained. The homology of these guinea-pig sequences to other species was assessed using BLAST. The most homologous species to the guinea-pig species protein sequence was selected for cross-reactivity screening. Table 2 shows a summary of the results. For full sequence data please see Appendix 3.

Cytokine	Role	Ab used	Homology
IL-4	Th2 differentiation	Mouse	54%
IL-5	Th2 derived, eosinophil recruitment and activation	Human	81%
IL-13	Th2 derived, AHR	Human	79% (predicted)
IL-12 p70	Pro-inflammatory, Th1 differentiation	Human	85%
IFN-γ	Th1 effector cytokine, anti-intracellualar pathogen	Human	71%
IL-17 (a)	'exacerbation' linked	Human	81% (predicted)
IL-10	Anti-inflammatory	Human	92%
IL-8	Neutrophil chemoattractant	Human	82%
TNF-α	Pro-inflammatory	Guinea pig	N/a

Table 2: Shows function of cytokines assessed, the species with the closet sequence to the guinea-pig protein and the percentage homology between the two.

Special consideration for the results obtained using the human IL-8 ELISA kit is necessary for rodents. In mice and rats, the functional ortholog CXCL1 (KC) is the main neutrophil chemoattractant (Mestas J, Hughes, 2004). Unlike other rodents used in research, in the guinea-pig it appears that CXCL8 (IL-8) is the main neutrophil chemoattractant, as in humans (Lyons *et al*, 2004; Gorden *et al*, 2005). Guinea-pig IL-8 also signals through the same receptors: CXCR1 and CXCR2 as in humans (Catusse et al, 2003; Takahashi et al, 2007). In rats and mice CXCR1 is not present. This would suggest that a antibody against human IL-8 would be capable of reacting with the guinea-pig homologue.

2.2.3.2 PERFORMING AN ELISA

Approximately 100mgs of lung lobes snap frozen in liquid nitrogen and stored at -80°C were homogenised in Precellys tubes containing 1ml of lysis buffer (see Appendix 1.3) for 2x 50 seconds in a Precellys tissue homogeniser. The resulting homogenate was then spun at 13,000rpm for 15 minutes at 4°C, aliquoted into tubes and stored at -80°C until use. Lavage fluid was stored at -80°C until use. ELISA assays were performed using Duoset kit reagents supplied by R&D systems, UK. The major steps in this process are shown in Figure 12. In all assays a 96 well maxisorb plate was coated overnight at 24°C with 100µl of capture antibody. The following day the plate was washed 3 times with 400µl phosphate-buffered saline with tween-20 (PBST) and blotted dry. To prevent non-specific binding, plates were blocked for 90 minutes at 24°C with 1x reagent diluent (1% BSA in PBS, R&D systems). Plates were washed as previously described. 100µl of samples, standards or 1x reagent diluent were added in duplicate to wells and incubated for 2 hours at 24°C. A 7 point standard curve with 2-fold serial dilutions were used (refer to Table 4 for details for specific cytokines). A wash step was performed as above. Next, 100µl of the detection antibody was added to each well and incubated for 2 hours at 24°C. A wash step was again performed as described above. Next, 100µl of enzyme labelled (HRP-Stepavidin, diluted per manufacturer's instructions) was added to each well and incubated for 20 minutes at room temperature, avoiding direct sunlight. A wash

step was then performed. 100µl of enzyme substrate (TMB) was added to each well and incubated away from direct sunlight at room temperature for 20-40 minutes until the standard curve colour had developed sufficiently. 50µl of stop solution (H_2SO_4) was added to each well to stop the reaction and then gently mixed on a plate shaker. Optical densities were determined at 450nm using a plate reader. Results were calculated from a 7-point standard curve using softmax pro or Microsoft excel. Standard curves with R^2 values below 0.95 were excluded. Values below the lowest point of the standard curve and therefore indistinguishable from blanks were given a value of 0. Lung homogenate cytokine levels were adjusted for protein content (performed by BCA protein assay) and expressed as weight per mg of lung.

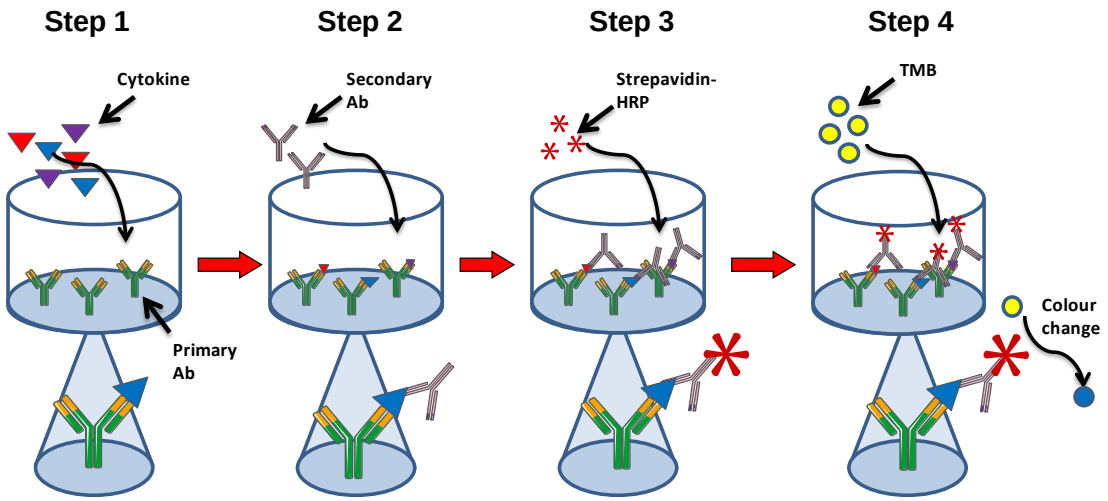


Figure 12: Diagram of the major steps in an enzyme-linked immunosorbent assay (ELISA). Step 1: the biological sample is added to a plastic well coated with primary antibody. Step 2: The secondary antibody is added and binds to the primary-antibody-sample complex. Step 3: Strepavidin-Horse radish peroxidase (HRP) is added and binds to the secondary antibody. Step 4: The substrate tetramethylbenzene (TMB) is added and is turned from yellow to blue by strepavidin-HRP. The more the colour changes the more protein in the sample there is present.

2.2.3.3 Initial screening of antibody cross reactivity.

Initial screening of antibody cross-reactivity was performed using neat lavage fluid and a 1:2 serial dilution of lung homogenate from acute saline or Ova challenged guinea-pigs. ELISAs were performed as per the manufacturer's instructions.

Antibodies were considered to cross-react if samples produced higher optical density values then blank values and fell within the range of the standard curve. Table 3 shows the results of these studies. Only IL-13, IL-17 and IL-8 antibodies were found to cross-react with guinea-pig proteins in lung homogenate. IL-17 and IL-13 were detectable in lavage fluid but not reliably so, only occasionally within the range of the standard curve. The lack of detection of the other cytokines could be due to lack of cross-reactivity or that the time point at which the lavage fluid and lungs were sampled was not optimal for specific cytokine detection. Cytokines differ in their temporal profile following allergen challenge. In particular, the levels of IL-8 have been shown to be lower at 24 hours post-allergen challenge compared to 4 hours post (Danahay *et al*, 1999).

Cytokine Ab	Lung detection	Lavage fluid detection
IL-4 (mouse)	✗	✗
IL-5 (human)	✗	✗
IL-13 (human)	✓	✗
IL-12 p70 (human)	✗	✗
IFN-γ (human)	✗	✗
IL-17 (a) (human)	✓	✗
IL-10 (human)	✗	✗
IL-8 (human)	✓	✗
TNF-α (guinea-pig)	✓	✓

Table 3: Shows which of the selected antibodies reacted with guinea-pig specific cytokines. Cytokines were considered to be detectable if levels were higher then background and fell within the standard curve. Lung homogenate was tested via serial dilution to remove protein matrix effects. Lavage fluid was tested neat.

2.2.3.4 ELISA OPTIMISATION

Capture and detection antibody concentrations were optimised for IL-8, IL-13, IL-17 and TNF-α. Grid optimisations using 3 different standard concentrations (+blanks) and varying concentrations of both capture and detection antibody were performed. Table 4 shows the final assay conditions for each cytokine assessed.

Cytokine	C: Ab conc	C: Ab diluent	D: Ab conc	D: Ab diluent	Standard range	Standard diluent
TNF-α	4ug/ml	PBS	4ug/ml	1% BSA in PBS	2000-32pg/ml	1% BSA in PBS
IL-8	8ug/ml	PBS	30ng/ml	0.1% BSA in TBS-T	2000-32pg/ml	0.1% BSA in TBS-T
IL-13	4ug/ml	PBS	300ng/ml	1% BSA in PBS	6000-94pg/ml	1% BSA in PBS
IL-17	4ug/ml	PBS	150ng/ml	1% BSA in PBS	1000-16pg/ml	1% BSA in PBS

Table 4: Shows the final ELISA antibody conditions after optimisation. C: Ab: Capture antibody, D: Ab detection antibody.

The dilution of lung homogenate used in ELISAs was also optimised by performing 1:5 serial dilution curves. This is necessary to overcome the interference of matrix factors present in lung tissue which can otherwise obscure the differentiation of protein concentrations between groups (Lavoie-Lamoureuxa *et al*, 2010). Figure 13 shows the results of these dilution curves. From these dilution curves a dilution in the linear range was selected: **IL-8**: 1/5, **IL-13**: 1/625, **IL-17**: 1/25, **TNFa**: 1/625.

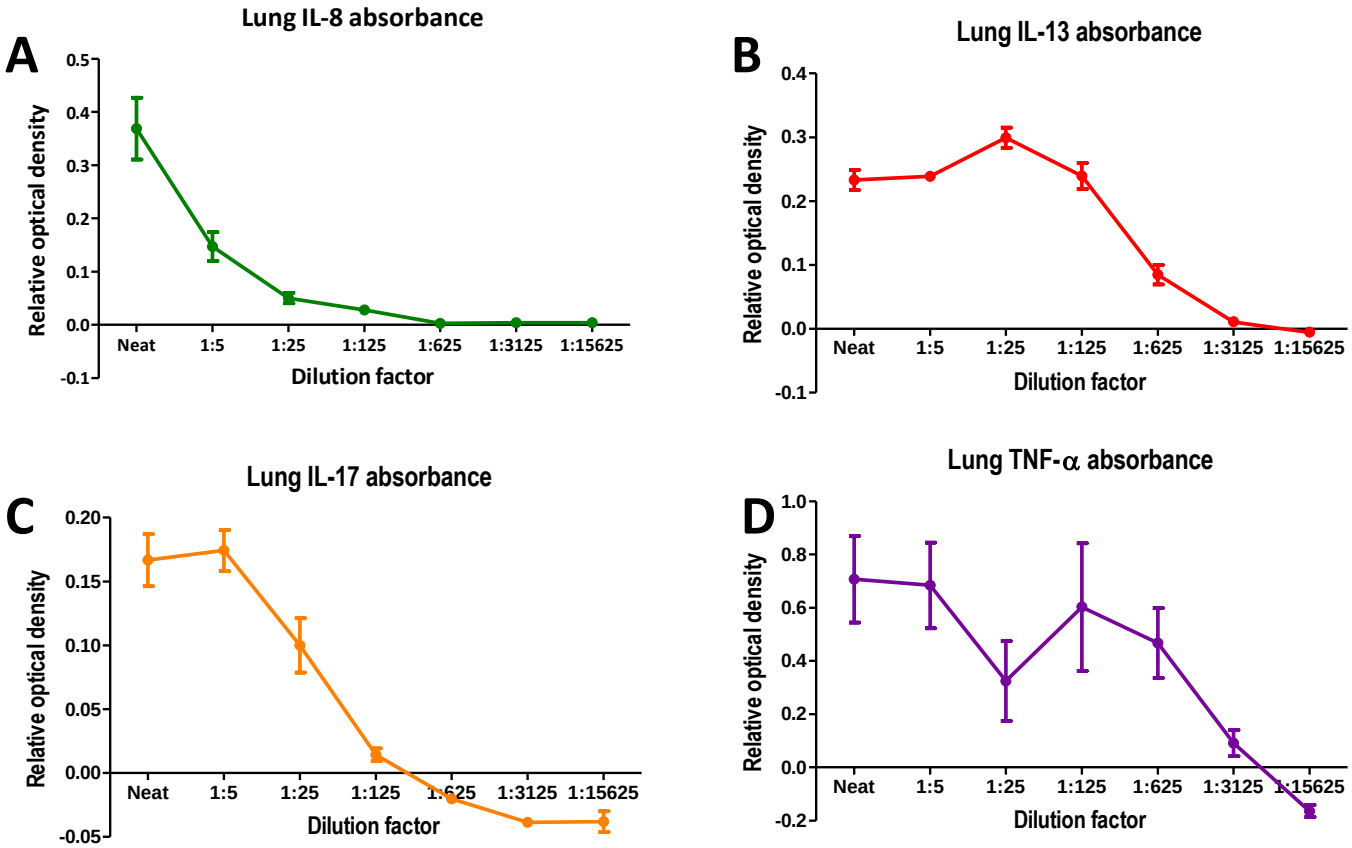


Figure 13: Shows the relative optical densities of A) IL-8 B) IL-13 C) IL-17 and D) TNF-α in lung homogenate. Lung homogenate from ovalbumin sensitised and challenged and LPS challenged guinea-pigs was serially diluted. Background absorbance was subtracted from absolute ODs. Data presented as the mean±SEM. N=3.

2.2.3 HISTOLOGICAL ANALYSIS OF LUNGS

Histological procedures were carried out on the un-lavaged large lobe of the right lung.

2.2.3.1 TISSUE PROCESSING

Tissues were removed from formaldehyde and sliced transversely, mid-way down the lobe, and the flat-edge of the dissected lobe used to obtain two 1-2mm sections. Each section was placed into a tissue cassette and stored in fresh phosphate-buffered (pH7.3) 10% formaldehyde.

Tissue samples were treated via the following protocol, without removal from cassettes.

50% ethanol (1 hour).
70% ethanol (1 hour).
90% ethanol (1 hour).
100% ethanol (1.5 hours).
100% ethanol (1.5 hours).
100% ethanol (1.5 hours).
Chloroform:100% ethanol 50:50 (overnight).
100% chloroform (1.5 hours).
100% chloroform (1.5 hours).
Molten paraffin (2 hours).
Molten paraffin (2 hours).
Molten paraffin (2 hours).

Tissue cassettes were removed from molten paraffin and samples placed into metal mounts. The mount was filled with molten paraffin, the tissue cassette backing applied under pressure and the set placed onto a cooling block at -20^{o}C for 10 minutes. Solidified sections were removed from mounts and excess wax trimmed.

Using a microtome, 5µm thick sections were obtained, placed immediately onto water (40^{o}C), and fixed onto Polysine® slides. Slides were dried overnight at 30^{0}C in an oven.

2.2.3.2 HISTOLOGICAL STAINING

The following protocol was followed for all samples and staining procedures. Stain-specific procedures are detailed in sections *2.2.3.2.1* and *2.2.3.2.2*

1. Histoclear® (5 minutes).
2. Histoclear® (5 minutes).
3. Histoclear® (5 minutes).
4. 100% ethanol (3 minutes).
5. 100% ethanol (3 minutes).
6. 90% ethanol (3 minutes).
7. 70% ethanol (3 minutes).
8. Stain-specific procedures (sections *2.2.3.1* and *2.2.3.2*).
9. 70% ethanol (3 minutes).
10. 90% ethanol (3 minutes).
11. 100% ethanol (3 minutes).
12. 100% ethanol (3 minutes).
13. Histoclear® (5 minutes).
14. Histoclear® (5 minutes).
15. Histoclear® (5 minutes).
16. Air-dry for 24 hours, mounted using Histomount® and a cover-slip placed on top.

Subsequent to this lung sections were analysed using a Leica DMRAZ microscope. Images were acquired using a Leica DC500 camera and Leica QWin software.

2.2.3.2.1 HAEMATOXYLIN AND EOSIN STAINING

1. Deparaffinise with Histoclear® and rehydrate through graded ethanol (section *2.2.3*.2 points 1-7).
2. Running tap water (5 minutes)

3. Rinse with distilled water.
4. Mayer's haematoxylin (2 minutes).
5. Running tap water (5 minutes)
6. Rinse with distilled water.
7. 1x scotts tap water
8. Running tap water (5 minutes)
9. Rinse with distilled water.
10. 1% Eosin Y (90 seconds).
11. Dehydrate through graded ethanol and clear with Histoclear® (section *2.2.3.2*, points 9-16).

A typical photomicrograph obtained with the HE staining procedure is illustrated in Figure 14. Haematoxylin & eosin stain allows for assessment of general lung morphology. Sections were blinded to prevent experimenter bias. A semi-quantitative scoring method was used to assess inflammation in and around the bronchi. Each sample was scored for the number of cells on the basis of 3 images. The scoring system used was 0=normal lung; 1= minor peribronchiolar (PB) inflammation, minimal PB inflammation; 2= slight inflammation in PB area; 3= moderate PB inflammation; 4= marked PB inflammation and cuffing. Slight loss of lung structure (alevoli etc); 5= severe PB inflammation, cufifng and infiltration. Loss of lung structure i.e. solid lung (Barends *et al*., 2004).

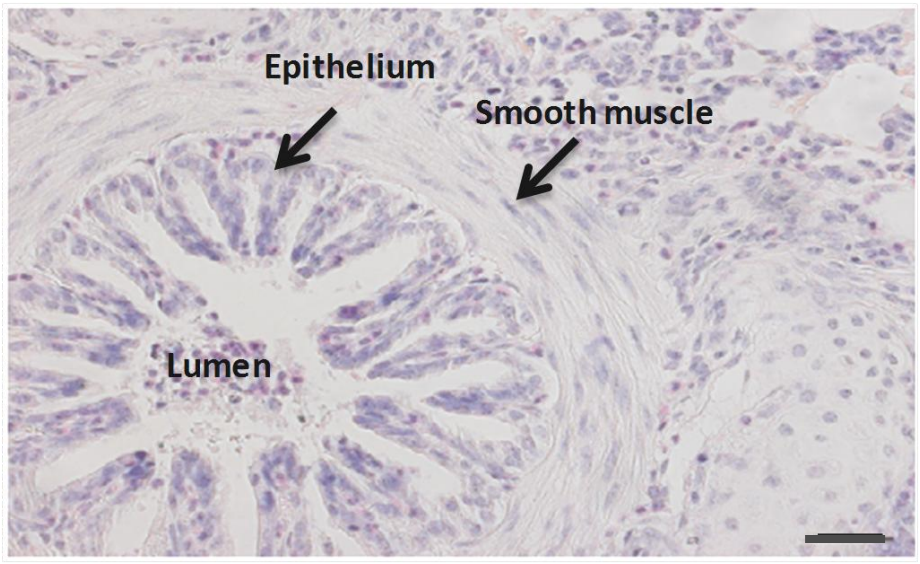


Figure 14: Haematoxylin and eosin stained section of guinea-pig lung following acute ovalbumin challenge (original magnification 200x). A large bronchiole exhibiting notable eosinophilia (bar = 30 µm).

2.2.3.2.2 ALCIAN BLUE/PERIODIC ACID SCHIFF STAIN

1. Deparaffinise with Histoclear® and rehydrate through graded ethanol (section *2.2.3*.2 points 1-7).
2. Distilled water (5 minutes)
3. 1% Alcian blue dissolved in 3% aqueous acetic acid (pH 2.5) (5 minutes)
4. Running tap water (5 minutes)
5. Periodic acid (0.5%) (5 minutes)
6. Running tap water (5 minutes)
7. Distilled water (5 minutes)
8. Schiff's reagent (10 minutes)
9. Running tap water (10 minutes)
10. Mayer's haematoxylin (20 seconds)
11. Running tap water (5 minutes)
12. Dehydrate through graded ethanol and clear with Histoclear® (section *2.2.3.2*, points 9-16).

A typical photomicrograph obtained with the Alcian blue/periodic acid Schiff staining procedure is illustrated in figure 15. The Alcian blue/ periodic acid Schiff stain allows for the identification of mucin and goblet cells.

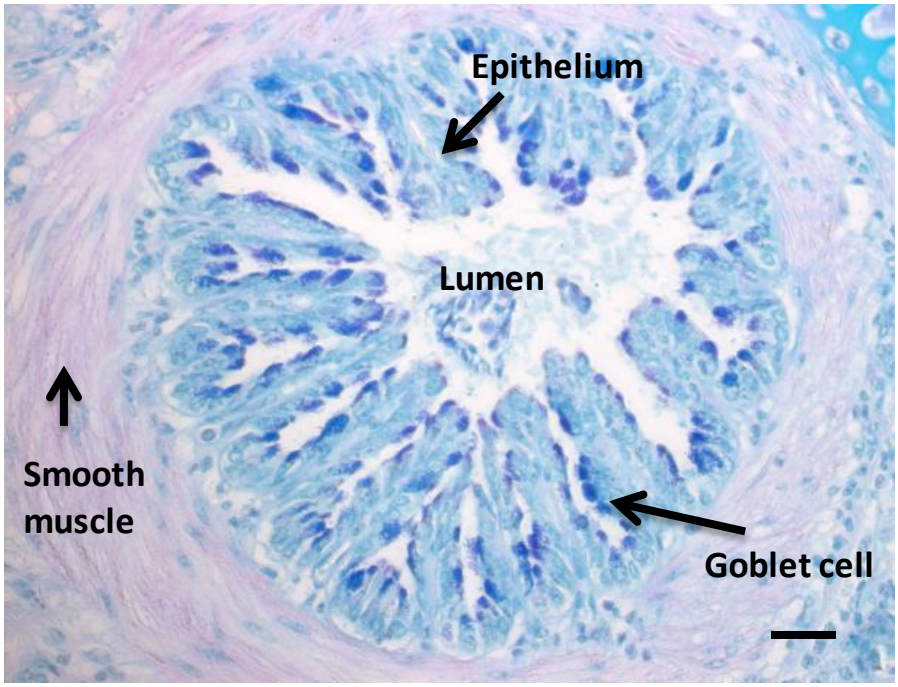


Figure 15: Alcian blue/periodic acid Schiff stain stained section following acute ovalbumin challenge and 2 pre-ovalbumin LPS inhalations (original magnification 200x). A large bronchiole exhibiting goblet cell hyperplasia (bar = 30 μm).

To quantify the number of mucin-associated goblet cells in the epithelium by light microscopy and using the program image J. First epithelial area was calculated by subtracting the lumen area from the total area of the epithelium and lumen combined (shown in Figure 16A). Next the number of goblet cells (Ab/PAS positive points, stained dark blue and purple and typical goblet cell morphology) were counted (Figure 16B). The following formula was applied to calculate the number of mucin-associated goblet cells per 10,000 epithelial pixels.

$$\text{Mucin per 10,000} = (\text{Number of AB/PAS+ points} \div \text{Epithelial area}) \times 10000$$

The resulting values allowed for determination of whether goblet cell hyperplasia and metaplasia had taken place.

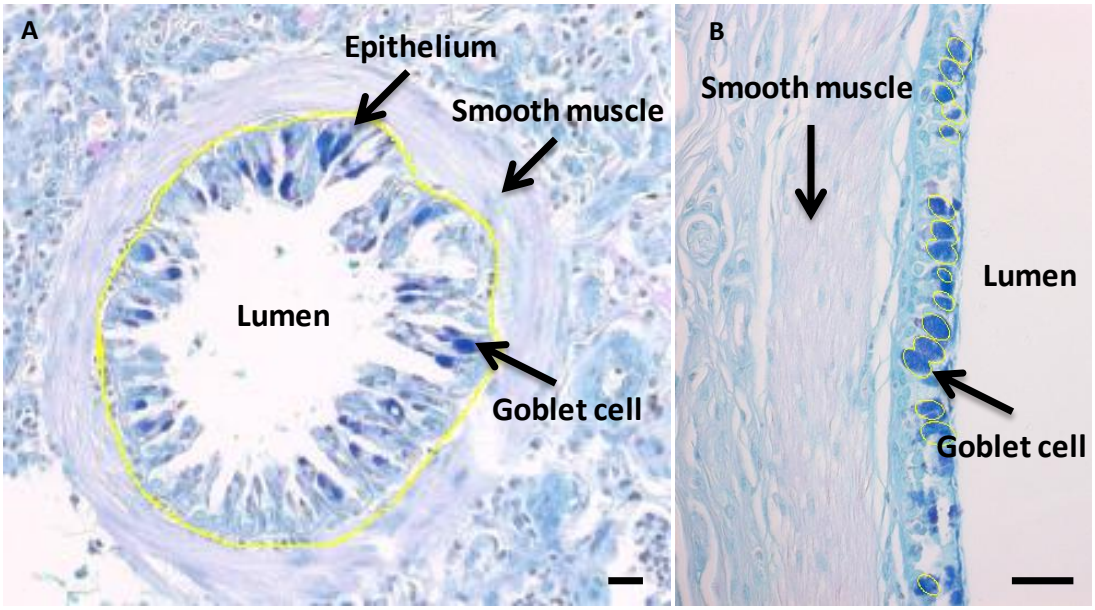


Figure 16: Alcian blue/periodic acid Schiff stain stained section following acute ovalbumin challenge and a single pre-ovalbumin LPS inhalation. **A)** A large bronchiole exhibiting goblet cell hyperplasia (original magnification 100x) B) Airway epithelium with numerous goblet cells (original magnification 200x); (bar = 30 µm).

2.2.3.2.3 SIRIUS RED STAIN

1. Deparaffinise with Histoclear® and rehydrate through graded ethanol (section *2.2.3*.2 points 1-7).
2. Mayer's haematoxylin (2 minutes).
3. Running tap water (2 minutes).
4. Rinse with 100% ethanol.
5. Sirius red (2 hours).
6. Running tap water (2 minutes).
7. Dehydrate through graded ethanol and clear with Histoclear® (section *2.2.3.2*, points 9-16).

A typical photomicrograph obtained with the Sirius Red staining procedure is illustrated in Figure 17. Sirius red staining allows for the differentiation of eosinophils

from neutrophils (Mayerholz *et al*, 2009). For analysis, 3 bronchioles of comparable size were selected and each centered within the field of view at a 200x magnification. The number of eosinophils was analysed as the number per field of view and per bronchiole area. For the latter, bronchiole perimeter was defined by a line around the adventitia and all eosinophils within that area counted (Figure 18). Counting the number of eosinophils per bronchiole area minimises the confounding effect of bronchiole size on eosinophil counts. For counting, eosinophils were defined as cells demonstrating a cytoplasm staining an intense red with dark bi-lobed nuclei. Neutrophils demonstrated less cytoplasmaic staining and different nuclear morphology. Investigator blinding was performed to minimise investigator bias.

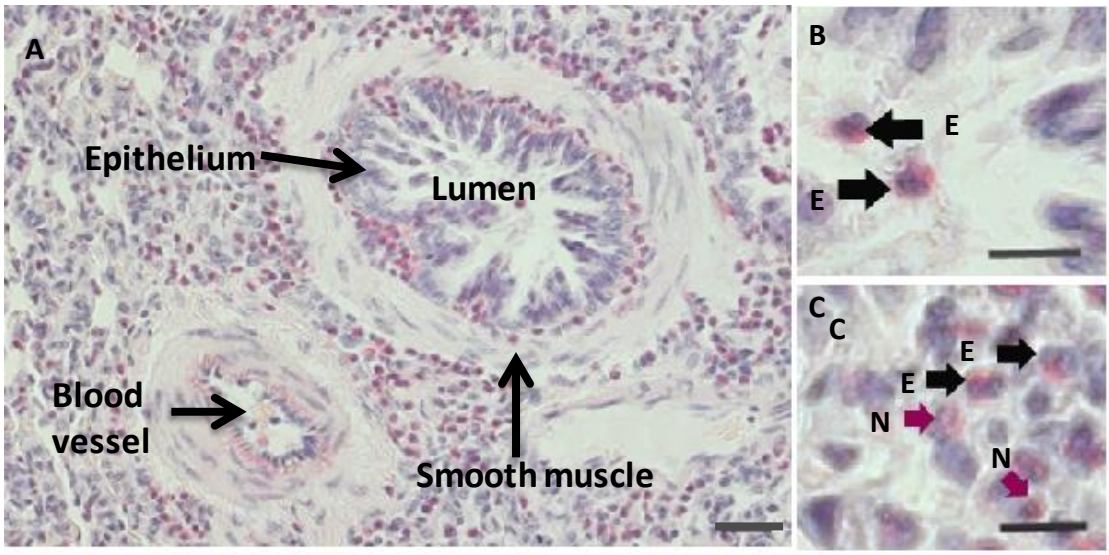


Figure 17: Sirius red stained section of guinea-pig lung following acute ovalbumin challenge (original magnification 200x). **A** An artery in close proximity to a heavily eosinophil-infiltrated bronchiole (bar = 30 µm). **B** Two eosinophils with clearly-defined bi-lobed nuclei (black arrows; bar = 15 µm). **C** Clearly differentiated eosinophils (black arrows) and neutrophils (purple arrows; bar = 15 µm).

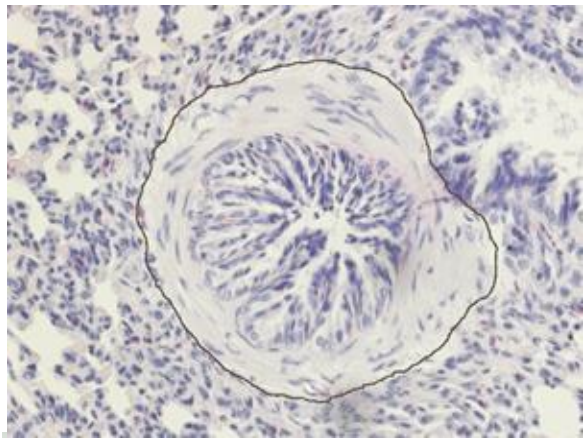
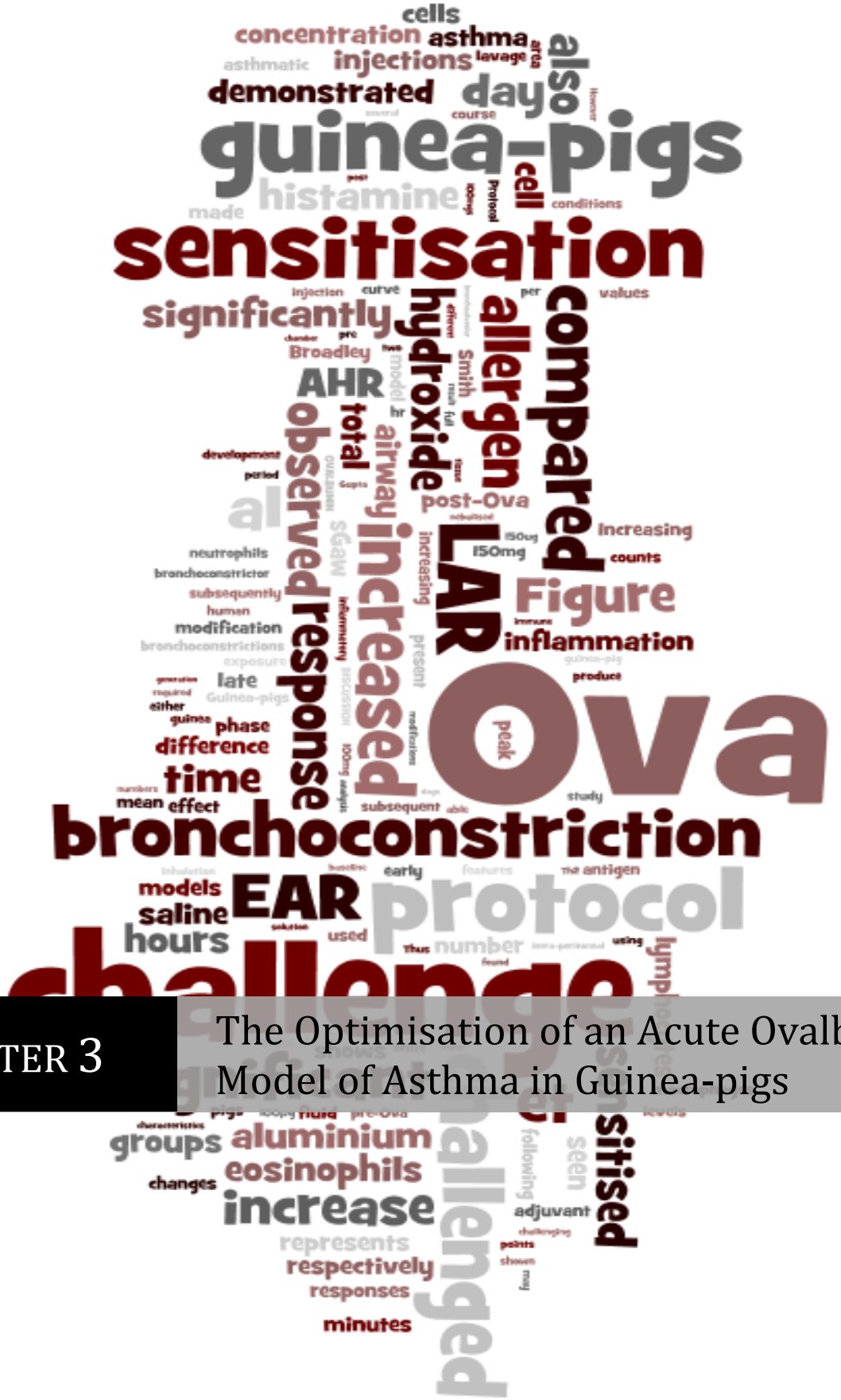


Figure 18: Sirius red stained section of guinea-pig lung tissue showing the area defined as bronchiole perimeter. Eosinophils within or in contact with the bronchiolar perimeter were eligible for counting.

2.3 STATISTICAL ANALYSIS

Student's t-tests were used for the comparison of differences between groups or data points. One way analysis of variance (ANOVA) followed by Dunnet post hoc test were used when 2 or more groups were being compared to a control group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Results are plotted as the mean±standard error of the mean (SEM). All lung function data were plotted as a percentage of baseline to take into account the individual differences in guinea-pig baseline sGaw values. Graphs were drawn using GraphPad Prism 5 and results analysed using Graphpad Instat 3.



CHAPTER 3

The Optimisation of an Acute Ovalbumin Model of Asthma in Guinea-pigs

Chapter 3

3.1 Introduction

3.1.1 Ovalbumin models of asthma

Allergen challenge models of asthma are created through the initial sensitisation to an allergen and subsequent re-encounter of the same allergen. A variety of different allergens have been used in a range of species including ragweed, cockroach extract, *Aspergillus fumigatus*, house dust mite and ovalbumin (Ova) (Canning & Chou, 2008). The most common allergen used is ovalbumin (Ova), especially in guinea-pigs (Smith & Broadley, 2007). A variety of different protocols have been used but generally all follow a basic structure. Commonly Ova is administered intraperitoneally with an adjuvant such as aluminium hydroxide. Animals are then given several weeks to develop an immune response. Re-exposure to Ova, generally by the inhaled route then triggers the effector phase. The intraperitoneal route of administration in these models has been criticised for its lack of relevance to the likely route of allergen sensitisation in humans, the respiratory mucosa. This could lead to differences in the immune response (Cates *et al*, 2007). However, no direct comparison between models that use intraperitoneal and respiratory mucosa (house dust mite) sensitisation have been performed, so currently this is unknown. Protocols involving sensitisation by aerosolised Ova exist but they take up to 4 weeks to achieve sensitisation (Nabe *et al*, 1997).

Aluminium adjuvants exist as many different salts including aluminium phosphate and aluminium hydroxide. The latter is the most commonly used in allergy research and exists as several different preparations including powders and gels. Differences in the amount of other salts including magnesium hydroxide and the method of preparation can alter the immunestimulating capabilities of the adjuvants (Lindblad, 2004). Aluminium hydroxide performs its action as an adjuvant by several mechanisms. It forms bonds with Ova when in solution, which then act as a depot for the slow release of antigen. This prolongs the time that antigen, antigen presenting cells (APC) and lymphocytes have to interact (Gupta *et al*, 1993). Additionally, aluminum hydroxide is reported to increase the efficiency of antigen uptake by APCs

by aiding the formation of particles of optimal size (< 10 µm) for APCs (Gupta *et al*, 1995). Additionally, adjuvant-allergen pairings act as an inflammatory focus for immunocompetent cells (Gupta *et al*, 1995). Aluminium hydroxide has also been shown to favour development of Th2 lymphocyte responses (Cooper, 1994). Th2 responses in asthmatic patients favour the development of early and late asthmatic (EAR and LAR respectively) responses. Finally, aluminium hydroxide activates complement and aids the development of B cell memory (Gupta *et al*, 1995).

3.1.2 Features of acute ovalbumin models of asthma

Both acute and chronic Ova models of asthma have been developed. Acute models of asthma involve either a single or several allergen challenges. Chronic models of asthma typically involve at least 8 allergen challenges over several weeks (Evans *et al*, 2012). Both acute and chronic models typically display an EAR, LAR to the final allergen challenge, airways hyperresponsiveness (AHR) and airway inflammation. Chronic models also display additional features seen in human asthma such as airway remodelling (Rennard, 1996). This makes chronic models useful for investigating remodelling processes in asthma. The length of time they take to develop (typically over 6 weeks) is a drawback for drug screening. Acute models have the advantage of reproducing key functional and inflammatory responses in asthma while taking less time to develop. Thus, they represent a good starting point for the investigation of the effect of viruses and LPS on these features.

Early and late asthmatic responses are seen in humans following allergen challenge (Booij-Noord *et al*, 1971). The EAR is an immediate bronchoconstriction to allergen and usually resolves within the first couple of hours. The LAR is a delayed bronchoconstriction to an allergen and is observed in the majority of asthmatic patients. In humans, this is typically seen 4-8 hours after allergen challenge (Galli *et al*, 2008). Airways hyperresponsiveness (AHR) is another key feature of asthma. AHR is an increased response to a bronchoconstrictor stimulus such as histamine (Cockcroft & Davis, 2006). The final key feature that acute Ova models demonstrate is airway inflammation. In particular eosinophils, macrophages and lymphocytes are

increased in human asthma (Laitinen *et al*, 1996). Neutrophils are also important; especially in more severe forms of the disease (Taha *et al*, 2001). Airway inflammation can be assessed by bronchoalveolar lavage or histological staining of lung sections (Walters and Gardiner, 1991; Grootendorst *et al*, 1997). Dissociation between these indices of airway inflammation has been observed making it useful to investigate inflammation using both methods (Barends *et al*, 2004).

Several groups have demonstrated isolated characteristics such as AHR, EAR and LAR in guinea-pig models (Hutson *et al*, 1990; Danahay *et al*, 1999). However, not all groups have been able reproduce these features. In particular the LAR has proved difficult to achieve under a range of different sensitisation conditions (Everitt & Moore, 1992; Underwood *et al*, 1992). Within this laboratory a model demonstrating an EAR, LAR, AHR and airway inflammation to Ova challenge in guinea-pig has been developed (Evans *et al*, 2012). However this model has required optimisation on several occasions over the years to continue to produce these features. Lewis *et al*, (1996) modified the allergen challenge conditions to stop the need for mepyramine, which prevents fatal anaphylaxis. Smith & Broadley, (2007) modified the sensitisation conditions because of the loss of key features over time, increasing the amount of Ova used and the number of injections given. This restored the EAR, LAR and AHR to Ova challenge. In the present study, modifications to both the challenge and sensitisation conditions were made to restore EAR, LAR, AHR and airway inflammation. That had again waned by the start of this PhD project.

3.2 Hypothesis

Modifications to the ovalbumin sensitisation and challenge protocol will restore functional and inflammatory responses in an acute model of asthma

3.3 Aims and objectives

The aim of this chapter was to re-establish an acute guinea-pig model of asthma displaying an early and late asthmatic responses, airway hyperresponsiveness and airway inflammation as demonstrated by Smith & Broadley, (2007) and Evans *et al*, (2012). By producing these features, subsequent work to investigate the effect of viruses, LPS and anti-asthma drugs on these characteristics can be performed.

3.4 Methods

These methods are described in more detail in chapter 2.

3.4.1 Sensitisation

Guinea pigs (Dunkin-Hartley, male) were sensitised by an bilateral intra-peritoneal injection of a solution containing ovalbumin (Ova) and aluminium hydroxide ($Al(OH)_3$) in normal saline on days 1 and 5 (protocol 1 and 2) or 1, 4 and 7 (protocol 3-7). Please refer to Table `1 or details on the amount of Ova and ($Al(OH)_3$) used in each protocol. The solution was stirred for 2 hours previous to injection to ensure the formation of Ova-aluminium hydroxide complexes.

3.4.2 Ovalbumin challenge

Allergic responses were triggered by inhalation of Ova in a Perspex exposure chamber using a DeVilbiss nebuliser for 1 hour. Please refer to Table 1 for information on the day of Ova challenge and the concentration.

3.4.3 Acute challenge protocols

An acute challenge protocol as per Smith & Broadley, (2007) was used initially as described below (protocol 1). Subsequently, modifications were made to the protocol to produce an EAR, LAR, AHR and total and differential cell numbers.

Protocol	Ova Challenge Dose	No. of sensitisations	Ova Sensitisation dose	(Al (OH)3) sensitisation dose	Injection volume	Challenge day
Protocol 1 *	0.01%	2	100µg/ml	100mg/ml	1ml	Day 15
Protocol 2	0.03%	2	100µg/ml	100mg/ml	1ml	Day 15
Protocol 3	0.03%	3	100µg/ml	100mg/ml	1ml	Day 15
Protocol 4	0.03%	3	150µg/ml	100mg/ml	1ml	Day 15
Protocol 5	0.03%	3	75µg/ml	50mg/ml	2ml	Day 15
Protocol 6	0.03%	3	75µg/ml	75mg/ml	2ml	Day 15
Protocol 7	0.03%	3	150µg/ml	100mg/ml	1ml	Day 21

Table 1: Variations made to the acute ovalbumin guinea-pig model as established in Smith & Broadley, (2007). * Protocol of Smith and Broadley, (2007). Modifications for each protocol are shown in red.

3.4.4 Measurement of lung function

Guinea-pig airway function was measured using non-invasive double chamber plethysmography, using specific airway conductance (sGaw) as a measure of airway conductance (Griffiths-Johnson *et al*, 1988). Lung function was recorded following Ova challenge, hourly for 12 hours and every 15 minutes during the first hour of measurements. A final measurement was also taken 24 hours post-challenge. All values from these readings were expressed as a percentage of the baseline reading, taken before Ova challenge. A negative percentage change in baseline value represents a bronchoconstriction. To account for differences in the timing of allergen responses during the early (0-6 hours) and late (6-12 hours) phases, sGaw was also expressed as the peak bronchoconstriction during that period (displayed as a histogram next to a time course plot) and area under the curve.

3.4.5 Measurement of Airways hyperresponsiveness (AHR)

AHR was determined using whole body plethysmography. This was performed by measuring the bronchoconstrictor response to histamine pre- (day 15) and post-Ova challenge (24 hours after Ova challenge). Histamine (0.3mM) was delivered to the guinea-pigs by the use of a Buxco nebuliser chamber at a rate of 0.5 LPM per, two minutes nebulisation, and 1 minute drying period with a 10% duty setting per chamber. Lung function was measured before histamine inhalation and at 0, 5 and 10 minutes post-histamine exposure.

3.4.6 Measuring Airway Inflammation

Following final histamine challenge, guinea-pigs were sacrificed by an intra-peritoneal overdose of sodium pentobarbitone, the lungs excised and lavaged. Total and differential cell counts were then performed.

3.4.7 Histological analysis of lungs

Lung lobe samples were stored in formaldehyde and 1-2mm bilateral sections cut. Samples were dehydrated in increasing concentrations of alcohol and then chloroform. Tissue sections were then set into wax blocks using molten paraffin. 5µm sections were cut using a microtome and fixed to polysine coated slides. Slides were stained using the Sirius red staining protocol which allows the identification of eosinophils. The number of eosinophils was counted per field of view and per bronchiole. Eosinophils within the perimeter of a bronchiole were included in the latter. Eosinophils were defined as cells demonstrating a cytoplasm staining an intense red with dark bi-lobed nuclei.

3.5 Results

3.5.1 Protocol 1

Sensitisation: 2 intra-peritoneal injections of 100µg Ova and 100mg $Al(OH)_3$

Ova challenge: 0.01% Ova or saline

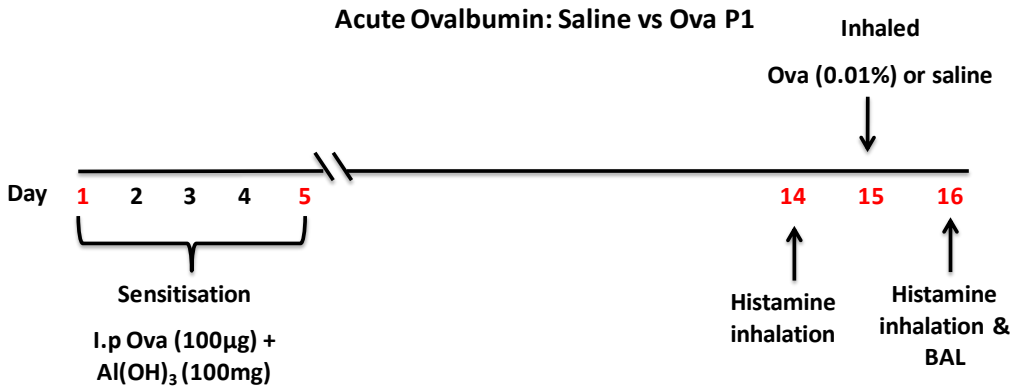


Figure 1: The acute ovalbumin protocol for guinea-pigs exposed to saline or Ova protocol 1.

In Ova challenged guinea-pigs a significant bronchoconstriction was observed immediately (-45.6± 6.2%), which did not return to saline challenged levels until 2 hours post-Ova challenge. This bronchoconstriction was the early asthmatic response. No further significant differences between saline and Ova challenged sGaw values were observed. Therefore no late asthmatic response was observed (Figure 2, time-course). A significant increase in the peak bronchoconstriction during the EAR was observed in Ova challenged guinea-pigs, as shown by a significant decrease in sGaw compared to saline challenged guinea pigs (-45.6±6.2 compared with 2.0±2.6 respectively). No significant difference in the peak sGaw values was seen between 6-12 hours (Figure 2, histogram). Throughout the total time course, Ova challenged guinea-pigs demonstrated significantly greater area under the curve then saline challenged guinea-pigs (97.6±11.1%.hr compared to 10.0±6.1%.hr). This

was also the case for the EAR time points (80.3±9.4%.hr compared to 8.5±5.4%.hr). A significant increase in area under the curve (AUC) during the LAR was also seen with Ova challenge (17.7±5.0%.hr compared to 1.6±1.0%) (Figure 3). 24 hours after saline challenge, guinea-pigs show no significant change in the bronchoconstrictor response to histamine compared to before saline challenge (Figure 4A). This was also the case for Ova challenged animals (Figure 4B).

Ova challenge significantly increased total lavage cells ($3.2\pm0.5x10^6$/ml compared to saline, $1.6\pm0.13x10^6$/ml). Eosinophils made up most of this increase ($1.3\pm0.3x10^6$/ml compared to saline, $0.05\pm0.01x10^6$/ml). No changes in any other cell type were observed (Figure 5).

3.5.1.1 Summary

Guinea-pigs sensitised with 2 injections of 100µg Ova and 100mg aluminium hydroxide and subsequently challenged with 0.01% Ova (protocol 1) demonstrated an EAR, an increase in total cells and eosinophils in lavage fluid. The protocol did not lead to the development of a clear LAR, AHR and increase in macrophages and lymphocytes. These results are inconsistent with Smith and Broadley, (2007) and Evans *et al*, (2012). In both studies this protocol induced an EAR, LAR, AHR and airway inflammation.

To restore these features, modifications to the sensitisation and challenge protocol were made. Previously, protocol modification has been shown to restore these responses (Smith and Broadley, 2007). The first modification made was an increase in the allergen challenge concentration from 0.01% to 0.03% Ova, to ensure the full allergen response was being triggered.

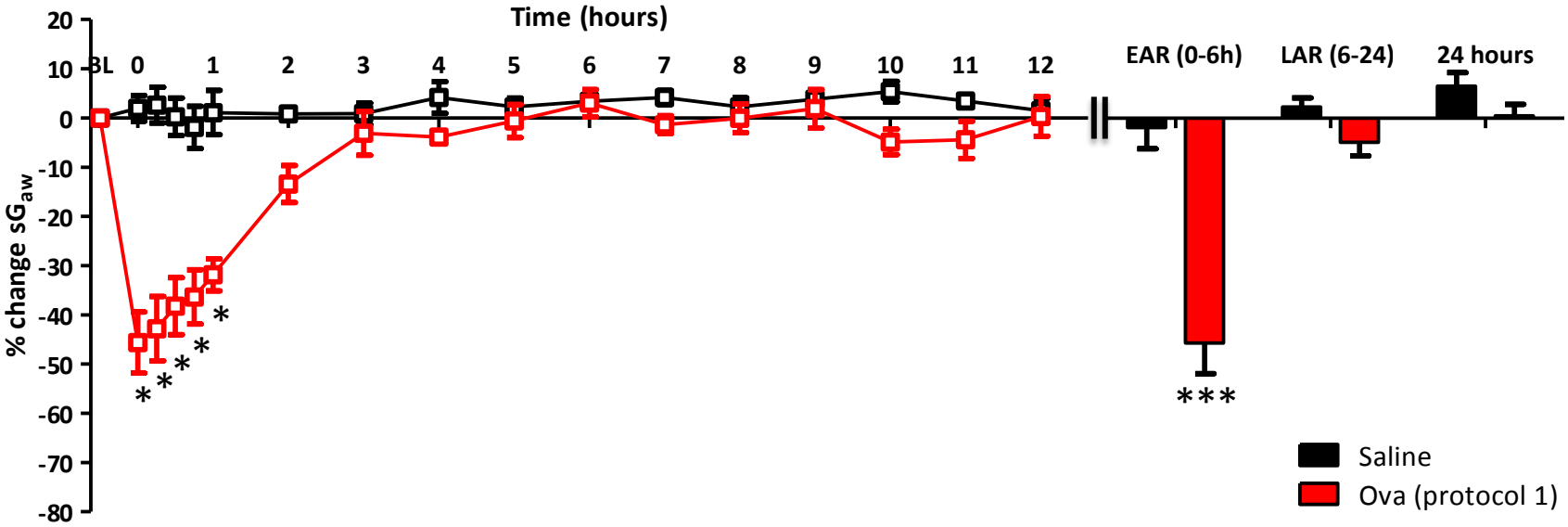


Figure 2: The mean time-course values of sGaw in guinea-pigs sensitised with 2 injections of a suspension of 100µg ovalbumin (Ova) and 100mg aluminium hydroxide and challenged with 0.01% Ova or saline. The histogram represents the maximum bronchoconstriction values recorded during early asthmatic response (EAR) (0-6 hours), late asthmatic response (LAR) (6-12 hours) and 24 hours readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=6 (saline), N=5 (Ova); *Significantly different from saline treatment $p<0.05$, *** $p<0.001$; performed with a two tailed T-test.

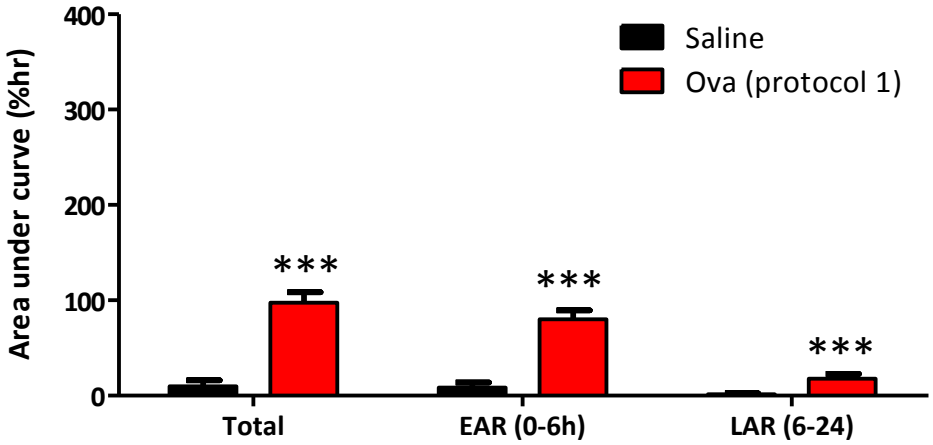


Figure 3: Area under the curve analysis of bronchoconstrictor responses in guinea-pigs sensitised with 2 injections of a suspension of 100µg ovalbumin (Ova) and 100mg aluminium hydroxide and challenged with 0.01% Ova or saline. For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-24 hours, early asthmatic response (EAR) includes values from 0-6 hours and late asthmatic response (LAR) includes values from 6-12 hours. Area under the curve is measured in %.hour. N=6 (saline), N=5 (Ova). ***Significantly different from saline treatment $p<0.001$; performed with a two tailed T-test.

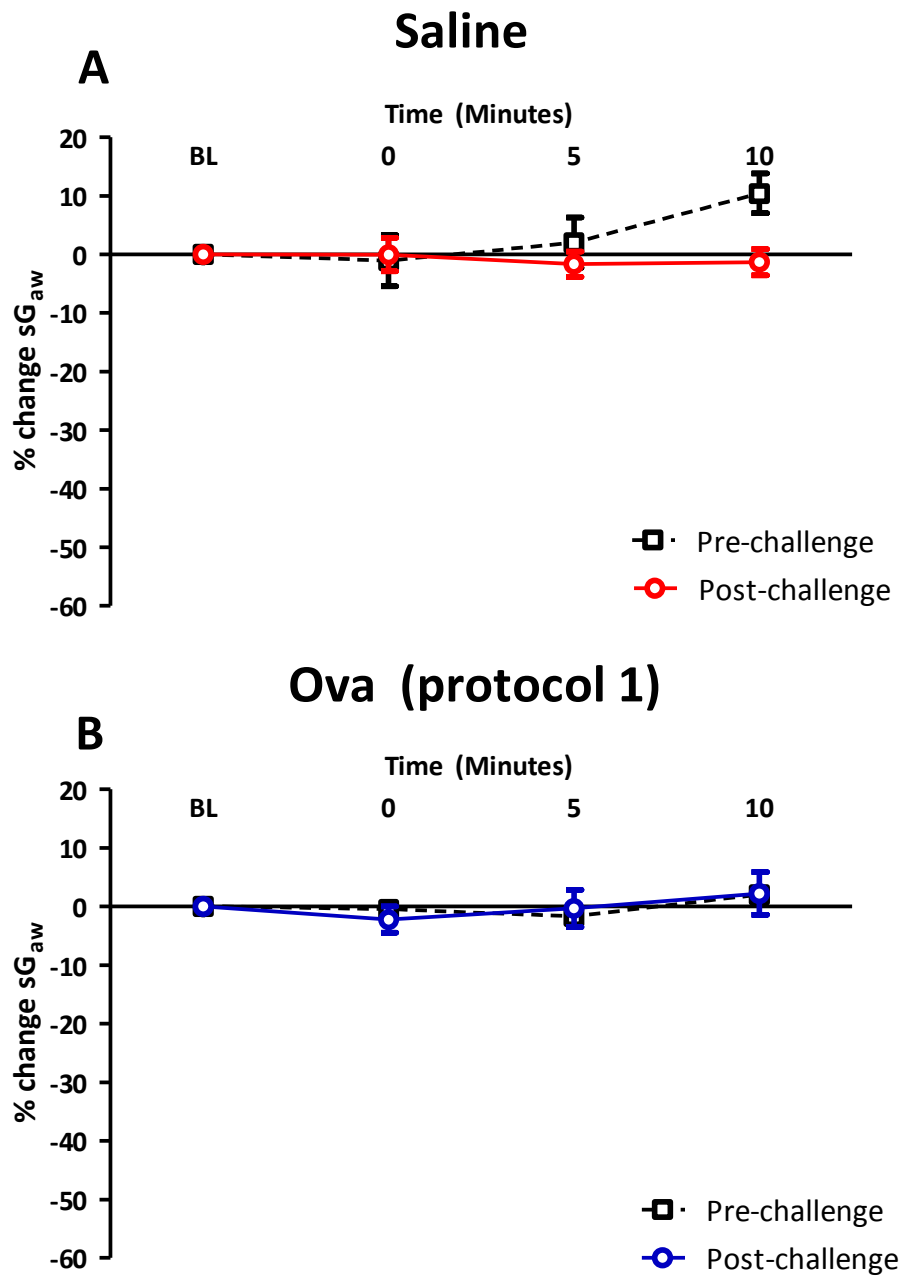


Figure 4: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles with 0.5LPM flow per chamber over 2 minutes, 1 minute drying period) in guinea-pigs sensitised with 2 injections of a suspension of 100µg ovalbumin (Ova) and 100mg aluminium hydroxide and challenged with A) saline or B) 0.01% Ova. Values were recorded 24 hours pre- and post-saline or Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=6 (saline), N=5 (Ova); performed with a two tailed T-test.

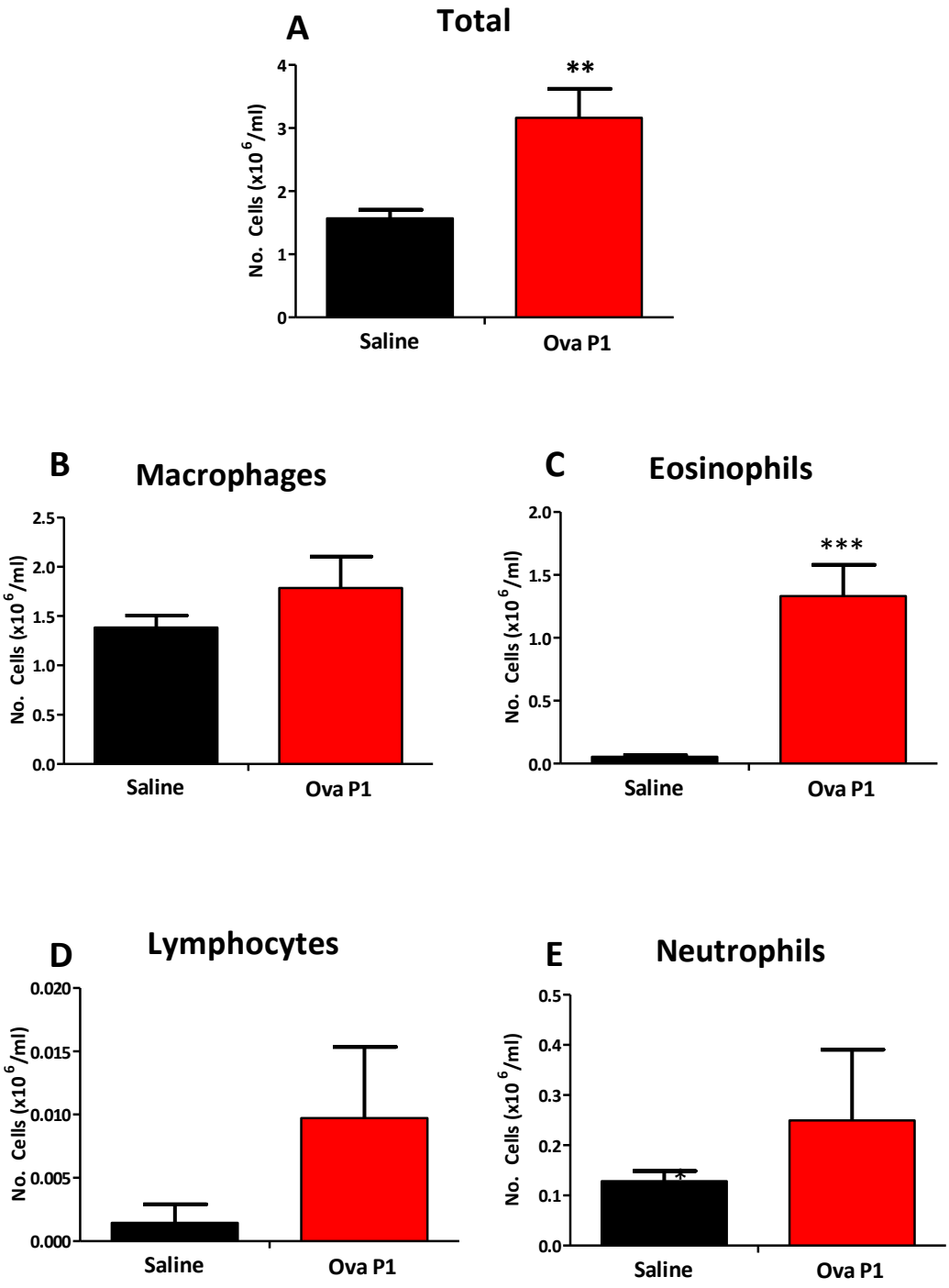


Figure 5: The total cell (A), macrophages (B), eosinophils (C), lymphocytes (D) and neutrophils (E) counts in bronchoalveolar fluid in guinea-pigs sensitised with 2 injections of a suspension of 100µg ovalbumin (Ova) and 100mg aluminium hydroxide and challenged with 0.01% Ova or saline. N=6 (saline), N=5 (Ova); **Significantly different from saline treatment $p<0.01$; *** $p<0.001$; performed with a two tailed T-test.

3.5.2 Protocol 2

Sensitisation: 2 intra-peritoneal injections of 100µg Ova and 100mg $Al(OH)_3$

Ova challenge: 0.01% (Protocol 1) or 0.03% Ova (protocol 2).

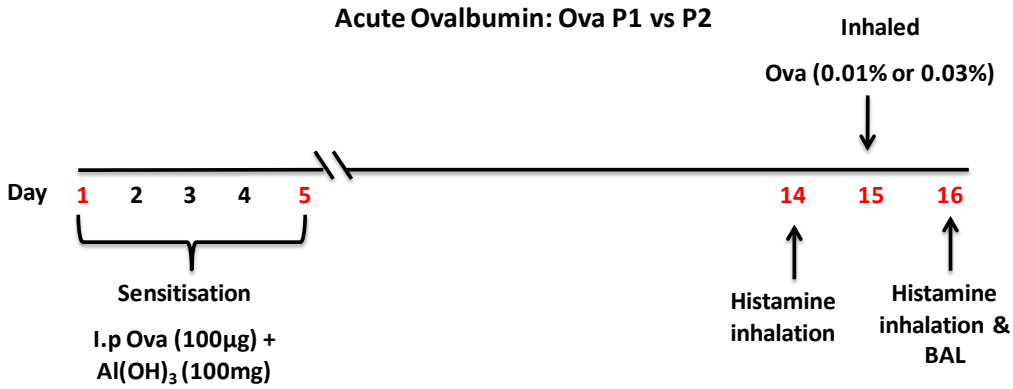


Figure 6: The acute ovalbumin protocol for guinea pigs exposed to Ova protocol 1 or 2.

In both 0.01% and 0.03% Ova challenged guinea-pigs a significant bronchoconstriction was observed immediately (-45.6± 6.2% and -60.9±2.1%). No significant difference was seen between the times either group took to return to baseline levels following Ova challenge. Both these bronchoconstrictions constitute the EAR. No further bronchoconstrictions representing the LAR were seen in either of the Ova challenged groups (Figure 7, time course). The peak EAR of the 0.03% Ova challenged guinea-pigs was significantly increased compared to the 0.01% Ova challenged guinea-pigs (-45.6±6.2 and -60.9±2.1% respectively). No significant difference in peak sGaw values was seen 6-12 hours post-Ova challenge (Figure 7, histogram). Throughout the total time course 0.03% Ova challenged guinea-pigs demonstrated significantly greater area under the curve then 0.01% Ova challenged guinea-pigs (165.8±15.3%.hr compared to 97.5±9.1%.hr). This was also the case for the EAR time point (141.3±6.8%.hr compared to 80.3±7.7%.hr). No difference was observed between the two groups at LAR time points (Figure 8)

24 hours after 0.03% Ova challenge a significant increase in the immediate bronchoconstriction to histamine inhalation compared to the pre-Ova challenge sGaw values was observed (-38.5±7.9% compared to -4.1±2.3%). A significant bronchoconstriction to histamine was still present 5 minutes post-histamine challenge (-2.2±3.1% compared to -18±4.2%). At 10 minutes post-histamine exposure sGaw values were no different from pre-Ova challenge values (Figure 9).

0.03% Ova challenge significantly increased the total cells present in lavage fluid ($5.3\pm0.4x10^6$/ml compared to 0.01% Ova, $3.2\pm0.5x10^6$/ml). Eosinophils were significantly elevated ($2.0\pm0.2x10^6$/ml compared to 0.01% Ova, $1.3\pm0.3x10^6$/ml). No changes in any other cell type with Ova challenge were observed (Figure 10).

3.5.2.1 Summary

Guinea-pigs sensitised with 2 injections of 100µg Ova and 100mg aluminium hydroxide and subsequently challenged with 0.03% Ova (protocol 2) demonstrated an EAR, slightly increased in duration, AHR and an increase in total cells, eosinophils and macrophages. Thus increasing the allergen challenge concentration 3-fold induced AHR and increased airway inflammation. However, a clear LAR was not demonstrated so further modification to the Ova protocol were made. Increasing the number of sensitisation injections was shown by Smith & Broadley, 2007 to induce a LAR. Therefore the next modification made to the protocol was to increase the number of sensitisation injections from 2 to 3.

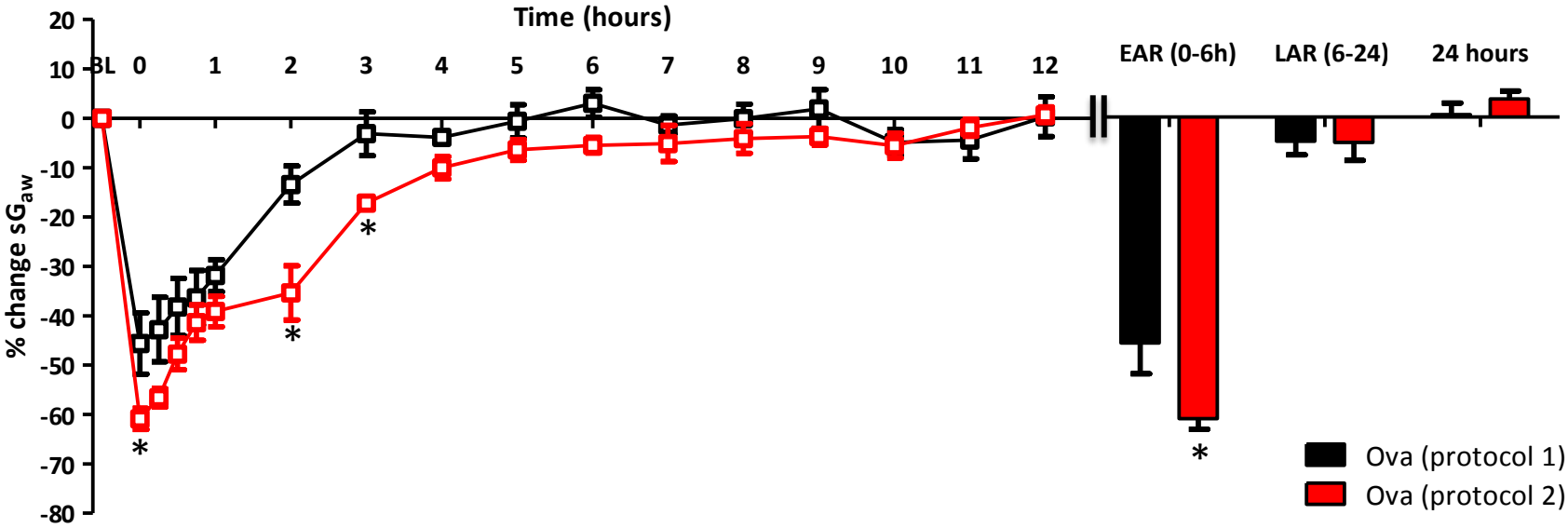


Figure 7: The mean time-course sGaw values in guinea-pigs sensitised with 2 injections of a suspension of 100µg ovalbumin (Ova) and 100mg aluminium hydroxide and challenged with 0.01% (protocol 1) or 0.03% Ova (protocol 2). The histogram represents the maximum bronchoconstriction values recorded during the early asthmatic response (EAR) (0-6 hours), late asthmatic response (LAR) (6-12 hours) and 24 hours readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=5 (protocol 1), N=6 (protocol 2) *significantly different from protocol 1 $p<0.05$; performed with a two tailed T-test.

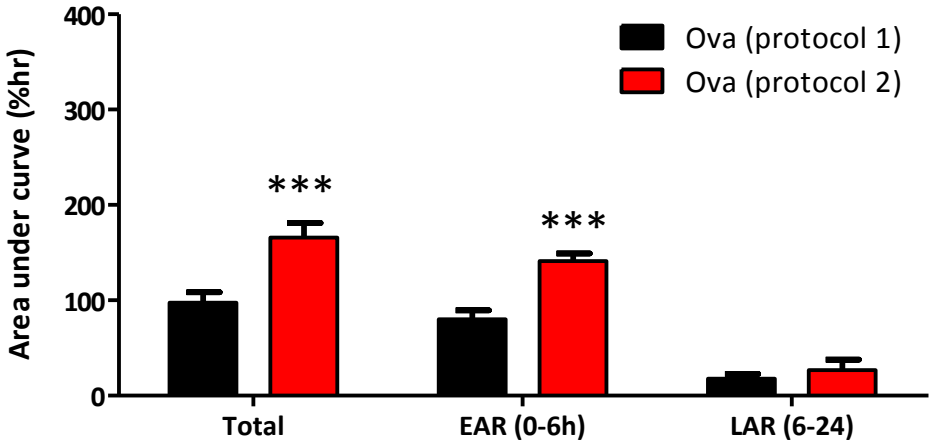


Figure 8: Area under the curve analysis of bronchoconstrictor responses in guinea-pigs sensitised with 2 injections of a suspension of 100ug ovalbumin (Ova) and 100mg aluminium hydroxide and challenged with 0.01% (protocol 1) or 0.03% Ova (protocol 2). For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-24 hours, early asthmatic response (EAR) includes values from 0-6 hours) and late asthmatic response (LAR) includes values from 6-12 hours. Area under the curve is measured in %.hour. N=5 (protocol 1), N=6 (protocol 2) ***significantly different from protocol 1 $p<0.001$; performed with a two tailed T-test.

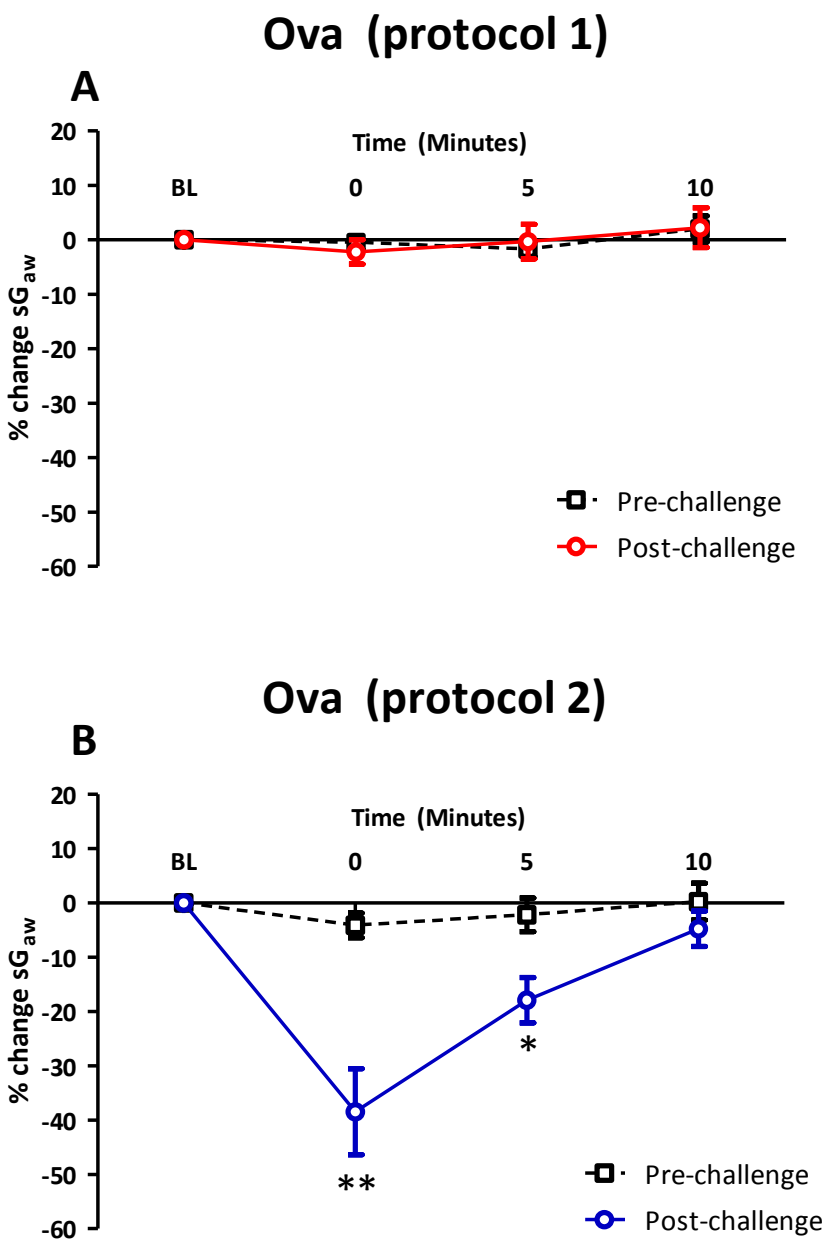


Figure 9: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles with 0.5LPM flow per chamber over 2 minutes, 1 minute drying period) in guinea-pigs sensitised with 2 injections of a suspension of 100ug ovalbumin (Ova) and 100mg aluminium hydroxide and challenged with A) 0.01% (protocol 1) or B) 0.03% Ova (protocol 2). Values were recorded 24 pre- and post-Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=5 (protocol 1), N=6 (protocol 2) *Significantly different from time paired pre-Ova challenge values $p<0.05$, ** $p<0.01$; performed with a two tailed T-test.

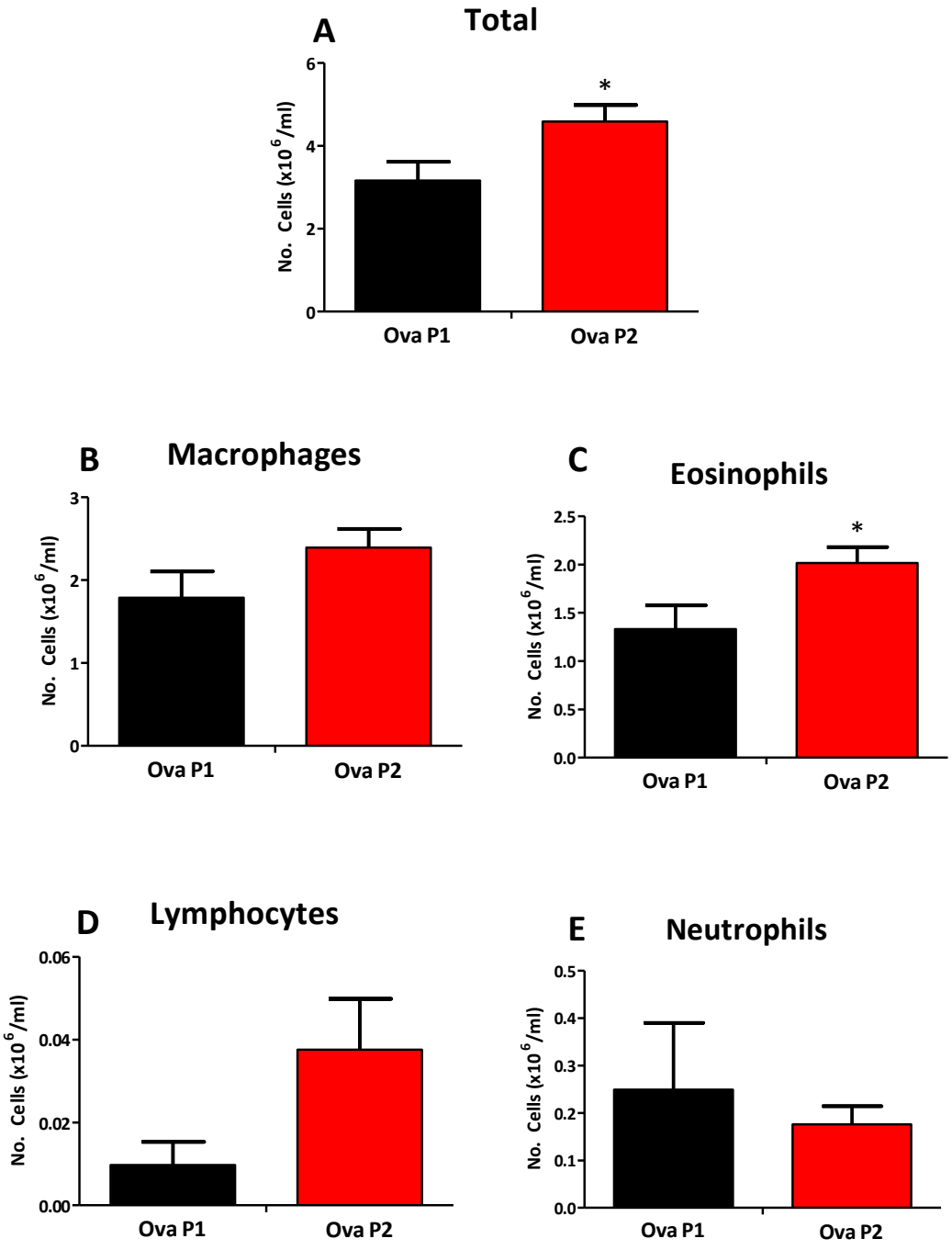


Figure 10: The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in bronchoalveolar fluid in guinea-pigs sensitised with 2 injections of a suspension of 100ug ovalbumin (Ova) and 100mg aluminium hydroxide and challenged with 0.01% (protocol 1) or 0.03% Ova (protocol 2). N=5 (protocol 1), N=6 (protocol 2) *Significantly different from protocol 1 $p<0.05$; performed with a two tailed T-test.

3.5.3 Protocol 3

Sensitisation: 3 intra-peritoneal injections of 100µg Ova and 100mg $Al(OH)_3$

Ova challenge: 0.03% Ova.

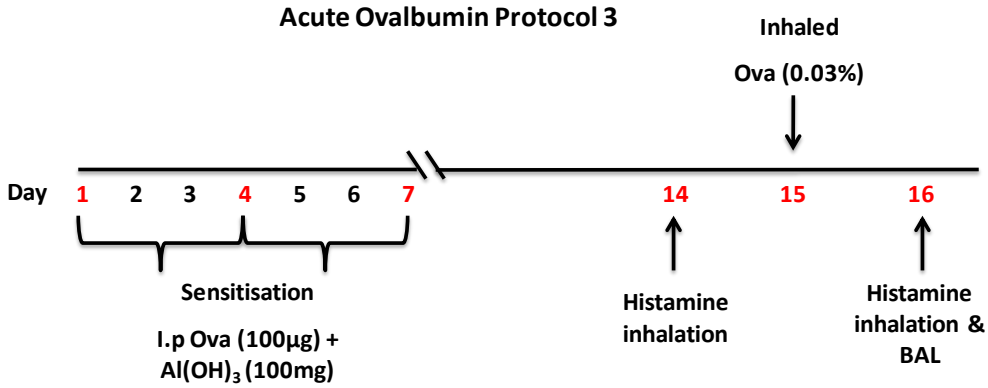


Figure 11: The acute ovalbumin protocol for guinea-pigs exposed to Ova protocol 3.

In both guinea-pigs sensitised with protocol 2 & 3 (2 and 3 I.p sensitisation injections respectively) and subsequently challenged with Ova a significant bronchoconstriction was observed immediately (-60.9±2.15% and -61.9±2.1% respectively). No significant difference was observed between the times either group took to return to baseline sGaw values following Ova challenge. Both these bronchoconstrictions constitute the EAR. No further bronchoconstrictions representing the LAR were seen in either of the Ova challenged groups (Figure 12, time course). No significant difference between the peak EAR and LAR bronchoconstrictions was seen between groups (Figure 12, histogram). No difference in area under the curve was observed between the two groups at total, EAR and LAR time points (

Figure **13**)

Guinea-pigs sensitised with 3 intra-peritoneal injections of Ova demonstrated a significant increase in the bronchoconstrictor response to histamine inhalation 24 hours after Ova challenge (-28.3±6.0% compared to pre-Ova challenge, -3.9±2.8%). This bronchoconstriction to histamine was still present 5 minutes post-exposure (-

11.8±3.7%) compared to pre-challenge (-2.8±3.7%). At 10 minutes post-histamine exposure the guinea-pig sGaw values had recovered to pre-Ova exposure levels. The extent of the increased bronchoconstrictor response to histamine was not significantly different between groups (Figure 14).

The addition of a 3rd sensitisation injection did not significantly change total cells or any other specific cell populations measured (Figure 15).

3.5.3.1 Summary

Guinea-pigs sensitised with 3 injections of 100µg Ova and 100mg aluminium hydroxide and subsequently challenged with 0.03% Ova (protocol 3) demonstrated an EAR, AHR and airway inflammation. However, a LAR was not observed. Therefore, further modifications to Ova protocol were made. Smith & Broadley, 2007 demonstrated that an increase in the number of sensitisation injections, increasing the Ova concentration in each was can promote a LAR. Therefore the next modification made to the protocol was to increase the concentration of Ova in each sensitisation injection from 100µg to 150µg/ml.

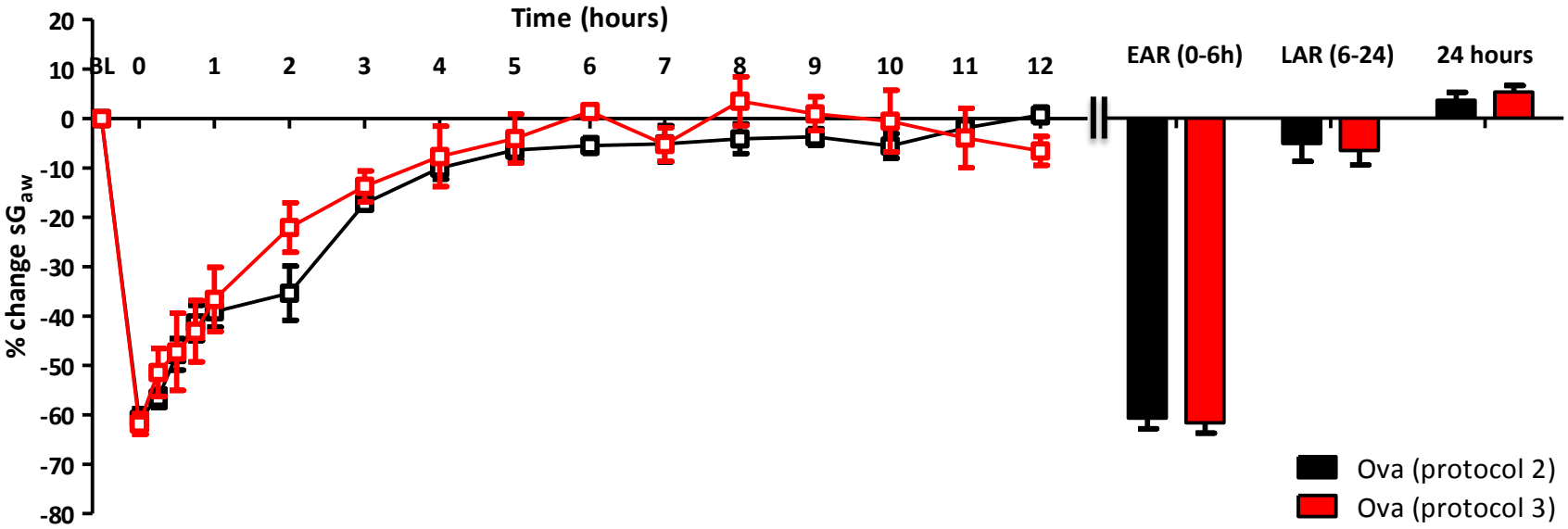


Figure 12: The mean time-course values of sGaw in guinea-pigs sensitised with 2 (protocol 2) or 3 (protocol 3) injections of a suspension of 100µg Ova and 100mg aluminium hydroxide and challenged with 0.03% Ova. The histogram represents the maximum bronchoconstriction values recorded during the early asthmatic response (EAR) (0-6 hours), late asthmatic response (LAR) (6-12 hours) and 24 hours readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=6 (protocol 2)' N=4 (protocol 3; performed with a two tailed T-test.

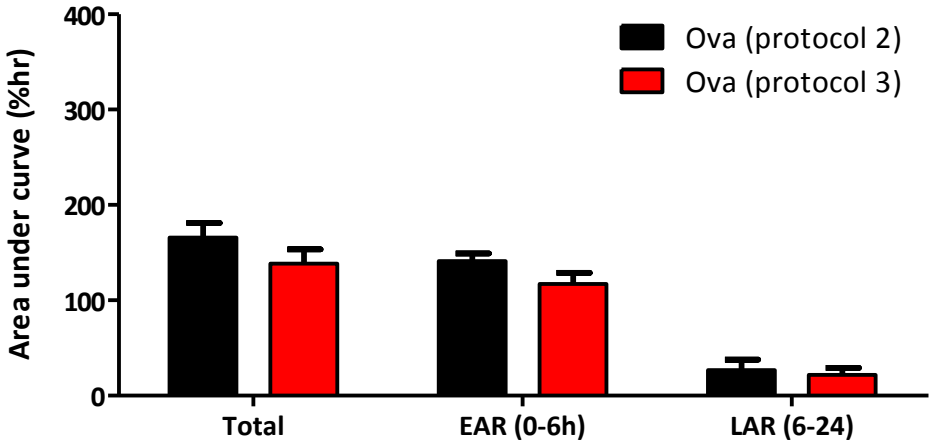


Figure 13: Area under the curve analysis of bronchoconstrictor responses in guinea-pigs sensitised with 2 (protocol 2) or 3 (protocol 3) injections of a suspension of 100µg Ova and 100mg aluminium hydroxide and challenged with 0.03% Ova. For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-24 hours, early asthmatic response (EAR) includes values from 0-6 hours) and late asthmatic response (LAR) includes values from 6-12 hours. Area under the curve is measured in %.hour. N=6 (protocol 2)' N=4 (protocol 3); performed with a two tailed T-test.

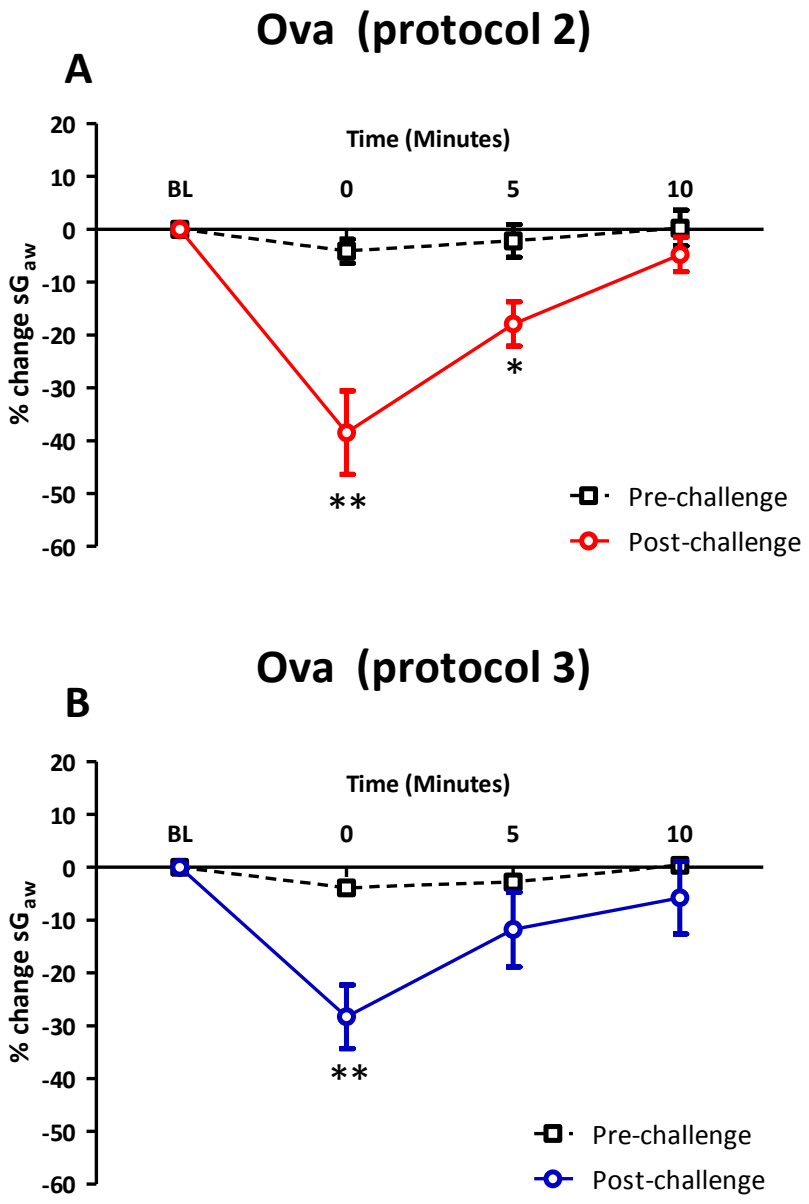


Figure 14: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles with 0.5LPM flow per chamber over 2 minutes, 1 minute drying period) in guinea-pigs sensitised with A) 2 (protocol 2) or B) 3 (protocol 3) injections of a suspension of 100µg Ova and 100mg aluminium hydroxide and challenged with 0.03% Ova. Values were recorded 24 hours pre- and post-Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=6 (protocol 2), N=4 (protocol 3); *significantly different from pre-Ova challenge values $p<0.05$, ** $p<0.01$; performed with a two tailed T-test.

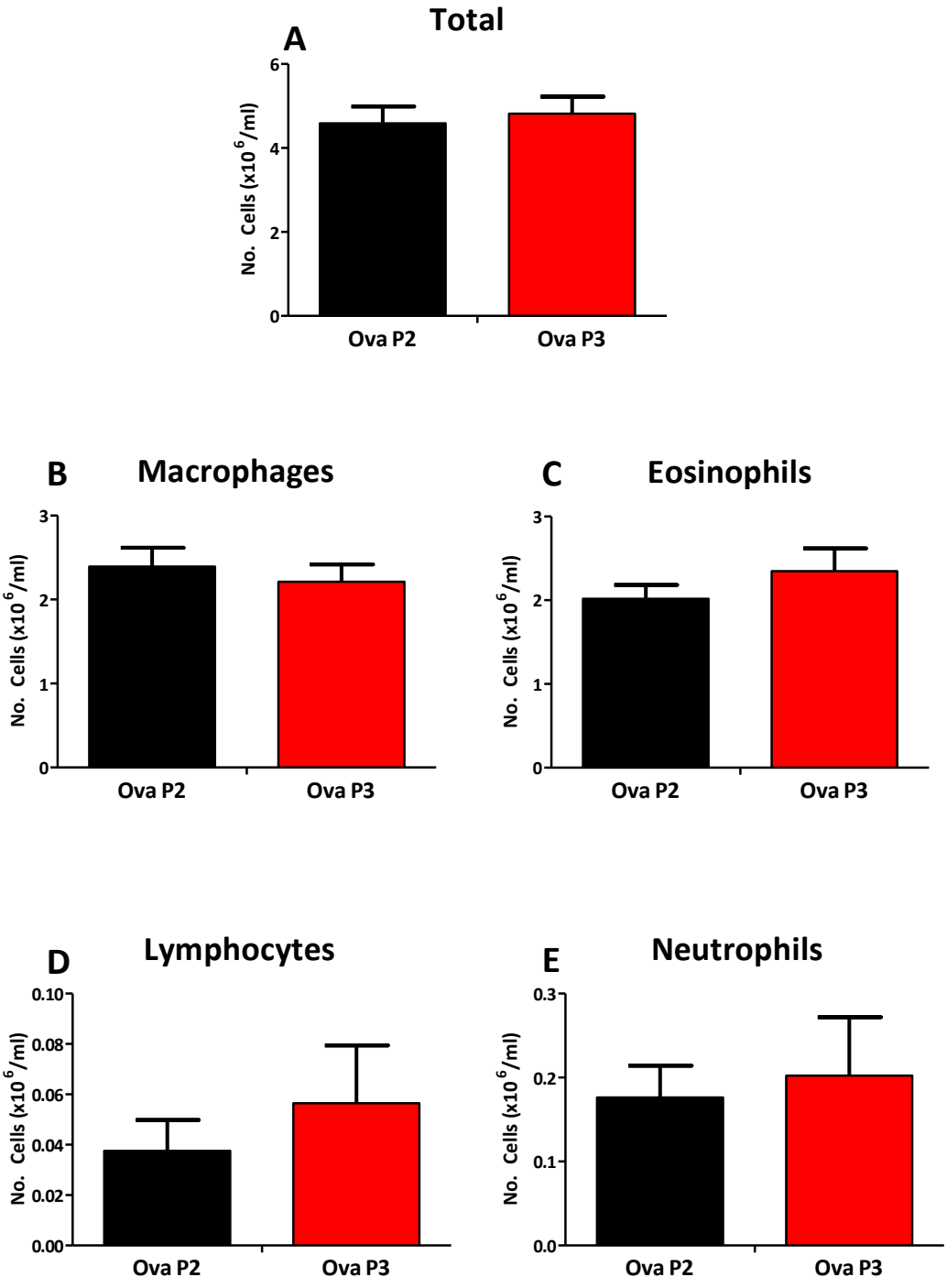


Figure 15: The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in bronchoalveolar fluid in guinea-pigs sensitised with 2 (protocol 2) or 3 (protocol 3) injections of a suspension of 100ug Ova and 100mg aluminium hydroxide and challenged with 0.03% Ova. N=6 (protocol 2), N=4 (protocol 3); performed with a two tailed T-test.

3.5.4 Protocol 4

Sensitisation: 3 intra-peritoneal injections of 150µg Ova and 100mg $Al(OH)_3$

Ova challenge: 0.03% Ova

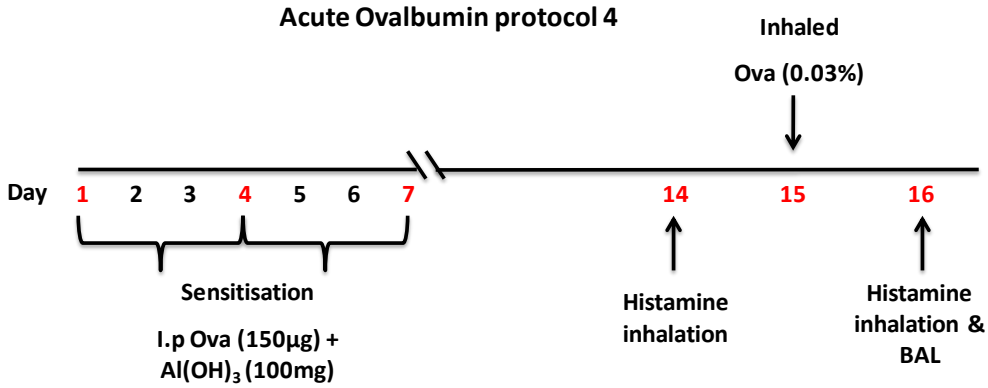


Figure 16: The acute ovalbumin protocol for guinea-pigs exposed to Ova protocol 4.

Guinea-pigs sensitised with 100µg and 150µg of Ova (protocol 3 & 4 respectively) demonstrated a significant bronchoconstriction immediately following Ova challenge (-61.9±2.1% and -60.7±4.1%). No significant difference between groups was observed during the early phase responses. No significant late phase bronchoconstrictions were observed in either group (Figure 17, time course). No significant difference in peak early phase bronchoconstrictions was observed between groups. No significant late phase bronchoconstriction was observed with an increase in the Ova sensitisation dose. (Figure 17, histogram). No significant difference in area under the curve was observed between the two groups at total, EAR and LAR time points (Figure 18)

Guinea-pigs sensitised with 150ug of Ova demonstrated a significant bronchoconstrictor response to histamine 24 hours after Ova inhalation challenge (-35.7±8.9% compared to pre-Ova values, -1.4±2.8). A significant bronchoconstriction was still present 5 minutes post-histamine challenge (-29.2±6.4% compared to -0.3±4.5%). At 10 minutes post-histamine exposure guinea-pigs sGaw values were not

significantly different from pre-Ova challenge values. No significant difference in the post-Ova bronchoconstrictor response to histamine was observed between the 2 groups (Figure 19).

Increasing the sensitisation dose of Ova from 100 to 150ug significantly increased the total cells present in lavage fluid ($8.3\pm0.9x10^6$/ml compared to $4.8\pm0.4x10^6$/ml). Eosinophils and macrophages were also significantly increased with increased Ova during sensitisation ($3.9\pm0.3x10^6$/ml compared to $2.4\pm0.3x10^6$/ml; $3.5\pm0.3x10^6$/ml compared to, $2.2\pm0.2x10^6$/ml respectively) (Figure 20).

3.5.4.1 Summary

Guinea-pigs sensitised with 3 injections of 150µg Ova and 100mg aluminium hydroxide and subsequently challenged with 0.03% Ova (protocol 4) demonstrated an EAR, AHR and airway inflammation. An increase in total cells was observed but no LAR. Therefore, further modifications to Ova protocol were made. To further enhance sensitisation to Ova, the concentration of aluminium hydroxide was increased to 150mg.

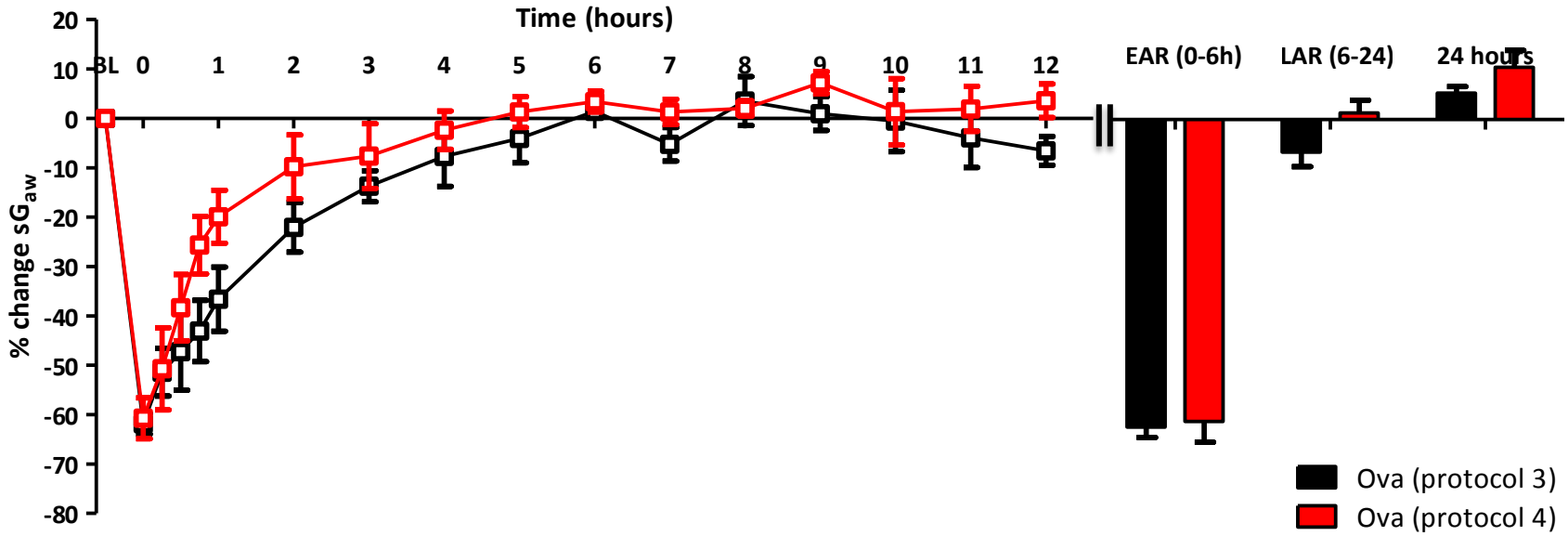


Figure 17: The mean time-course values of sGaw in guinea-pigs sensitised with 3 injections of a suspension of 100µg or 150ug ovalbumin (Ova) and 100mgs of aluminium hydroxide (protocol 3 & 4 respectively) and challenged with 0.03% Ova. The histogram represents the maximum bronchoconstriction values recorded during the early asthmatic response (EAR) (0-6 hours), late asthmatic response (LAR) (6-12 hours) and 24 hour readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=4 (protocol 3), N=5 (protocol 4); performed with a two tailed T-test.

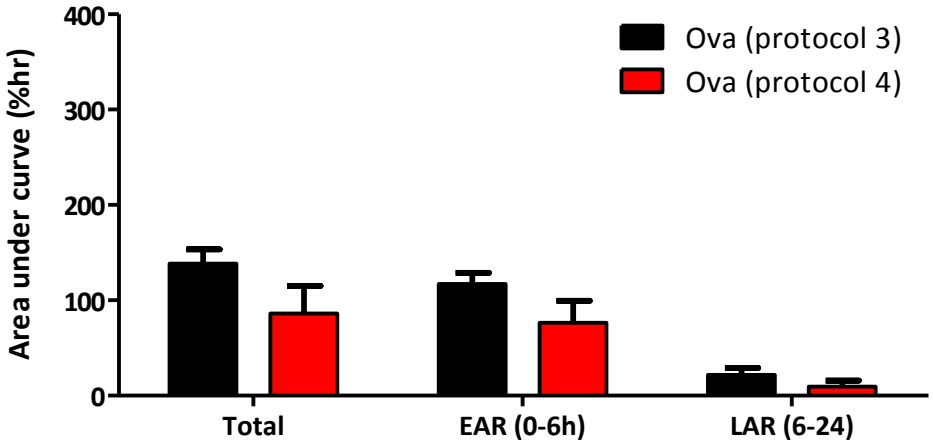


Figure 18: Area under the curve analysis of bronchoconstrictor responses in guinea-pigs sensitised with 3 injections of a suspension of 100µg or 150ug ovalbumin (Ova) and 100mgs of aluminium hydroxide (protocol 3 & 4 respectively) and challenged with 0.03% Ova. For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-24 hours, early asthmatic response (EAR) includes values from 0-6 hours and late asthmatic response (LAR) includes values from 6-12 hours. Area under the curve is measured in %.hour. N=4 (protocol 3), N=5 (protocol 4); performed with a two tailed T-test.

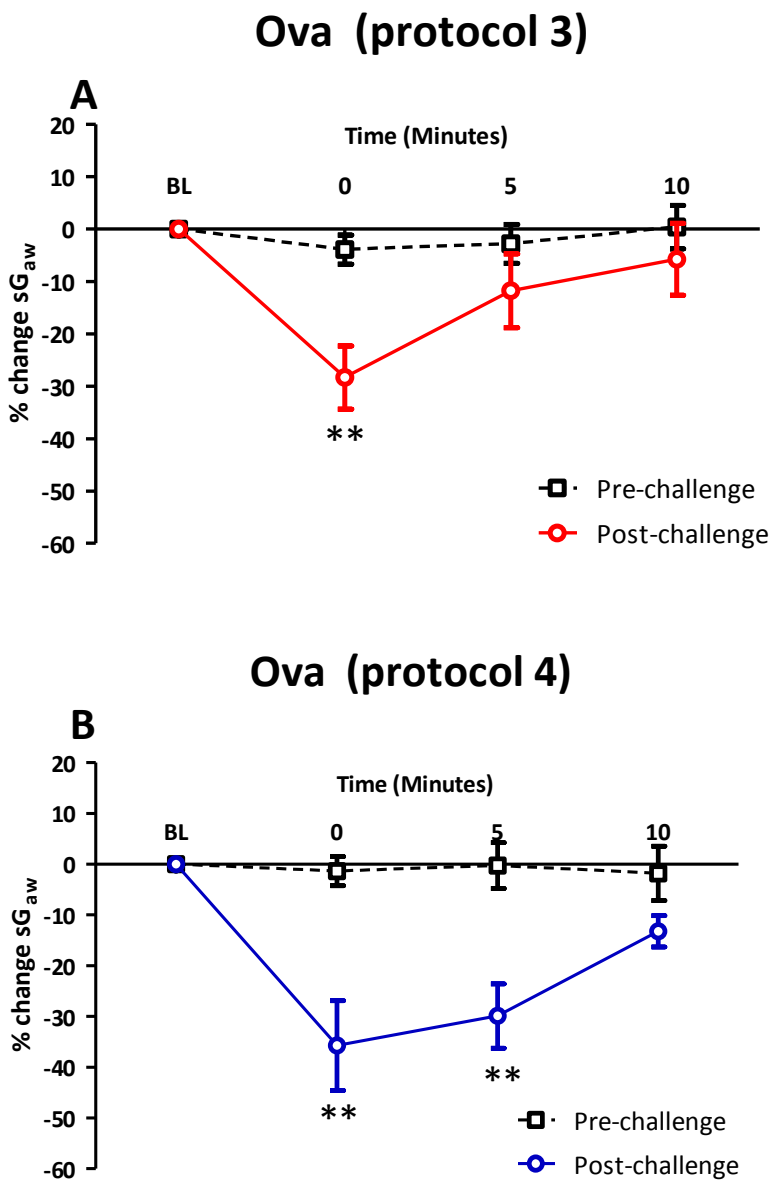


Figure 19: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles with 0.5LPM flow per chamber over 2 minutes, 1 minute drying period) in guinea-pigs sensitised with A) 3 injections of a suspension of 100µg or B) 150ug ovalbumin (Ova) and 100mgs of aluminium hydroxide (protocol 3 & 4 respectively) and challenged with 0.03% Ova. Values were recorded 24 pre- and post-Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=4 (protocol 3), N=5 (protocol 4); **significantly different from time paired pre-Ova challenge values $p<0.01$; performed with a two tailed T-test.

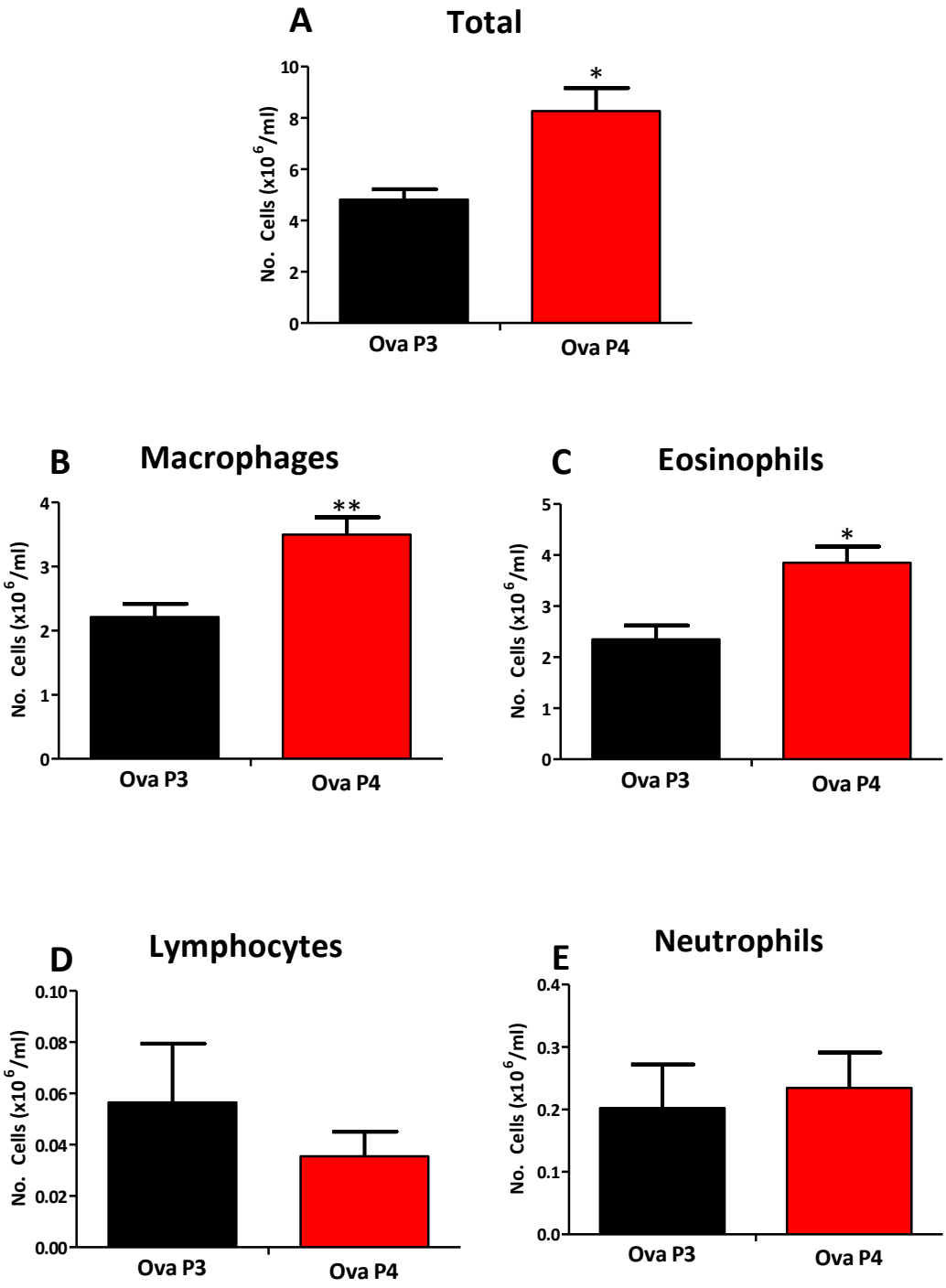


Figure 20: The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in bronchoalveolar fluid in guinea-pigs sensitised with 3 injections of a suspension of 100µg or 150ug ovalbumin (Ova) and 100mgs of aluminium hydroxide (protocol 3 & 4 respectively) and challenged with 0.03% Ova N=4 (protocol. 3) N=5 (protocol 4); *significantly different from protocol 3 $p<0.05$, ** $p<0.01$; performed with a two tailed T-test.

3.5.5 Protocol 5 & 6

Sensitisation: 3 intra-peritoneal injections of 150µg Ova and 100mg or 150mg $Al(OH)_3$ in normal saline (protocols 5 & 6 respectively). For protocol 5 and 6, 2mls of sensitisation solution was injected bilaterally on days 1, 4 and 7. This prevented clogging of the injection syringe due to the increased viscosity of solution. Protocol 5 was the control for the volume change of the sensitisation solution used in protocol 6. Increasing the sensitisation solution volume from 1 to 2ml did not significantly alter any parameter. These results of the comparison between protocol 4 and 5 can be found in Appendix 4.

Ova challenge: 0.03% Ova

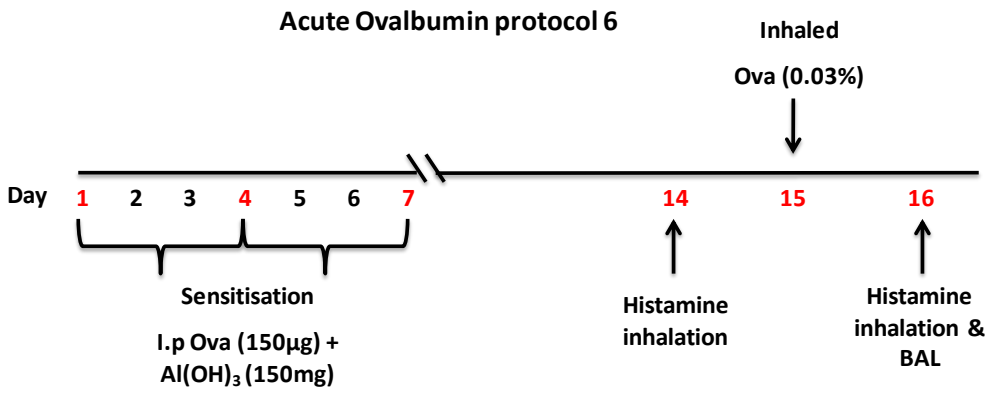


Figure 21: The acute ovalbumin protocol for guinea-pigs exposed to Ova protocol 6.

Guinea-pigs sensitised with 100mg or 150mg of aluminium hydroxide (Protocol 5 & 6 respectively) demonstrated an immediate bronchoconstriction to Ova (-61.0±5.5% and -53.6±4.3%, protocol 5 and 6 respectively). The bronchoconstrictions returned to baseline sGaw values 4 hours post-Ova challenge. These bronchoconstrictions constitute the EAR. In guinea-pigs sensitised with an increased amount of aluminium hydroxide (protocol 6), a second significant bronchoconstriction was observed at 6 hours (-17.6±4.6%) compared to protocol 5, (-3.8±4.2%). The bronchoconstriction returned to baseline sGaw levels by 8 hours. This bronchoconstriction represents a

late asthmatic response (Figure 22, time course). Both groups demonstrated similar peak EAR. Increasing the sensitisation dose of aluminium hydroxide from 100 to 150mg significantly increased the peak late phase bronchoconstriction (-17.6±4.6%) compared to protocol 5, (-3.8±4.2%) (Figure 22, histogram). No difference in area under the curve (AUC) was observed between the two groups at total and EAR time points. Although AUC for the late phase showed a trend for an increase this was found to be non-significant (Figure 23).

Guinea pigs sensitised with 150mg of aluminium hydroxide demonstrated a significant bronchoconstrictor response to histamine exposure 24 hours post-Ova challenge (-37.0±8.1%) compared to pre-Ova challenge, (-5.9±2.1%). A bronchoconstriction was still present 5 minutes after histamine exposure (-19.2±4.7% compared to -9.4±2.2%). 10 minutes after histamine exposure, sGaw values had returned to baseline. No significant difference in the bronchoconstrictor response to histamine between groups was observed (Figure 24).

Increasing the aluminium hydroxide sensitisation dose from 100 to 150mg did not significantly increase total cell counts. A significant increase in eosinophils was observed ($6.9\pm0.8x10^6$/ml compared to protocol 5, $4.6\pm0.5x10^6$/ml). Both neutrophils and lymphocytes were also significantly elevated ($0.15\pm0.02x10^6$/ml) compared to protocol 5 ($0.3\pm0.01x10^6$/ml) (Figure 25).

3.5.5.1 Summary

Guinea-pigs sensitised with 3 injections of 150µg Ova and 150mg aluminium hydroxide and subsequently challenged with 0.03% Ova (protocol 6) demonstrated an EAR, AHR and airway inflammation, with a significant increase in eosinophils, lymphocytes and neutrophils. Most importantly, a clear LAR was seen at 6 hours post-allergen challenge. This result was further confirmed by the significant increase in the peak bronchoconstriction 6-12 hours after Ova challenge. This protocol produced all characteristics of asthma desired in an acute model of asthma. Despite this, the protocol was found to be unsuitable for further use due to the high dose of

aluminum hydroxide and injection volume. The protocol also produced high levels of distress to guinea-pigs during Ova challenge. Thus one further modification to the protocol was made. The time between the allergen sensitisation and challenge was extended from approximately 1 to 2 weeks.

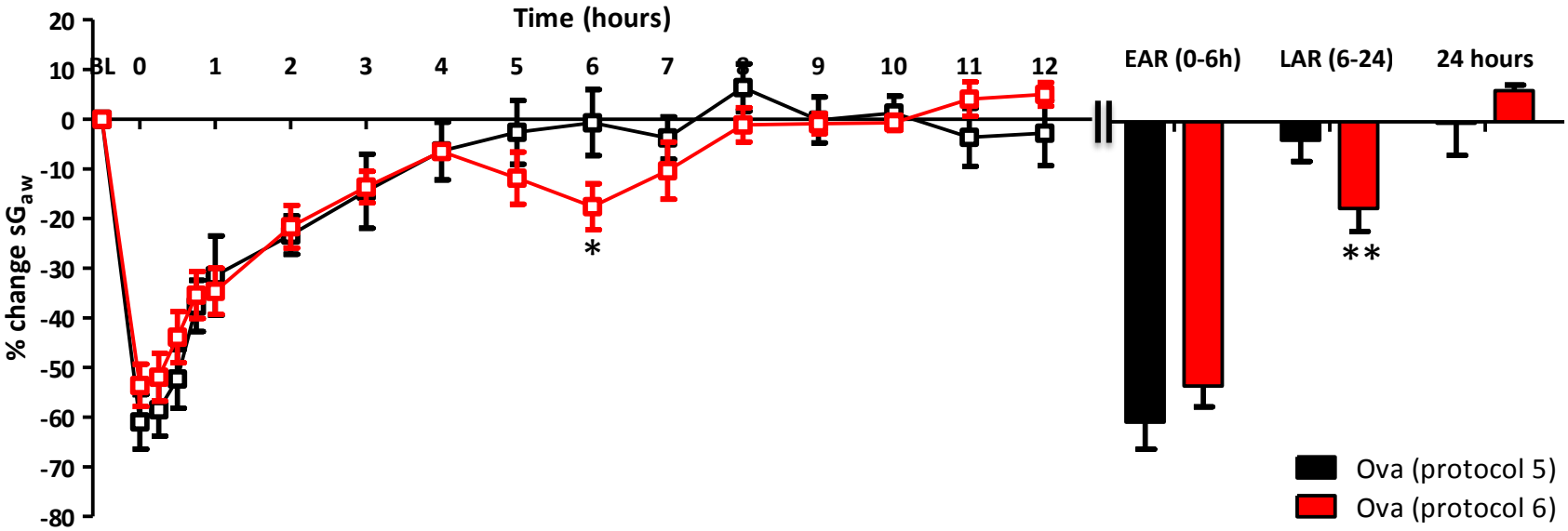


Figure 22: The mean time-course values of sGaw in guinea-pigs sensitised with 3 injections of 2ml of a suspension of 150µg Ova and 100mg or 150mg of aluminium hydroxide and challenged with 0.03% Ova (protocol 5 and 6 respectively). The histogram represents the maximum bronchoconstriction values recorded during the early asthmatic response (EAR) (0-6 hours), late asthmatic response (LAR) (6-12 hours) and 24 hour readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=5; *significantly different from protocol 5 $p<0.05$, ** $p<0.01$; performed with a two tailed T-test.

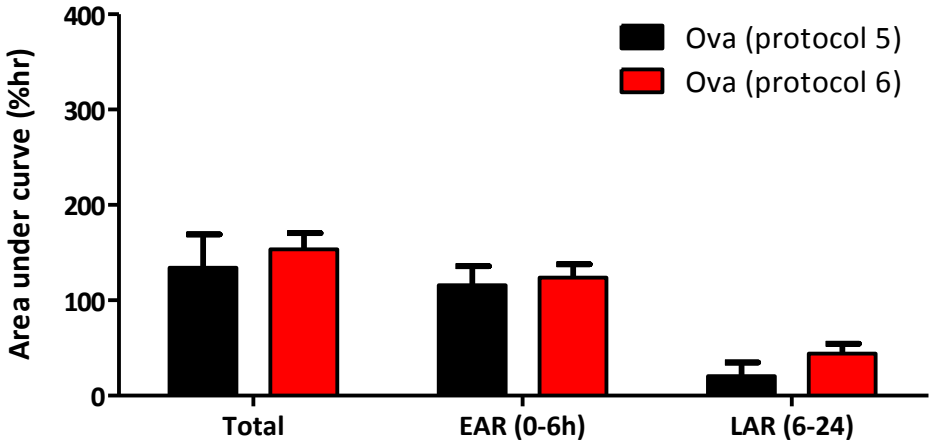


Figure 23: Area under the curve analysis of bronchoconstrictor responses of in guinea-pigs sensitised with 3 injections of 2ml of a suspension of 150µg Ova and 100mg or 150mg of aluminium hydroxide and challenged with 0.03% Ova (protocol 5 and 6 respectively). For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-24 hours, early asthmatic response (EAR) includes values from 0-6 hours and late asthmatic response (LAR) includes values from 6-12 hours. Area under the curve is measured in %.hour. N=5; performed with a two tailed T-test.

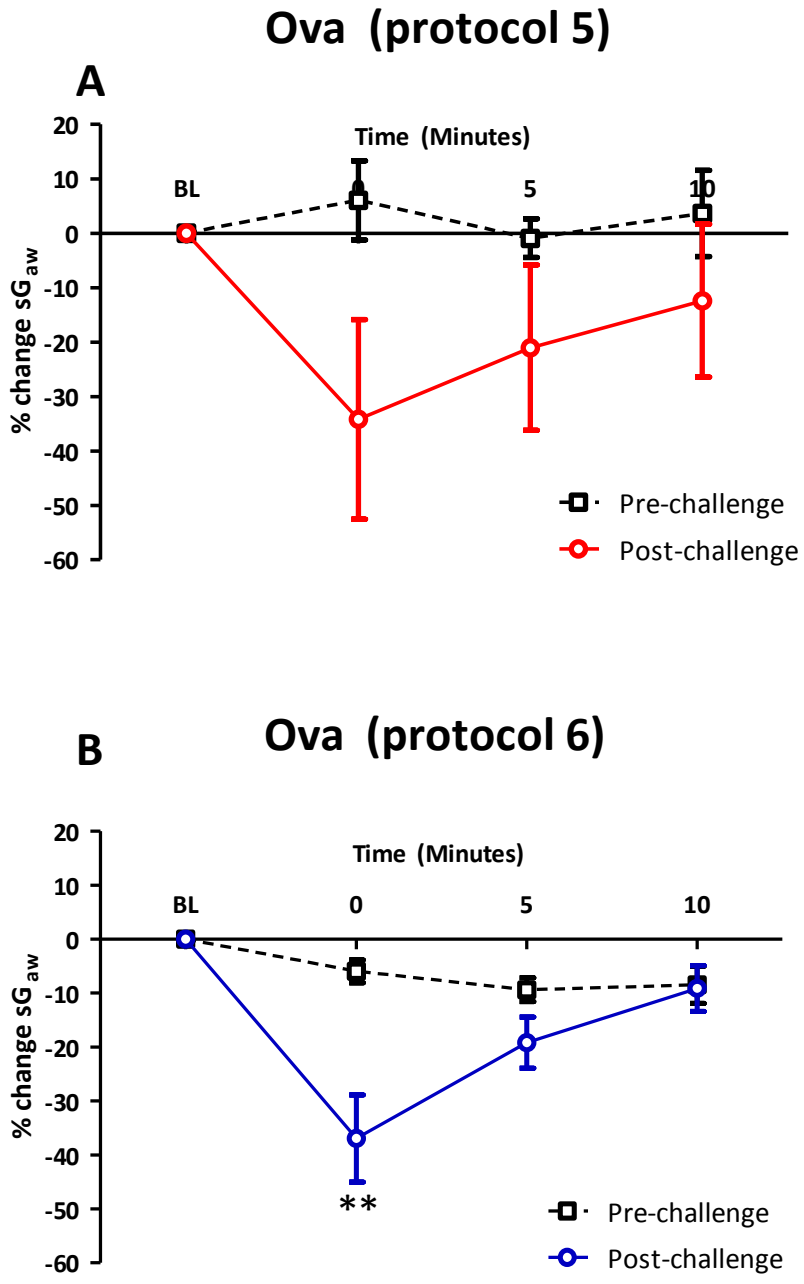


Figure 24: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles with 0.5LPM flow per chamber over 2 minutes, 1 minute drying period) in guinea-pigs sensitised with 3 injections of 2ml of a suspension of A) 150µg Ova and 100mg or B) 150mg of aluminium hydroxide and challenged with 0.03% Ova (protocol 5 and 6 respectively). Values were recorded 24 pre- and post-Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=5; **significantly different from time paired pre-Ova challenge values ** $p<0.01$; performed with a two tailed T-test.

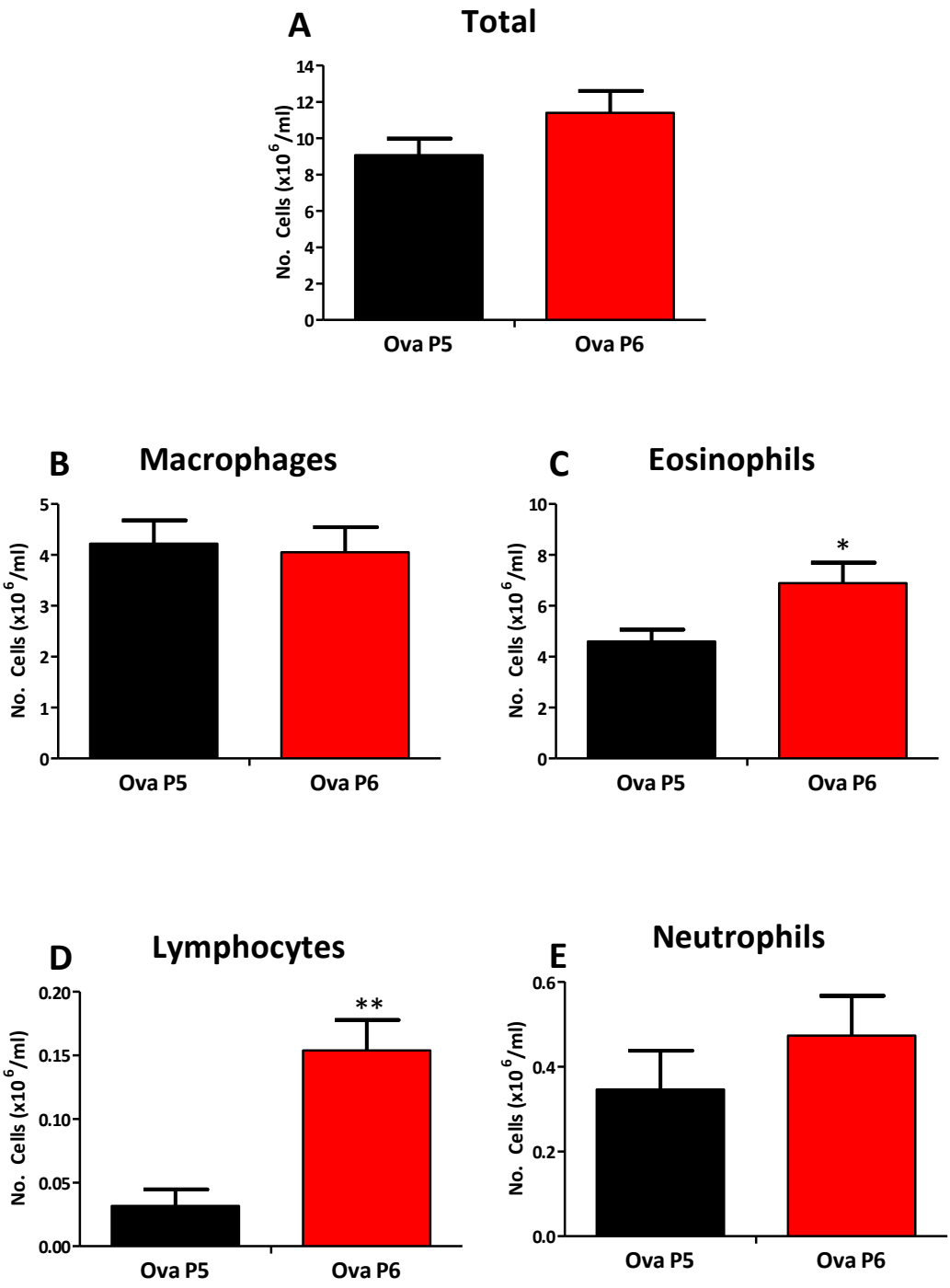


Figure 25: The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in bronchoalveolar fluid in guinea-pigs sensitised with 3 injections of 2ml of a suspension of 150µg Ova and 100mg or 150mg of aluminium hydroxide and challenged with 0.03% Ova (protocol 5 and 6 respectively); N=5; *significantly different from protocol 5 $p<0.05$, ** $p<0.01$; performed with a two tailed T-test.

3.5.7 Protocol 7

Sensitisation: 3 intra-peritoneal injections of 150µg Ova and 100mg $Al(OH)_3$ in 1ml saline.

Ova challenge: 0.03% Ova, day 15 (protocol 4) or day 21 (protocol 7)

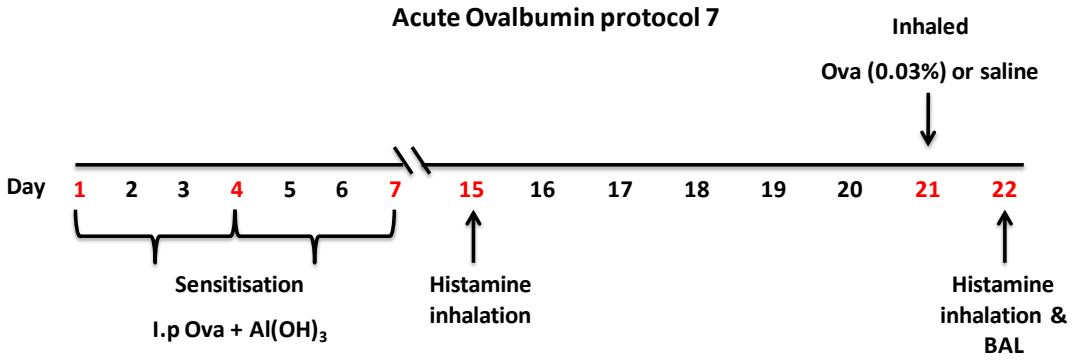


Figure 26: The acute ovalbumin protocol for guinea-pigs exposed to Ova protocol 7.

Both guinea-pigs challenged on day 15 (-60.7±4.1%) and day 21 (-66.7±4.0%) demonstrated a significant bronchoconstriction at 0 minutes post-Ova challenge. In guinea-pigs challenged on day 21 the bronchoconstriction to Ova was significantly increased at all time points from 45 minutes to 5 hours post-Ova compared to guinea-pigs challenged on day 15. In guinea-pigs challenged with Ova on day 21 a further bronchoconstriction was observed from 7-9 hours. At 8 and 9 hours this bronchoconstriction was significantly increased compared to guinea-pigs challenged with Ova on day 15 (-10.7±3.9% compared to 2.0±1.6%; -13.2±7.0% compared to 7.2±2.2%). This second bronchoconstriction represents a late asthmatic response (Figure 26, time course). No significant difference in peak early phase bronchoconstrictions was observed between guinea-pigs challenged on day 15 and day 21. A significant increase in the peak LAR was observed in guinea pigs challenged with Ova on day 21 (-19.9±4.9%) compared to challenge 15, (1.3±2.6%). No significant difference in sGaw values was observed 24 hours post-Ova challenge (Figure 26, histogram).

Figure 28 represents the area under the curve (AUC) analysis for sensitised guinea-pigs challenged with Ova on day 15 or day 21. A significant increase in the total (246±45.9%.hr compared to 86.2±23.7%.hr), EAR (194.2±25.1%.hr compared to 76.6±18.6%.hr) and LAR (53.1±21.0%.hr compared to 9.6±5.1%.hr) AUC were seen in guinea pigs challenged on day 21 compared to day 15.

Guinea-pigs challenged with Ova day 15 and 21 demonstrated a significant increase in the bronchoconstrictor response to histamine, 24 hours post-Ova challenge (-35.7±8.9% and -53.9.4±11.4%, respectively) compared to pre-Ova challenge (-10.1±2.4%). In guinea-pigs challenged with Ova on day 15 the bronchoconstriction to histamine was not significantly different from pre-Ova sGaw values at 10 minutes. In guinea pigs challenged on day 21 a significant bronchoconstriction was still present at 10 minutes (-26.7±11.4% compared to 1.6±2.7%) (Figure 29).

Total cells were not significantly different between groups. Macrophage and neutrophils also remained unchanged. Lymphocytes were significantly increased in guinea-pigs challenged with Ova on day 21 compared to day 15 ($0.37\pm0.07x10^6$/ml, compared to $0.04\pm0.01x10^6$/ml respectively). Eosinophils were also significantly increased ($5.5\pm0.2x10^6$/ml compared to $3.9\pm0.3x10^6$/ml) (Figure 30).

3.5.5.1 Summary

Guinea-pigs sensitised with 3 injections of 100µg Ova and 150mg aluminium hydroxide and subsequently challenged with 0.03% Ova on day 21 (protocol 7) demonstrated an EAR, LAR, AHR and airway inflammation with a significant increase in lymphocytes. The duration of the EAR increased with the lengthening of the sensitisation period. Thus this protocol was able to produce all the required characteristics of an acute Ova model. Unlike protocol 6, the allergen challenge was well tolerated. On this basis, the protocol was used in all subsequent chapters.

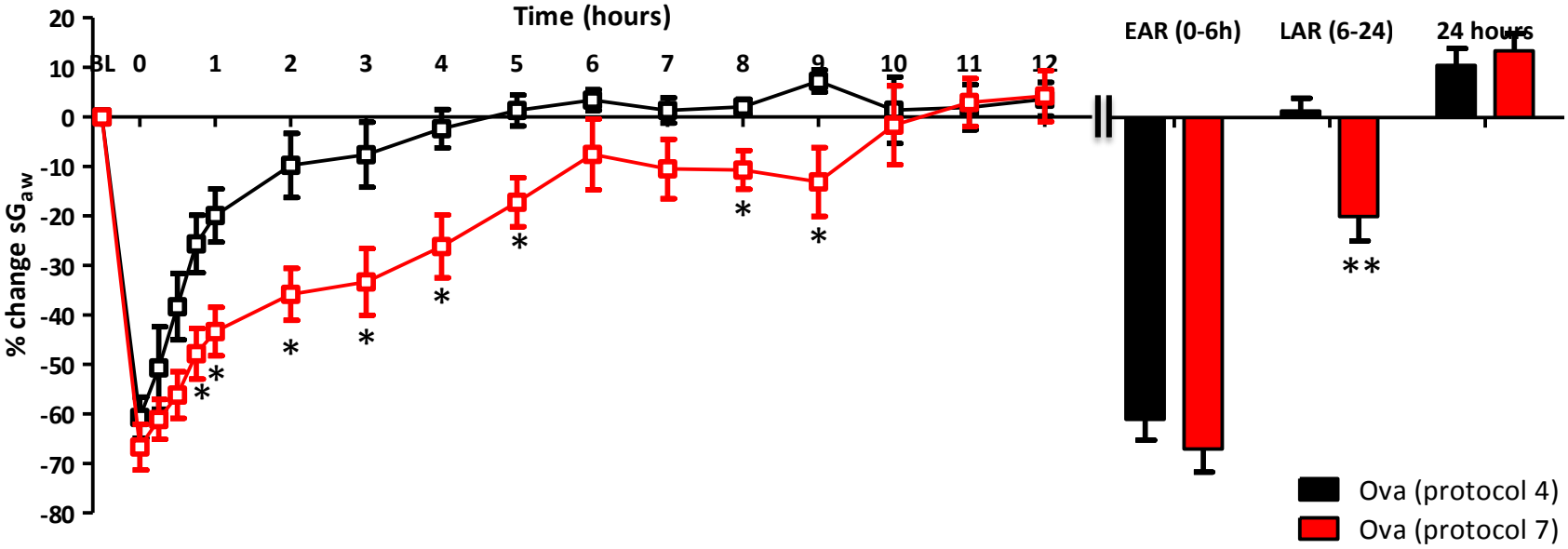


Figure 27: The mean time-course values of sGaw in guinea-pigs sensitised with 3 injections of a suspension of 150µg Ova and 100mg aluminium hydroxide and challenged with 0.03% Ova on day 15 (protocol 4) or day 21 (protocol 7). The histogram represents the maximum bronchoconstriction values recorded during baseline, early asthmatic response (EAR) (0-6 hours), late asthmatic response (LAR) (6-12 hours) and 24 hours readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction; N=6 *Significantly different to protocol 4 $p<0.05$, ** $p<0.01$; performed with T-test.

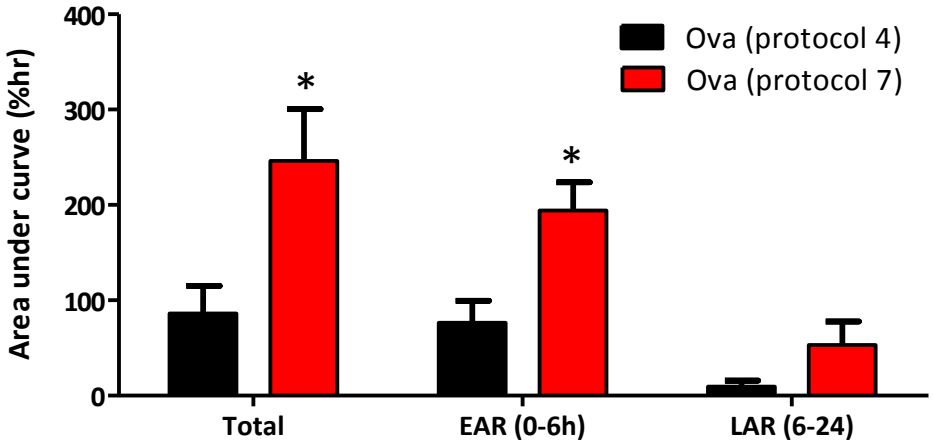


Figure 28: Area under the curve analysis of bronchoconstrictor response in guinea-pigs sensitised with 3 injections of a suspension of 150µg Ova and 100mg aluminium hydroxide and challenged with 0.03% Ova on day 15 (protocol 4) or day 21 (protocol 7). For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-24 hours, early asthmatic response (EAR) includes values from 0-6 hours and late asthmatic response (LAR) includes values from 6-12 hours. Area under the curve is measured in %.hour. N=6 *Significantly different to protocol 4 $p<0.05$; performed with T-test.

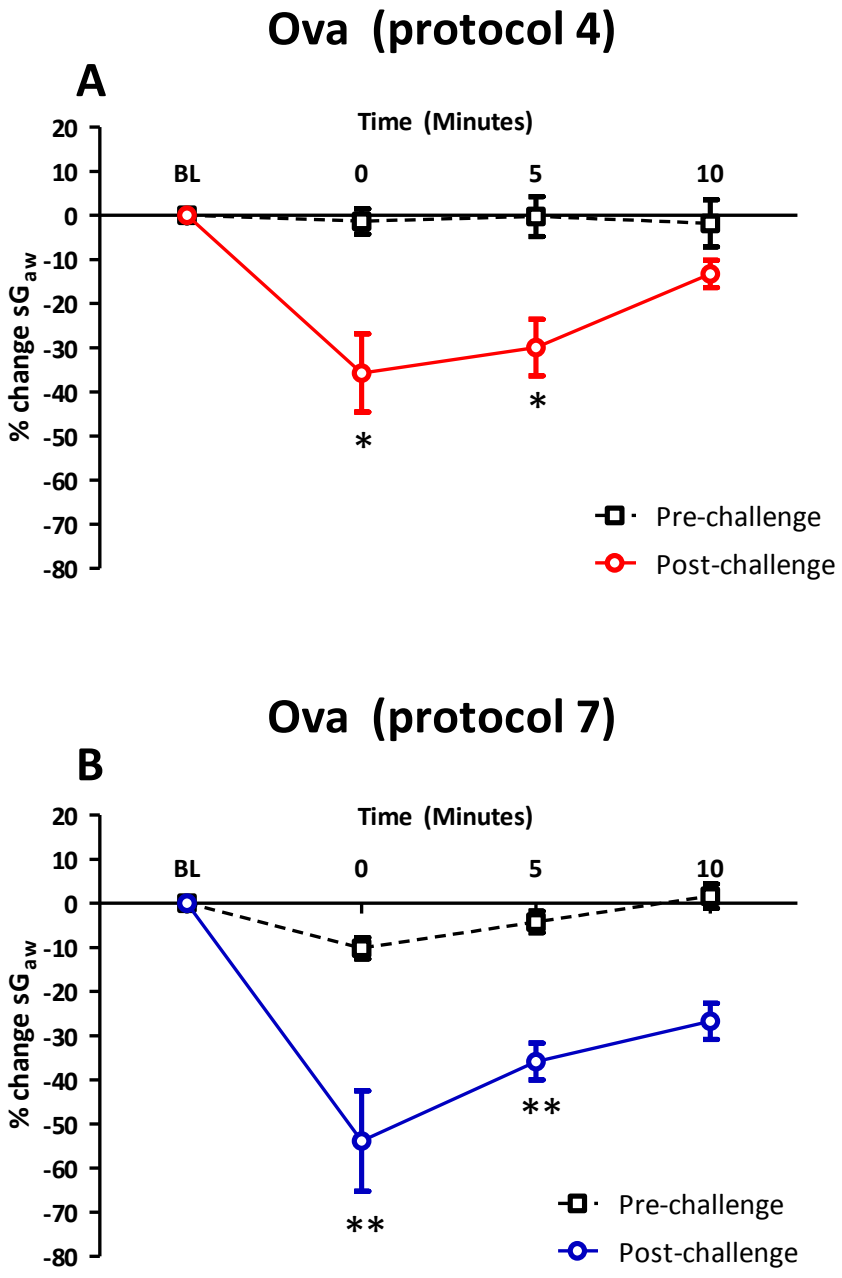


Figure 29: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles with 0.5LPM flow per chamber over 2 minutes, 1 minute drying period) in guinea-pigs sensitised with 3 injections of a suspension of 150µg Ova and 100mg aluminium hydroxide and challenged with 0.03% Ova on A) day 15 (protocol 4) or B) day 21 (protocol 7). Values were recorded 24 pre- and post-Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=6; *Significantly different from pre-Ova challenge values $p<0.05$; ** $p<0.01$; performed with T-test.

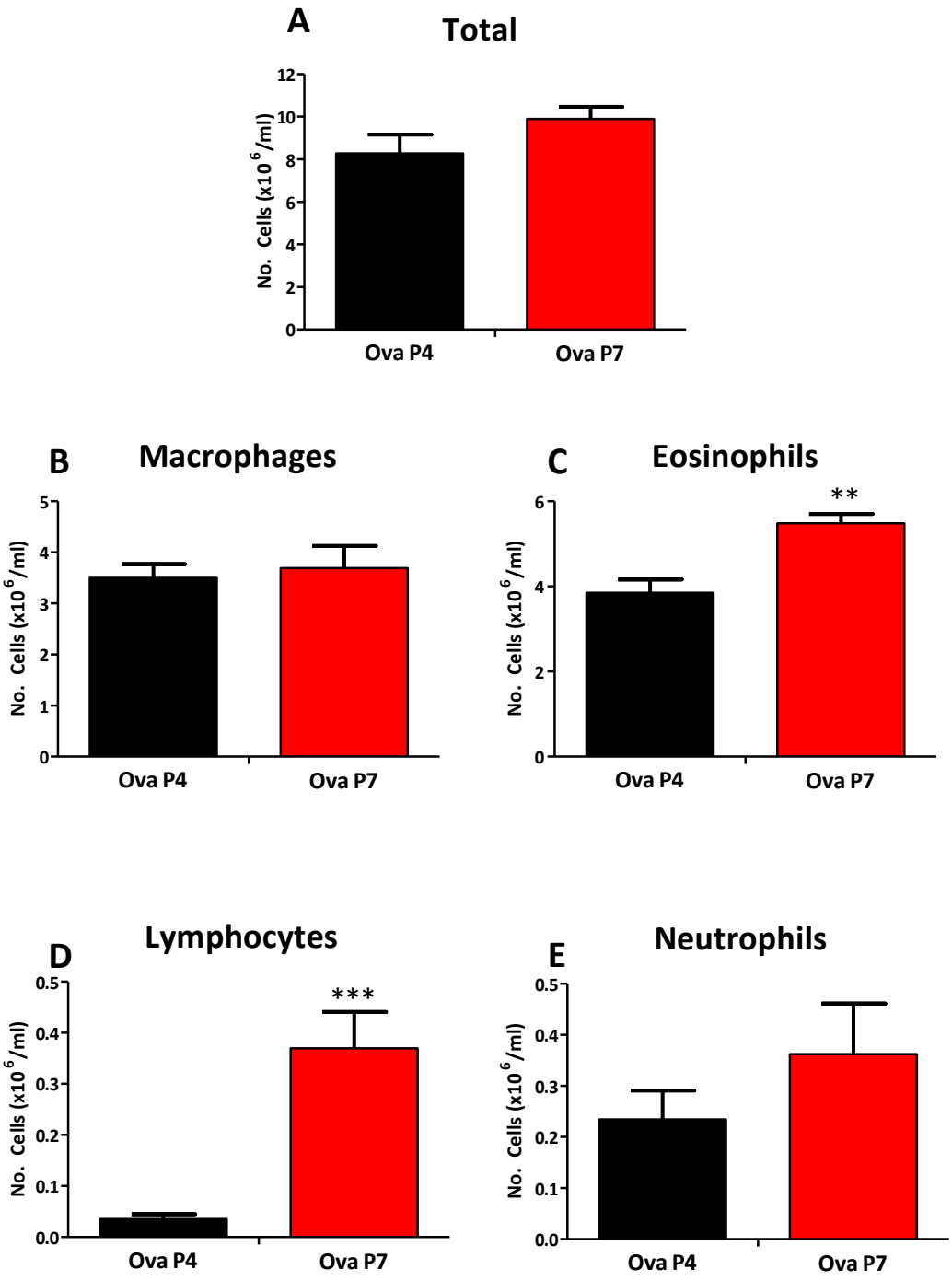


Figure 30: The total cell (A), macrophages (B), eosinophils (C), lymphocytes (D) and neutrophils (E) counts in bronchoalveolar fluid in guinea-pigs sensitised with 3 injections of a suspension of 150µg Ova and 100mg aluminium hydroxide and challenged with 0.03% Ova on day 15 (protocol 4) or day 21 (protocol 7). N=6; **significantly different from protocol 4 $p<0.01$; *** $p<0.001$; performed with a two tailed T-test.

3.5.7 Tissue eosinophilia

Figure 31 shows typical photomicrographs for lung sections stained with Sirus red (eosinophil stain). Figure 32A shows the number of eosinophils counted per field of view. A progressive trend for increased eosinophil numbers was observed with cumulative modifications to the Ova sensitisation and challenge protocol. This reached significance with protocol 3 (187.4±40.2) compared to saline, (27.0±7.4). Protocols 4-7 also significantly increased tissue eosinophilia (173.7±29.1, 180.2±13.0 and 185.8±20.5 respectively). Figure 32B shows the number of eosinophils per bronchiole area. Similar results to eosinophils per field of view were observed. Protocol 3-7 all significantly increased tissue eosinophilia compared to saline (90.8±5.9, 99.8±5.9, 89.7±15.3, 73.7±8.8 respectively, compared to saline, 9.4±3.4).

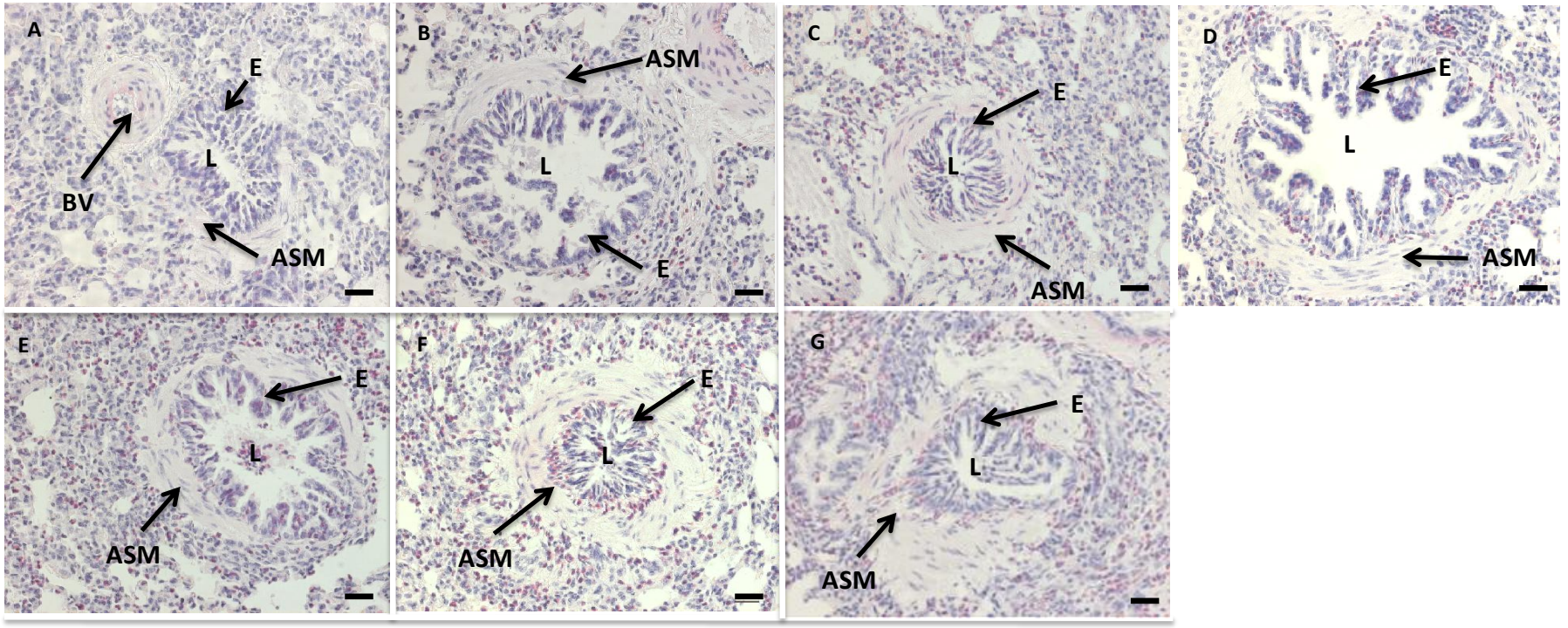


Figure 31: Guinea-pig lung tissue stained with Sirius red. Sensitisation and challenge varied accordingly **A** 2 x 100µg Ova/100mg $Al(OH)_3$ with saline challenge. **B** 2 x 100µg Ova/100mg $Al(OH)_3$ with 0.01% Ova challenge (protocol 1). **C** 2 x 100µg Ova/100mg $Al(OH)_3$ with 0.03% Ova challenge (protocol 2). **D** 3 x 100µg Ova/100mg $Al(OH)_3$ with 0.03% Ova challenge (protocol 3). **E** 3 x 150µg Ova/100mg $Al(OH)_3$ with 0.03% Ova challenge (protocol 4). **F** 3 x 150µg Ova/150mg $Al(OH)_3$ with 0.03% Ova challenge (protocol 6). **G** 3 x 100µg Ova/100mg $Al(OH)_3$ with 0.03% Ova challenge on day 21 (protocol 7). Original magnification 200x; bar = 50 µm). L: lumen; ASM: airway smooth muscle; E: epithelium. Eosinophils were defined as cells demonstrating a cytoplasm staining an intense red with dark bi-lobed nuclei.

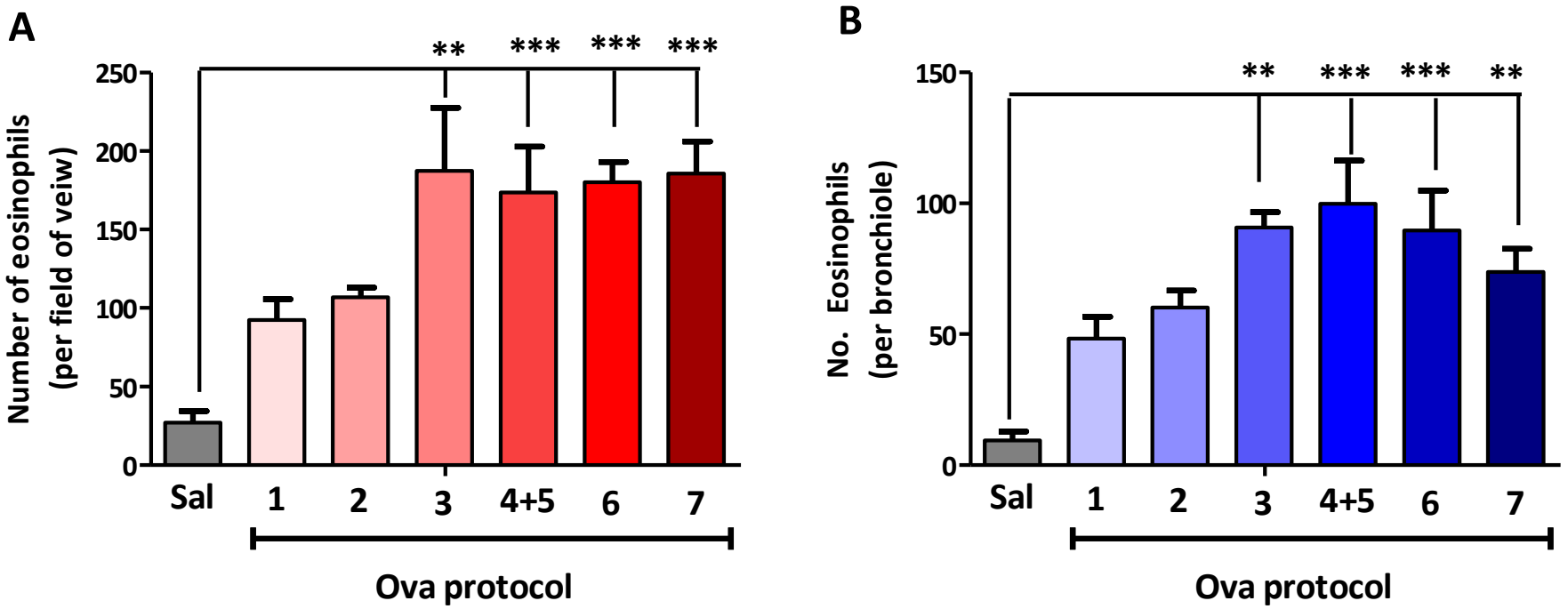


Figure 32: A) the number of tissue eosinophils (per field of view) and **B)** (per bronchiole) following varying ovalbumin (Ova) sensitisation and challenge protocols. **Sal:** 2 x 100µg Ova/100mg $Al(OH)_3$ with saline challenge. **Protocol 1:** 2 x 100µg Ova/100mg $Al(OH)_3$ with 0.01% Ova challenge. **Protocol 2:** 2 x 100µg Ova/100mg $Al(OH)_3$ with 0.03% Ova challenge. **Protocol 3:** 3 x 100µg Ova/100mg $Al(OH)_3$ with 0.03% Ova challenge (protocol 3). **Protocol 4 & 5:** 3 x 150µg Ova/100mg $Al(OH)_3$ with 0.03% Ova challenge. **Protocol 6:** 3 x 150µg Ova/150mg $Al(OH)_3$ with 0.03% Ova challenge. **Protocol 7:** 3 x 100µg Ova/100mg $Al(OH)_3$ with 0.03% Ova challenge on day 21; N=4-6; **Significantly different to saline $p<0.01$; *** $p<0.001$; performed with one way analysis of variance.

3.5.8 Results Summary

Protocol	EAR	LAR	AHR	Lavage cells	Tissue eos
Saline	✗	✗	✗	✗	✗
Protocol 1	✓	✗	✗	Total, eos	✗
Protocol 2	✓	✗	✓	Total, eos	✗
Protocol 3	✓	✗	✓	Total, eos,	✓
Protocol 4 & 5	✓	✗	✓	Total, eos, macro	✓
Protocol 6	✓	✓	✓	Total, eos, macro, lympho	✓
Protocol 7	✓	✓	✓	Total, eos, macro, lympho	✓

Table 2: Shows a summary of the results of ovalbumin protocol modification in guinea-pigs. EAR: early asthmatic response; LAR: late asthmatic response; AHR: airways hyperresponsiveness; macro: macrophages; eos: eosinophils; lympho: lymphocytes.

Figure 33 demonstrates the variability between guinea-pigs in the timing of the early and late asthmatic response to ovalbumin challenge protocol 7. The parallels the situation in humans where individuals demonstrate wide differences in the timing and duration of early and late responses.

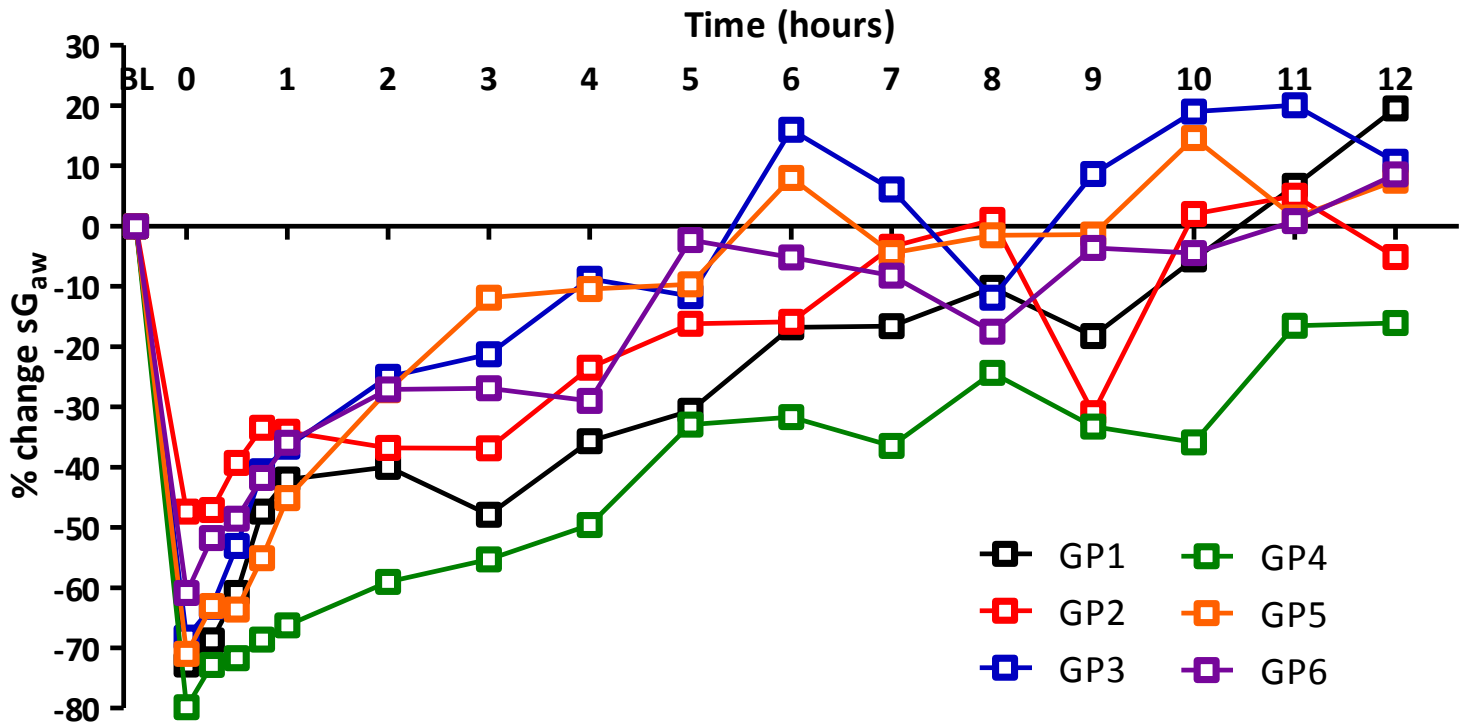


Figure 33: The time-course values of sGaw in individual guinea-pigs sensitised with 3 injections of a suspension of 150µg Ova and 100mg aluminium hydroxide and challenged with 0.03% Ova on day 21 (protocol 7). Changes in sGaw are expressed as percentage change from baseline. A negative value represents a bronchoconstriction. The figure illustrates the individual variability in the early and late asthmatic responses to Ova challenge in guinea-pigs.

3.6 Discussion

This study has shown that by making modifications to the allergen sensitisation and challenge conditions, it is possible to induce an early and late asthmatic response, AHR and airway inflammation. Sensitisation of guinea-pigs with 2 injections of 100µg Ova and 100mg aluminium hydroxide and subsequent Ova challenge on day 15 with 0.01% Ova (protocol 1) was unable to produce a LAR and AHR. Total cells were increased but of the subtypes measured only eosinophils were elevated. Increasing the Ova challenge concentration increased the peak of the EAR, induced AHR and increased airway inflammation. Increasing the number of sensitisation injections resulted in an increase in tissue eosinophilia. Similarly, increasing the Ova sensitisation concentration resulted in an increase in total lavage cell counts. Either increasing the aluminium hydroxide sensitisation concentration or increasing the time between Ova sensitisation and challenge was able to produce a LAR and a full inflammatory cell response. The latter modification was better tolerated by guinea-pigs and thus selected for further work.

3.6.1 Loss of sensitivity

There are several possible reasons for the decreased responsiveness over time of guinea-pigs to the allergen sensitisation and challenge protocol successfully employed by Smith & Broadley, 2007 and Evans *et al*, 2012.

Inbreeding which is employed in maintaining the homogeneity of guinea-pig strains such as Dunkin-Hartley is one potential cause. Successive generations of inbreeding could lead to an accumulation of certain traits, including decreased activity of the immune response. Differences in the various strains of guinea-pigs have been shown to significantly alter their immune response to antigen (Ellman *et al*, 1971; Stone, 1962). However, given the time frame in which decreased sensitivity to allergen was seen (approximately 1 year) it is unlikely that any trait that decreased sensitivity to allergen would have sufficient time to become widespread in the suppliers guinea-pig colony.

This loss appears to be unrelated to infection. An intracellular infection could skew the T lymphocyte populations in favour of Th1 responses, which do not favour allergen sensitisation (Galli *et al*, 2008). This is unlikely as animals are routinely screened for common infections upon arrival from suppliers and none were found. The change does not seem to be associated with alterations to the guinea-pig diet or environment at both the supplier and animal house in Cardiff. Seasonal changes also don't seem to be a factor as the lack of sensitivity was observed across nearly an entire year.

3.6.2 Model Optimisation

3.6.2.1 Increasing the ovalbumin challenge concentration

All guinea-pigs sensitised and challenged with Ova demonstrated an immediate bronchoconstriction, indicative of an EAR. Increasing the Ova challenge dose 3-fold from 0.01% to 0.03% increased the magnitude of this bronchoconstriction. The EAR is mediated by mast cells. Allergen challenge in sensitised animals causes mast cell degranulation by the crosslinking of FcεR1 receptors (Gould and Sutton, 2008). Degranulation products include histamine, prostaglandins, PAF and leukotrienes which cause bronchoconstriction. The increase in bronchoconstriction with increasing allergen challenge concentration is possibly a result of either increased release of granule products or an increase in the number of mast cells degranulating (Dvorak, 2005). A similar increase in the peak EAR bronchoconstriction was seen in Smith and Broadley, (2007). This was achieved by increasing the sensitisation concentration of Ova 10-fold, indicating that altering sensitisation conditions can also increase mast cell degranulation. This was possibly as a result of enhanced sensitisation and IgE production (Galli *et al*, 2008).

Increasing the Ova challenge concentration also induced AHR. This may be due to increased degranulation of mast cells during the EAR. Mast cell products produced during the EAR play a role in triggering later events such as lymphocyte activation and eosinophil influx. Eosinophils are able to release granule products such as ECP which can cause epithelial damage and exposure of underlying sensory nerves,

increasing sensitivity to bronchoconstrictor stimuli like histamine (Gleich *et al*, 1993; Laitinen *et al*, 1987). Concordantly, in the present study eosinophil numbers increased along with the development of AHR.

AHR has been linked to the LAR and also airways inflammation, in particular eosinophils (Hargreave *et al*, 1986). Smith and Broadley, (2007) study supports this as AHR was only seen in association with the LAR. In addition, the AHR increased with increasing size of the LAR bronchoconstriction. In contrast, in the current study dissociation between AHR and the LAR was observed. Dissociation between AHR and the LAR has also been observed in humans, although is sometimes due to the presence of a hyperresponsive state before allergen challenge (Ward *et al*, 1987). The result in the present study may indicate that alternative mechanisms of AHR exist in the model or that higher numbers of eosinophils are required for a LAR. In support of the latter, tissue eosinophilia was not increased with this Ova challenge protocol.

3.6.2.2 Increasing the ovalbumin and aluminum hydroxide sensitisation concentrations and number of sensitisation injections

The general effect of altering the sensitisation conditions was to increase airway inflammation. Increasing the number of sensitisation injections from 2 to 3 increased the number of tissue eosinophils, whereas increasing the Ova sensitisation concentration also increased the total number of lavage fluid cells. Increasing the concentration of aluminium hydroxide during sensitisation also produced this effect, increasing lavage eosinophils and lymphocytes. This modification also induced the development of a LAR.

These changes are likely the result of increased immune stimulation during sensitisation. Increased immune stimulation during sensitisation increases the priming of lymphocytes, which produce stronger Th2 responses when re-encountering an allergen. In accordance with this, increased lymphocyte numbers were observed with increased adjuvant. Additionally, eosinophil numbers also increased, characteristic of Th2 responses. The adjuvant used, aluminium hydroxide

stimulates the immune system during sensitisation via a variety of mechanisms including acting as a depot for the slow release of antigen, optimising allergen size for APC uptake, forming an inflammatory focus site and promoting Th2 type lymphocyte responses (Gupta *et al*, 1993; Gupta *et al*, 1995; Cooper, 1994). Aluminium hydroxide produces these effects in a concentration dependent manner. A small amount of adjuvant may be required for antigen absorption but may not provide optimal immune stimulation. Jenson and Koch, (1998) showed that there needs to be an excess of free adjuvant for these effects. However, there is also a maximal concentration at which the adjuvant works. It is speculated that this is because only a certain concentration of aluminium hydroxide is necessary for the formation of a depot site and stimulation of antigen presenting cells (APC). Beyond this the excess crystals may obstruct antigen access by APCs or be toxic to macrophages (Munder *et al*, 1969).

An increased immune response during sensitisation may also have contributed to the development of a LAR. A link between airway inflammation and the LAR is known to exist. Asthmatics displaying a LAR and EAR to allergen tend to have higher levels of inflammatory infiltrate then asthmatics that only demonstrate an EAR (Silvestri *et al*, 1997). The development of the LAR has been linked specifically to increased lymphocyte activity. In humans demonstrating both an EAR and LAR, lymphocyte trafficking and IL-5 secretion is increased (Yoshida *et al*, 2005). Eosinophil numbers and activation also increase in asthmatics who demonstrate a LAR (Gauvreau *et al*, 2000). In the present study, the development of a LAR coincided with increased lymphocytes and eosinophils. These cells may contribute to this delayed bronchoconstriction through the release of substances including PAF, leukotrienes and eosinophilic basic proteins (Bradley *et al*, 1991; Durham and Kay, 1985).

3.6.2.3 Increasing the time between allergen sensitisation and challenge

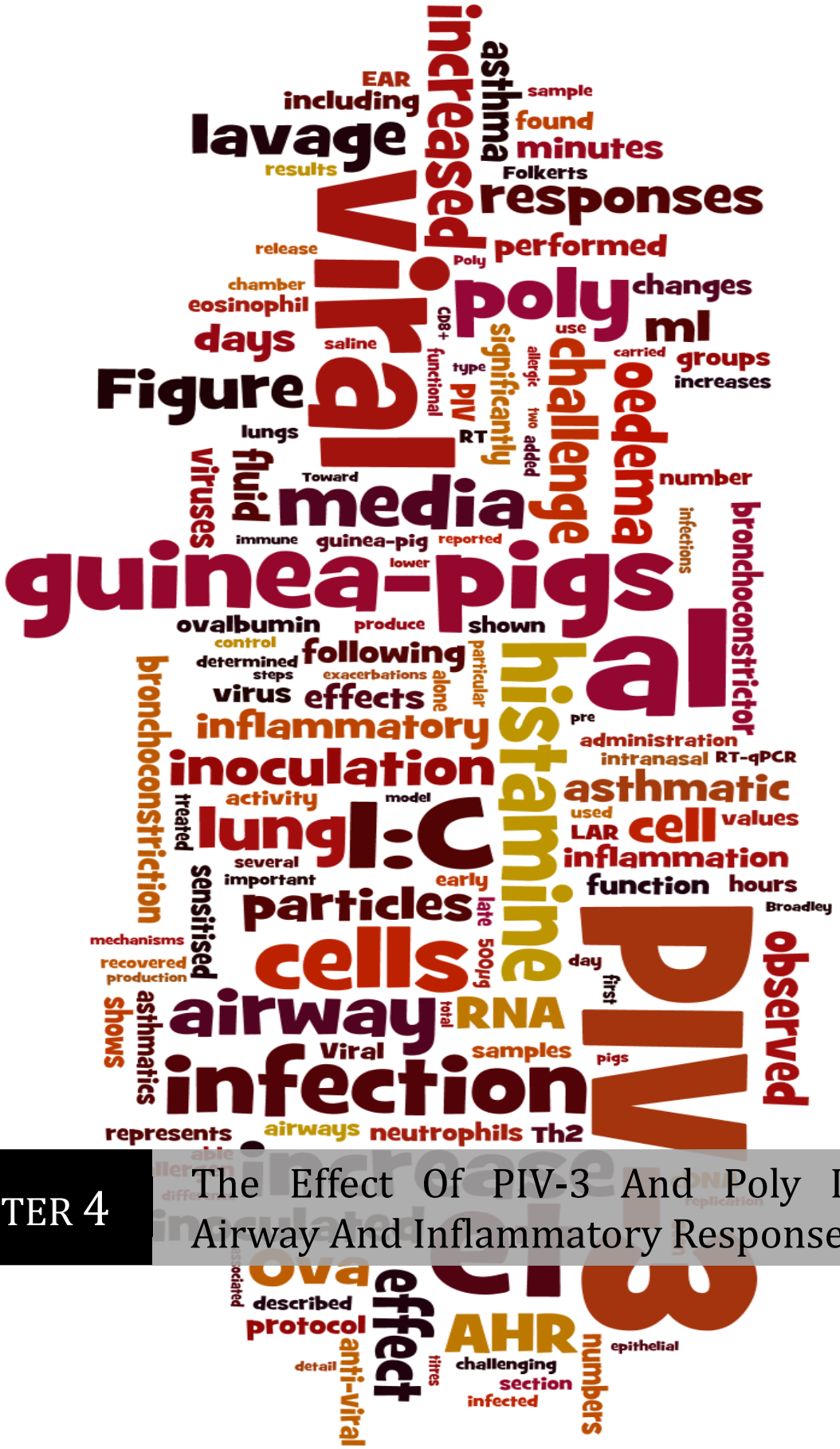
Increasing the length of time between final sensitisation and allergen challenge increased the length of the bronchoconstriction during the EAR. It also increased the

number of cells in lavage fluid, especially lymphocytes and eosinophils and produced a LAR. This suggests that a longer period between allergen sensitisation and challenge is required to generate a full response to allergen. This modification restores the gap between sensitisation and challenge to the length of time used in this laboratory's original sensitisation protocol (Lewis *et al*, 1996). In this protocol, one sensitisation injection was given 2 weeks before Ova challenge. Subsequent modifications made to the protocol with the addition of a second injection on day 5 reduced the antibody generation period to 10 days (Smith and Broadley, 2007). The addition of 3rd sensitisation injection on day 7 resulted in a further shortening of this period to 8 days from the final sensitisation.

The increase in time between the final allergen sensitisation and challenge likely allowed for the development of a fuller immunological response. This response involves the production of IgE, which is important in mast cell degranulation. The activation of lymphocytes, which act as the orchestras' of Th2 responses is also important (Matsumoto *et al*, 2002). The generation of a response to sensitisation in animal models can take several weeks (Nials & Uddin, 2008). 8 days between allergen sensitisation and challenge may not be enough time to produce a full response, except when the sensitisation conditions are increased to a certain extent, as seen in guinea-pigs sensitised with increased adjuvant concentration. Thus extending the length of time between allergen sensitisation and challenge allows more time for the development of this response.

3.6.3 Conclusions

This study indicates that it is possible to produce an acute Ova model of asthma in guinea-pigs displaying a EAR, LAR, AHR and airway inflammation. This model will be useful in investigating the effects of virus and LPS on these asthmatic responses.



CHAPTER 4

The Effect Of PIV-3 And Poly I:C On Airway And Inflammatory Responses

Chapter 4

4.1 INTRODUCTION

4.1.1 VIRAL EXACERBATIONS OF ASTHMA

Viral infections have been implicated in both exacerbations and the development of asthma. How viruses promote the development of asthma is unknown but a range of factors including the age of infection and temporal proximity of infection to allergen exposure have been implicated (Hansbro *et al*, 2008). Viruses are also involved in asthma exacerbations which are characterised by a worsening of symptoms such as wheezing, cough, chest tightness and shortness of breath (Hogg, 1997). Viral infections are the leading cause of asthma exacerbations and have been found in the lungs of as many as 80% of patients with an asthma exacerbation (Johnston *et al*, 1995). Many types of virus have been found including rhinovirus (RV), influenza viruses, parainfluenza and respiratory syncytial virus (RSV) (Atmar *et al*, 1998). RV is the most commonly detected virus but parainfluenza viruses (PIV) are also frequently found and are the second leading cause of hospitalisation for respiratory tract infection in children (Hall, 2001).

4.1.2 THE EFFECTS OF VIRAL INFECTION ON ASTHMA

Viral infections in asthmatics induce a range of changes which underlie the worsening of clinical symptoms. These changes include increased airway hyperresponsiveness (AHR), increased bronchoconstriction to allergen, β-adrenoreceptor insensitivity, altered neuronal control of the airways and increased airway inflammation (Folkerts *et al*, 1998). Viral infections increase the risk of hospitalisation in asthmatics with sensitisation to 1 or more allergens such as house dust mite or animal dander. Asthmatics with viral infection but no sensitisation show lower rates of hospital admission (Green *et al*, 2002). This effect is due to synergism between allergens and viruses.

4.1.2.1 FUNCTIONAL AND STRUCTURAL EFFECTS

Clinically, AHR is determined by the response to a bronchoconstrictor such as histamine or methacholine. Mild asthmatics, inoculated with RV demonstrate an increased sensitivity and maximal response to methacholine compared to sham infection. These effects persist for 1 week post inoculation (Cheung *et al*, 1995). Changes are also observed in the bronchoconstriction in response to allergen. In atopic individuals who only demonstrate an early asthmatic response (EAR) to allergen, viral infection increases the likelihood of a late asthmatic response (LAR) (Lemanske *et al*, 1989). This may be due to increases in total and viral specific IgE, which facilitate mast cell degranulation (Welliver *et al*, 1982). One proposed mechanisms of these functional effects is a change of the neural control of the airways. Several mechanisms have been suggested including an enhancement of parasympathetic innervation and decreased activity of non-adrenergic, non-cholinergic (NANC) neurons. The former constricts the airways and the latter relaxes the airways by the release of nitric oxide (Folkerts *et al*, 1995; Buckner *et al*, 1985). The increased release of neuropeptides, which upregulate leukotriene synthesis and mast cell mediator release has also been implicated (Saban *et al*, 1987).

Viral infection in asthma can also promote structural changes in the airway. Studies of the effects of viral infections have revealed extensive epithelial desquamination, thickening of the basement membrane, hylinisation and a distortion of its airway structure (Walsh *et al*, 1961). Mucus plugging is also commonly observed and can lead to hyperinflation of the lungs due to air trapping. Viral infection also increases the percentages of degranulated mast cells, mucus gland area and numbers of neutrophils in the submucosal glands (Carroll *et al*, 2002). Ciliary function has been shown to be decreased in asthmatics following influenza infection and thus may serve to facilitate mucus plugging (Camner *et al*, 1973). In guinea pigs, epithelial disruption by parainfluenza-3 leads to significant increases in epithelial permeability to allergens and with it, increased allergen exposure (Riedel *et al*, 1996).

4.1.2.2 INNATE IMMUNE RESPONSES

The innate immune system is the first line of defence against viruses, which are recognised by pathogen recognition receptors (PPRs). These receptors are able to recognise pathogen associated molecular patterns (PAMPs) that are present on viruses. There are several different groups of these receptors including RNA helicases and the toll like receptors (TLR). TLRs are predominantly expressed on APCs such as dendritic cells and macrophages but are also found on a range of other cell types (See and Wark, 2008). Once recognition of a virus has occurred, an anti-viral response is mounted which recruits macrophages and neutrophils. Interferons (IFNs) are key mediators in this response and induce an anti-viral state in infected cells and their neighbours. Interferons are subdivided into two groups: type 1 IFNs include IFN-α and IFN-β; type 2 IFNs only include IFN-γ and are only produced during Th1 adaptive immune responses by CD4+ lymphocytes, CD8+ lymphocytes and natural killer cells. The anti-viral activity of IFNs is mediated through the activation of IFN stimulated genes (ISG). ISG have a range of effects including negative regulation of cell proliferation and inhibition of viral replication (Katze *et al*, 2002).

Several changes in the cellular and cytokine profile occur during viral infection in asthmatic individuals. The number of neutrophils increase significantly compared with asthmatics without infection (Fahy *et al*, 1995). Respiratory viruses can also enhance superoxide responses, chemotaxis and adhesion of neutrophils, which likely contribute to a worsening of symptoms (Folkerts *et al*, 1998). Respiratory infection is also able to increase allergen-induced eosinophil recruitment (Calhoun *et al*, 1994; Toward *et al*, 2005). However, unlike in a non-infected asthmatic where eosinophils are the predominant cell type, eosinophil levels can be up to 8x lower than neutrophils (Ordonez *et al*, 2000). Increases are also seen in eosinophil granule products including eosinophil cationic protein (ECP), which may contribute to the development of AHR (Grünberg *et al*, 1997).

4.1.2.3 ADAPTIVE IMMUNE RESPONSES

In asthmatics there is a deficiency in the antiviral response, independent of corticosteroid use and increases susceptibility to viral infection. Epithelial cells from asthmatic airways demonstrate enhanced viral replication and cell lysis. In addition, apoptosis which is important in limiting viral replication and the release of inflammatory mediators is impaired in the cells of asthmatic subjects. Furthermore, production of IFN-β is also reduced in asthmatic airways (Wark *et al*, 2006). A deficient anti-viral defence allows for enhanced viral replication, production of inflammatory cytokines and cell damage.

Asthmatics may demonstrate a deficient anti-viral response for a number of reasons. The balance between the two CD4+ subtypes, Th1 and Th2 may be important. Th1 cells coordinate cytotoxic and antibody responses to viral infection. However, in allergic disease a Th2 lymphocyte response predominates and has weak anti-viral activity (Moran *et al,* 1996; Legg *et al*, 2003). Additionally viruses can also induce Th2 responses themselves, facilitating their own replication (van Rijt *et al*, 2005). Changes in CD8+ T cells may also contribute to the weak anti-viral immunity. CD8+ T cells' main role during viral infection is the induction of apoptosis of infected cells and the secretion of IFN-γ (Cox *et al*, 2013). However, on a Th2 inflammatory background, CD8+ cells switch to a Th2 cytokine secretion pattern, producing IL-4 and IL-5 (Erard *et al*, 1993). This phenotypic switch further reduces anti-viral activity of CD8+ T cells and contributes to a Th2 polarised response.

4.1.3 PARAINFLUENZA-3

4 types of parainfluenza viruses (PIV) are known and are accordingly numbered PIV 1-4. PIV is an enveloped RNA virus belonging to the family of Paramyxoviridae with single stranded, non-segmented negative sense genomes (Voyles, 1993). The basic structure of PIV is shown in Figure 1. The envelope glycoproteins consisting of fusion protein (F) and hemagglutinin neuraminidase are integral to immunity and pathogenesis of these viruses. Like all respiratory viruses, PIV enter and replicate in

airway epithelial cells. PIV has an incubation period of 2-8 days, after which it replicates in the nasopharyngeal epithelium and 1-3 days later spreads to the lower respiratory tract (Hall, 2001).

Common features of PIV infections in humans are sloughing of the epithelium in the small airways, increased mucus secretion, oedema and associated small airway obstruction. These manifest themselves in the clinical symptoms of atelectasis, hyperinflation and wheezing. Immunological recognition of PIV is mainly via TLR7/8 (Heil *et al*, 2004). In guinea-pig models, PIV-3 alone can induce non-specific AHR and an increase in airway inflammation (Folkerts *et al*, 1992b; Toward *et al*, 2005). Within my laboratory, PIV-3 inoculation superimposed on ovalbumin challenge in sensitised guinea-pigs has been shown to cause an exacerbation of the responses to allergen. In particular, the early asthmatic response to allergen is prolonged and merges with the late asthmatic response. Increased airway inflammation, especially neutrophils is also seen (Broadley *et al*, 2008). Studies from this laboratory have suggested that this model may be steroid insensitive (Broadley *et al*, 2009; Broadley *et al*, 2010). This model may serve as a good basis for further investigation into the mechanisms of viral exacerbations of asthma and their effect on asthma treatment.

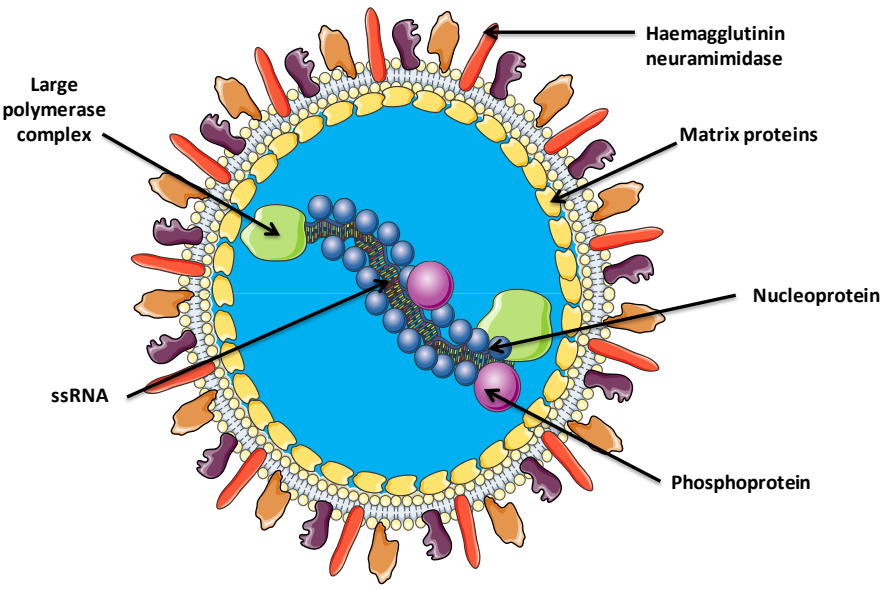


Figure 1: Diagram of the structure of the parainfluenza virus (PIV). Adapted and redrawn from Hall, 2001.

4.1.3 Polyinosinic-polycytidylic acid (Poly I:C)

Polyinosinic-polycytidylic acid (Poly I:C) is synthetic double stranded (ds) RNA which can mimic certain aspects of viral infection. RNA viruses such as PIV synthesise dsRNA during replication, which is recognized by several PAMPs including MDA5 and TLR3 (Jiang *et al*, 2003; Kato *et al*, 2006). Both signaling pathways are important in viral induction of airways inflammation and AHR without a pre-existing allergic background (Wang *et al*, 2011). In particular, signalling through TLR3 creates a local cytokine burst which is specific to the cell type and microenvironment, but generally consists of type 1 interferons, TNF-α, CCL-2 (MCP-1) and IL-12 (Nicodemus & Berek, 2010).

On an allergic background, poly I:C mimics aspects of viral infection and asthma exacerbations including increased cellular influx (neutrophils especially), an increased Th2 response, AHR and structural remodeling (increased smooth muscle mass and goblet cell hyperplasia) in mice and rats (Torres *et al*, 2010; Takayama *et al*, 2011). The effects of poly I:C in guinea-pigs alone or in parallel with allergen challenge have not been investigated. Pilot data from my laboratory suggests that poly I:C can induce AHR and invoke a cellular inflammatory response (unpublished). Furthermore, the effect of poly I:C on functional allergic responses including the early and late asthmatic responses (EAR and LAR respectively) remains to be investigated.

The advantage of using agents such as poly I:C is that they present no risk of infection, making them easier to work with. However, agents such as poly I:C are not able to replicate themselves so fail to model this aspect of an viral infection.

4.2 HYPOTHESIS

'PIV-3 infection in sensitised guinea-pigs will exacerbate the functional and inflammatory responses to ovalbumin challenge'.

4.3 AIMS AND OBJECTIVES

The aim of this chapter was to investigate the functional and inflammatory response of PIV-3 and poly I:C in guinea-pigs. Furthermore, the effect of these agents in an ovalbumin model of asthma developed in the previous chapter will be determined. In particular, the effect of these agents on the early asthmatic response, late asthmatic response to Ova challenge, airway hyperresponsiveness, airway oedema and airway inflammation will be investigated.

4.4 METHODS

4.4.1 IN VITRO

4.4.1.1 GENERATION OF PARAINFLUENZA-3 VIRAL TITRES

BSC-1 cells from African green monkey kidney epithelium were cultured in 75cc flasks containing cell media (EMEM, 2mM glutamine, 1% non-essential amino acids (NEAA), 10% foetal bovine serum) at 37°c in a CO_2 incubator until a confluent monolayer formed. At this stage cells were infected with PIV-3 as follows. PIV-3 stocks were defrosted rapidly from -80°c freezer stocks. BSC-1 culture medium was removed from the culture flask, PIV-3 viral stock added and the cells incubated for 5 minutes at 37°c. BSC-1 culture medium was then re-added and cells returned to the incubator. Cells were checked daily for signs of cytopathic effect, when cells start to fuse, form multi nucleated bodies (syncytia) and change shape. This first becomes visible 24 hours after PIV-3 inculcation and should reach full prominence 2-3 days post-inoculation. At this point viral harvest was performed. This was carried out by scraping cells from the bottom of the flask, resulting in cell lysis and the release of viral particles. Viral media was centrifuged at 1000rpm for 5 minutes at 20°C to pellet cell debris, the supernatant aliqouted and subsequently stored at -80°c. Viral tire was determined on samples immediately following viral harvest.

4.4.1.2 DETERMINATION OF VIRAL TITRE

Viral titre of PIV-3 harvested from BSC-1 cells and the homogenised lungs and lavage fluid of PIV-3 infected guinea-pigs (see section 4.4.2 for information on *in vivo* protocols) were determined. Quantitative assessment of viral RNA titres was performed in 2 steps, first by isolating and purifying viral nucleic acids and then performing reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) to convert RNA to cDNA and then amplify it.

4.4.1.2.1 ISOLATION OF VIRAL RNA

Lungs required homogenisation before viral nucleic acid isolation. This was performed as follows: ~100mg of lung lobe stored on ice was weighed and added to

tissue homogeniser tubes (containing microbeads) with 1ml of dPBS. The lung/dPBS mixture was homogenised with the use of an automated homogeniser (3x 5 second bursts, with 15 second gaps in between).

Viral RNA was isolated from lavage fluid, lung and cell media using a high pure viral nucleic acid kit supplied by Roche. All reagents in this section were made up as per the manufacturer's instructions and are described in more detail in Appendix1.3. Briefly, 200µl of sample (viral media, lung homogenate or lavage fluid) was added to sterile, nuclease free tubes, followed by 200µl of working solution (50µl Poly A, 2.5ml binding buffer) and 50µl of proteinase K (dissolved in 500µl of elution buffer) to digest any remaining protein in the sample. The contents were mixed and incubated at 72^{o}C for 10 minutes. Immediately, 100µl of binding buffer was added to the sample. Subsequently, the sample was run through a series of filter tube centrifugation steps to remove contaminating cellular components. At each step the filter tube (containing a glass fibre fleece which binds viral nucleic acid) was kept and flow through liquid discarded. Initially the full sample solution was pipetted into the upper reservoir of the filter tube. Samples were centrifuged for 1 minute at 8,000g in between each step. These steps are shown in Figure 2. The flow through liquid from the last step contained isolated viral nucleic acid and was stored at -80^{o}C until RT-qPCR was carried out.

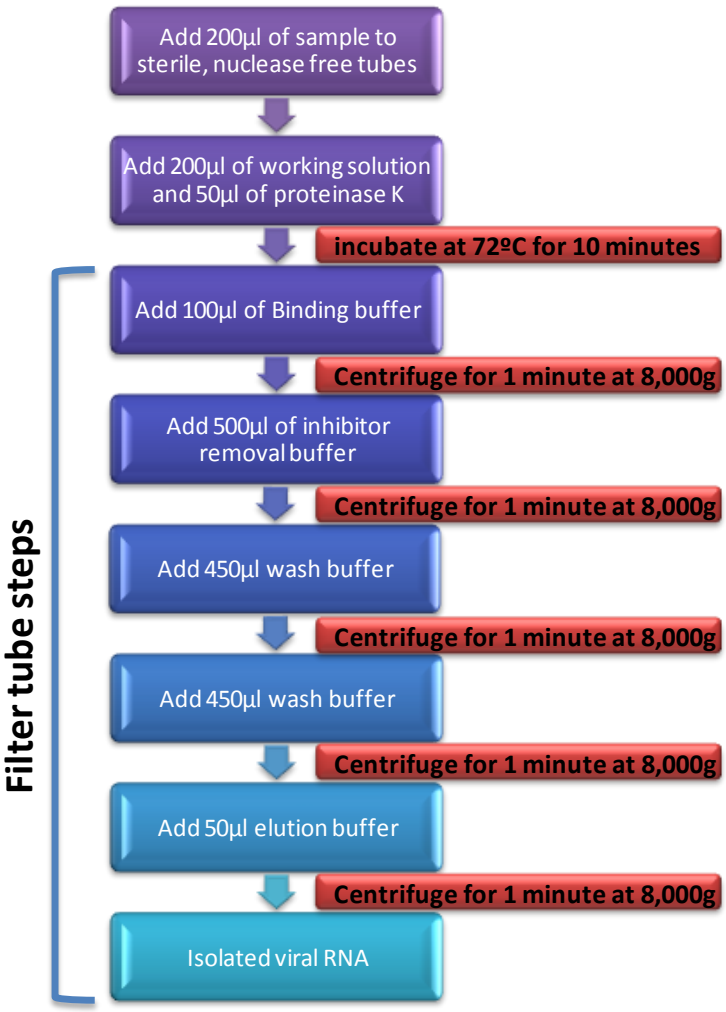


Figure 2: Schematic of the method used to isolate viral nucleic acid from cell culture medium, lavage fluid and homogenised lung.

4.4.1.3 RT-qPCR

Reverse transcriptase- quantitative polymerase chain reaction (RT-qPCR) was carried out to convert viral RNA into cDNA for amplification and quantitative determination of viral titres. Conversion of viral RNA into cDNA was carried out using a superscript first-strand synthesis system kit supplied by Invitrogen, which is capable of converting 1ng-5ng of RNA into 1st strand cDNA. Samples were defrosted on ice and RNA/primer mixtures prepared as described in Table 1. This primer mix provides the substrates and catalysts necessary for the production of cDNA from RNA. An RT negative control was used to show that cDNA amplification is not due to DNA contamination before the reverse transcriptase step. An RT positive control was used

to demonstrate a successful reverse transcription step. The RT H_20 sample demonstrates the purity of RT water.

Components	Sample	-RT Control	+RT Control	RT H_20
<5µg total RNA	8 µl	8µl	-	-
Control RNA (50ng/µl)	-	-	1µl	-
10mM dNTP mix	1µl	1µl	1µl	1µl
RT primer mix	1µl	1µl	1µl	1µl
DEPC-treated water	-	-	7µl	8µl

Table 1: Reagents used to produce cDNA from RNA for viral samples and controls. dNTP: deoxyribonucleotide triphosphate; DEPC : deionised, diethylpyrocarbonate, RT: reverse transcription.

RNA/primer mixtures were added to their respective nuclease free, sterile tube and run through a series of thermal cycling steps using a thermocycler. The conditions used at each step are shown in Figure 3. During step 2, samples were placed on ice and a reaction mix containing 2µl of 10x RT buffer, 4µl 25mM $MgCl_2$, 2µl 0.1mM dithiothreitol (DTT) and 1µl of RNase out. This mixture aids the work of the superscript enzyme and prevents the cleavage of RNA by RNAses. After step 3, 1µl (50U) of superscript was added to all tubes except the RT negative control. Following the conclusion of thermal cycling, samples were briefly centrifuged and chilled on ice for commencement of cDNA amplification.

Figure 3: The timing and temperatures used on a thermocycler to convert RNA to cDNA.

cDNA was amplified using q-PCR performed in a thermo light-cycler. Reaction mixes were prepared by the addition of 10µl PCR grade water, 4µl of 5x conc master mix (both from Roche, Switzerland) and PIV-3/β-actin cDNA primers (Primer design, Ltd). To 5µl of DNA template in a chilled capillary tube and centrifuged for 5 seconds at 700g. Samples were run using the following parameters: 1 pre-incubation cycle at 95^{o}c for 10mins, 50 amplification cycles at 95ºc for 10 seconds, 60^{o}c for 60 seconds and finally a cooling step at 40^{o}c for 30 seconds. The light cycler software calculates the individual virus particles per µl which is then converted into virus particles per ml.

4.2.2 In vivo methods

4.2.2.1 Parainfluenza-3 infection protocol

Guinea-pigs (300-400g, male, Dunkin Hartley) were inoculated intranasally with 125µl per nostril of PIV-3 ($1x10^7$-10^9 viral particles per ml) or media on day 2 and 3. The airway response to histamine was tested on days 1 and 7 of the protocol (described in more detail in section 4.2.2.5). Bronchoalveolar lavage (BAL) was performed on day 7 of the protocol, following post-virus AHR assessment (Figure 4).

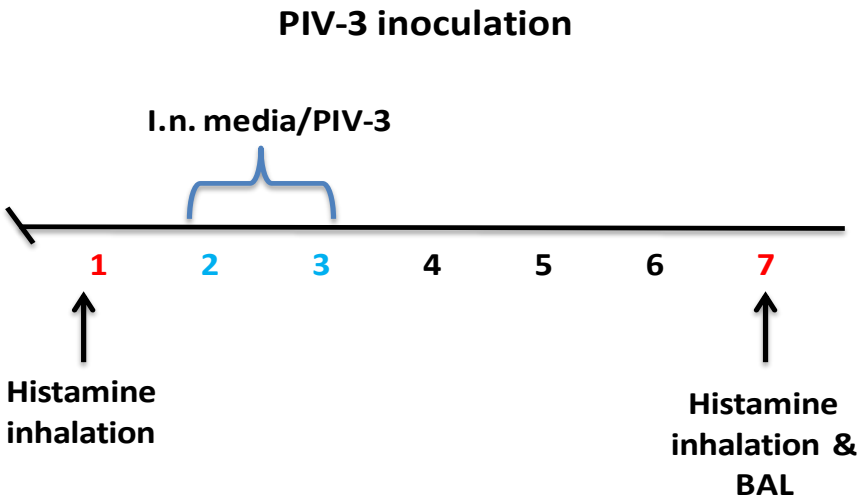


Figure 4: Diagram of the 7 day PIV-3 protocol. I.n: intra-nasal; BAL: bronchoalveolar lavage.

4.2.2.2 Acute ovalbumin and PIV-3 protocol

Guinea-pigs (300-400g, male, Duncan Hartley) were sensitised on days 1, 3 and 7 with 150µg ovalbumin (Ova) and 100mg aluminium hydroxide. The airway response to histamine was tested on days 15 and 22 of the protocol (described in more detail in section 4.2.2.5). Guinea-pigs were Intranasally administered 125µl per nostril of PIV-3 ($1x10^7$-10^9 viral particles per ml) or media on days 17 and 18. The effector phase of asthma was elicited on day 21 by challenging sensitised guinea-pigs to 0.03% Ova for 1 hour in an exposure chamber. Lung function was then measured over 12 hours (described in more detail in section 4.2.2.4).

BAL was performed on day 22 of the protocol, following post-Ova AHR assessment (Figure 5).

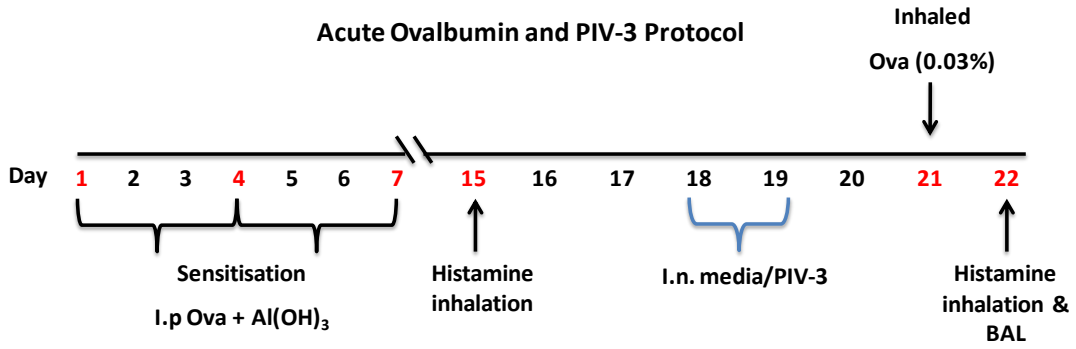


Figure 5: Diagram of the 22 day ovalbumin (Ova) and PIV-3 protocol. I.n: intra-nasal; BAL: bronchoalveolar lavage.

4.2.2.3 Poly I:C protocol

Guinea pigs (300-400g, male, Duncan Hartley) were inoculated with poly I:C (125 or 500µg) intranasally on day 2, 3 and 4. The airway response to histamine was tested on days 1 and 5 (described in more detail in section 4.2.2.5). BAL was performed on day 5 of the protocol, following post-poly I:C AHR assessment (Figure 6).

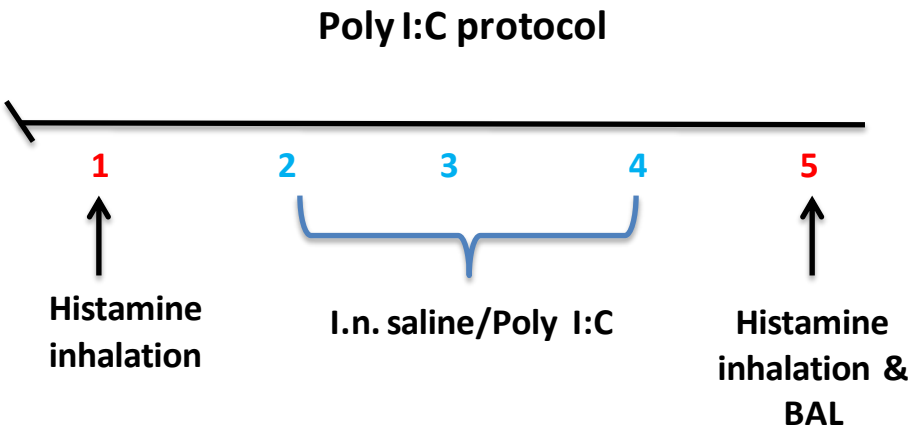


Figure 6: Diagram of the 5 day Poly I:C protocol. I.n: intra-nasal; BAL: bronchoalveolar lavage.

4.2.2.4 MEASUREMENT OF LUNG FUNCTION

Guinea-pig airway function was measured using non-invasive double chamber plethysmography, using specific airway conductance (sGaw) as a measure of airway conductance (Griffiths-Johnson *et al*, 1988). In Ova challenged groups, lung function was recorded hourly for 12 hours and every 15 minutes during the first hour of measurements following Ova challenge. A final measurement was also taken 24 hours post-challenge. All values from these readings were expressed as a percentage of the baseline reading, taken before Ova/poly I:C challenge. A negative percentage change in baseline value represents a bronchoconstriction. To account for differences in the timing of allergen responses between animal values for the early (0-6 hours) and late (6-12 hours) phases were expressed as the peak bronchoconstriction during that period (displayed as a histogram next to a time course plot) and area under the curve.

4.2.2.5 MEASUREMENT OF AIRWAYS HYPERRESPONSIVENESS (AHR)

AHR was determined using whole body plethysmography. This was performed by measuring the bronchoconstrictor response to histamine pre- and post-PIV-3/Poly I:C exposure. Histamine (0.3mM) was delivered to the guinea-pigs by the use of Buxco nebuliser chamber at a rate of 0.5 LPM, two minute nebulisation, and 1 minute drying period with a 10% duty setting per chamber. Lung function was measured just before histamine inhalation and at 0, 5 and 10 minutes post-histamine exposure.

4.2.2.6 MEASURING AIRWAY INFLAMMATION

Following the final histamine inhalation challenge, guinea-pigs were sacrificed by an intra-peritoneal overdose of sodium pentobarbitone, the lungs excised and lavaged. Total and differential cell counts were then performed.

4.2.2.7 MEASURING AIRWAY OEDEMA

Lung oedema was determined by comparison of lung wet weight with dry weight. The 3rd largest lobe of the right lung was weighed following excision from the guinea-pig and then dried overnight in an oven at 40^{o}C. The lobe was again weighed. The difference between the wet and dry weight was expressed as a percentage of the dry

weight according to the following formula [Airway oedema= ((wet weight-dry weight)/dry weight x 100)].

4.5 RESULTS

4.5.1 THE EFFECT OF PIV-3 ON AIRWAY RESPONSES TO HISTAMINE, LUNG OEDEMA AND INFLAMMATORY CELLS.

Intranasal media inoculation did not cause a significant increase in the bronchoconstrictor response to histamine. PIV-3 inoculation resulted in a non-significant immediate increase in the bronchoconstrictor response to histamine, reaching significance 5 minutes post-histamine challenge (-25.9±5.7% compared to pre-PIV-3 values, -9.5±1.1% respectively). A bronchoconstriction was still present 10 minutes after the histamine challenge (-25.3±4.3% compared to 0.5±3.0% respectively) (Figure 7).

PIV-3 inoculation significantly increased lung oedema compared to media inoculated control animals (410±13.2% compared to 317.0±13.4% respectively) (Figure 8).

PIV-3 inoculation resulted in a significant increase in airway inflammation. Total lavage cells were significantly increased in PIV-3 treated guinea-pigs compared to media (3.5±0.23x10^6/ml, 1.6±0.27x10^6/ml respectively). This was also true for macrophage numbers (3.04±0.19x10^6/ml compared to 1.44±0.26x10^6/ml respectively), eosinophil numbers (0.20±0.05x10^6/ml compared to 0.09±0.02x10^6/ml respectively), neutrophil numbers (0.09±x0.02x10^6/ml compared to 0.01±0.003x10^6/ml respectively) and lymphocyte numbers (0.02±0.06x10^6/ml compared to 0.01±0.004x10^6/ml respectively) (Figure 9).

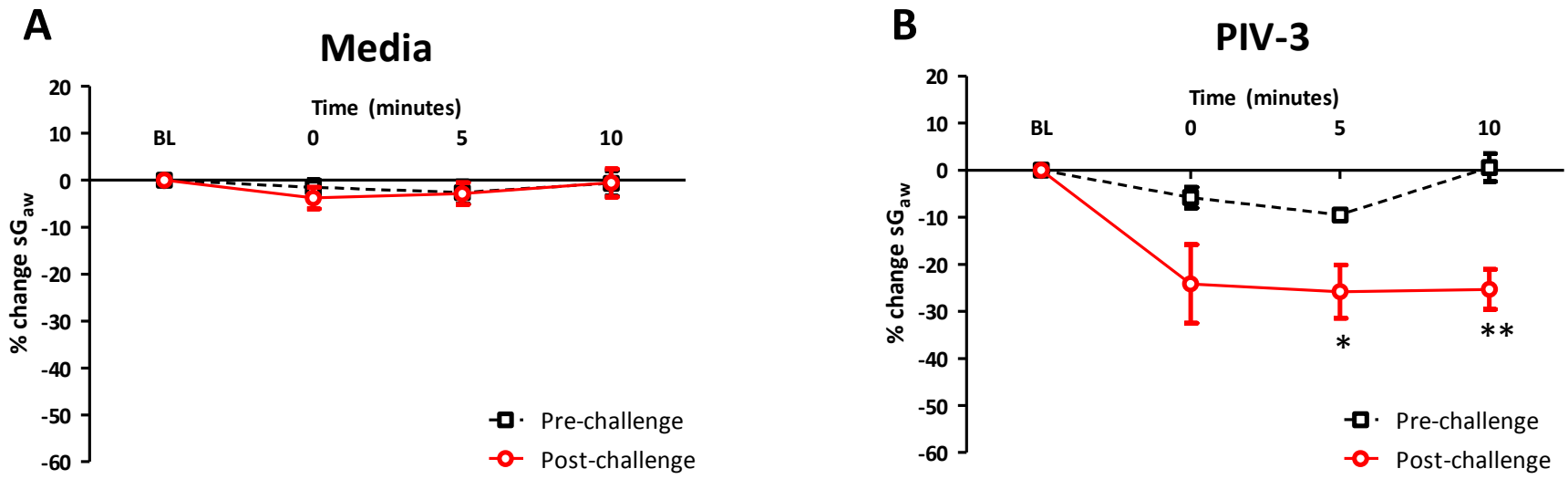


Figure 7: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles with 0.5LPM flow per chamber over 2 minutes, 1 minute drying period) in guinea-pigs inoculated with 2 intranasal doses of **A)** PIV-3 ($1x10^7$-10^9 viral particles per ml) or **B)** media. Values were recorded 24 hours pre- and 4 days post- first inoculation. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=12 (media), N=5 (PIV-3); *Significantly different from time paired pre-challenge values $p<0.05$, ** $p<0.01$; performed with a two tailed T-test.

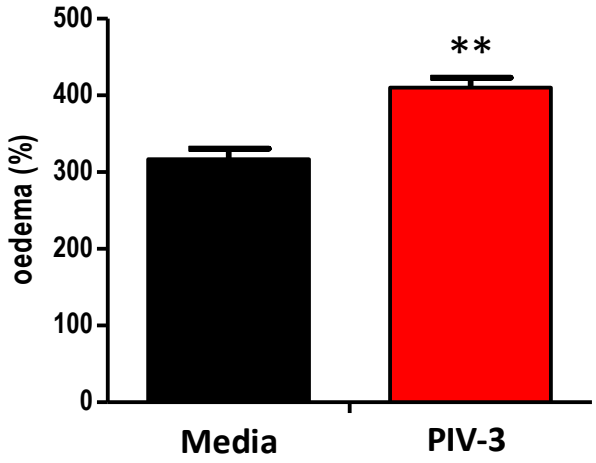


Figure 8: Lung oedema in guinea-pigs inoculated with 2 intranasal doses of PIV-3 ($1x10^7$-10^9 viral particles per ml) or media, 4 days after the initial inoculation. Oedema is expressed as ((lung wet weight- lung dry weight)/dry weight x100). Lung weight measured following lung excision (wet) and overnight drying in an oven at 40^oC (dry). N=12 (media), N=5 (PIV-3) **Significantly different from time media $p<0.01$; performed with a two tailed T-test.

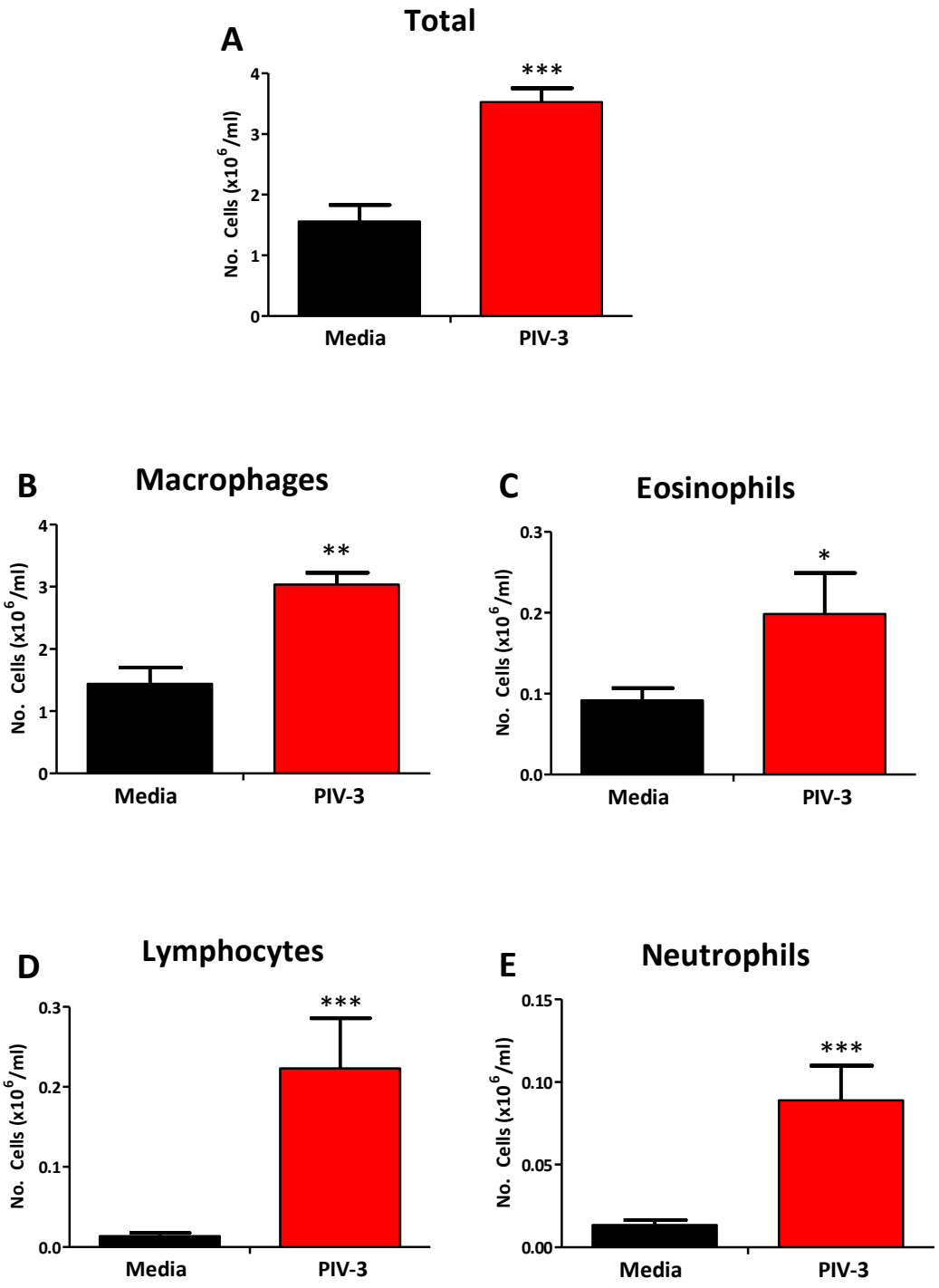


Figure 9: The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in bronchoalveolar fluid in guinea-pigs inoculated with 2 intranasal doses of PIV-3 ($1x10^7$-10^9 viral particles per ml) or media, 4 days after the initial inoculation. N=12 (media), N=5 (PIV-3); *significantly different from media $p<0.05$, ** $p<0.01$; *** $p<0.001$; performed with a two tailed T-test.

4.5.2 The effect of PIV-3 on Ova-induced functional and inflammatory responses

In both media and PIV-3 treated guinea-pigs a bronchoconstriction was observed at 0 minutes post-Ova challenge (-58.8±4.0% and –55.1±3.7% respectively). This bronchoconstriction was the early asthmatic response. Both groups also displayed evidence of a late asthmatic response between 6-12 hours. No significant difference was observed between the 2 groups at the 0 minute time point, or during the further 12 hours of lung function measurement (Figure 10, time course. No significant difference between the EAR and LAR peak values was found for media and PIV-3 inoculated guinea-pigs (Figure 10, histogram). Similarly, no difference in area under the curve was observed between the two groups at total, EAR and LAR time points (Figure 11).

Ova challenge with media inoculation produced a significant increase in the bronchoconstrictor response to histamine, when compared to sG_{aw} values pre-Ova challenge (-20±4.2% compared to 0.6±3.5%). At 5 minutes post-histamine challenge, this bronchoconstriction was no longer significantly different from pre-Ova challenge values. In guinea-pigs challenged with Ova, inoculated with PIV-3 a significant bronchoconstriction was observed at 0 minutes, compared to pre-Ova challenge values (-15.5±3.9% compared to 0.6±2.3%). A bronchoconstriction was still present at 5 (-16.7±3.2% compared to -3.1±2.6%) and 10 minutes (-20.2±2.9% compared to 3.1±3.1%) (Figure 12).

No significant difference in oedema in the two groups was observed (Figure 13). PIV-3 inoculated guinea-pigs challenged with Ova demonstrated a non-significant trend for increased total lavage cell number compared to media inoculated guinea-pigs. There was a significant increase in eosinophil numbers, compared to media inoculated guinea-pigs ($3.8±0.34x10^6$/ml compared to $2.7±0.23x10^6$/ml respectively). Neutrophil, macrophage and lymphocyte numbers were unchanged (Figure 14).

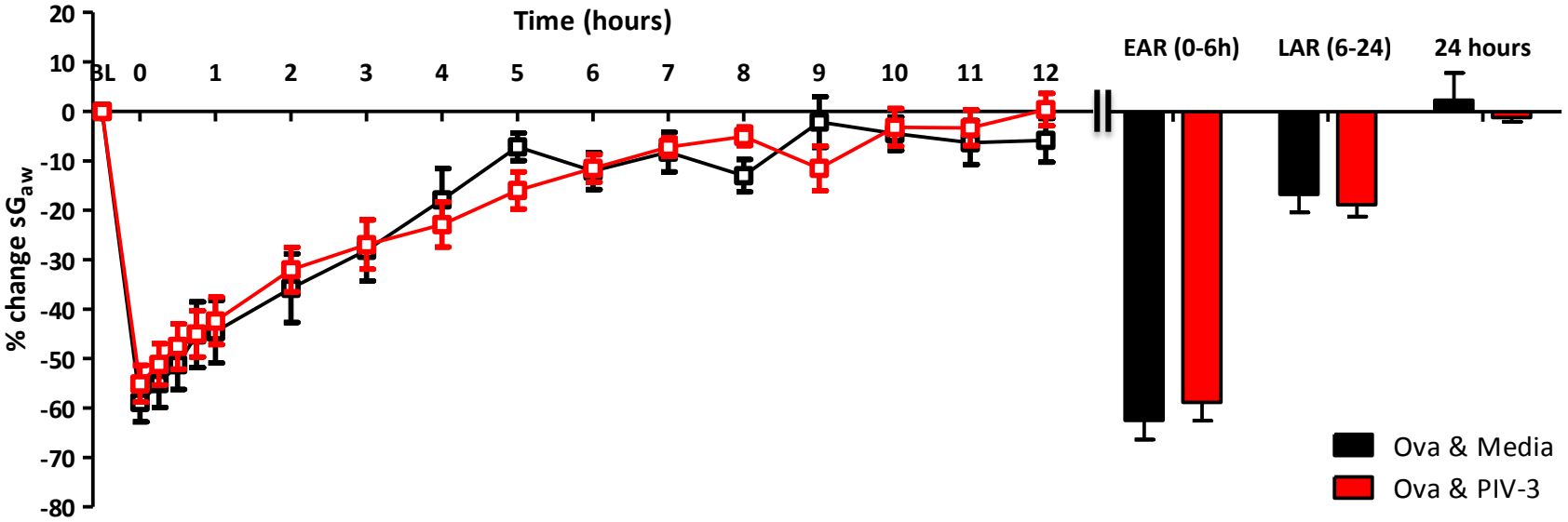


Figure 10: The mean time-course values of sGaw in ovalbumin (Ova) sensitised guinea-pigs inoculated with 2 intranasal doses of PIV-3 ($1x10^7$-10^9 viral particles per ml) or media, 3 days before subsequent inhalation Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline prior to Ova challenge. A negative value represents a bronchoconstriction. N=10 (Ova + media), N=16 (Ova + PIV-3); performed with a two tailed T-test.

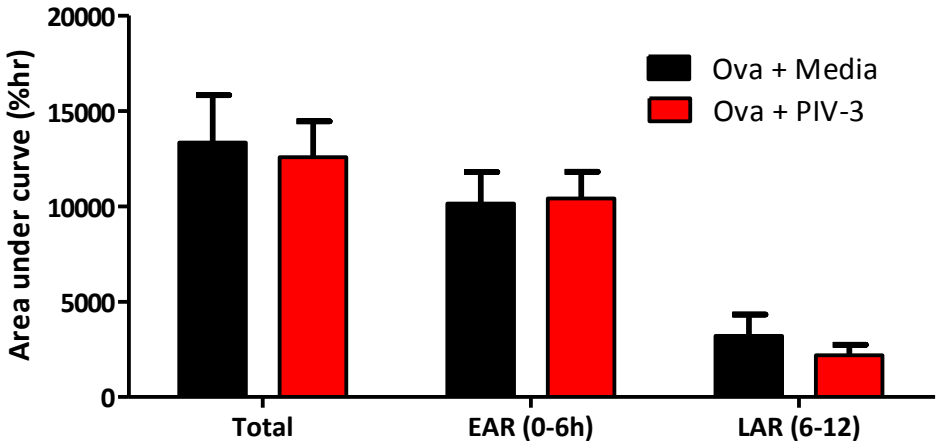


Figure 11: Area under the curve analysis of sGaw values over 12 hours following ovalbumin (Ova) inhalation challenge in guinea-pigs inoculated with 2 intranasal doses of PIV-3 ($1x10^7$-10^9 viral particles per ml) or media. For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-24 hours, early asthmatic response (EAR) includes values from 0-6 hours and late asthmatic response (LAR) includes values from 6-12 hours. Area under the curve is expressed as %.hour; N=10 (Ova + media), N=16 (Ova + PIV-3); performed with a two tailed T-test.

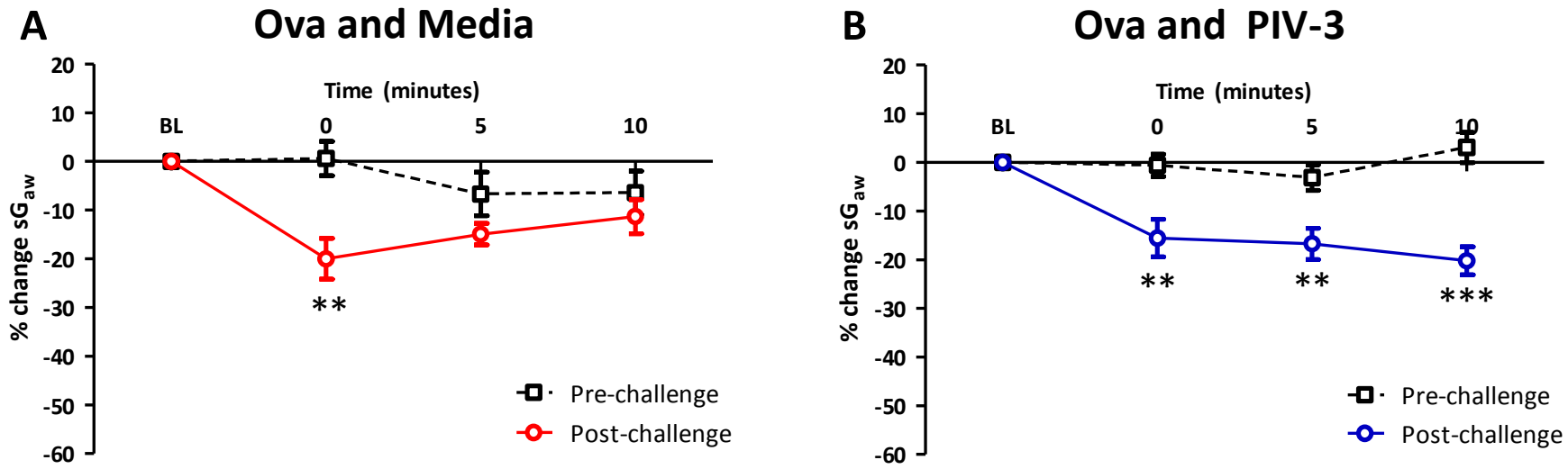


Figure 12 Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles with 0.5LPM flow per chamber over 2 minutes, 1 minute drying period) in ovalbumin (Ova) sensitised guinea-pigs inoculated with A) 2 intranasal doses of PIV-3 ($1x10^{7}$-10^{9} viral particles per ml) or B) media, 3 days before subsequent Ova inhalation challenge. Values were recorded 24 hours before the first inoculation and 24 hours post- Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. * Significantly different from time paired pre-challenge values; N=10 (Ova + media), N=16 (Ova + PIV-3); performed with a two tailed T-test.

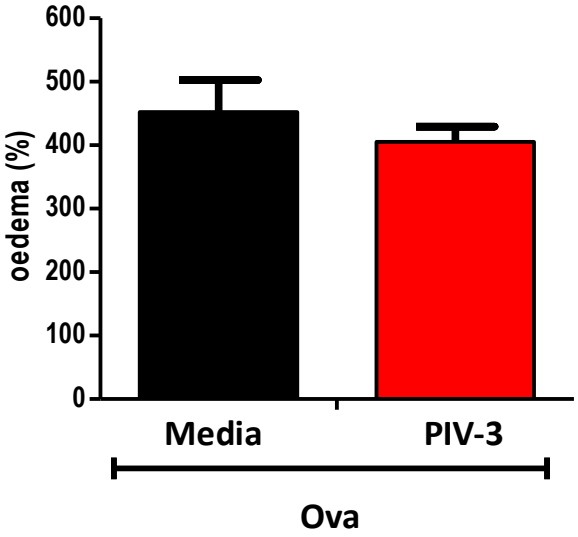


Figure 13: Lung oedema in ovalbumin (Ova) sensitised guinea-pigs inoculated with 2 intranasal doses of PIV-3 ($1x10^7$-10^9 viral particles per ml) or media, 3 days before subsequent Ova inhalation challenge. Oedema is expressed as ((lung wet weight- lung dry weight)/dry weight x100). Lung weight measured following lung excision (wet) and overnight drying in an oven at 40^{o}C (dry). N=10 (Ova + media), N=16 (Ova + PIV-3); performed with a two tailed T-test.

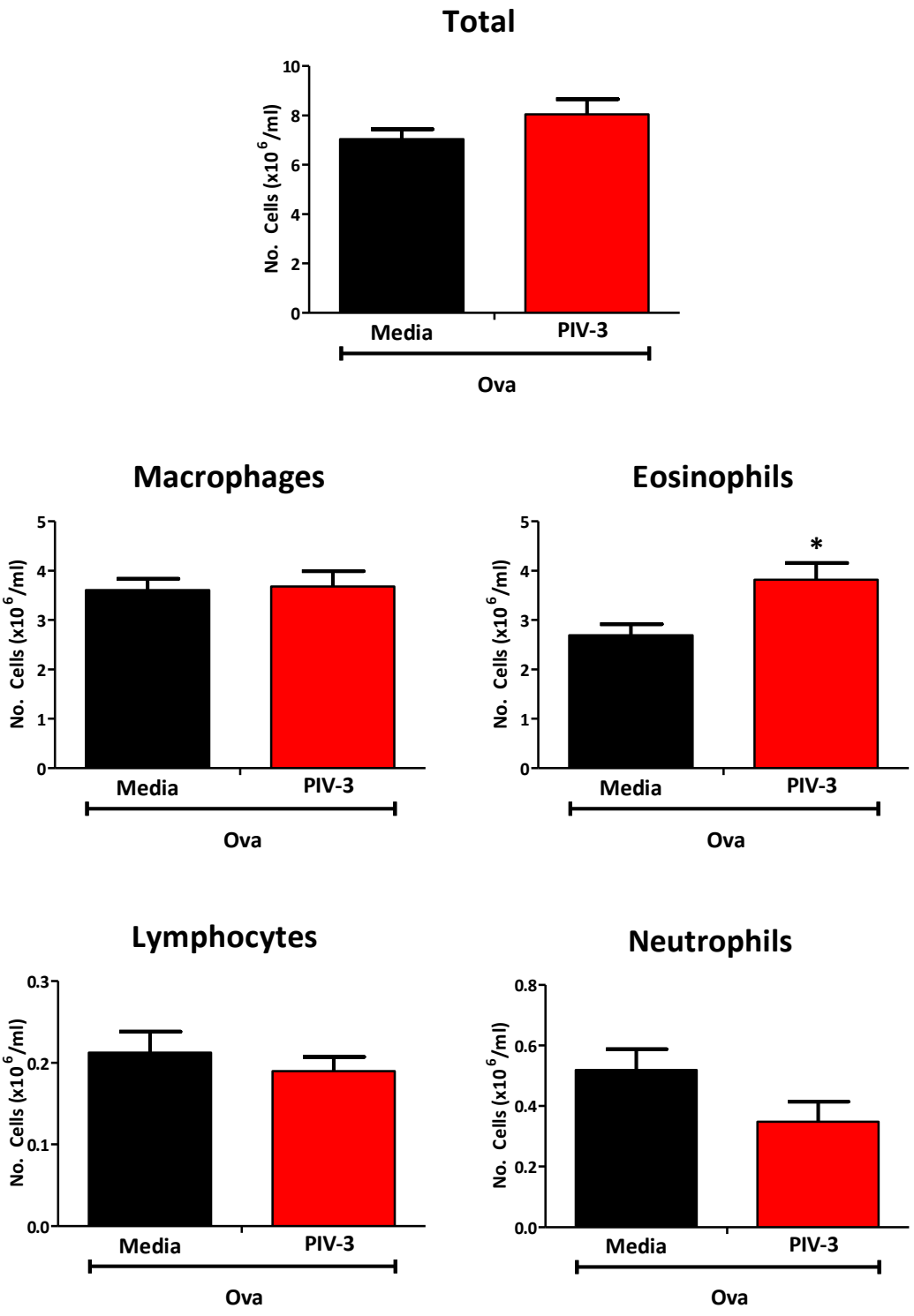


Figure 14: The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in bronchoalveolar fluid in ovalbumin (Ova) sensitised guinea-pigs inoculated with 2 intranasal doses of PIV-3 ($1x10^7$-10^9 viral particles per ml) or media, 3 days before subsequent to Ova inhalation challenge. N=10 (Ova + media), N=16 (Ova + PIV-3); performed with a two tailed T-test.

4.5.3 A RE-EVALUATION OF THE EFFECT OF PIV-3 ALONE ON AIRWAY LUNG FUNCTION AND AIRWAY INFLAMMATION.

Because PIV-3 failed to enhance the responses to Ova, it was decided to re-examine the effects of PIV-3 alone. Media inoculation failed to produce a significant increase in bronchoconstriction in response to histamine, when compared to sG_{aw} values prior to media inoculation. PIV-3 inoculation also did not change the bronchoconstrictor response to histamine (Figure 15). PIV-3 inoculation produced a significant increase in oedema when compared to media inoculated animals (404.8±10.6% compared to 317.0±13.4%) respectively (Figure 16). PIV-3 inoculation did not significantly increase any of the cell types in lavage fluid measured when compared to media inoculated guinea-pigs (Figure 17).

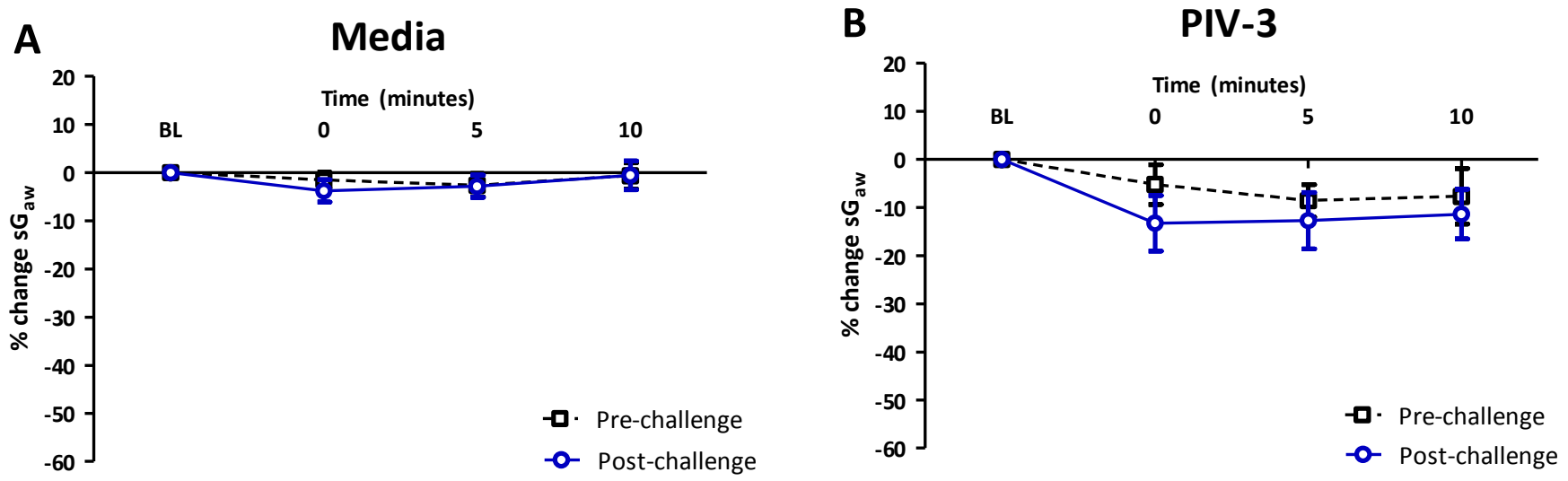


Figure 15: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles with 0.5LPM flow per chamber over 2 minutes, 1 minute drying period) in guinea-pigs inoculated with 2 intranasal doses of PIV-3 ($1x10^7$-10^9) or media. Values were recorded 24 hours pre- and 4 days post- first inoculation. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=6; performed with a two tailed T-test.

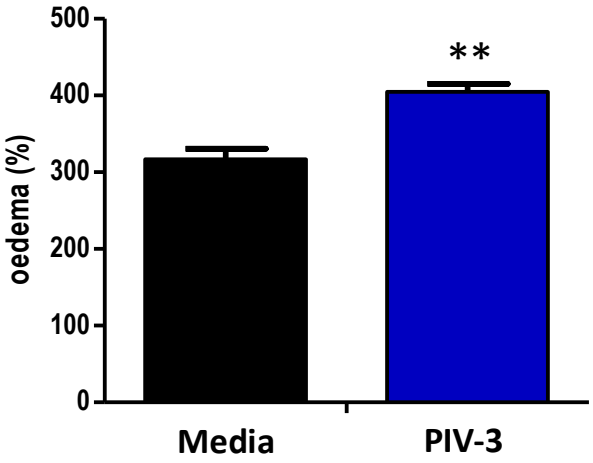


Figure 16: lung oedema in guinea-pigs inoculated with 2 intranasal doses of PIV-3 ($1x10^7$-10^9) or media, 4 days after the initial inoculation. Oedema is expressed as ((lung wet weight- lung dry weight)/dry weight x100). Lung weight measured following lung excision (wet) and overnight drying in an oven at 40^{o}C (dry). **Significantly different from media $p<0.01$; N=6; performed with a two tailed T-test.

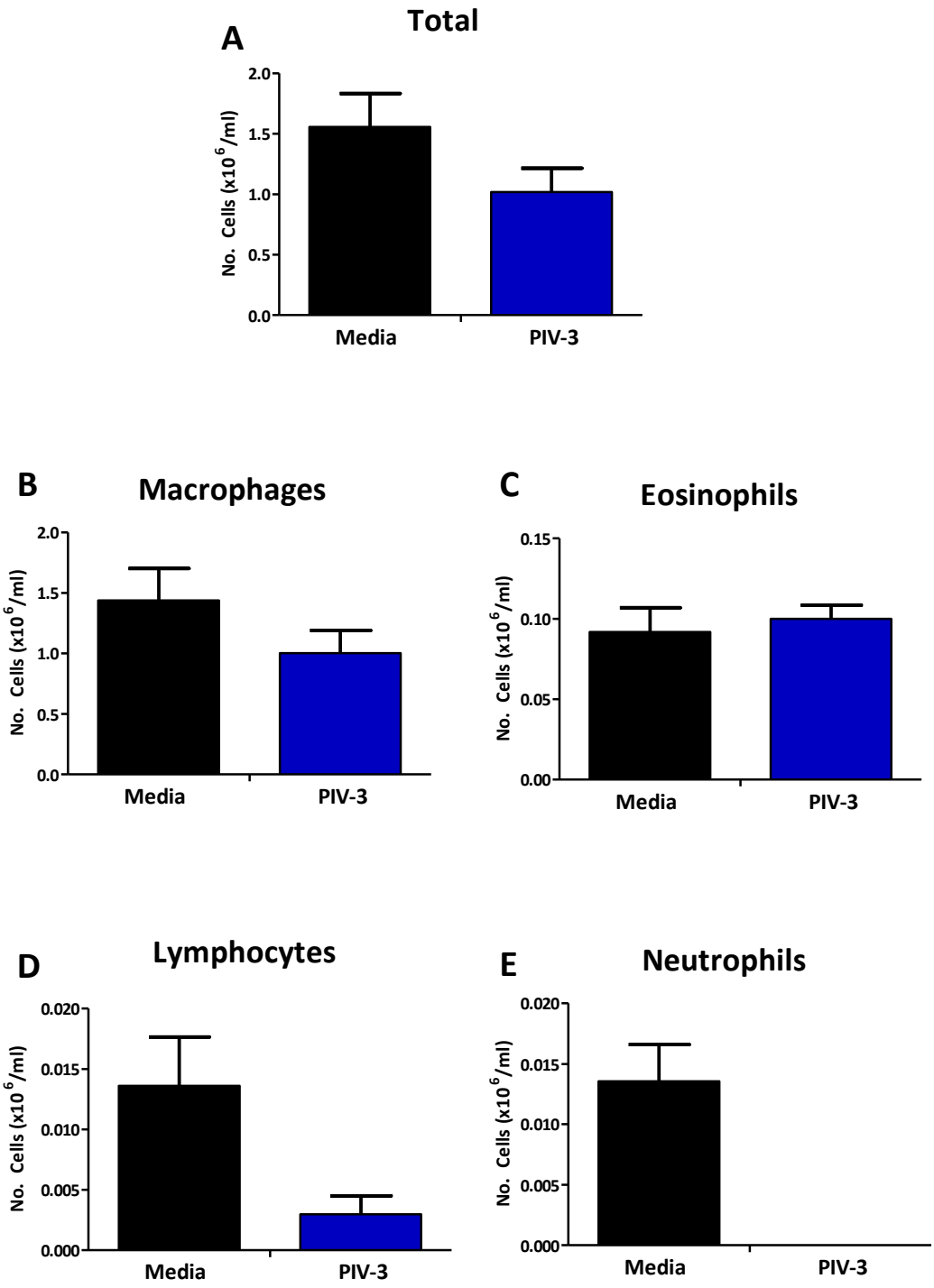


Figure 17: The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in bronchoalveolar fluid in guinea-pigs inoculated with 2 intranasal doses of PIV-3 ($1x10^7$-10^9 viral particles per ml) or media, 4 days after the initial inoculation N=6; performed with a two tailed T-test.

4.5.4 Results of RT-qPCR analysis on lung and lavage fluid samples

Table 2 shows the results of RT-qPCR analysis of lung and lavage samples from PIV-3 and media inoculated guinea-pigs. Large numbers of viral particle per ml were found in guinea-pigs inoculated with PIV-3 in the first experiment in the chapter. In subsequent experiments (Ova + PIV-3 and PIV-3 alone (repeat) viral particles per ml were considerably reduced. The reference product, β-actin displayed consistent levels for both lung and lavage fluid samples.

Per x10^6/ml of PCR product	Media	PIV-3 1st exp	PIV-3 2nd exp	Ova + PIV-3
Lung PIV-3	0.023±1.489	62.49± 39.13	0.287±0.108	0.69±0.304
Lung β-actin	41.25±9.02	43.31±11.06	52.79±27.95	32.46±3.967
Lavage fluid – PIV3	0.018 ±1.244	2.92± 1.58	0.35±0.24	0.45±0.158
Lavage fluid - β-actin	0.35±0.08	0.38±0.04	0.428±0.1	0.33±0.03

Table 2: Shows the amount of PCR product recovered from lung and lavage fluid for PIV-3, media and PIV-3 and ovalbumin treated guinea-pigs. β-actin represents a reference gene product. Data are displayed as PCR product x10^6 per ml.

4.5.5 The effect of Poly I:C on airway hyperresponsiveness to histamine, lung oedema and inflammatory cells.

Saline and the lower dose of poly I:C did not significantly increase the bronchoconstrictor response to histamine. 500µg of poly I:C (125µg) did not significantly increase the immediate response to histamine challenge post-poly I:C administration. At 10 minutes post-histamine inhalation a small increase in this response was observed (-9.4±2.2% compared to pre-poly I:C, -1.9±1.9%) (Figure 18).

A significant increase in lung oedema was observed in guinea-pigs treated with 125µg poly I:C compared to saline treated guinea-pigs (512±75% compared to 313±17%). Although a trend for increased oedema was observed in guinea-pigs treated with 500µg poly I:C, this was not significant (Figure 19).

Although total cells were unchanged between all groups, an increase in neutrophils in guinea-pigs treated with low dose poly I:C compared to saline was seen ($0.3\pm0.13x10^6$ compared to $0.03\pm0.01x10^6$ respectively). No other significant difference in cell numbers was observed (Figure 20).

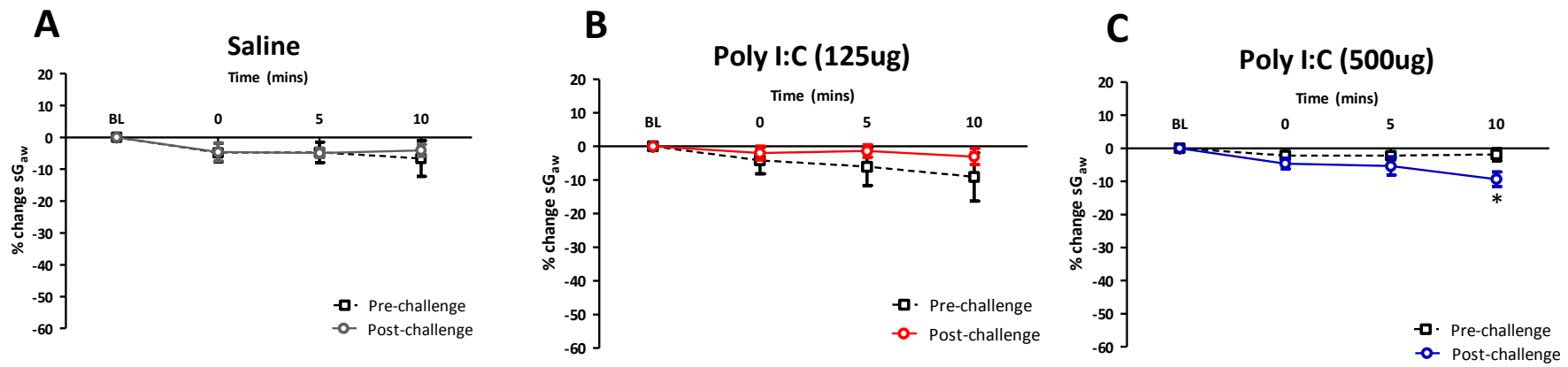


Figure 18: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles with 0.5LPM flow per chamber over 2 minutes, 1 minute drying period) in guinea-pigs pre-and post-.administration of 3 intranasal doses of A) saline, B) 125µg poly I:C or 500µg poly I:C. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=6 (Saline), N=6 (125µg poly I:C), N=9 (500µg poly I:C); *significantly different from time paired pre-Ova challenge values $p<0.05$; performed with a two tailed T-test.

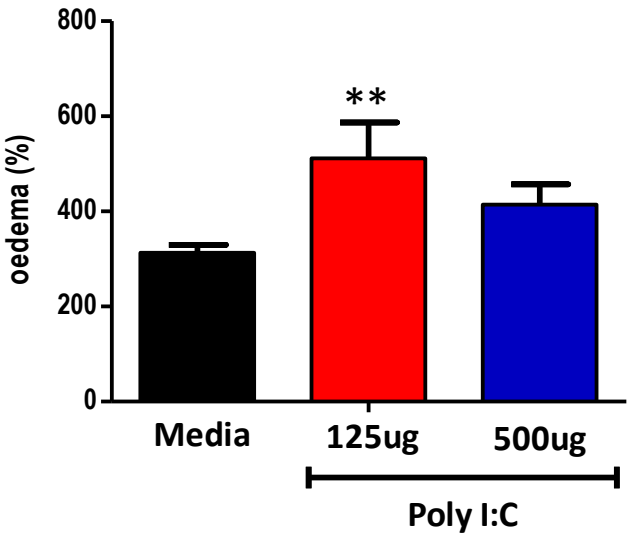


Figure 19: Lung oedema in guinea-pigs administered 3 intranasal doses of poly I:C (125µg or 500µg) or saline. Oedema is expressed as ((lung wet weight- lung dry weight)/dry weight x100). Lung weight measured following lung excision (wet) and overnight drying in an oven at 40^{o}C (dry). N=6 (Saline), N=6 (125µg poly I:C), N=9 (500µg poly I:C); **Significantly different from saline $p<0.01$; performed with one-way analysis of variance followed by Bonferroni post-test.

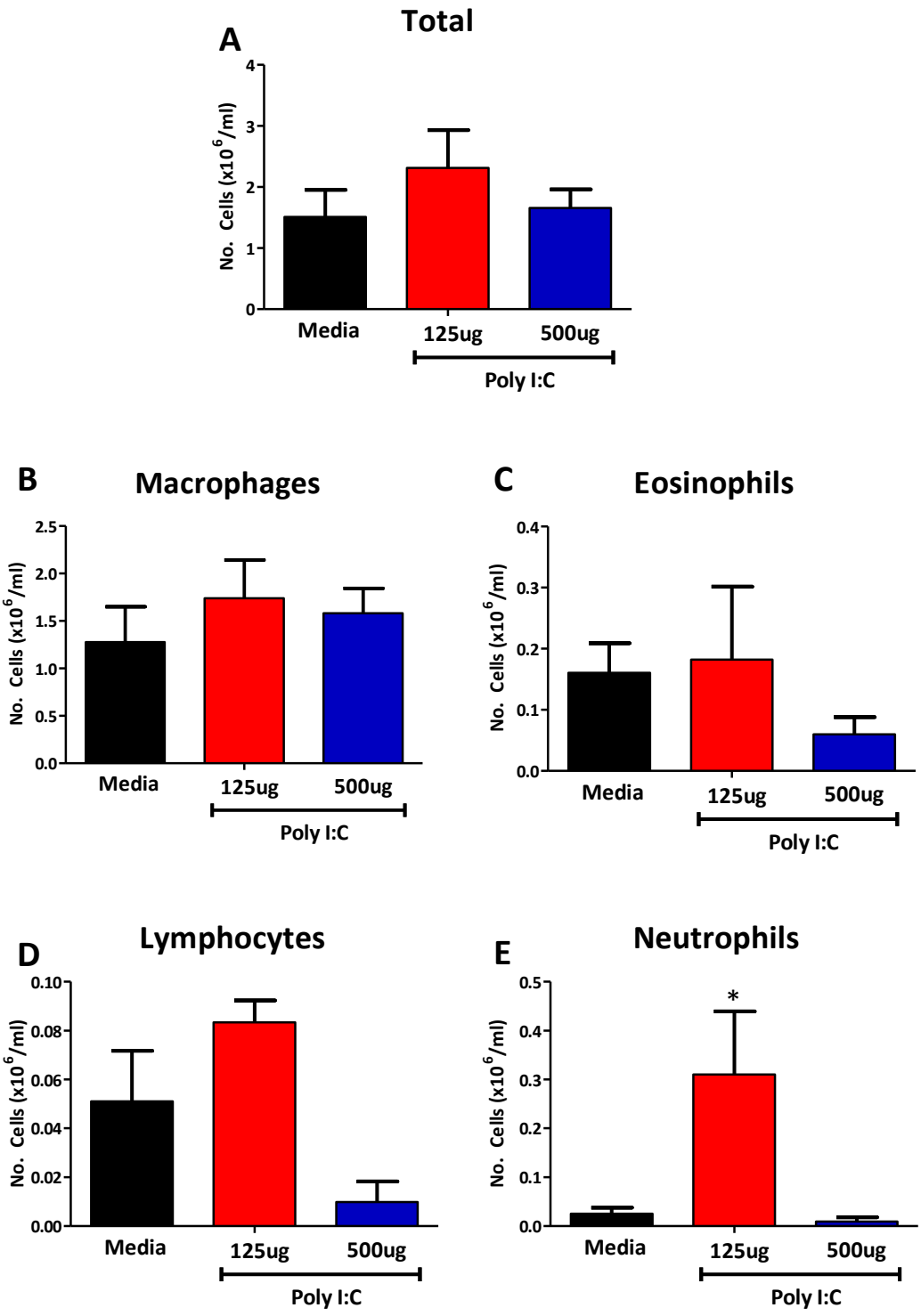


Figure 20: The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in bronchoalveolar fluid in guinea-pigs administered 3 intranasal doses poly I:C (125µg or 500µg) or saline. N=6 (Saline), N=6 (125µg poly I:C), N=9 (500µg poly I:C); *Significantly different from saline $p<0.05$; performed with one-way analysis of variance followed by Bonferroni post-test.

4.6 DISCUSSION

4.6.1 EFFECT OF PIV-3 IN GUINEA-PIGS

PIV-3 infection in guinea-pigs resulted in an increase in the bronchoconstrictor response to histamine, airway oedema and airway inflammation. These results are in accordance with Toward *et al*, (2005) and Broadley *et al*, (2009). Increases in airway response to bronchoconstrictor stimulus such as histamine have been reported by several other groups and various mechanisms implicated. Folkerts *et al*, (1995) reported that the development of AHR following PIV-3 infection is related to a deficiency in nitric oxide. This was prevented by the administration of nitric oxide donor compounds. Inflammatory cells have also been implicated in AHR to PIV-3. Folkerts *et al*, (1992A) demonstrated that *ex vivo* contractility to histamine in tracheal spirals increases when incubated with lavage cells from PIV-3 treated guinea-pigs, In particular, incubation with eosinophils which increase significantly with PIV-3 infection. Treatment with an antibody to IL-5, a key cytokine in eosinophil biology is able to abolish AHR (van Oosterhout *et al*, 1995). In the present study, a significant increase in eosinophilia was also observed but also in several other inflammatory cells including lymphocytes, neutrophils and macrophages. *In vitro,* macrophages can significantly increase their production of reactive oxygen species which can directly cause epithelial damage (Henricks *et al*, 1993). This can expose the sensory nerves, smooth muscle and also remove a source of spasmogen metabolisers, promoting AHR (Folkerts & Nijkamp, 1998). Depletion of spamogen metabolisers such as diamine oxidase which metabolises histamine may lengthen the response to histamine. In the present study a prolonged bronchoconstrictor response to histamine was observed suggesting a decrease in diamine oxidase activity. Disruption of sensory nerves due to inflammation may also promote AHR. In particular, the M2 receptor has been reported to lose its function with PIV-3 infection, decreasing pre-synaptic feedback inhibition of cholinergic responses (Adamko *et al*, 1999).

4.6.2 Effect of PIV-3 in an acute ovalbumin model of asthma

PIV-3 infection superimposed on ovalbumin challenge increased the duration of the bronchoconstrictor response to histamine and the number of eosinophils recovered in lavage fluid. However, it did not significantly alter the EAR and LAR. Previously in this laboratory, this protocol resulted in an increase in the length of the EAR, merging with the LAR. This effect is possibly mediated by increased histamine release from basophils and mast cells, which is increased by PIV (Graziano *et al*, 1989). Alternatively, an increase in the uptake and response to allergens induced by viral infection may mediate this effect (Freihorst *et al*, 1989). Also unlike Broadley *et al*, 2008, no overall increase in total lavage cells and neutrophils was found. An increase in neutrophils is commonly reported during viral infection in asthmatics (Fahy *et al*, 1995). Increases in eosinophil numbers have been observed in both humans and animal models (Calhoun *et al*, 1994; Toward *et al*, 2005). Eosinophil numbers may be increased due to CCL11 (eotaxin-1), which is reported to increase 2-3 days post-PIV-3 infection (Scheerens *et al*, 1999). Respiratory viruses can also promote Th2 lymphocyte development which favours eosinophilic inflammatory responses (Openshaw & O'Donnell, 1994). The results of this study were confirmed on several repeat studies. The lack of a full exacerbation response to PIV-3 was considered to be due to a diminished responsiveness to PIV-3. Therefore the effects of PIV-3 alone were re-examined with a new batch of virus. This repeat showed no AHR, oedema and airway inflammation. This loss of response was found to be associated with decreased viral titres recovered from the lavage fluid and lungs of PIV-3 infected animals.

4.6.3 Loss of response to PIV-3

The reasons for a diminished response to PIV-3 are unknown although there are several possible explanations. The first is a delivery problem with the intranasal method of administration, without anaesthetic. The distribution of fluid deposition

between the lungs can differ widely depending on small changes in animal handling and the use of anaesthetic (Southam *et al*, 2002). PIV-3 inoculation was unable to be carried out under anaesthetic due to anti-viral effects of common inhaled anaesthetics such as isoflorane (Belvisi, 2009). Problems with this route of administration are unlikely though as the change in response to PIV-3 occurred with the same animal handler, employing the same inoculation method throughout experiments. In addition, this route has previously been shown to result in active infection under a number of investigators (Toward *et al*, 2005, Broadley *et al*, 2008; Broadley *et al*, 2010).

Another possibility is that the virus had mutated, resulting in it no longer being able to infect guinea-pigs. This is also unlikely as several different stocks were tried and completely new PIV-3 brought from the health protection agency. Also, viral titres remained high ($1x10^{7-9}$.viral particles per ml) throughout the experiments. Furthermore, PIV-3 continued to display normal infective and plaque forming ability in BSC-1 cells, not suggestive of mutations.

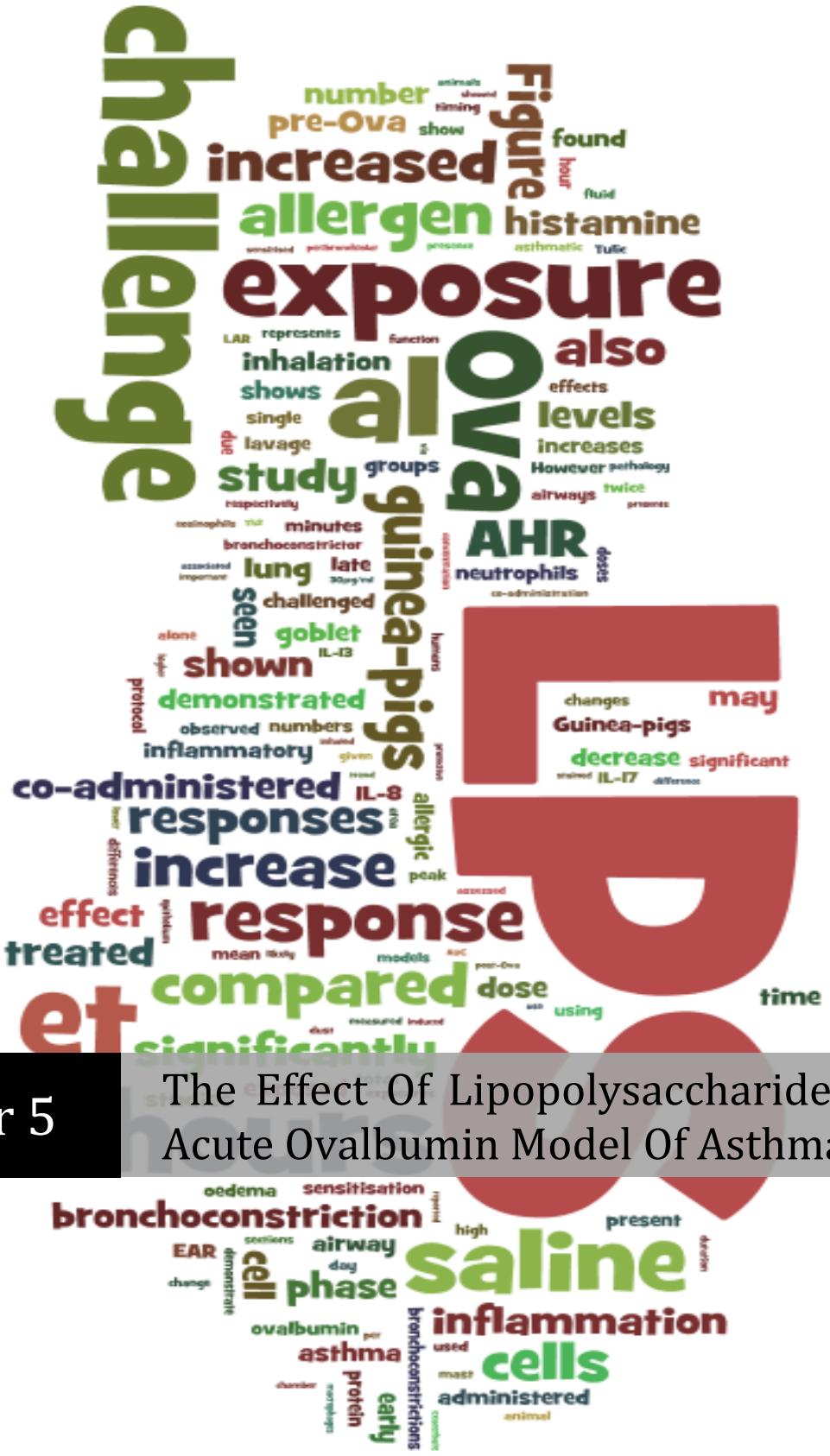
The third possibility is that a change in the guinea-pig population has occurred. The guinea-pig population is known to have a high prevalence of PIV-3 antibodies due to endemic infection in some colonies (Blomgvist *et al*, 2002). PIV-3 infection spreads quickly across a colony, making it possible for the widespread development of antibodies to parainfluenzas. These antibodies may account for a faster and more specific immune response, eliminating PIV-3 quickly and thus preventing the usual responses. This is supported by the observation that PIV-3 viral titres measured by RT-qPCR in lavage fluid and lungs were lower in experiments where limited or no response to PIV-3 was observed. However, this method is not ideal for detecting active viral infection, since it does not distinguish between infectious and non-infectious particles. Determination of the levels of infectious viral particles by cell plaque assay would give a better indication of this. Further studies involving the detection of PIV-3 specific antibodies in the blood would be required to confirm decreased response to PIV-3 due to increased adaptive immunity.

4.6.4 Effect of poly I:C in guinea-pigs

The unreliability of PIV-3 infection and failure to produce a full exacerbation of Ova-induced responses led to the investigation of the TLR-3 agonist poly I:C as a suitable alternative exacerbating agent. Lower doses of poly I:C increased airway oedema and neutrophils. Higher doses of poly I:C did not produce these effects but did slightly increase the bronchoconstrictor response to histamine.

Previously, in the laboratory 500µg poly I:C administered intranasally for 3 days increased inflammatory cell influx, oedema and AHR. Additionally, on an allergic background, poly I:C increased cellular influx, Th2 response and AHR in mice and rats (Torres *et al*, 2010; Takayama *et al*, 2011). It is unknown why the highest dose of poly I:C used did not reproduce the effects of previous work. However, this may again suggest changes in the guinea-pig population and their response to antigen. Another possibility is that a change in poly I:C is responsible. Differences between the *in vivo* responses to various manufacturers of poly I:C have been observed, along with differences between poly I:C batches from the same manufacturer (verbal communication).

The failure of PIV-3 infection and Poly I:C to satisfactorily cause exacerbation of Ova-induced functional and inflammatory responses led to the search for more reliable and robust alternative exacerbating agents. The next chapter examines lipopolysaccharide (LPS).



Chapter 5

The Effect Of Lipopolysaccharide On An Acute Ovalbumin Model Of Asthma

5.1 INTRODUCTION

5.1.1 LIPOPOLYSACCARIDE (LPS) AND ENDOTOXIN

Endotoxin is a lipopolysaccharide (LPS) derived from the outermost cell wall layer of gram negative bacteria such as *E.coli* and *H.influenzae*. Endotoxin is comprised of several structural domains including o-antigen, core phospholipid and Lipid A. The structure of LPS is shown in Figure 1. The polysaccharide component carries an organism-specific antigen pattern, whereas the lipid component facilitates cell adhesion and the stimulation of innate immunity (Liu, 2002). Endotoxin is ubiquitous in the environment, in part due to the high temperatures (>100ºC) and length of time (>4 hours) it takes to destroy its immune stimulatory capacity. LPS is found in grain dust, livestock, textiles, pets, carpeting, milk, tobacco smoke, house dust-mite, particulate air pollution and indoor ventilation systems (Medvedev *et al*, 2000). Thus, the airways are constantly exposed to LPS but the response invoked depends on a range of factors discussed below.

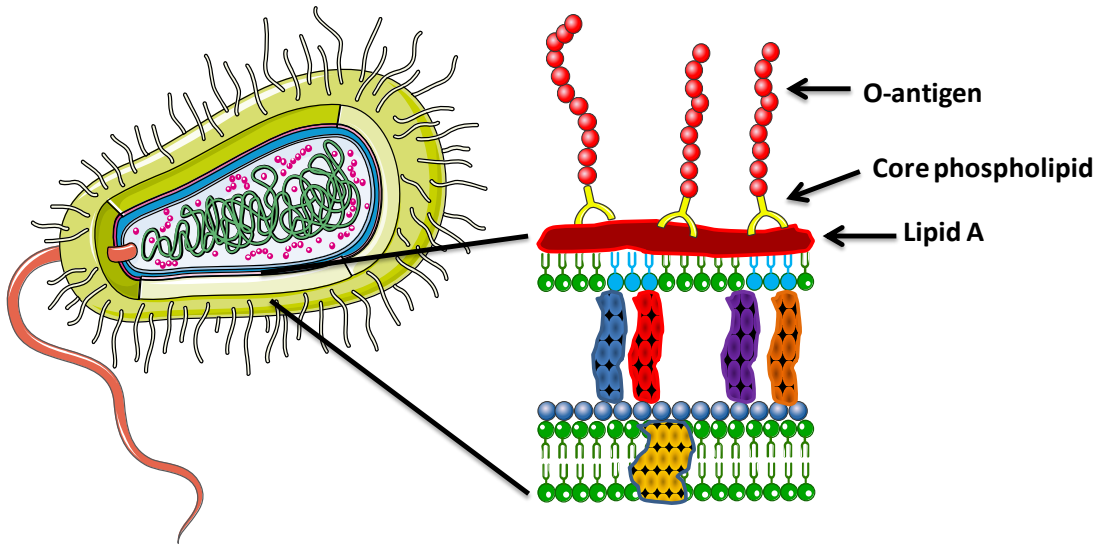


Figure 1: The structure of a gram-negative bacteria cell wall and the 3 components of lipopolysaccharide (endotoxin).

5.1.2 Airway Response to LPS

A typical response to LPS in a susceptible individual includes increased chest tightness, chills and fever, dyspnea and myalgia. The pathophysiology underlying these symptoms includes an increase in airflow obstruction, oedema, reduced alveolar diffusion capacity and airways hyperresponsiveness. An innate and adaptive immune response is also evident and is mediated by epithelial cells, alveolar macrophages, B and T lymphocytes which release numerous chemokines, cytokines, adhesion molecules and inflammatory mediators. Key mediators include TNF-α, IL-1β, IL-6 and IL-8, the latter of which is important in neutrophil recruitment (reviewed in Reed and Milton, 2001). Another cardinal effect of the inflammatory milieu is the increased production of mucus. LPS is also a potent inducer of nitric oxide synthase 2 (NOS2, aka iNOS), which produces nitric oxide and contributes to LPS-induced vasodilation (Stuehr &, Marletta, 1984). LPS produces additional effects when exposure coincides with IgE-mediated events including increased histamine release from mast cells, CCL5 (RANTES) and GM-CSF from fibroblasts (Warren & Holford-Strevens, 1986; Nonaka *et al*, 1999).

The recognition of LPS and the initiation of a response involves several proteins. LPS-binding protein is released locally from the airway epithelium and acts as a high affinity transporter, bringing LPS in contact with both soluble and membrane bound CD14 (Dentener *et al*, 2001). The LPS-CD14 complex then associates with TLR4 and MD2 on monocyte-macrophages and epithelial cells and initiates a signalling cascade which activates nuclear factor- κB (NF-κB) and activator protein 1 (AP1). Interferon regulatory factor 3 (IRF3) is also activated via a separate pathway independent of NF-κB and can lead to the expression of type-1 interferons (Perros *et al*, 2011). These nuclear factors subsequently translocate to the nucleus to initiate transcription of inflammatory cytokines (Medvedev *et al*, 2000) (Figure 2). LPS can also signal through non-NF-κB pathways to induce expression of the anti-oxidant manganese superoxide dismutase and COX-2 (White *et al*, 2000; Wadleigh *et al*, 2000). Systemically, exposure to LPS induces tolerance to subsequent doses via

suppression of expression and function of signalling intermediates (Medvedev *et al*, 2000). LPS tolerance is a compartmentalised phenomenon and in the lungs actually primes alveolar macrophages for further exposures. Subsequent LPS exposures increase pro-inflammatory signalling (Hoogerwerf *et al*, 2010; Reino *et al*, 2012). An increased response to LPS may be due to upregulation of TLR4 expression and downstream messengers. (Lin *et al*, 2006; Hoogerwerf *et al*, 2010).

Neutrophil accumulation in the airways is mediated by the chemoattractants IL-8 and MIP-1α and the binding molecules P-selectin and ICAM-1, all of which are upregulated by LPS exposure (Kamochi *et al*, 1999). LPS also decreases the apoptosis of neutrophils, prolonging the airways exposure to products such as elastase. Neutrophil elastase breaks down lung elastic fibres and encourages the development of emphysema (Nolan *et al*, 1999). Neutrophil elatase also promotes mucus secretion which is further augmented by LPS-induced epithelial growth factor (EGF) driven proliferation of goblet cells (Harkema *et al*, 1992; Takeyama *et al*, 1999). Evidence also suggests that LPS can recruit and activate eosinophils depending on the dose and section of airway sampled (Medvedev *et al*, 2000). LPS inhalation produces an immediate bronchoconstriction corresponding to a decrease in FEV_1 and increased bronchoconstrictor response to histamine in asthmatic individuals (Jagielo *et al*, 1996; Pauwels *et al*, 1990). However not all studies have confirmed these functional effects, possibly on account of lower doses of LPS being used (Nightingale *et al*, 1999).

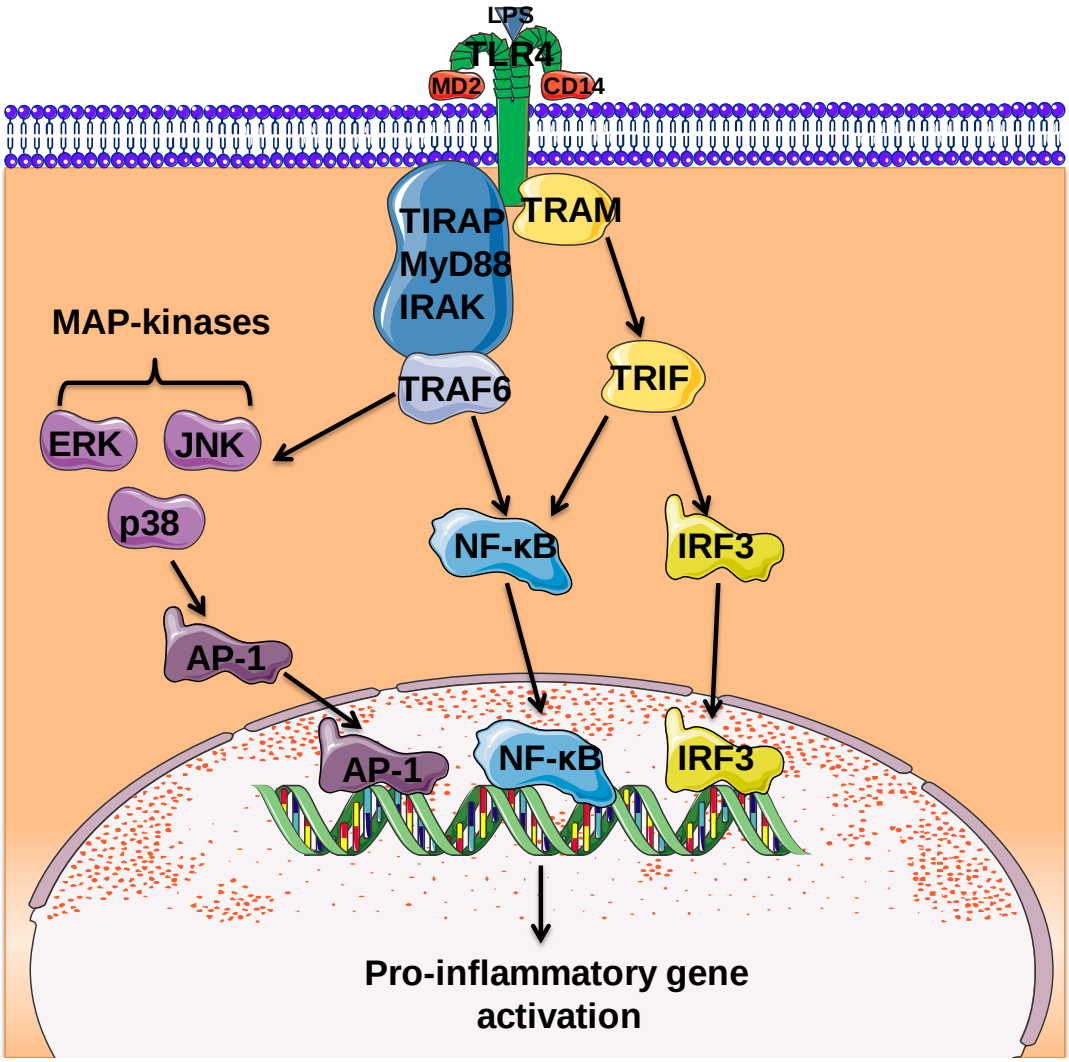


Figure 2: Simplified diagram of LPS signalling via toll like receptor 4 (TLR4). LPS can signal through several different intracellular pathways to increase pro-inflammatory gene transcription in the nucleus. TIRAP (Toll/IL-1R domain containing adapter protein); MyD88 (myeloid differentiation primary response protein 88); IRAK (IL-1 receptor-associated kinase); TRAF6 (tumor necrosis factor receptor-associated factor 6); NF-κB (nuclear factor-kappa B); JNK (c-Jun N-terminal kinase); ERK (extracellular signal-regulated kinase); TRAM (TRIF-related adaptor molecule); TRIF (TIR domain-containing adaptor inducing IFN-β). IRF: Interferon regulatory factor; Diagram redrawn and adapted from Gribar *et al*, 2008.

5.1.3 Factors that determine airway response to LPS in asthma

LPS has paradoxically been shown to be both protective and exacerbating in asthma. These differences are due to a range of factors including the concentration of LPS,

host genetics, the timing of exposure and temporal proximity to other agents such as viruses, pollution and allergen.

5.1.3.1 TIMING

The timing of LPS exposure is likely key to its dual role as both protective and exacerbating in asthma. Early LPS exposure attenuates allergic responses and later LPS exposure augments it. A study by Tulic *et al*, 2000 demonstrated this experimentally in rats using an ovalbumin sensitisation and challenge model. Inhaled LPS either before or 4 days after ovalbumin sensitisation decreased the sensitisation response. Whereas LPS inhalation 1-5 days before ovalbumin challenge increased allergic responses

5.1.3.2 DOSE

The dose of LPS individuals encounter varies widely. Individuals that experience LPS through factories or farms are reported to be exposed to doses' 100-1000 times greater than that of a typical home environment (Liu, 2002). However there are many problems with sampling LPS levels due to variability between measurement techniques, room to room differences, dust vs airborne LPS, seasonal variability, geographical location and other co-factors such as the presence of dogs (Park *et al*, 2000). *In vitro*, low levels of LPS (pg/ml) prime macrophages and neutrophils for Th1 cytokine production, whereas higher levels (>ng/ml) result in the production of free radical generating substances such as nitric oxide.

5.1.3.3 GENETICS

Genetic variability is also likely to account for differences in LPS response. In particular, polymorphisms in CD14 one of the receptors necessary for LPS response has been implicated. Other candidates for genetic variability in LPS response include TLR4 and TLR2, both of which recognise gram-negative bacteria cell products (reviewed in Doreswamy & Peden, 2010).

5.1.3.4 ENVIRONMENTAL CO-FACTORS

Many other factors can alter the response of the airways to LPS including the presence of bacterial DNA, viruses and allergens. In particular, allergen and LPS can

act synergistically to enhance each other's effects. Allergen responses are enhanced by the presence of LPS (Tsuchiya *et al*, 2012). Allergen may also be able to enhance the effect of LPS by causing plasma extravasations of proteins such as LBP which can enhance LPS induced pro-inflammatory signalling (Dubin *et al*, 1996). Other factors such as differences in gastroinstestinal colonisation, antibiotic use, breast feeding, pets and ingestion of fermented vegetables have been shown to influence the coincidence of atopy (Liu, 2002).

5.1.4 The Role Of LPS In Asthma

5.1.4.1 LPS As An Asthma Protective Factor

The protective benefits of LPS are thought to be associated with exposure in early life before allergic sensitisation. This is in accordance with the 'hygiene hypothesis' which suggests that exposure to microbial infections and products such as LPS in early life may inhibit the development or pathogenesis of allergic disease (Strachan, 1989; Schaub *et al*, 2006). The mechanism of this protection is thought to be via the promotion of Th1 lymphocyte responses over allergy promoting Th2 responses (Martinez & Holt, 1999). Several studies support the protective benefits of LPS. Children raised in rural environments such as farms, where higher levels of endotoxin are present are less affected by allergy (von Mutius *et al*, 2000; Riedler *et al*, 2000). Furthermore, this protective benefit has been shown to have a critical period. Reider *et al*, (2001) showed that children who were exposed to farm milk and stables (both having high endotoxin levels) before the age of 1 had a lower incidence of asthma and atopy then non-farming children. Farming children exposed between 1-5 years showed no protective benefit over their non-farming equivalents. In the urban setting, houses with lower levels of house dust mite endotoxin inversely correlate with an increasing incidence of asthma (Gereda *et al*, 2000).

5.1.4.2 LPS As An Asthma Exacerbating Factor

LPS has also been implicated in exacerbations asthma. These effects are thought to occur in individuals with pre-existing asthma and sensitisation to allergens. A correlation between an increased medication use, incidence of wheezing episodes

and LPS exposure in asthmatics has been demonstrated (Douwes *et al*, 2006). The concentration of LPS in the domestic environment is also correlated with the severity rating of asthma (Michel *et al*, 1996). Clinically, LPS exposure can cause a persistent bronchoconstriction over 5 hours (Michel *et al*, 1989). Asthmatics bronchoconstrict more readily to low doses of LPS (Cavagna *et al*, 1969). Underlying the clinical worsening of symptoms is range of cellular and molecular changes. A significant increase in eosinophil and poly mononuclear cell counts is observed following dust mite and LPS challenge (Eldridge & Peden, 2000). LPS can modify the response to allergen challenge, favouring a late phase response with a greater number of neutrophils then seen with allergen alone (Hunt *et al*, 1994). Associated with neutrophilia are increases in cytokines such as IL-8 and IL-17 (Doe *et al*, 2010). However, not all studies have shown these results. A study by Nightingale *et al*, 2000 found no increase in airway inflammation or decrease in FEV_1 in asthmatic subjects. Thus the effects of LPS remain controversial.

5.1.4 Animal Models Of Asthma Using LPS

The role of LPS in asthma has been assessed in several animal models. These models can be broadly divided into those that use LPS during the sensitisation phase and those that use it during the allergen challenge phase. LPS exposure in rats prior to Ova sensitisation and up to 4 days after protects against the development on Ova specific IgE and allergic responses to the allergen (Tulic *et al*, 2000). Another study has shown that LPS induced attenuation of allergic responses is associated with a decrease in total and Ova-specific IgE (Delayre-Orthez *et al*, 2004). This process may involve IL-12 as anti-IL-12 antibodies administered with LPS, prior to Ova sensitisation were able to prevent LPS induced attenuation of allergic responses to Ova (Gerhold *et al*, 2002). These attenuating effects of LPS seem to be dose-dependent. At higher LPS doses (>100µg) allergic sensitisation is suppressed but at lower concentrations (0.1µg) allergic sensitisation is boosted and dependent on the presence of LPS (Eisenbarth *et al*, 2002). More recently the low levels of LPS present in house dust mite extract have been shown as critical for dendritic cell response.

The absence of TLR4 on epithelial cells prevents this response via the secretion of IL-25, IL-33 and CM-CSF and TSLP (Hammad *et al*, 2009).

Other animal models have administered LPS around the time of allergen challenge. Studies are split between those that have shown an exacerbation and those that demonstrate an attenuation of allergic responses. Delayre-Orthez *et al*, (2004) showed that intra-nasal LPS co-administered with Ova in mice produced a dose-dependent increase in eosinophilia, IL-4, IL-5 and AHR. Tulic *et al,* (2000) showed an increase in Ova-induced neutrophils, eosinophils and IgE with LPS. The LPS-induced exacerbation was shown in part to be dependent on mast cells as mast cell deficient mice did not demonstrate an increase in IgE, total cells or eosinophlia (Murakami *et al,* 2006). LPS administered 24 hours before Ova challenge has also been demonstrated to increase cellular inflammation and nitric oxide levels but reduce IL-4, IL-13 and AHR (Komlosi *et al*, 2006). Other studies have shown opposite effects. A study by Rodriguez *et al*, (2003) found that Intra-nasal LPS administered shortly before intra-nasal Ova challenge prolonged AHR to methacholine but did not alter inflammation, although neutrophils were increased and eosinophils decreased. Contrastingly, intravenous LPS administered with intra-nasal Ova challenge significantly attenuated inflammation and AHR. Another study has shown that guinea-pigs administered Intravenous LPS 24 hours before intra-tracheal Ova challenge demonstrate significantly less bronchoconstriction. This was associated with partially degranulated mast cells (Vannier *et al*, 1991). LPS administered after Ova inhalation also seems to attenuate allergic responses. LPS administered 18 hours after Ova challenge in rats reduced lymphocyte, macrophages, eosinophils and AHR (Tulic *et al*, 2000). The same group showed that the co-administration of a high concentration of LPS with Ova decreases AHR, inflammation and oedema (Tulic *et al*, 2002). The effect of LPS on goblet cells in asthma models seems uncertain. LPS alone causes goblet cell hyperplasia (Harkema & Hotchkiss, 1992). In combination with allergen it has been reported to both not change, increase and decrease goblet cell numbers depending on the LPS dose (Dong *et al*, 2009; Tsuchiya *et al*, 2012).

In summary, in animal models of asthma LPS seems to have an attenuating effect on allergic responses when administered close to or with sensitisation. The effect of LPS administered close to allergen challenge is less clear but it appears that it may act as an exacerbating agent if a high dose is administered with the correct timing. These differences probably stem from the range of Ova protocols (single vs repeat challenge), dose and timing of LPS administration, route of LPS administration (systemic vs local), species and even the strain i.e. BALB/C vs C57BL/6 mice used to perform these studies.

5.2 HYPOTHESIS

'*A high dose of LPS when administered with or close to allergen challenge, subsequent to allergen sensitisation will exacerbate functional and allergic responses to the allergen ovalbumin.'*

5.3 AIMS AND OBJECTIVES

The aim of this chapter was to use an acute ovalbumin model and exacerbate at least one of the following parameters: early asthmatic response, late asthmatic response, airways hyperresponsiveness to histamine, airways inflammation and oedema using LPS inhalation. Since data from other animal models suggests that LPS exposure close to sensitisation attenuates allergic responses, whereas exposure near allergen challenge may exacerbate allergic responses, the latter time point was chosen. However, the specific timing of LPS exposure in relation to allergen challenge has not been examined in a single study so several LPS exposure time points were assessed. A high dose of LPS was selected as both human and animal studies suggest that exacerbations of asthma are more likely than with lower doses. The effect of single or multiple LPS challenges on responses to allergen challenge have not been examined so the present study will assess this. This study will also be the first to examine the effect of LPS on both early and late asthmatic responses to allergen challenge, along with the more commonly measured airways hyperresponsiveness and airway inflammation. The effect of LPS alone was examined as a comparison to its effects on allergen challenge. IL-8 and IL-17 levels were investigated as both are implicated in neutrophilia. IL-13 was used to investigate changes to Th2 responses. Lung histology was assessed to investigate changes in airway inflammation and goblet cell numbers.

5.4 METHODS

Methods describing the measurement of lung function, AHR, airway oedema, airway inflammation, cytokine levels and lung histology can be found in more detail in chapter 2.

5.4.1 Ovalbumin Protocols

5.4.1.1 Sensitisation

Guinea-pigs (200-300g, Dunkin-Hartley, male) were sensitised by a bilateral intra-peritoneal injection of a solution containing 150µg ovalbumin (Ova) and 100mg aluminium hydroxide ($Al(OH)_3$) in normal saline on days 1, 4 and 7.

5.4.1.2 LPS Pre-Acute Ovalbumin Exposure Protocol

Sensitised guinea-pigs were exposed to inhaled saline or LPS (30µg/ml) both 72 and 24 hours before Ova challenge for 1 hour in a Perspex box. On day 21 guinea-pigs were exposed to Ova (0.03%) for one hour. The bronchoconstrictor response to histamine was assessed on day 15 and day 22. Figure 3 shows a diagram of this protocol.

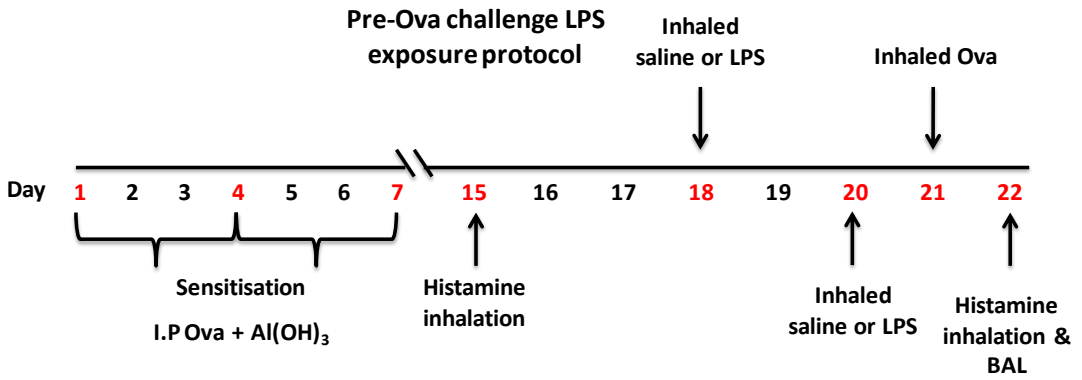


Figure 3: Diagram of the pre-ovalbumin (Ova) challenge LPS exposure protocol. Guinea-pigs were challenged with saline or LPS (30µg/ml) both 24 and 72 hours pre-Ova exposure.

5.4.1.3 LPS And Acute Ovalbumin Co-Exposure Protocol

Sensitised guinea-pigs were exposed to inhaled saline or LPS (30µg/ml) 48 hours before Ova challenge in an exposure chamber and both LPS (30µg/ml) and Ova (0.03%) co-administered on day 21. The bronchoconstrictor response to histamine was assessed on day 15 and day 22. Figure 4 shows a diagram of this protocol.

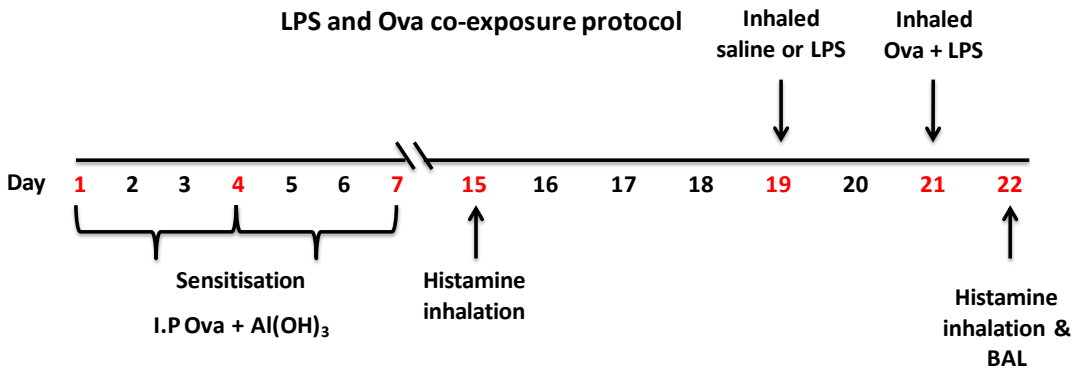


Figure 4: Diagram of LPS and Ova co-exposure protocol. Guinea-pigs were exposed to saline or LPS (30µg/ml) on day 19 and both Ova and LPS co-administered on day 21.

5.4.2 LPS Exposure Protocol

Non-sensitised guinea-pigs were exposed to saline or LPS (30µg/ml) on day 5 and 7 of the protocol. The bronchoconstrictor response to histamine was assessed on day 1 and day 8. Figure 5 shows a diagram of this protocol.

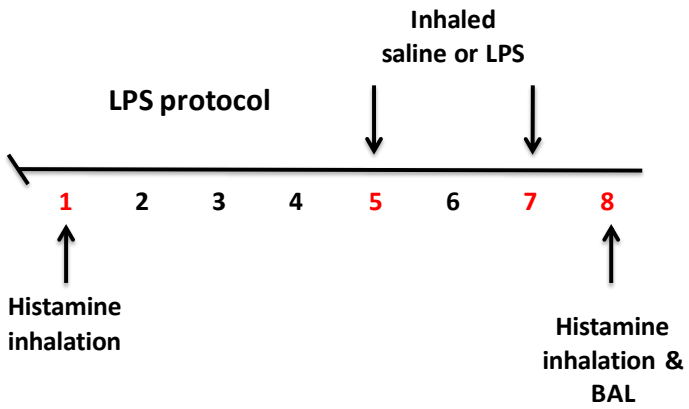


Figure 5: Diagram of the LPS alone exposure protocol. Guinea-pigs were exposed to 30µg/ml of LPS on day 5 and 7.

5.4.3 Measurement Of Lung Function

Lung function was measured by whole body plethysmography following final saline, Ova, LPS or Ova and LPS exposure in all protocols. Lung function was recorded hourly for 12 hours and every 15 minutes during the first hour of measurements. A final measurement was also taken 24 hours post-final challenge. All values from these readings were expressed as a percentage of the baseline reading, taken before the final challenge. A negative percentage change in baseline value represents a bronchoconstriction. In Ova protocols early (0-6 hours) and late (6-12 hours) phases were expressed as the peak bronchoconstriction during that period (displayed as a histogram next to a time course plot) and area under the curve. The duration of the early phase was expressed as the time taken to recover to 50% of peak early phase bronchoconstriction values. In the LPS only protocol lung function was measured for 12 hours after the second LPS exposure. The peak bronchoconstriction during this period was expressed on a histogram next to the time course plot.

5.4.4 Measurement of Airways Hyperresponsiveness

AHR was determined using whole body plethysmography. This was performed by measuring the bronchoconstrictor response to histamine before and 24 hours post-final saline, Ova, LPS or Ova and LPS exposure challenge. Histamine (0.3mM) was delivered to the guinea-pigs by the use of a Buxco nebuliser chamber and lung function was measured just before histamine inhalation and at 0, 5 and 10 minutes post histamine exposure. The peak bronchoconstriction during this period was expressed on a histogram.

5.4.5 Measuring Airways Inflammation

Following post-saline/Ova/LPS/Ova and LPS histamine challenge guinea-pigs were sacrificed by an intra-peritoneal overdose of sodium pentobarbitone, the lungs excised and lavaged. Total and differential cell counts were then performed as described in chapter 2.

5.4.6 Quantification of cytokine levels using enzyme-linked immunosorbent assay (ELISA)

Levels of IL-8, IL-17 and IL-13 were measured in diluted homogenised lung samples using ELISA supplied by R&D systems. Cytokine levels were adjusted for total lung protein and expressed as weight per mg of lung as described in section 2.2.3.2.

5.4.7 Assessment Of Airway Oedema

Protein content in lavage fluid was determined as a measure of airway oedema by BCA protein assay as per the manufacturer's instructions (Pierce protein biology).

5.4.8 Histological analysis of lungs

Lung lobe samples were stored in formaldehyde and 1-2mm bilateral sections cut. Samples were dehydrated in increasing concentrations of alcohol and then chloroform. Tissue sections were then set into wax blocks using molten paraffin. 5µm sections were cut using a microtome and fixed to polysine coated slides. Slides were stained with haematoxylin and eosin or Alcian blue/periodic acid Schiff stain (Ab/PAS). Haematoxylin and eosin staining allows for assessment of general lung morphology and inflammation. Semi-quantitative assessment of airway inflammation was performed using a scoring method. 0=normal lung; 1= minor peribronchiolar (PB) inflammatory cell infiltration; 2= slight inflammatory cell infiltration in PB area; 3= moderate PB inflammatory cell infiltration; 4= marked PB inflammatory cell infiltration and cuffing. Slight loss of lung structure (alevoli etc); 5= severe PB inflammatory cell infiltration, cuffing and infiltration. Loss of lung structure i.e. solid lung. Ab/PAS staining allowed assessment of goblet cell numbers in airway epithelium using Image J software.

5.5 RESULTS

5.5.1 PRE-ACUTE OVA LPS EXPOSURE

Figure 6 represents the mean time course changes in sGaw in guinea-pigs exposed to inhaled saline or LPS 72 hours and 24 hours pre-ovalbumin (Ova) challenge. All 3 groups demonstrated an EAR but this was attenuated in guinea-pigs treated with LPS 24 hour pre-Ova (-25.0±6.6%) and 72 and 24 hours pre-Ova (-12.5±11.4%) compared to saline (-72.2±2.0%) at 0 minutes. Both LPS treated groups maintained significantly attenuated bronchoconstrictions compared to saline treatment until 3 hours post-Ova challenge. Whereas in saline treated animals sGaw values had returned to baseline by about 6 hours (-10.0±5.5%), the bronchoconstriction in both LPS treated guinea-pigs remained relatively constant across the first 6 hours. Evidence of a LAR was seen in saline treated guinea-pigs at 8 and 9 hours (-13.3±3.7% and -20±5.5%, respectively). A well defined LAR in both LPS treated groups was not seen although bronchoconstrictions were present in both guinea-pigs treated with LPS 24 hours pre-Ova (-10.9±4%) and LPS 72 and 24 hours pre-Ova (-15.7±7.0%) at 9 hours. These bronchoconstrictions were resolved at 12 hours post-Ova challenge in both saline (-5.8±6.2%) and LPS 24 hours pre-Ova (-4.4±5.5%) treated guinea-pigs. Animals treated with LPS twice still had a clear bronchoconstriction present at this same time point (-20±6.6%).

The peak early phase response of guinea-pigs treated with LPS 24 hours (-34.4±4.6%) and LPS 72 and 24 hours (-25.2±27.7%) pre-Ova was significantly attenuated compared to saline treated animals (-73.5±1.9%). No significant difference in peak late asthmatic responses between groups was observed. Although there was a trend for guinea-pigs treated with LPS twice to demonstrate a bronchoconstriction at 24 hours post-Ova challenge, this did not reach significance (Figure 6, histogram). Guinea-pigs treated once or twice with LPS demonstrated significantly lower early phase area under the curve compared to those treated with saline (124±18.3%.hr and 100.7±51.9%.hr compared to 249.8±11.8%.hr respectively). No significant

differences were found in LAR area under the curve (Figure 7). No difference between the duration of the EAR between groups was observed (Figure 8).

Ova challenge with saline treatment produced a significant increase in immediate bronchoconstriction to histamine, when compared to the sG_{aw} values prior to Ova-challenge (-24.1±7.7% compared to 0.6±3.2%). The bronchoconstriction was no longer significant 5 minutes post-histamine challenge. Guinea-pigs challenged with Ova and treated with LPS once also demonstrated an increased response to histamine at 0 minutes post-Ova challenge (-12.9±4.7% compared to -0.18±1.1%), which returned to baseline sGaw values after 5 minutes. No change in the response to histamine was seen in guinea-pigs challenged with Ova and treated with LPS twice; the AHR to Ova was eliminated by LPS (Figure 9).

No difference in lavage protein levels was found between groups (Figure 10). A trend for an increase in total cell numbers was seen with Ova challenge and treatment with 1 dose of LPS, although this failed to reach significance. Ova challenge with 2 doses of LPS significantly increased total cell numbers (18.3±3.5x10^6/ml compared to saline, 8.9±0.4x10^6/ml respectively). In particular macrophages (7.7±x1.0x10^6 /ml compared with saline, 4.2±0.19x10^6/ml) and neutrophils (6.1±1.5x10^6/ml compared with saline, 0.6±0.05x10^6/ml) were increased. Neutrophils were also increased in the lavage fluid of guinea-pigs treated with LPS once (5.2±0.8x10^6/ml). All other cell types remained unchanged (Figure 11).

IL-8 levels in lung increased in guinea-pigs treated with Ova and LPS once compared to other groups where IL-8 was not detectable. IL-13 levels increased significantly in guinea-pigs challenged with Ova compared to sensitised guinea-pigs challenged with saline (6.2±0.8ng/mg compared to 1.4±0.5ng/ml). LPS exposure did not significantly change IL-13 levels. IL-17 levels were not significantly increased in Ova challenged

guinea-pigs. Exposure to LPS once significantly increased IL-17 compared to sensitised, saline challenged guinea-pigs (126.1±19.7pg/ml compared to 61.8±3.2pg/ml respectively). LPS exposure twice did not significantly change IL-17 levels (Figure 12).

Figure 13 shows lung sections stained with haematoxylin and eosin for general morphology of the bronchioles. Guinea-pigs challenged with Ova show increased inflammatory cell numbers in the peribronchiolar area. Treatment with LPS did not alter the inflammatory cell presence. Figure 14 presents the mean pathology scores for the number of inflammatory cells in the peribronchiolar area in these guinea-pigs. Ovalbumin challenge with saline pre-treatment significantly increases mean pathology score compared to saline challenge (2.64±0.34 compared to 0.58±0.19). LPS treatment 72 hours pre-Ova challenge also significantly increases the mean pathology score (2.29±0.5 compared to saline challenge, 0.58±0.19) but not significantly more than Ova alone. 2 pre-Ova LPS challenges did not significantly increase the mean pathology score, despite demonstrating a trend, probably due to the low N.

Figure 15 shows the lung sections stained with Alcian blue/ periodic acid to reveal mucus containing goblet cells. Guinea-pigs challenged with Ova showed an increase in the number of goblet cells. Treatment with LPS further increased the number of goblet cells. Figure 16 presents the number of mucin associated-goblet cells per 10,000 pixels of epithelium. Single saline or LPS exposure 24 hours pre-Ova challenge increased the number of goblet cells, although not significantly. Treatment with LPS 72 and 24 hours pre-Ova challenge increased goblet cells significantly compared to saline challenge (769.8±154.3 compared to 182.4±65.2).

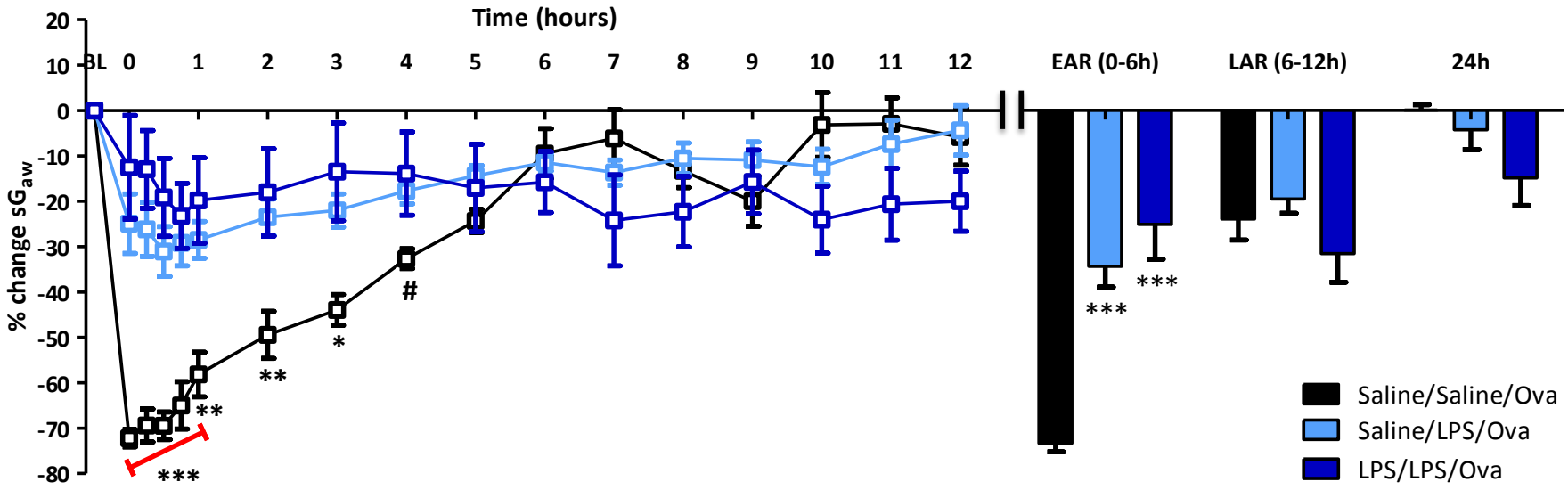


Figure 6: The mean time-course values of sGaw in ovalbumin (Ova) sensitised guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour, 72 hours and 24 hours pre-Ova challenge. The histogram represents the maximum bronchoconstriction values recorded during the early asthmatic response (EAR) (0-6 hours), late asthmatic response (LAR) (6-12 hours) and the 24 hours reading. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline prior to Ova challenge. A negative value represents a bronchoconstriction. N=6 (saline/saline/Ova), N=8 (saline/LPS/Ova), N=4 (LPS/LPS/Ova). *Significantly different from saline treatment $p<0.05$, ** $p<0.01$; *** $p<0.001$; # single LPS significantly different from saline treatment $p<0.05$; performed with one-way analysis of variance followed by Bonferroni post-test.

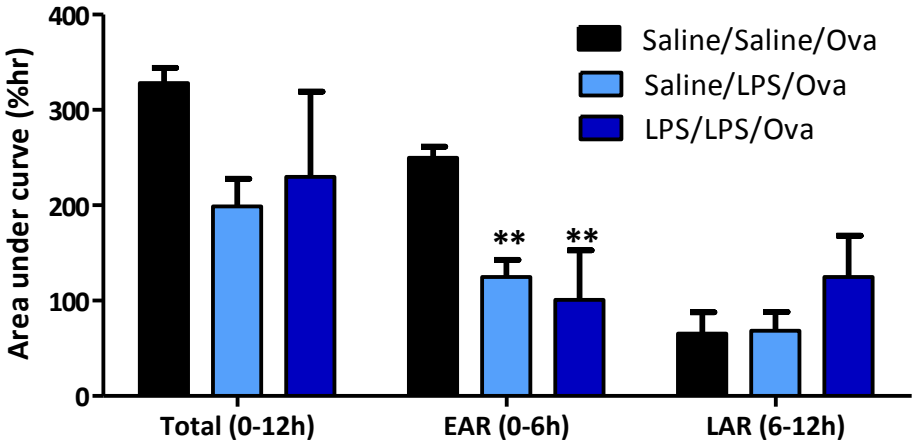


Figure 7: Area under the curve analysis of sGaw values over 12 hours following ovalbumin (Ova) challenge in guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour, 72 hours and 24 hours pre-Ova challenge. For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-24 hours, early asthmatic response (EAR) includes values from 0-6 hours and late asthmatic response (LAR) includes values from 6-12 hours. Area under the curve is expressed as %.hour. N=6 (saline/saline/Ova), N=8 (saline/LPS/Ova), N=4 (LPS/LPS/Ova). **Significantly different from saline treatment $p<0.01$; performed with one-way analysis of variance followed by Bonferroni post-test

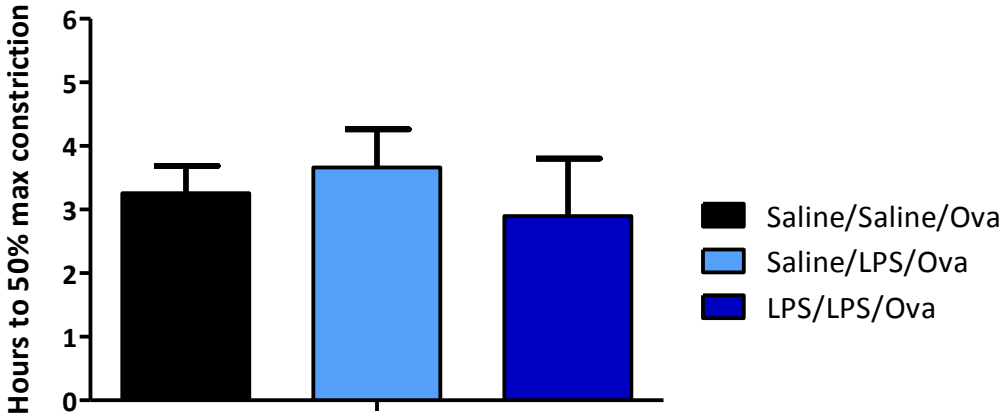


Figure 8: Analysis of the time taken for early asthmatic responses (EAR) to recover to 50% of peak bronchoconstriction values in guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour, 72 hours and 24 hours pre-Ova challenge. Results are expressed as mean±SEM; N=6 (saline/saline/Ova), N=8 (saline/LPS/Ova), N=4 (LPS/LPS/Ova); performed with one-way analysis of variance followed by Bonferroni post-test

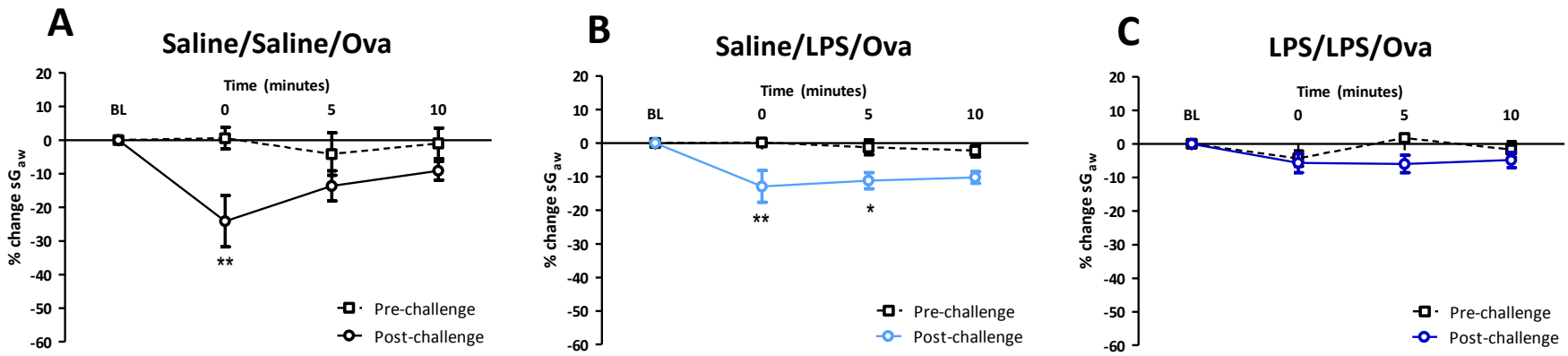


Figure 9: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles and 0.5 LPS per chamber over 2 minutes, 1 minute drying period) in ovalbumin (Ova) sensitised guinea-pigs treated with either A) saline or 30µg/ml LPS for 1 hour B) 24 hours pre-Ova, C) 72 hours and 24 hours pre-Ova challenge. Values were recorded 7 days pre- and 24 hours post-Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=6 (saline/saline/Ova), N=8 (saline/LPS/Ova), N=4 (LPS/LPS/Ova). *Significantly different from time paired pre-Ova challenge values $p<0.05$, ** $p<0.01$; performed with a two tailed T-test.

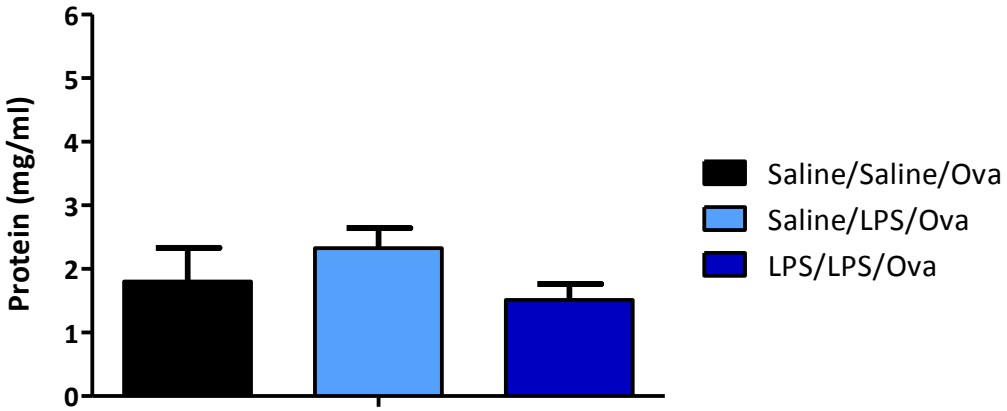


Figure 10: Total lavage fluid protein in ovalbumin (Ova) sensitised guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour, 72 hours and 24 hours pre-Ova challenge. Protein content determined by BCA protein assay. Results are expressed as mean±SEM; N=6 (saline/saline/Ova), N=8 (saline/LPS/Ova), N=4 (LPS/LPS/Ova); performed with one-way analysis of variance followed by Bonferroni post-test

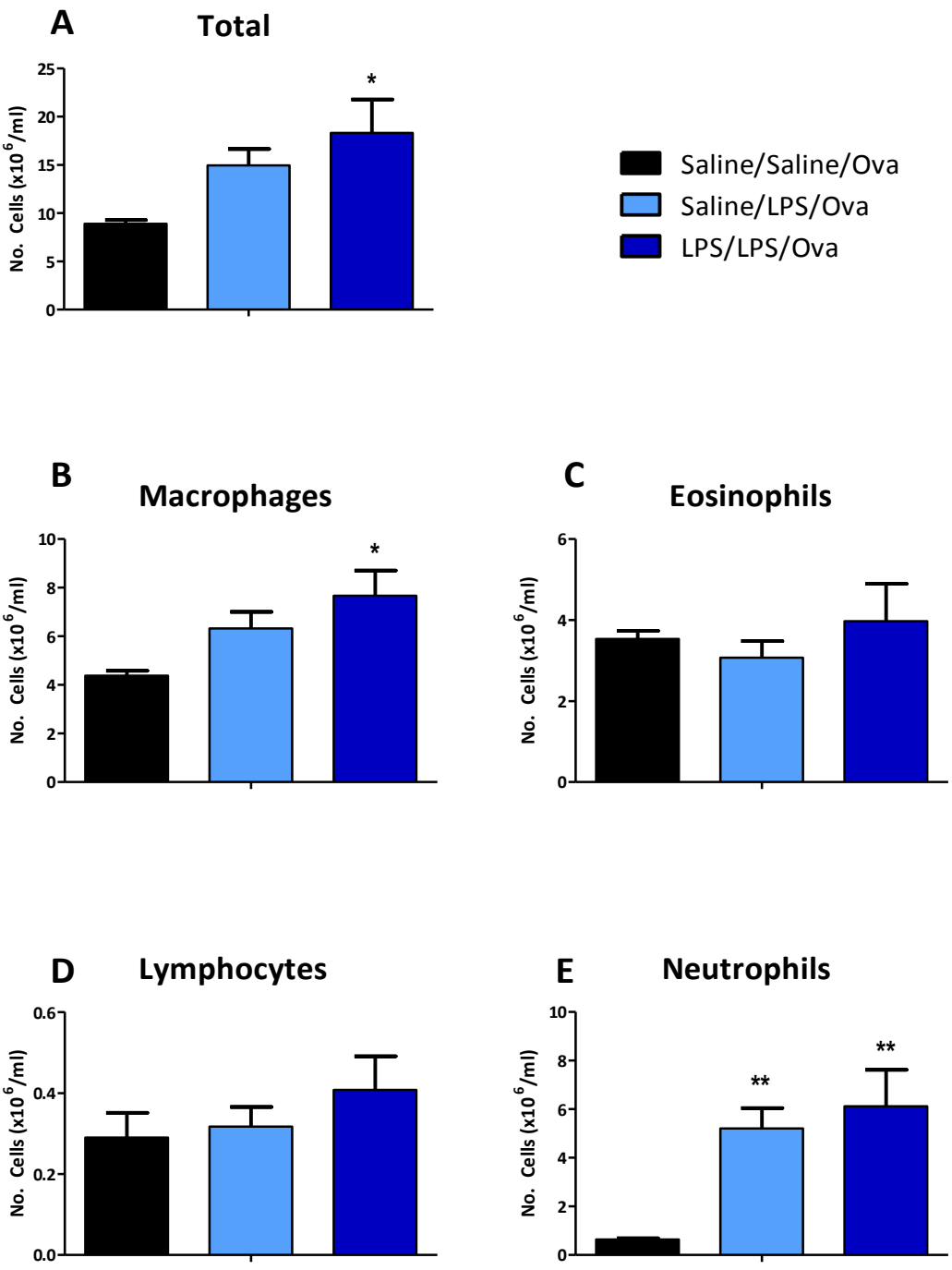


Figure 11: The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in bronchoalveolar fluid in ovalbumin (Ova) sensitised guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour, 72 hours and 24 hours pre-Ova challenge. Results are expressed as mean±SEM; N=6 (saline/saline/Ova), N=8 (saline/LPS/Ova), N=4 (LPS/LPS/Ova). *Significantly different from saline treatment $p<0.05$, ** $p<0.01$; *** $p<0.001$; performed with one-way analysis of variance followed by Bonferroni post-test.

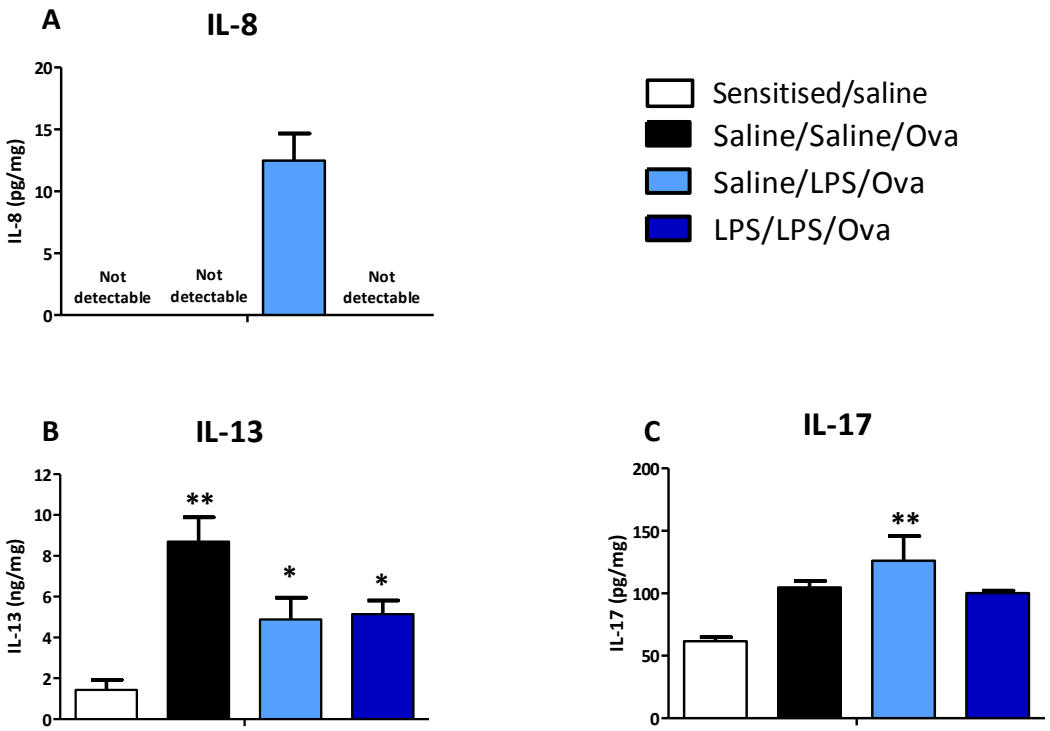


Figure 12: The concentration of A) IL-8 B) IL-13 C) IL-17 in the lungs of ovalbumin (Ova) sensitised guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour, 72 hours and 24 hours pre-Ova challenge or saline challenge alone. Results are expressed as mean±SEM; N=6 (saline/saline/Ova), N=8 (saline/LPS/Ova), N=4 (LPS/LPS/Ova). *Significantly different from sensitised/saline $p<0.05$, ** $p<0.01$; *** $p<0.001$; performed with one-way analysis of variance followed by Bonferroni post-test.

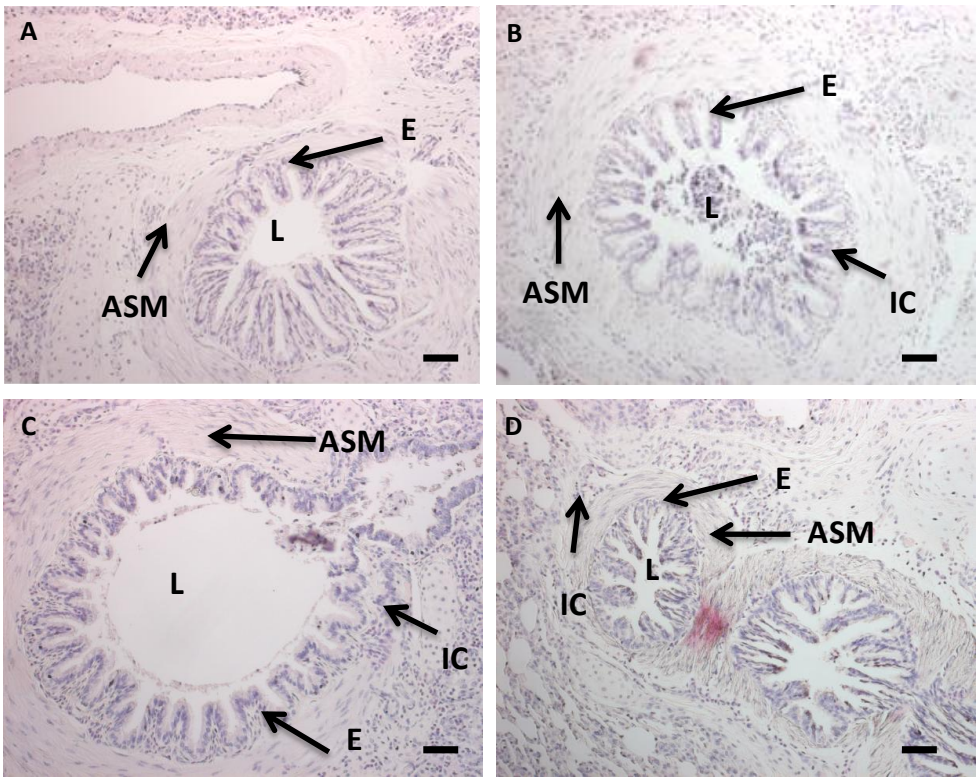


Figure 13: Bronchiolar changes in ovalbumin (Ova) sensitised guinea-pigs exposed to A) saline or B) Ova C) 30µg/ml LPS for 1 hour, 24 hours pre-Ova challenge D) 30µg/ml LPS, 72 hours and 24 hours pre-Ova challenge. L: lumen; ASM: airway smooth muscle; E: epithelium, IC: inflammatory cell. Stained with haematoxylin and eosin. Original magnification 100x (bar = 25 µm).

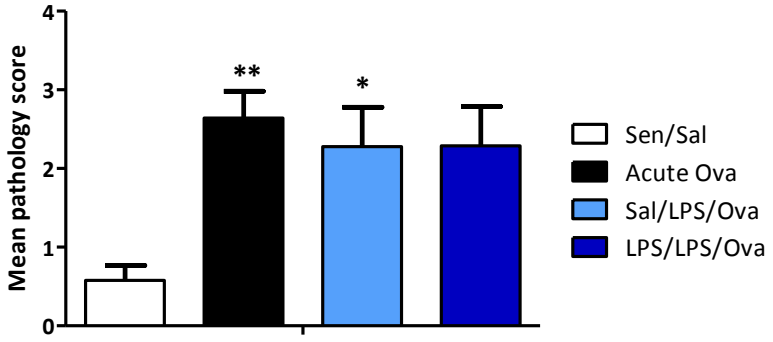


Figure 14: The mean pathology score of the peribronchiolar area in ovalbumin (Ova) sensitised guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour, 72 hours and 24 hours pre-Ova challenge. Results are expressed as mean±SEM; N=6 Sen/saline (sensitised, exposed to saline), (saline/saline/Ova), (saline/LPS/Ova), N=4 (LPS/LPS/Ova). *Significantly different from sensitised/saline treated group treatment $p<0.05$, ** $p<0.01$; performed with one-way analysis of variance followed by Bonferroni post-test.

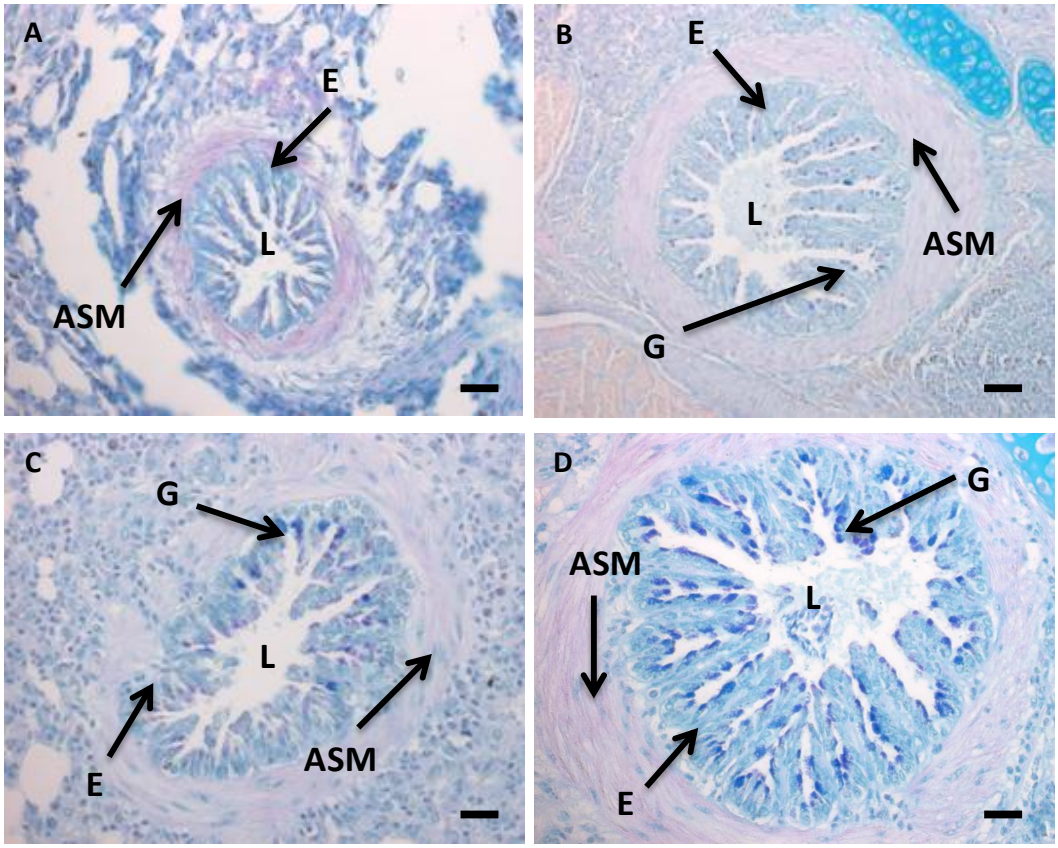


Figure 15: Bronchiolar changes in ovalbumin (Ova) sensitised guinea-pigs exposed to A) saline or B) Ova C) 30µg/ml LPS for 1 hour, 24 hours pre-Ova challenge D) 30µg/ml LPS, 72 hours and 24 hours pre-Ova challenge. L: lumen; ASM: airway smooth muscle; E: epithelium; G: goblet cell; Stained with alcian blue/periodic acid Schiff stain Original magnification 200x (bar = 50 µm).

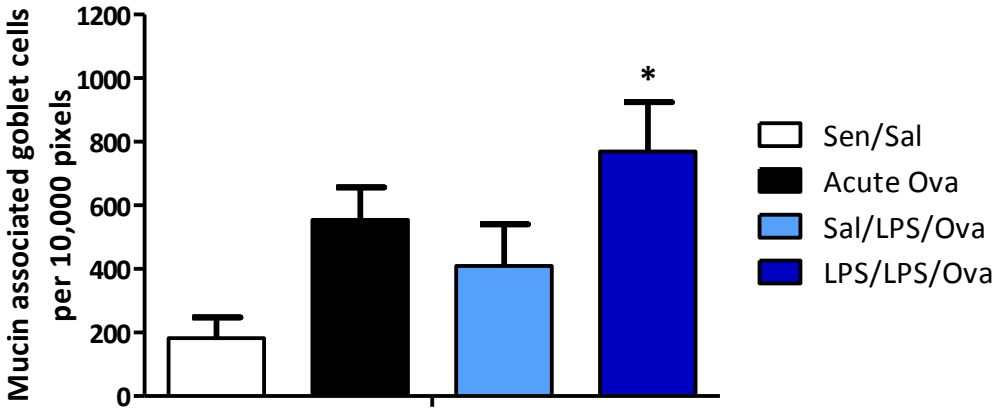


Figure 16: The number of mucin–associated goblet cells per 10,000 epithelial pixels in ovalbumin (Ova) sensitised guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour, 72 hours and 24 hours pre-Ova challenge or saline challenge alone. Results are expressed as mean±SEM; N=6 Sen/saline (sensitised, exposed to saline), (saline/saline/Ova), (saline/LPS/Ova), N=4 (LPS/LPS/Ova). *Significantly different from sensitised/saline treated group treatment $p<0.05$, performed with one-way analysis of variance followed by Bonferroni post-test.

5.5.2 Acute Ova And LPS Co-Exposure

Figure 17 represents the mean time course changes in sGaw in ovalbumin (Ova) challenged guinea-pigs exposed to inhaled saline (saline/Ova), or LPS co-administered with Ova (Saline/LPS+Ova) or LPS 48 hours before Ova challenge and co-administered with Ova (LPS/LPS+Ova). All three groups demonstrated an immediate bronchoconstriction, similar in degree; with LPS inhalation once (-47.5±9.5%), LPS inhalation twice (-52.1±11.6%) and saline inhalation (-60.1±3.4%). Guinea-pigs co-administered LPS with Ova but receiving saline pre-treatment showed a similar recovery from EAR bronchoconstriction to Ova challenged animals. Guinea-pigs exposed to LPS twice demonstrated a slower recovery from EAR bronchoconstriction, being significantly more bronchoconstricted at 5 hours (-37.3±7.6%) compared to saline (-9.7±2.8%). All 3 groups demonstrated late asthmatic responses. A LAR was seen in both guinea-pigs exposed to saline (-14.9±4.4%) and LPS once (-36.6±8.8%) at 8 and 7 hours respectively. A delayed LAR at 10 hours (-18.3±2.9) was observed in guinea-pigs that inhaled LPS twice.

The peak EAR responses to Ova were not altered by LPS treatment. LPS treated animals demonstrated a trend for an increase in the peak LAR but this did not reach statistical significance (Figure 17). No significant differences between total, EAR or LAR area under the curve were found between groups (Figure 18). LPS co-administered with Ova increased the duration of the EAR, which reached significance when LPS was both co-administered with Ova and given 48 hours before (4.8±0.6h compared to saline, 2.3±0.7h) (Figure 19).

Guinea-pigs challenged with only Ova demonstrated a bronchoconstriction to histamine at 0 minutes (-21.6±4.6 compared to pre-Ova, 0.7±4.0%) which returned to baseline sGaw values by 5 minutes. Ova challenge and LPS exposure once (-19.8±4.9% compared to pre-Ova challenge, -0.2±1.9%) and twice (-19.1±10.6%

compared to pre-Ova challenge, -0.2±1.9%) also produced a bronchoconstriction to histamine at 0 minutes. These bronchoconstrictions continued to increase over the 10 minutes. At 10 minutes post-histamine challenge in guinea-pigs exposed to LPS once the bronchoconstriction was -27.0±5.0% compared to pre-Ova, 0.9±3.3% and for LPS twice it was -41.1±7.0% compared to 7.3±2.7% (Figure 20).

LPS co-administered and given 48 hours before Ova challenge significantly increased protein levels compared with guinea-pigs challenge with Ova and Ova and LPS co-administered (4.2±0.6mg/ml compared to 2.0±0.3mg/ml and 2.2±0.3mg/ml respectively) (Figure 21).

A trend for an increase in total cell numbers was seen when Ova and LPS were co-administered, although this failed to reach significance. LPS co-administered with Ova and given 48 hours before significantly increased total cell numbers ($20.9\pm0.9x10^6$/ml compared to saline, $10.5\pm0.8x10^6$/ml respectively). Neutrophils were increased in both groups exposed to LPS once and twice compared to saline treatment ($4.0\pm0.9x10^6$/ml, $7.4\pm0.5x10^6$/ml compared with saline, $0.5\pm0.1x10^6$/ml). All other cell types remained unchanged (Figure 22).

Both single and 2 LPS exposures significantly increased the concentration of IL-8 compared to saline pre-treatment and Ova challenge (11.4±1.1pg/mg, 11.5±1.6pg/mg compared to 5.2±0.34pg/mg respectively). IL-13 and IL-17 levels were significantly increased in guinea-pigs challenged with Ova and exposed to saline (10.5±1.2ng/mg compared to 1.4±0.5ng/mg; 130.5±11.4pg/mg compared to 61.8±3.2pg/mg respectively). LPS exposure did not significantly change IL-13 and IL-17 levels compared with Ova alone (Figure 23).

Figure 24 shows lung sections stained with haematoxylin and eosin for general morphology of the bronchioles. Guinea-pigs challenged with Ova show increased inflammatory cell numbers in the peribronchiolar area. Treatment with LPS does not alter the inflammatory cell presence. Figure 25 presents the mean pathology scores for the number of inflammatory cells in the peribronchiolar area in these guinea-pigs. Ovalbumin challenge alone significantly increases the mean pathology score compared to saline challenge (2.64.±0.34 compared to 0.58±0.19). A single exposure to LPS co-administered pre-Ova challenge and also in guinea-pigs exposure 48 hours pre-Ova challenge also significantly increases the mean pathology score (2.71±0.2, 3.1±0.2 compared to saline challenge, 0.58±0.19 respectively) but not significantly more than Ova alone.

Figure 26 shows lung sections stained with Alcian blue/ periodic acid Schiff to reveal the number of goblet cells in the airway epithelium. Guinea-pigs challenged with Ova show an increased number of goblet cells. Treatment with LPS further increases the number of goblet cells. Figure 27 presents the number of mucin associated-goblet cells per 10,000 pixels of the epithelium. Ovalbumin challenge with a single saline exposure or LPS co-administrated with Ova increases the number of goblet cells, although not significantly. Treatment with LPS 48 pre-Ova and co-administered with Ova increases the number of goblet cells significantly compared to all other groups (974.4±98.8 compared to saline, 182.4±65.2, to Ova, 554±102.2 and to Sal/LPS+Ova, 485.1±175.0).

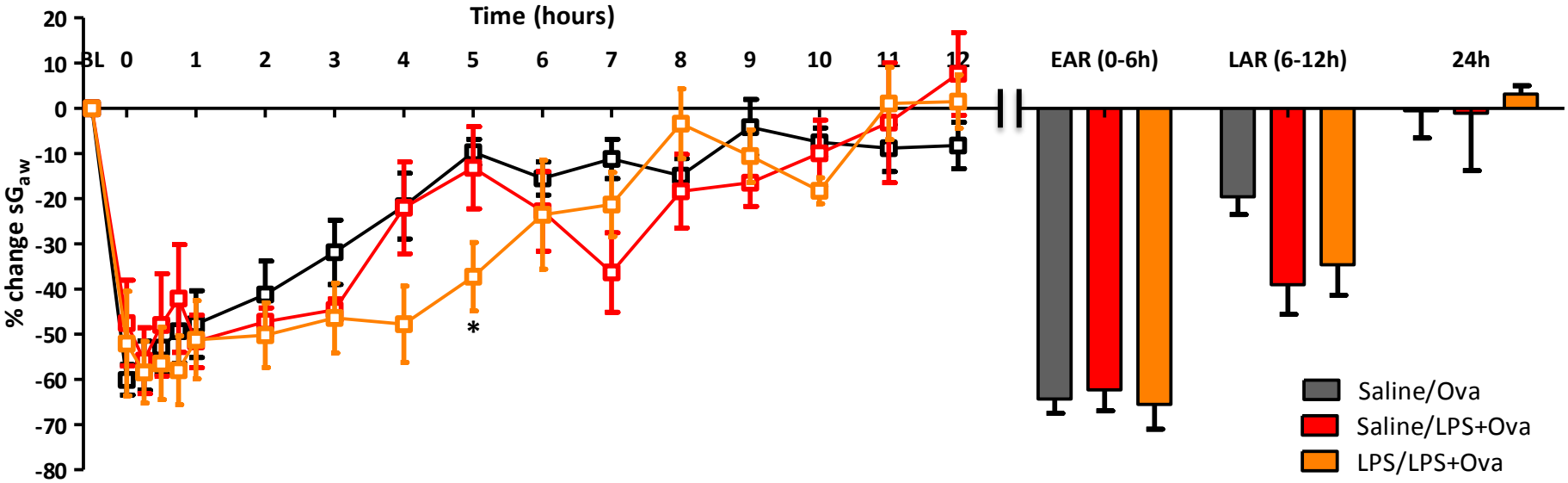


Figure 17: The mean time-course values of sGaw in ovalbumin (Ova) sensitised guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour, 48 hours before Ova challenge and co-administered with Ova. The histogram represents the maximum bronchoconstriction values recorded during the early asthmatic response (EAR) (0-6 hours), late asthmatic response (LAR) (6-12 hours) and 24 hours readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline prior to Ova challenge. A negative value represents a bronchoconstriction. N=5 (Saline/Ova), N=5 (Saline/LPS +Ova), N=6 (LPS/Ova+LPS).*significantly different from saline treatment $p<0.05$; performed with one-way analysis of variance followed by Bonferroni post-test.

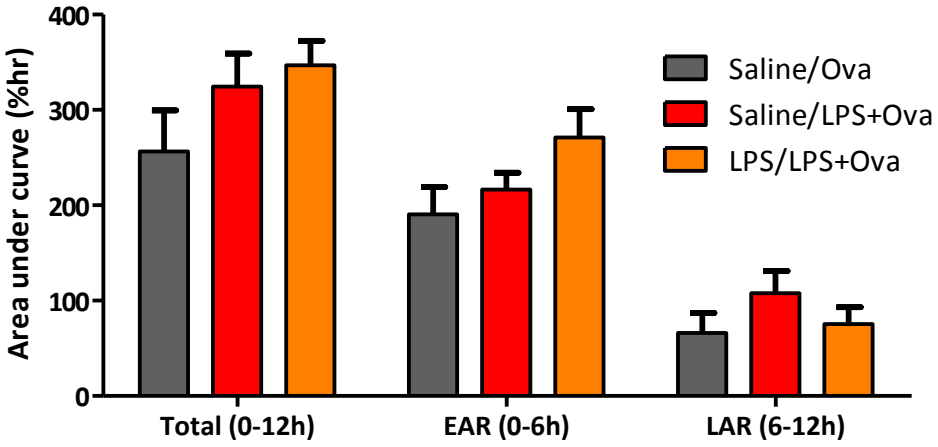


Figure 18: Area under the curve analysis of sGaw values over 12 hours following ovalbumin (Ova) in sensitised guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour, 48 hours before Ova challenge and co-administered with Ova. For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-24 hours, early asthmatic response (EAR) includes values from 0-6 hours) and late asthmatic response (LAR) includes values from 6-12 hours. Area under the curve is expressed as %.hour. N=5 (Saline/Ova), N=5 (Saline/LPS +Ova), N=6 (LPS/Ova+LPS); performed with one-way analysis of variance followed by Bonferroni post-test.

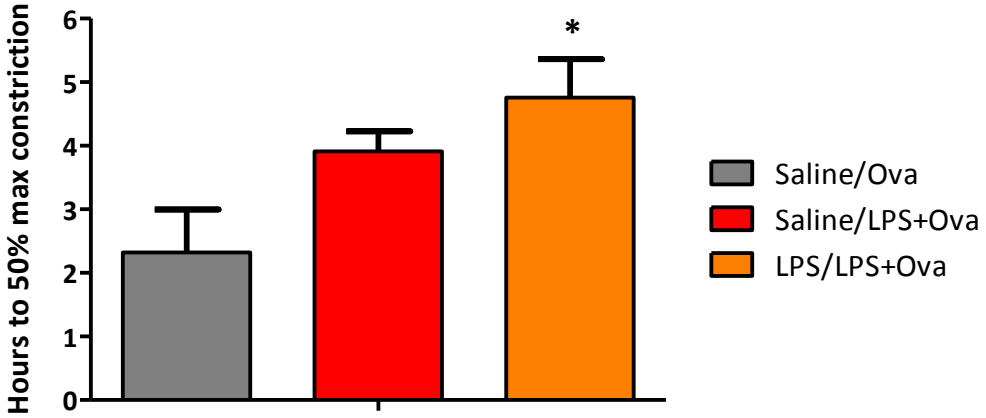


Figure 19: Analysis of the time taken for early asthmatic responses (EAR) to recover to 50% of peak bronchoconstriction values in guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour, 48 hours before Ova challenge and co-administered with Ova. Results are expressed as mean±SEM; N=5 (Saline/Ova), N=5 (Saline/LPS +Ova), N=6 (LPS/Ova+LPS).*significantly different from saline treatment $p<0.05$; performed with one-way analysis of variance followed by Bonferroni post-test.

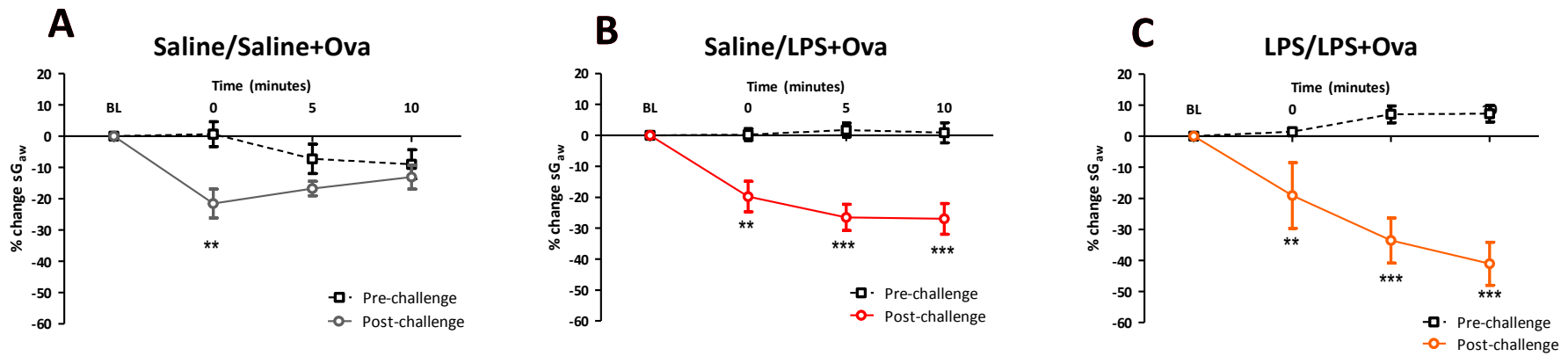


Figure 20: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles and 0.5 LPM, 2 minutes, 1 minute drying period) in ovalbumin (Ova) sensitised guinea-pigs treated with either A) saline or 30µg/ml LPS for 1 hour, B) co-administered with Ova or C) 48 hours before Ova challenge and co-administered with Ova. Values were recorded 7 days pre- and 24 hours post-Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=5 (Saline/Ova), N=5 (Saline/LPS +Ova), N=6 (LPS/Ova+LPS). **Significantly different from time paired pre-Ova challenge values p<0.01; *** p<0.001; performed with a two tailed T-test.

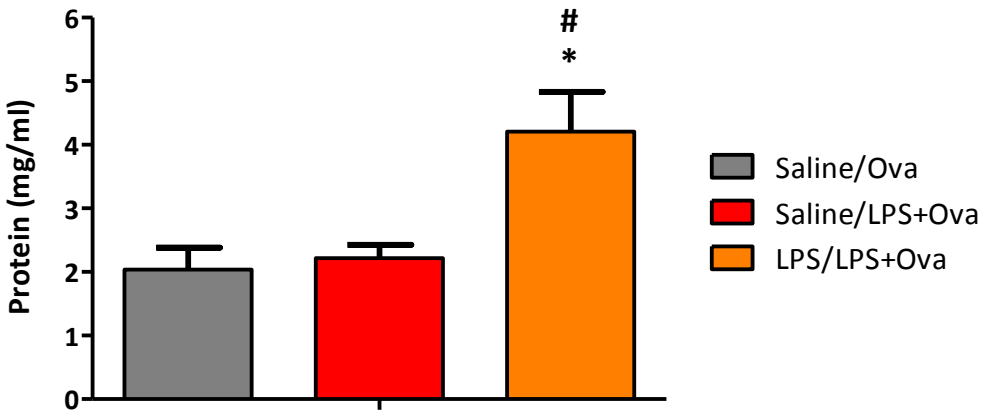


Figure 21: Total lavage fluid protein in ovalbumin (Ova) sensitised guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour, 48 hours before Ova challenge and co-administered with Ova. Protein content was determined by BCA protein assay. Results are expressed as mean±SEM; N=5 (Saline/Ova), N=5 (Saline/LPS +Ova), N=6 (LPS/Ova+LPS). *Significantly different from saline treatment $p<0.05$; # significantly different from saline/LPS+Ova $p<0.05$; performed with one-way analysis of variance followed by Bonferroni post-test

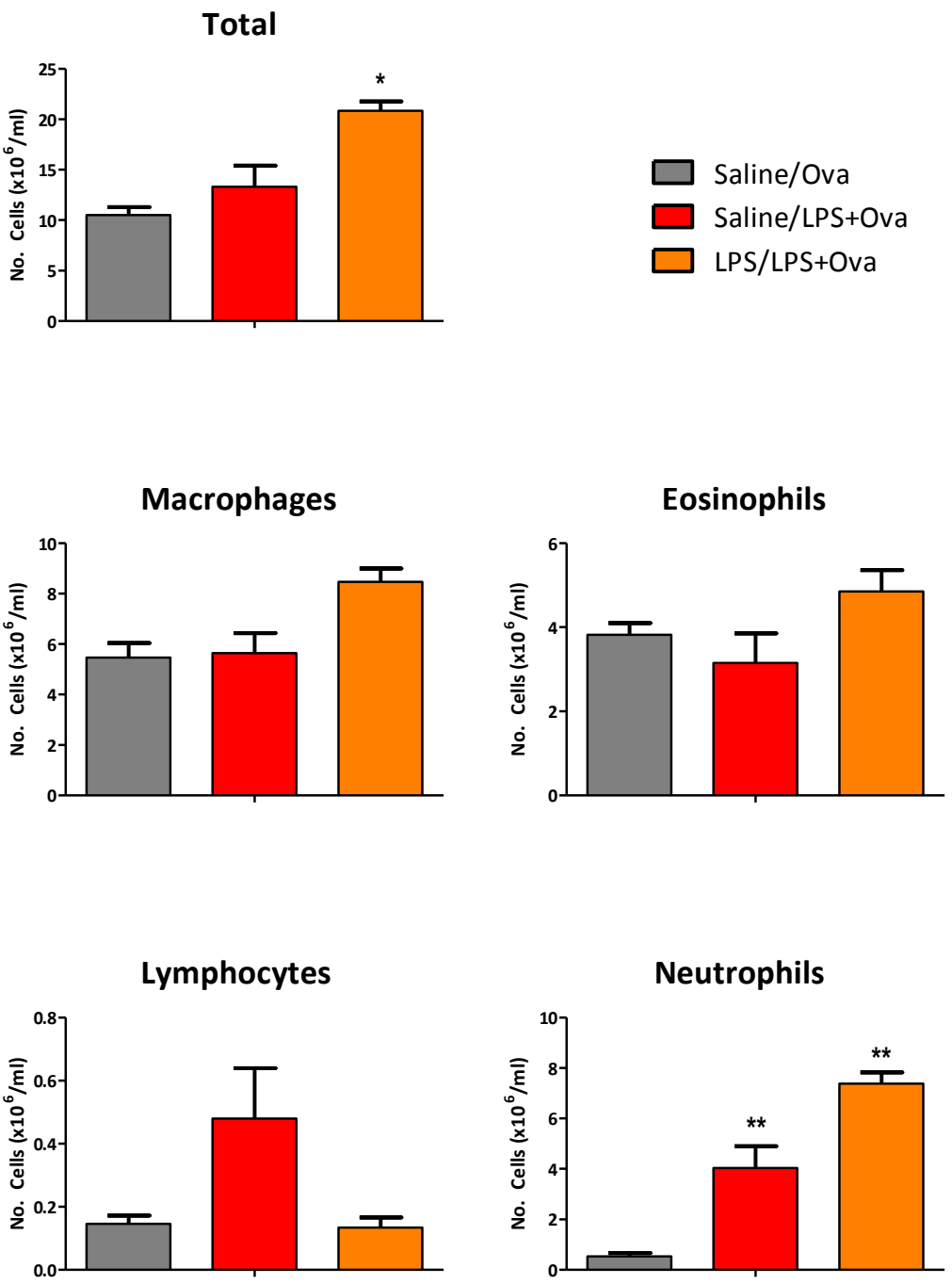


Figure 22: The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in the bronchoalveolar fluid of ovalbumin (Ova) sensitised guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour, 48 hours before Ova challenge and co-administered with Ova. Results are expressed as mean±SEM; N=5 (Saline/Ova), N=5 (Saline/LPS +Ova), N=6 (LPS/Ova+LPS). *Significantly different from saline treatment $p<0.05$, ** $p<0.01$; performed with one-way analysis of variance followed by Bonferroni post-test.

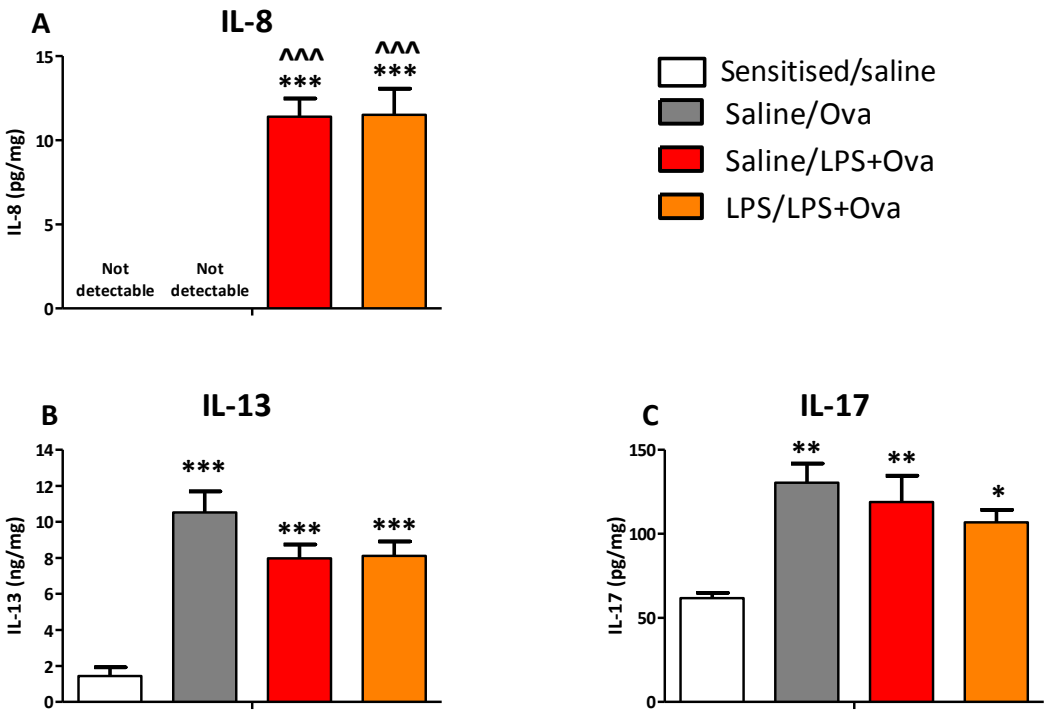


Figure 23: The concentration of A) IL-8 B) IL-13 C) IL-17 in the lungs of ovalbumin (Ova) sensitised guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour, 48 hours before Ova challenge and co-administered with Ova. Results are expressed as mean±SEM; N=5 (Saline/Ova), N=5 (Saline/LPS +Ova), N=6 (LPS/Ova+LPS). *Significantly different from sensitised/saline challenged guinea-pigs $p<0.05$, ** $p<0.01$; *** $p<0.001$; ^^^ significantly different from Sal/sal/Ova $p<0.001$. performed with one-way analysis of variance followed by Bonferroni post-test.

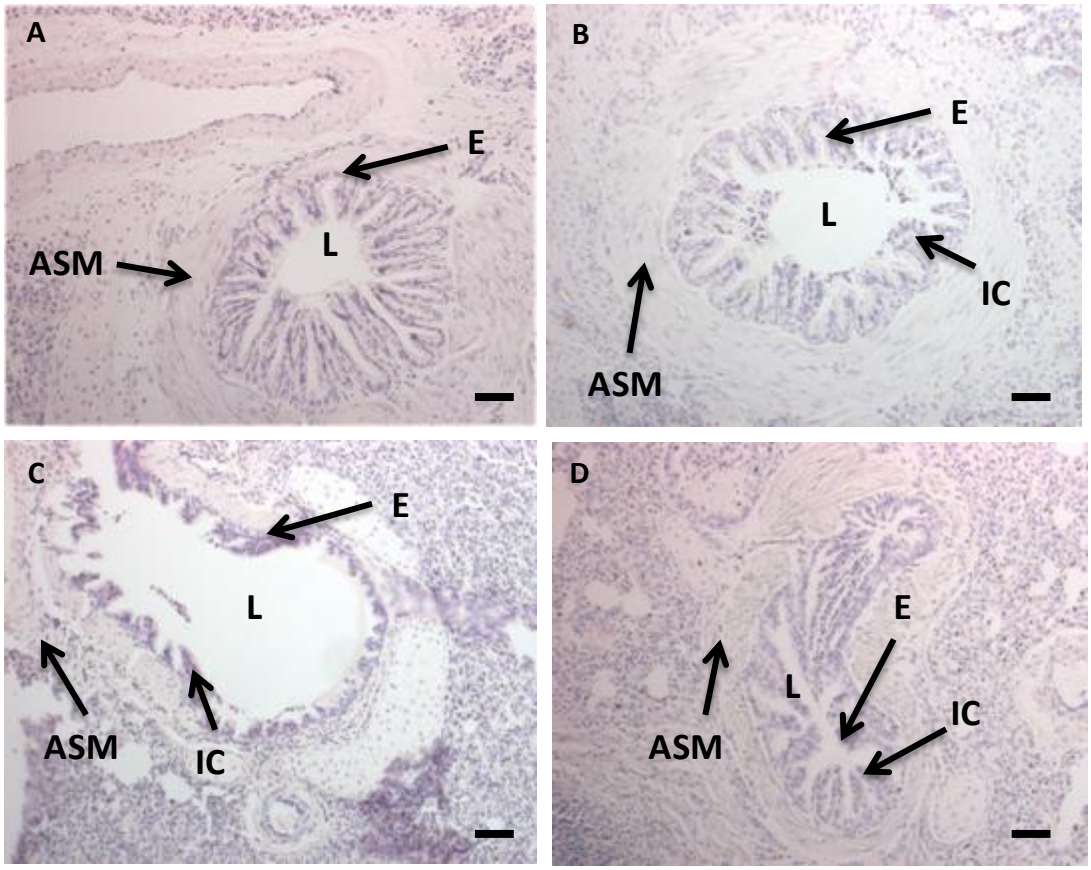


Figure 24: Bronchiolar changes in ovalbumin (Ova) sensitised guinea-pigs exposed to A) saline or B) Ova C) 30µg/ml LPS co-administered with Ova challenge D) 30µg/ml LPS 48 hours pre-Ova challenge and co-administered. L: lumen; ASM: airway smooth muscle; E: epithelium, IC: inflammatory cell. Stained with haematoxylin and eosin. Original magnification 100x (bar = 25 µm).

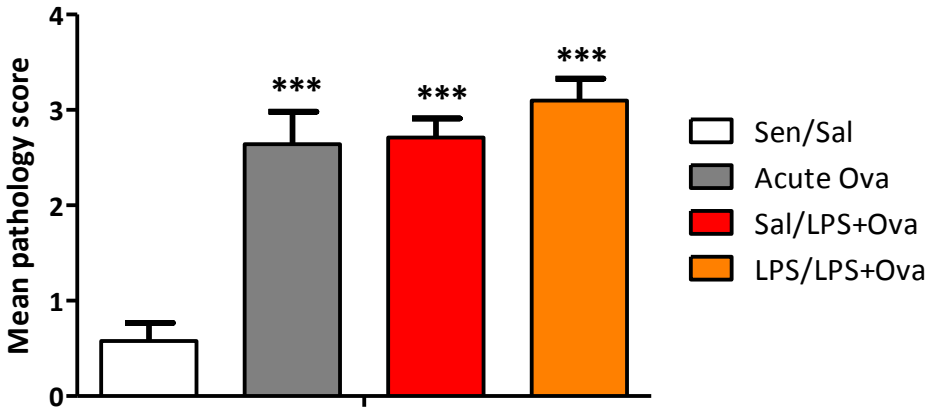


Figure 25: The mean pathology score of the peribronchiolar area in ovalbumin (Ova) sensitised guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour, 48 hours before Ova challenge and co-administered with Ova. Results are expressed as mean±SEM; N=6 Sen/saline (sensitised, exposed to saline), (saline/saline+Ova), (saline/LPS+Ova), N=4 (LPS/LPS+Ova). *** Significantly different from sensitised/saline treated group treatment $p<0.001$; performed with one-way analysis of variance followed by Bonferroni post-test.

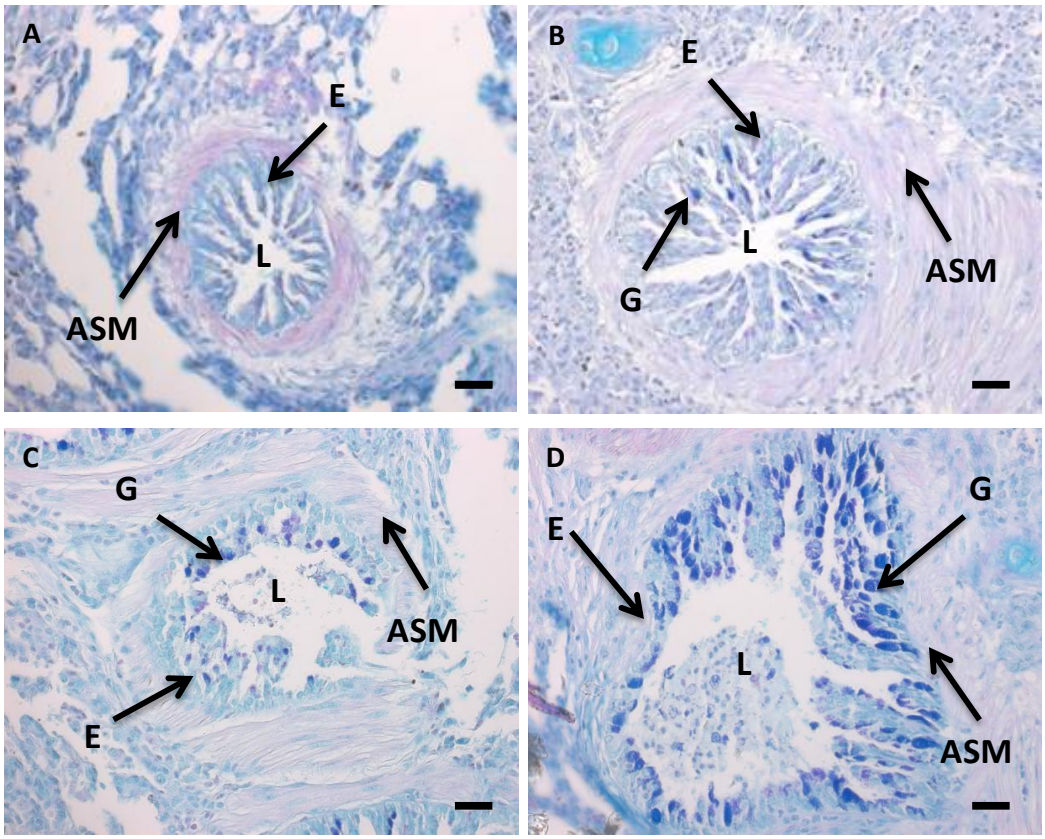


Figure 26: Bronchiolar changes in ovalbumin (Ova) sensitised guinea-pigs exposed to A) saline or B) Ova C) 30µg/ml LPS co-administered with Ova challenge D) 30µg/ml LPS 48 hours pre-Ova challenge and co-administered; L: lumen; ASM: airway smooth muscle; E: epithelium; G: goblet cell; Stained with alcian blue/periodic acid Schiff stain. Original magnification 200x (bar = 50 µm).

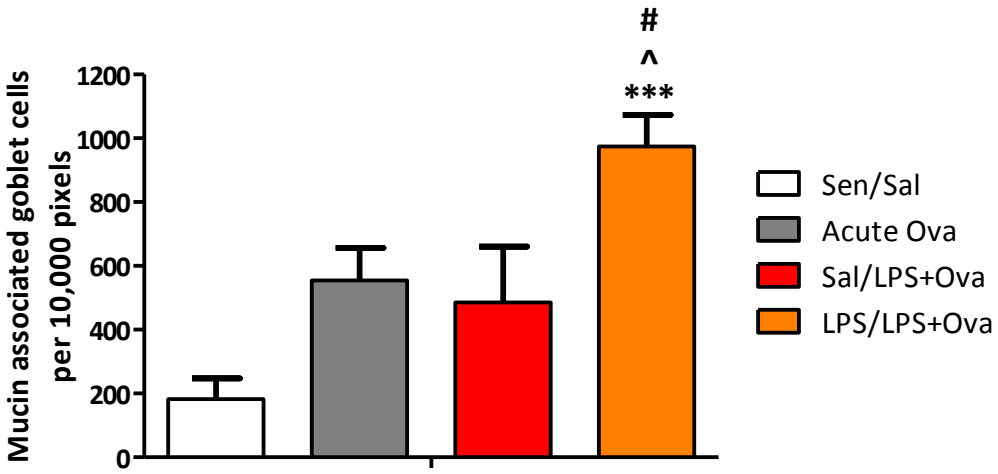


Figure 27: The number of mucin–associated goblet cells per 10,000 epithelial pixels in ovalbumin (Ova) sensitised guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour, 48 hours before Ova challenge and co-administered with Ova. Results are expressed as mean±SEM; N=6 Sen/saline (sensitised, exposed to saline), (saline/saline+Ova), (saline/LPS+Ova), N=4 (LPS/LPS+Ova). *** Significantly different from sensitised/saline treated group treatment $p<0.001$; ^ significantly different from Sal/Sal+Ova $p<0.05$; # significantly different from sal/LPS/Ova $p<0.05$; performed with one-way analysis of variance followed by Bonferroni post-test.

5.5.3 LPS EXPOSURE IN NON-SENSITISED GUINEA-PIGS

Lung function was assessed for 12 hours after the 1st LPS exposure and found to not produce an airway response significantly different from saline (data not shown).

Both saline and LPS challenged animals demonstrated an immediate bronchoconstriction which peaked 1 hour post-challenge. The magnitude of this bronchoconstriction was significantly higher in LPS challenged animals (-41.3±2.7% compared to saline, -22.5±4.5%). In both groups sGaw values returned to baseline by 5 hours post-challenge. No further bronchoconstrictions were seen across the 12 hours of measurements (Figure 28). LPS significantly increased the peak bronchoconstriction during 12 hours of lung function assessment compared to saline (-44.1±2.7% compared to -25.4±4.1%) No difference was seen at the 24 hour time point (Figure 28). LPS also produced a significant increase in area under the curve during this period (144.6±12.0%.hr compared to 75.4±20.8%.hr) (Figure 29).

LPS significantly increased protein levels over saline (1.3±0.3mg/ml compared to 0.5±0.05mg/ml) (Figure 30). Guinea-pigs challenged with saline demonstrated no change in response to histamine. Guinea-pigs challenged with LPS demonstrated an increased bronchoconstrictive response to histamine at 0 minutes (-14.1±3.8% compared to pre-LPS, -0.9±1.0%). This bronchoconstriction persisted until 10 minutes post-histamine (-16.1±4.7% compared to pre-LPS, -1.0±2.5%) (Figure 31).

LPS significantly increased total cells (26.0±6.1 $x10^6$/ml compared to saline, 1.4±0.2$x10^6$/ml), macrophages (11.7±1.7$x10^6$/ml compared to 1.3±0.15$x10^6$/ml) and neutrophils (13.7±4.2$x10^6$/ml compared with 0.02±0.01$x10^6$/ml) (Figure 32).

LPS exposure significantly increased the amount of IL-8 in lungs (23.8±3.6pg/mg compared to saline non-detection, 0±0pg/mg respectively) (Figure 33).

Figure 34 shows the lung sections stained with haematoxylin and eosin for general morphology of the bronchioles. Guinea-pigs exposed to LPS show increased inflammatory cell numbers in the peribronchiolar area. Figure 35 presents the mean pathology scores for the number of inflammatory cells in the peribronchiolar area in these guinea-pigs. LPS exposure significantly increases the mean pathology score compared to saline exposure (2.5.±0.36 compared to 0.4±0.16).

Figure 36 shows the lung sections stained with Alcian blue/ periodic acid Schiff to identifiy number of goblet cells in the airway epithelium. Guinea-pigs challenged with LPS show increased number of goblet cells. Figure 37 presents the number of mucin associated-goblet cells per 10,000 pixels of the epithelium. LPS exposure significantly increases goblet cell numbers compared to saline (898.0±221.6 compared to saline, 163.6±89.7).

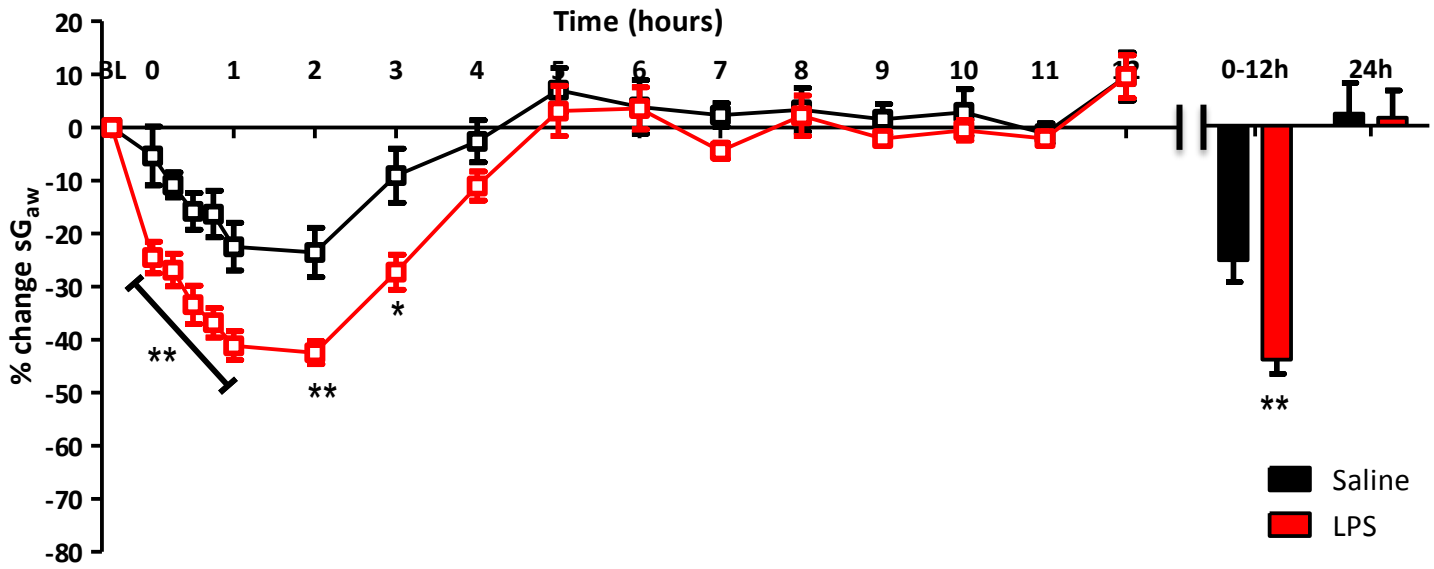


Figure 28: The mean time-course values of sGaw in guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour both 48 and 0 hours before lung function measurements. The histogram represents the maximum bronchoconstriction values recorded 0-12 hours and during the 24 hour reading. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline prior to saline or LPS challenge. A negative value represents a bronchoconstriction. Results shown are after the second LPS or saline exposure. N=6 *significantly different from saline treatment $p<0.05$, ** $p<0.01$; performed with a two tailed T-test.

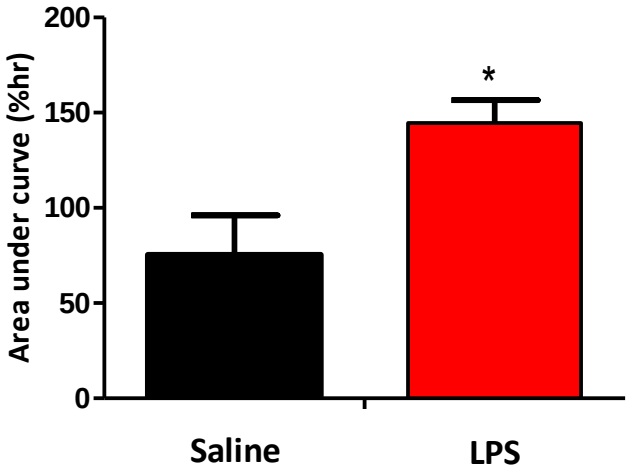


Figure 29: Total area under the curve analysis of sGaw values in guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour both 48 and 0 hours before lung function measurements. For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-12 hours. Area under the curve is expressed as %.hour. N=6 *significantly different from saline treatment $p<0.05$; performed with a two tailed T-test.

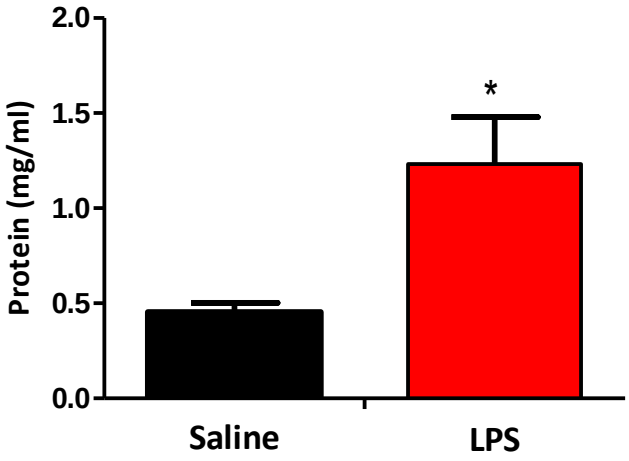


Figure 30: Total lavage fluid protein of guinea-pigs treated with either saline or 30µg/ml LPS for 1 hour, 72 and 24 hours before lavage. Protein content was determined by BCA protein assay. N=6 *Significantly different from saline treatment $p<0.05$; performed with a two tailed T-test.

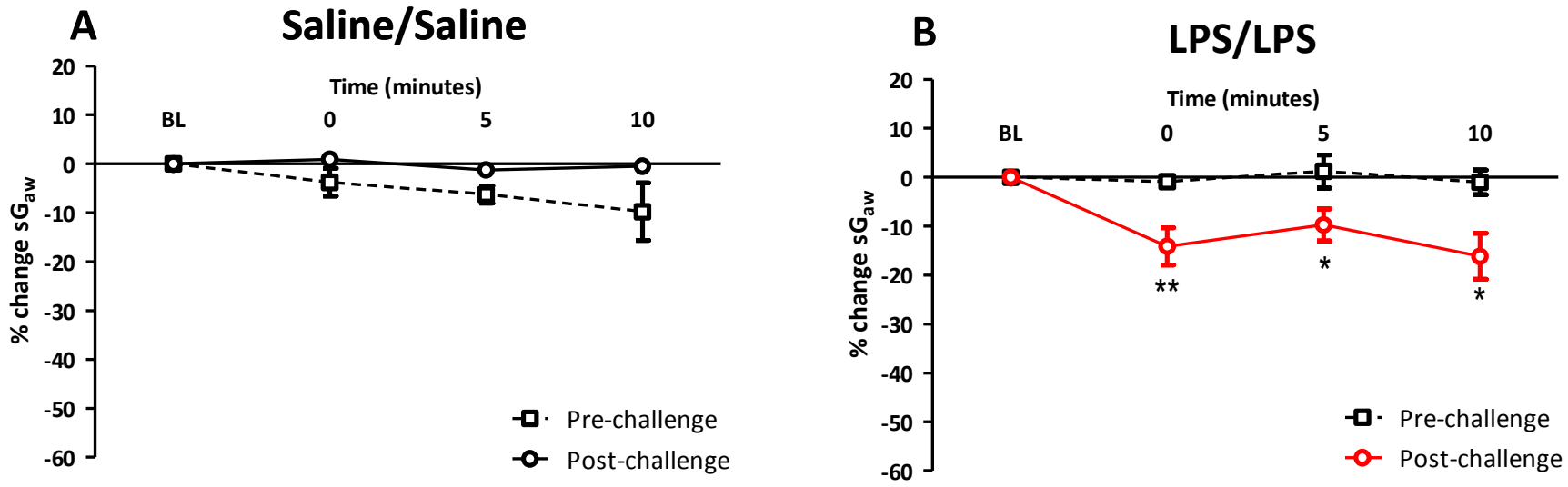


Figure 31: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles and 0.5 LPM per chamber over 2 minutes, 1 minute drying period) in guinea-pigs treated with either A) saline or B) 30µg/ml LPS for 1 hour, 72 and 24 hours before final AHR assessment. Values were recorded 7 days pre- and 24 hours post-2nd LPS challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=6 *Significantly different from time paired pre-LPS challenge values $p<0.05$, ** $p<0.01$; performed with a two tailed T-test.

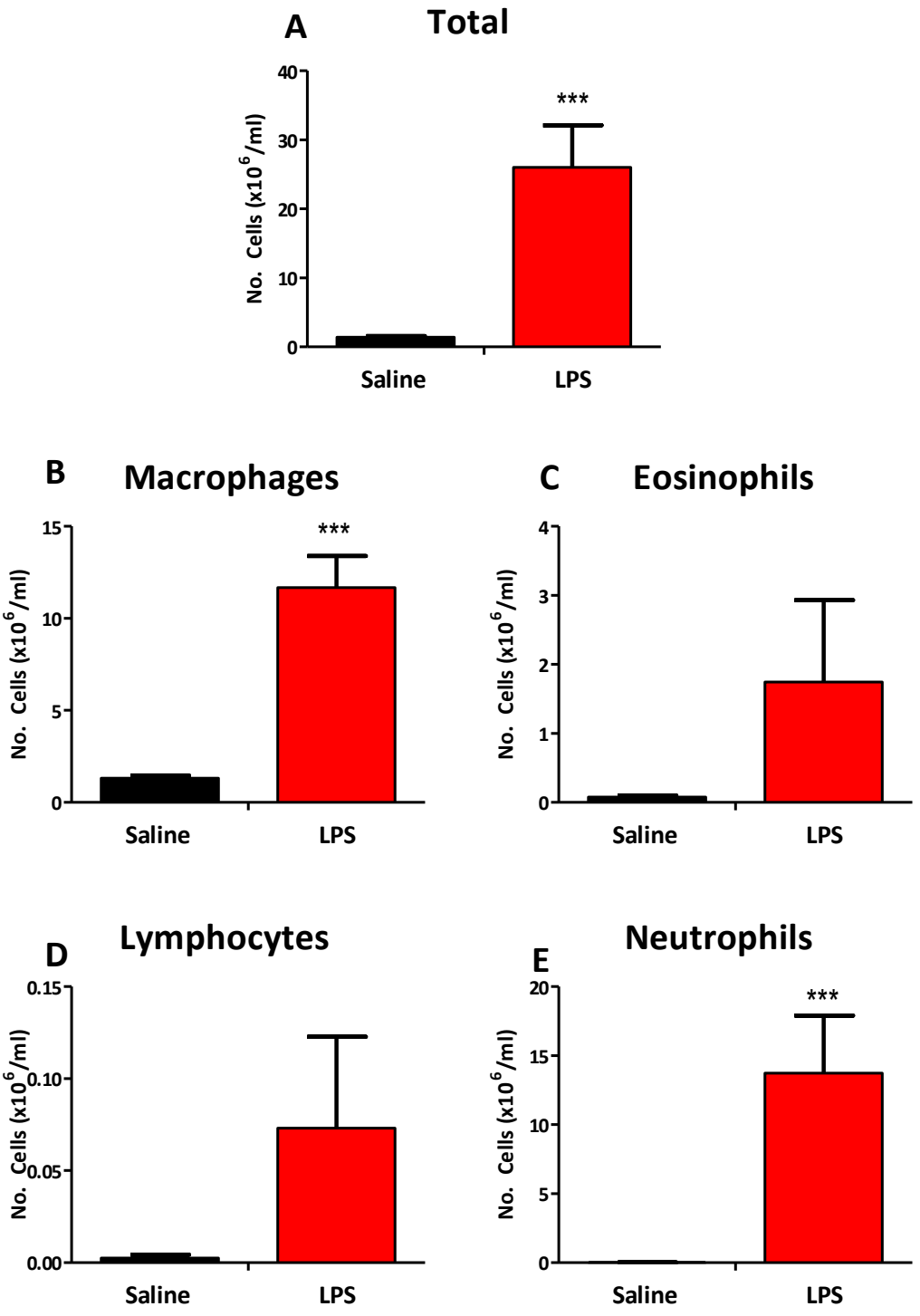


Figure 32: The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in bronchoalveolar fluid in guinea-pigs treated with saline or 30µg/ml LPS for 1 hour, 72 and 24 hours before lavage. Results are expressed as mean±SEM; N=6 ***significantly different from saline treatment $p<0.001$; performed with a two tailed T-test.

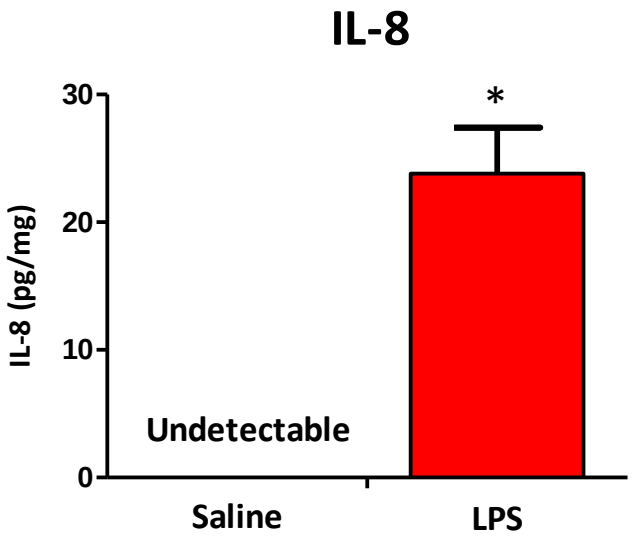


Figure 33: The concentration of IL-8 in the lungs of guinea-pigs treated with saline or 30µg/ml LPS for 1 hour, 72 and 24 hours before lavage. Results are expressed as mean±SEM; N=6 *significantly different from saline treatment $p<0.05$; performed with a two tailed T-test.

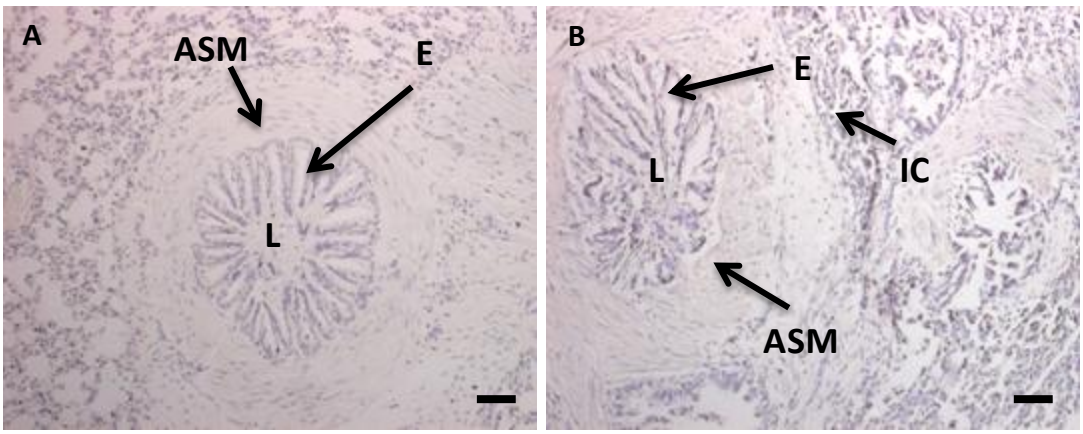


Figure 34: Bronchiolar changes in guinea-pigs treated with saline or 30µg/ml LPS for 1 hour, 72 and 24 hours before lavage. L: lumen; ASM: airway smooth muscle; E: epithelium, IC: inflammatory cell. Stained with haematoxylin and eosin. Original magnification 100x (bar = 25 µm).

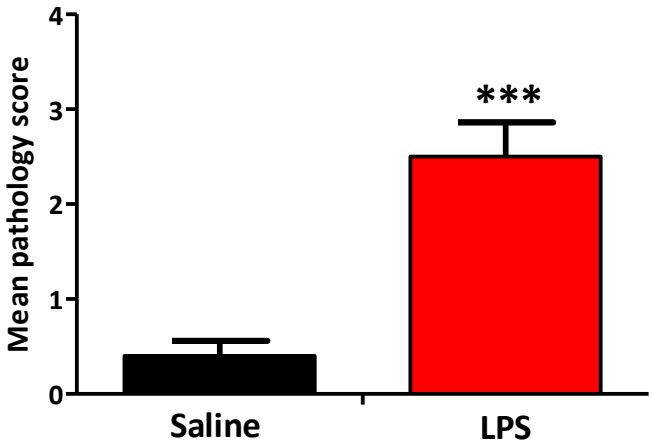


Figure 35: The mean pathology score of the peribronchiolar area in guinea-pigs treated with saline or 30µg/ml LPS for 1 hour, 72 and 24 hours before lavage. Results are expressed as mean±SEM; N=6 *** significantly different from saline treatment p<0.001; performed with a two tailed T-test.

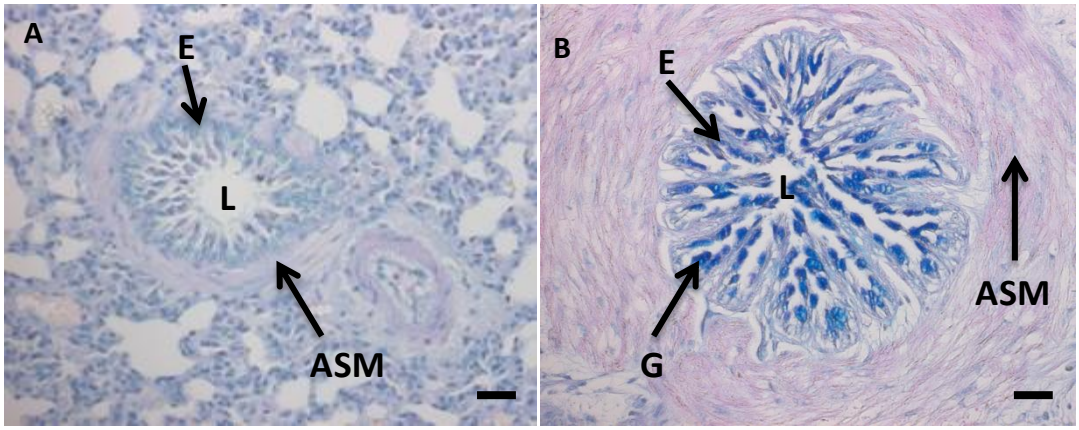


Figure 36: Bronchiolar changes in guinea-pigs treated with saline or 30µg/ml LPS for 1 hour, 72 and 24 hours before lavage. L: lumen; ASM: airway smooth muscle; E: epithelium; G: goblet cell; Stained with alcian blue/periodic acid Schiff stain Original magnification 200x (bar = 50 µm).

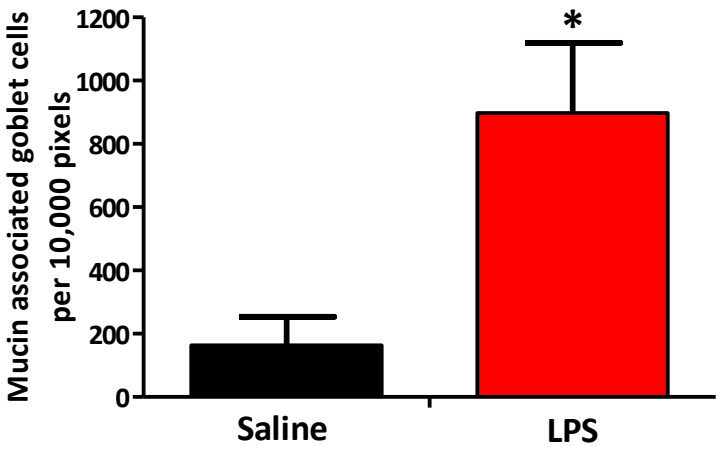


Figure 37: The number of mucin–associated goblet cells per 10,000 epithelial pixels in guinea-pigs treated with saline or 30µg/ml LPS for 1 hour, 72 and 24 hours before lavage. Results are expressed as mean±SEM; . Results are expressed as mean±SEM; N=6 *significantly different from saline treatment $p<0.05$; performed with a two tailed T-test.

5.6 DISCUSSION

LPS exposure has been shown to both attenuate and exacerbate responses to allergen in both humans and animal models. The range of different species, allergen sensitisation and challenge protocols, LPS dose and exposure timings in relation to allergen challenge in animal models probably account for these divergent responses. The experiments described in this chapter were performed to examine the effects of number and timing of LPS exposures on allergen-induced responses. The overall aim was to find a timing and number of LPS exposures that exacerbates allergen-induced responses as a model of asthma exacerbation.

5.6.1 LUNG FUNCTION

In the present study it was shown that LPS exposure 24 hours before allergen challenge significantly attenuates the early asthmatic response to ovalbumin. Contrastingly, when LPS was co-administered with ovalbumin there was no decrease in early phase response. A decrease in the immediate asthmatic response with LPS given 24 hours before allergen challenge has been demonstrated in both animal models and humans (Vannier *et al*, 1991; Sohy *et al*, 2006). The attenuation of the early asthmatic response may be due to effects on mast cells, which mediate early phase bronchoconstrictions via histamine and other granule product released. Mast cells respond to LPS via TLR4 on the cell membrane and become activated both *in vitro* (Masuda *et al*, 2002) and *in vivo* (Murakami *et al*, 2000). This activation results in a partial degranulation of stored histamine and decrease in the production of arachidonate acid metabolites such as LTC_4 and TXA_2, both of which are potent bronchoconstrictors (Vannier *et al*, 1991). Mast cells can take over 24 hours to fully recover from degranulation (Dvorak, 2005). Thus, the decrease in early phase bronchoconstriction to Ova could be due to insufficient time for mast cells to recover from LPS induced degranulation 24 hours before allergen challenge. The 48 hours between LPS exposure and allergen challenge in the Ova and LPS co-administration protocol likely gives mast cells sufficient time to recover from these effects.

Early phase responses are not typically measured in allergen challenge models and so there are few studies on which to compare responses. Using an ovalbumin and LPS co-administration protocol, Tulic *et al*, (2002) demonstrated an increase in the time taken to reach peak early phase bronchoconstriction. No elongation in the early phase response was reported. This is in accordance with the current study where a single dose of LPS co-administered with ovalbumin challenge did not significantly prolong the early phase bronchoconstriction. The addition of a second LPS exposure 48 hours before co-administration with ovalbumin significantly lengthened the duration of the early phase. To the best of my knowledge no other study has shown this effect. Other changes in the early phase response have been shown in humans. A further reduction in FEV_1 values after allergen was shown when co-administered with LPS (Boehlecke *et al*, 2003). Other studies in humans have failed to demonstrate this effect (Nightingale *et al*, 2000). In the latter study, the administration of salbutamol shortly before allergen challenge likely accounts for this difference.

The elongation of the EAR seen in this study could be due to enhanced activation of mast cells by LPS. Mast cells mediate other LPS-induced exacerbation effects such as enhanced allergic inflammation (Murakami *et al*, 2000). Also, mast cells have been shown *in vitro* to demonstrate increased IgE induced cross-linking in the presence of a single dose of LPS (Masuda *et al*, 2002). In the current study an increase in the length of the EAR was only observed in guinea-pigs in the co-administration protocol exposed to 2 doses of LPS. *In vivo* it might be necessary for LPS priming before an enhance response can be produced. LPS exposure upregulates the expression of TLR4, the main receptor involved in LPS signalling in addition to downstream messengers (Lin *et al*, 2006; Hoogerwerf *et al*, 2010). Subsequent LPS exposure in cells with increased TLR-4 expression may mediate a larger response (Nigo *et al*, 2006). LPS also primes airway neutrophils and macrophages for further exposures and the release of bronchoconstrictor substances either directly and indirectly (Reino *et al*, 2012; Hoogerwerf *et al*, 2010). This may also be the mechanism behind

the bronchoconstriction seen in guinea-pigs treated with LPS twice in the current study; although non-IgE mediated mast cell degranulation cannot be ruled out. The need for previous exposure to LPS to produce an increased bronchoconstrictor response is further highlighted by the results for LPS exposure on a non-allergic background. In the current study, a single LPS exposure does not cause a bronchoconstriction, whereas a second exposure does. LPS induced bronchoconstrictions have been observed in humans (Michel *et al*, 1989; Cavagna 1969). This process has been associated with the release of platelet activating factor, a potent bronchoconstrictor (Rylander & Beijer, 1987).

This study did not demonstrate any significant effect of LPS on late phase bronchoconstrictions to allergen. A lack of effect on the late phase bronchoconstriction following LPS exposure has also been shown in humans (Sohy *et al,* 2006). However it is difficult to draw many comparisons with this study as it only examined LPS exposure 24 hours before allergen challenge and failed to demonstrate a change in inflammatory response. This may be due to the low dose of LPS used. A study by Tulic *et al*, (2002) reported decreased late phase responses in rats following allergen and LPS co-administration. However, this study did not actually measure late phase bronchoconstrictions and instead inferred 'late phase response' from AHR and inflammation. As seen previously in chapter 3, dissociation between allergen responses can happen, making inferences difficult. Despite not having a significant effect on the late phase bronchoconstriction, the results suggest LPS may be having some effect on the late phase. A non-significant trend for an increase in the peak bronchoconstriction of the late phase with increasing number of LPS exposures in the co-administration protocol was observed. Even in the studies where the EAR was reduced by LPS, the response was prolonged with the EAR and LAR merging. Due to a lack of studies, especially in animal models examining 'true' late phase bronchoconstrictions it's not possible to be certain of the effect of LPS on the late phase.

5.6.2 Airways Hyperresponsiveness

The present study demonstrated that LPS has dichotomous effects on AHR depending on the timing of the exposure. When LPS exposure took place 24 hours before ovalbumin challenge there was decreased development of AHR. 2 exposures to LPS before Ova challenge completely abolished the development of AHR. Similarly, Komlosi *et al*, 2006 reported a decrease but not abolition of AHR when a single dose of LPS was given 24 hours before Ova challenge. Others have demonstrated no change in allergen-induced AHR with LPS exposure (Gerhold *et al*, 2002). However, in this particular study AHR was measured 48 hours after the final Ova challenge, a time point at which AHR to Ova alone would be expected to have diminished (Evans, 2009). The decreased AHR in this study does not seem to be due to the airways already being constricted. No significant bronchoconstriction was present at 24 hour post-Ova challenge, the time of AHR assessment.

LPS co-administered with Ova prolonged the bronchoconstrictor response to histamine. Several studies have demonstrated increases in AHR to allergen with LPS exposure. Delayre-Orthez *et al*, 2004 demonstrated an increase in AHR when repeat Ova challenges were co-administered with LPS. Rodriguez *et al*, (2003) showed that intra-nasal LPS immediately before Ova challenge prolongs the airway response to a bronchoconstrictor agent. This effect is only found when LPS is administered locally. LPS administered intravenously abolished the AHR after Ova challenge. Contrasting with the current study, LPS and allergen co-exposure has been shown to decrease AHR in rats (Tulic *et al*, 2002). However, this study also reported a decrease in inflammation suggesting that an alternative LPS response at this time point may have been triggered. Factors such as the species, dose and source of LPS and Ova protocol (the aforementioned study used ricin instead of alum as the adjuvant) may account for this discrepancy.

The mechanism behind both the increased and decreased AHR observed in this study is unknown. AHR is often associated with increases in airway inflammation (Cockcroft & Davis, 2006). Supporting this is the observation in the present study

that an increase in AHR was seen alongside an increase in inflammation in guinea-pigs co-administered LPS and Ova. Additionally, LPS alone induces AHR, 24 hours after LPS exposure alongside an increase in inflammation. This contrasts with Nevin & Broadley (2004) who demonstrated AHR only 1 hour after LPS exposure in guinea-pigs. However, this study only used a single LPS exposure, whereas the present study used 2, possibly increasing the duration of LPS-induced AHR. This supported by the observation that 2 LPS challenges induce AHR which is still present at 4 hours post-challenge, whereas with one it has dissipated (Toward & Broadley, 2001). Contrasting with the present study, LPS exposure 24 hours before Ova challenge has also been shown to increase inflammation but decrease AHR. Furthermore, the airway response to histamine was abolished with the addition of an exposure 72 hours before Ova challenge. This is not the first study to demonstrate dissociation between AHR and inflammation. AHR has been demonstrated with limited inflammation (Delayre-Orthez *et al*, 2004) and less AHR in the presence of increased inflammation (Komlosi *et al*, 2006). This suggests the importance of other factors in the development of AHR.

The nature of the inflammatory response may be a more important factor then the extent of it. In particular, neutrophil and macrophage activity may be an important factor in the development of AHR. LPS alone induces AHR in rats which correlate strongly with fluctuating levels of neutrophils (Pauwels *et al*, 1990). Neutrophils and macrophages release reactive oxygen species which cause epithelial damage and reduce the amount of enzymes such as diamine oxidase, important in breakdown of histamine (Folkerts & Nijkamp, 1998). This could extend the biological half-life of histamine, prolonging bronchoconstriction. In allergy, this may require a full allergen response to result in a worsening of AHR. The EAR and LAR are closely associated, the latter of which has been linked to the development of AHR (Hargreave *et al*, 1986). The observation that LPS did not increase AHR in pre-Ova LPS exposure groups, also not demonstrating a full EAR would support this. Alternatively, an increase in the production of nitric oxide may mediate increased

AHR. Nitric oxide levels and AHR both decrease in LPS binding protein deficient mice, which helps mediate LPS receptor binding (Stroheimer *et al*, 2001). However, an iNOS inhibitor effective on AHR to Ova alone, fails to reduce AHR in an Ova and LPS combination model questioning the role of this potential mechanism (Komlosi *et al*, 2006). Regardless of the mechanism behind prolonged AHR, this study has clearly highlighted the temporal nature of LPS's effect on functional responses. A study by Tulic *et al*, (2002) further demonstrates this; when LPS is administered 18 hours after allergen challenge it diminishes AHR. Thus it seems there is a key period around the time of allergen challenge where LPS will exacerbate AHR but given 18-42 pre- or post-allergen challenge will attenuate it.

5.6.3 Airway Inflammation And Cytokines

Leukocyte infiltration in lavage fluid increased with the number of LPS exposures but was unaffected by the timing of LPS exposure. Airway inflammation assessed by an H&E scoring method did not reveal this increase in inflammation with LPS treatment. This is probably because H&E scoring methods lack the same fidelity as lavage based methods of assessment. They are mainly used to assess morphological changes. The increase in lavage fluid inflammation with different LPS timings is in accordance with other studies which suggest that the route of LPS administration (intra-venous vs intra-nasal/inhaled) and the exposure dose are more important factors in the inflammatory response. A high dose of LPS administered locally to the airways increases total cell counts in both mice and rats. As in the present study, neutrophils were significantly increased (Murakami *et al*, 2006; Tulic *et al*, 2000). Unlike in the present study where no significant change in eosinophil was seen, an increase in eosinophils was also observed. This is similar to the situation in humans where neutrophil and/or eosinophils increase during asthma exacerbations (Ordonez *et al*, 2000). The lack of an increase in eosinophils in the present study could be due to variation in the protocols employed, with the former study using multiple allergen/LPS challenges. This may increase Th2 lymphocyte responses which would favour increases in eosinophils.

High doses of LPS have also been shown to result in an overall decrease in inflammatory cells (Gerhold *et al*, 2002). Most of the decrease was in eosinophils; whether neutrophils increased is unknown as the researchers were unable to detect them. Making comparisons between the results of these *in vivo* studies is always problematic. It is difficult to extrapolate the dose of LPS that an animal actually receives from what is reported in the literature. The time of exposure, route and pharmacokinetic properties likely all vary.

Underlying the increase in neutrophils seen in the present study was an increase in the amount of IL-8 in the lung of guinea-pigs where LPS was co-administered with Ova challenge. A similar increase was seen in guinea-pigs exposed to LPS twice. An increase in IL-8 with LPS treatment would be expected as LPS is known to be a potent inducer of the cytokine (Alexis *et al*, 2005). IL-8 is a key chemoattractant in the migration of neutrophils and unregulated by LPS (Kamochi *et al*, 1999). However, increases in neutrophilia were also seen in guinea-pigs treated with LPS 24 hours before Ova challenge but with no increase in IL-8. This is likely due to differences in the time point after LPS exposure that lungs were assessed. In the LPS co-administration groups, lungs were frozen 24 hours after final LPS exposure, whereas this was done 48 hours later In the 24 hour pre-Ova LPS exposure groups. IL-8 is a transient cytokine, not persistently increased over long time periods (Angrisano *et al*, 2010; Danahay *et al,* 1999). At the 48 compared to 24 hours post-LPS exposure, IL-8 levels would be expected to be lower.

Increased IL-13 was seen in guinea-pigs challenged with Ova. Ova induces Th2 lymphocyte responses which are characterised by increased levels of cytokines such as IL-4 and IL-13. LPS exposure did not further increase IL-13 levels in lung. This is in accordance with a study that also used a high dose of LPS (Murakami *et al*, 2006). Other studies have shown a decrease in IL-13 (Komlosi *et al*, 2006; Rodriguez *et al*, 2003). No increase in IL-13 levels indicates that LPS exposure is not further promoting Th2 allergic responses. This is more characteristic of lower doses of LPS,

whereas high doses tend to favour Th1 proinflammatory responses with increased IL-8, IL-1β and GM-CSF (Boehlecke *et al*, 2003).

IL-17 levels were increased in guinea-pigs exposed to LPS but not above that seen for Ova alone. The exception to this was in guinea-pigs exposed to a single dose of LPS 24 before Ova challenge. IL-17 is implicated in more severe forms of asthma and associated with increased neutrophils (Doe *et al*, 2010). Few studies examining the effect of LPS on IL-17 levels in allergic responses have been performed. One study using house dust mite as an allergen found that LPS increased IL-17 concentrations (de Boer *et al*, 2013). This study used repeat allergen challenge and a non-Ova allergen which may account for difference with the present study. In addition, Doe *et al*, (2010) noted that IL-17 can be difficult to detect and only secreted in certain lung compartments. Thus using lung homogenate may not have been the best way to detect changes in IL-17 levels. Ideally lavage fluid would have been used but IL-17 levels were too low to be consistently detected. Therefore the effect of LPS on IL-17 levels requires further investigation.

5.6.4 Airway Oedema

Airway oedema was observed to be significantly increased in guinea-pigs treated with LPS 48 hours pre- and co-administered with Ova challenge. This increase was also seen with 2 doses of LPS alone. This is characteristic of asthma exacerbations in humans (Hogg, 1997). LPS is well established at causing protein extravasation and oedema and has been shown to increase LPS binding protein levels, which may further amplify oedema (Dubin *et al*, 1996). An increase in airway oedema was not seen with a single dose of LPS co-administered. This indicates that 2 LPS exposures may be necessary to cause an increase in airway oedema. The lack of an increase in oedema in guinea-pigs treated with either 1 or 2 LPS exposures 24 hour pre- Ova challenge may be due to a failure to invoke the full allergic response. Alternatively, oedema levels may have decreased 48 hours after LPS challenge.

5.6.5 Goblet Cell Hyperplasia/Metaplasia

Goblet cell numbers increased significantly with 2 doses of LPS in either exposure protocol used but not with a single dose of LPS. Goblet cell numbers also increased with 2 doses of LPS on a non-allergic background. Other studies have confirmed this result (Toward & Broadley, 2002). The largest increase in goblet cells was seen with 2 LPS exposures in the Ova co-administration protocol. Importantly this was the protocol that also exacerbated other Ova-induced responses. Mucus secretion is one of the hallmarks of asthma exacerbations and is commonly seen in asthmatics that have died from status asthmaticus (Kuyper *et al*, 2003).

5.6.6 General Conclusions

This study has demonstrated the temporal relationship that LPS has on allergen challenge induced responses. It has shown that LPS exposure within 24 hours of allergen challenge diminishes functional responses, while increasing airway inflammation. It has also shown that LPS co-administered with allergen challenge is able to exacerbate functional and inflammatory responses to allergen challenge. From this study a model of asthma exacerbation demonstrating prolonged EAR, prolonged bronchoconstrictor response to histamine, increased airways inflammation, airway oedema and goblet cell hyperplasia has been developed. The sensitivity of this model to anti-asthma treatments will be investigated in the coming chapters.

CHAPTER 6

The Effect Of Inhaled Fluticasone Propionate On Functional And Inflammatory Responses To Ovalbumin And LPS

Chapter 6

6.1 INTRODUCTION

6.1.1 INHALED CORTICOSTEROIDS

Corticosteroids are a frontline therapy used in the treatment of asthma. Historically, when their ability to treat the symptoms of asthma were realised they were given systemically. However this caused extensive side effects including growth retardation and osteoporosis, so in the 1970s inhaled preparations were developed. Inhaled corticosteroids continued to be developed and many including budesonide, beclometasone and fluticasone propionate are still used in routine control of asthma. Inhaled corticosteroids have proven effective in providing symptomatic control, reducing exacerbations and preventing the development of irreversible airway remodelling (Barnes *et al*, 1998A). Corticosteroids are frequently used alone or in combination with other therapies such as theophylline, anti-leukotrienes and β_2-adrenoceptor agonists. These medicines are all complimentary to corticosteroid therapy. β_2-adrenoceptor agonists in particular are able to suppress early phase bronchoconstriction, one of the elements of asthma upon which corticosteroids have limited efficacy (Booij-Noord *et al*, 1971).

6.1.2 EFFECT OF CORTICOSTEROIDS IN ASTHMA

The effects of inhaled and systemic steroids are similar so will be discussed together. Corticosteroids have a wide range of actions on inflammatory cells, mediators, mucus secretion and AHR. Eosinophils, a hallmark of allergic disease, are reduced following treatment with steroids. This reduction is partly due to the suppression of eosinophil accumulation by decreased chemoattractants, adhesion molecules and increased eosinophil apoptosis (Underwood *et al*, 1997; Meagher *et al*, 1996). Macrophages can secrete both pro- and anti-inflammatory mediators. Corticosteroid treatment suppresses pro-inflammatory and increases anti-inflammatory secretion of mediators such as IL-10 (John *et al*, 1998). Corticosteroids also reduced mast cell numbers but have little effect on mast cell degranulation (Belvisi, 2004). This accounts for their lack of effect on the early asthmatic response which is mast cell

mediated. Dendritic cells are reduced in number by corticosteroids, decreasing allergen presentation and dampening the immune response (Nelson *et al*, 1995). T-lymphocyte activity is also blocked by corticosteroid treatment, resulting in a reduction in the secretion of Th2 cytokines such as IL-4, IL-5 and IL-13, which are important mediators in allergic disease (Krouwels *et al,* 1996). Epithelial cells are now recognised as an important source of inflammatory mediators including IL-8, CCL11 (eotaxin-1), GM-CSF, CCL4 (MIP-1α), CCL5 (RANTES) and nitric oxide. These are all decreased by corticosteroid treatment (Barnes *et al*, 1998a). Neutrophils are not commonly found in milder forms of asthma but can make up a significant portion of the airway inflammatory cell population in more severe and neutrophilic forms of the disease (Wenzel *et al*, 1999). Contrastingly with other inflammatory cell types, neutrophils are relatively unresponsive to corticosteroid and may even enhance their survival (Strickland *et al*, 2001). AHR to bronchoconstrictor agents is also reduced by corticosteroid treatment (Barnes, 1990). The late asthmatic response, seen in a majority of asthmatics is also reduced, likely because of corticosteroids anti-inflammatory actions (Palmqvist *et al*, 2005). Mucus secretion is reduced by several mechanisms including indirect action from a reduction in inflammatory mediators, direct action on the mucosal glands and inhibition of the mucin genes MUC5A and MUC2 (Shimura *et al*, 1990; Kai *et al*, 1996). Airway oedema is decreased by direct genomic effects but also by extra-genomic effects such as vasoconstriction (Mendes *et al*, 2003). A simplified diagram of some of the main cellular effects of corticosteroids is shown in Figure 1.

In addition to these desirable effects of corticosteroids many side effects are frequently encountered. Fewer side effects are encountered with inhaled than with oral corticosteroids due to lower systemic distribution (Wood *et al*, 1999). However, the incidence of side effects increases with higher inhaled doses, which are used in less corticosteroid-responsive asthma. Although rare with inhaled therapy, potential side effects include osteoporosis, cataracts, growth retardation in children and cardiovascular and gastrointestinal disturbances (Schäcke *et al*, 2002). Additional

side effects of inhaled steroids include hoarseness of voice, oral infections; particularly fungal and dysphonia due to laryngeal deposition (Barnes *et al*, 1998A).

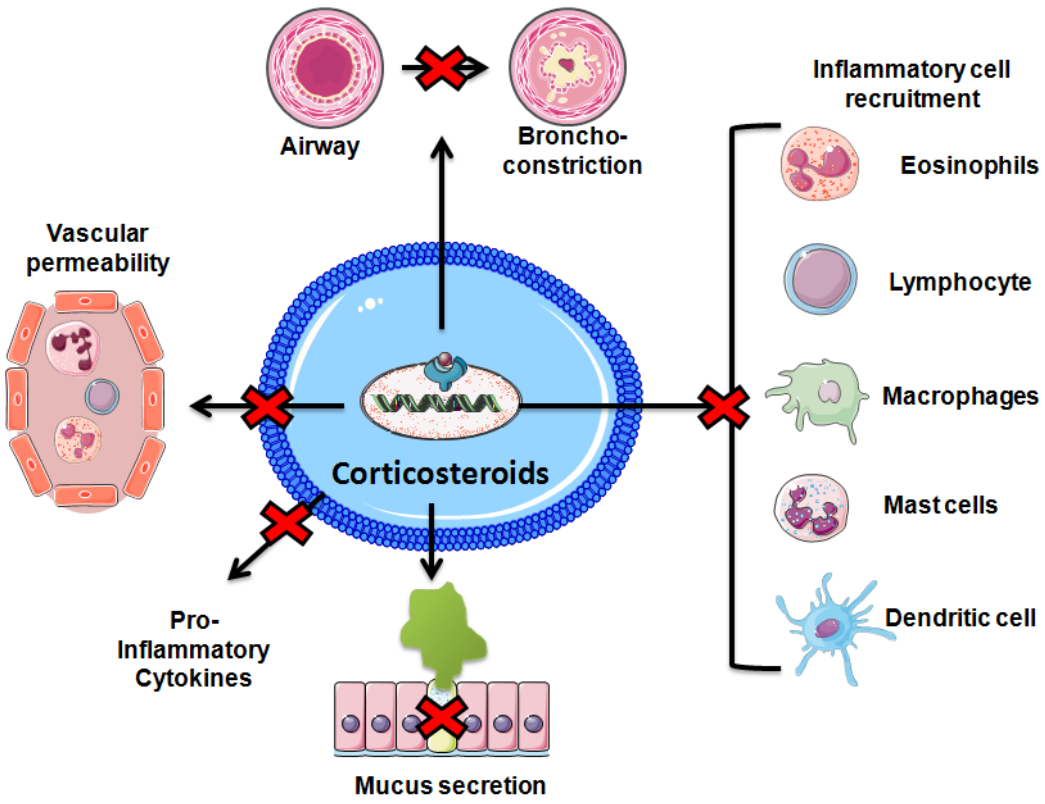


Figure 1: Simplified diagram of the major cellular effects of corticosteroids.

6.1.3 Mechanisms of corticosteroid action

Corticosteroids pass readily through the plasma membrane and bind to the cytoplasmic glucocorticoid receptor (GR). Several splice variants of the GR exist including GR-α which binds to corticosteroids and GR-β which does not. Thus the effects of corticosteroids are through GR-α, although GR-β may have a role in corticosteroid insensitivity (Leung and Bloom, 2003; Strickland *et al*, 2001). The binding of a corticosteroid to the GR results in dissociation of molecular chaperones such as heat shock protein (hsp-90) and FK-binding protein, resulting in nuclear localisation of the GR-corticosteroid complex. Corticosteroids exert their anti-inflammatory effects by several distinct mechanisms (Marwick *et al*, 2007). One

mechanism termed *trans*-activation increases the transcription of anti-inflammatory genes. Negative glucocorticoid response elements sites also exist which can suppress gene expression (*Cis*-repression) and likely mediate some of the side effects of steroids (Schäcke *et al*, 2002). Another anti-inflammatory effect of steroids is *trans*-repression which suppresses pro-inflammatory gene transcription. These processes are believed to take place at different concentrations of corticosteroid. Corticosteroids can also modify proteins post-transcriptionally by decreasing the stability of mRNA (Barnes, 2006).

6.1.3.1 *TRANS*-ACTIVATION

GR-corticosteroid complexes are able to dimerise at specific DNA sites called glucocorticoid response elements (GRE) in the promoter regions of corticosteroid-responsive genes. This allows GR to interact with a variety of co-activator molecules such as GRIP-1, pCAF and CREB binding protein (CBP). These proteins have intrinsic histone acetyltransferase (HAT) activity and thus can induce histone acetylation, which unwinds DNA and increases gene transcription. Inflammatory genes activated by corticosteroids include IL-10, MAP kinase phosophatase-1 (MKP-1), CBP, annexin-1 and the inhibitor of NF-κB (IκB). This is unlikely to be the main mechanism of corticosteroids as *in vitro* 100 fold higher concentrations then for *trans*-repression are required (Ito *et al*, 2000). Most of the side effect profile of steroids at high doses such as cataracts, osteoporosis, growth retardation, metabolic effects and fragility may be caused by *trans*-activation. This process is shown in Figure 2.

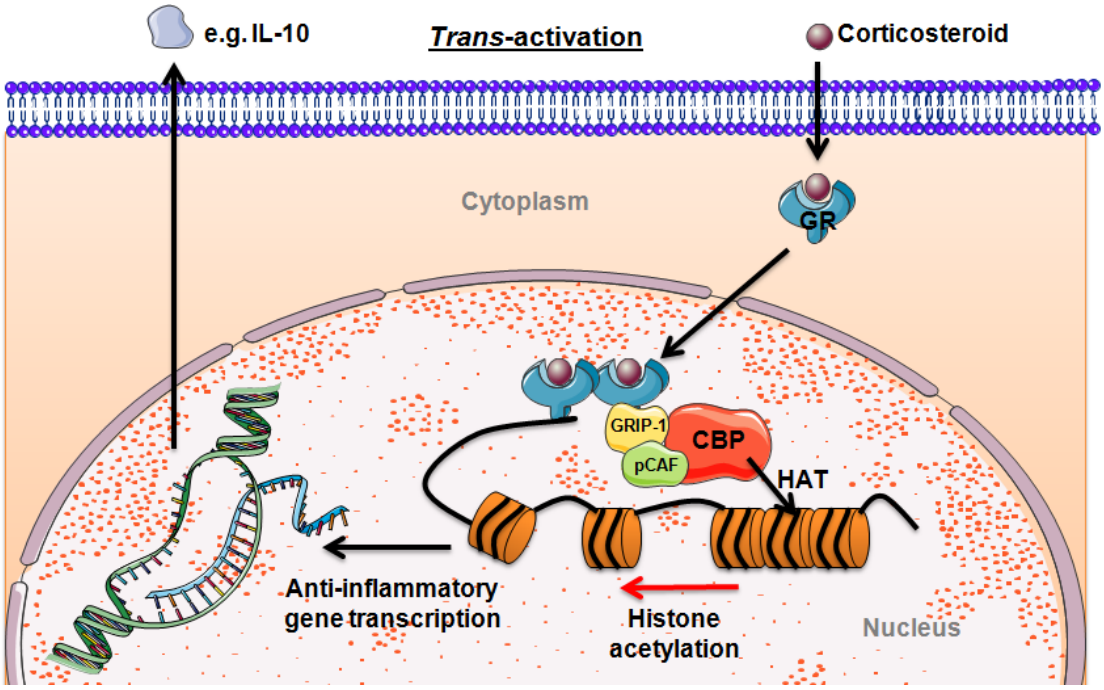


Figure 2: Transactivation of anti-inflammatory gene expression by corticosteroids. Corticosteroids pass through the cell membrane and bind to glucocorticoid receptors (GR) in the cytoplasm and translocate to the nucleus. In the nucleus they increase transcription by binding to glucocorticoid response elements (GRE) and interactions with co-activators such as pCAF, GRIP-1 and CBP, inducing histone acetylation and anti-inflammatory gene trascription. pCAF= p300 and p300-CBP associated factor; CBP= cyclic AMP response element binding (CREB) binding protein; GRIP-1= GR interacting protein-1. Diagram redrawn and adapted from Barnes, 2006.

6.1.3.2 *TRANS*-REPRESSION

Trans-repression is the major mechanism by which corticosteroids exert their anti-inflammatory effect. GR monomers are able to form co-repressor complexes which in turn can inhibit activated transcription factors such as NF-κB and AP-1 via protein-protein interaction. This prevents their natural HAT activity, suppressing pro-inflammatory gene transcription (Glass & Ogawa, 2006). Corticosteroids can also recruit histone deacetylases (HDAC) which remove acetyl groups from histone protein, causing DNA to become less accessible for transcription (Ito *et al*, 2000). This process is shown in Figure 3. Other modifications such as phosphorylations, methylations and ubiquitinations are also important in corticosteroid gene suppression effects.

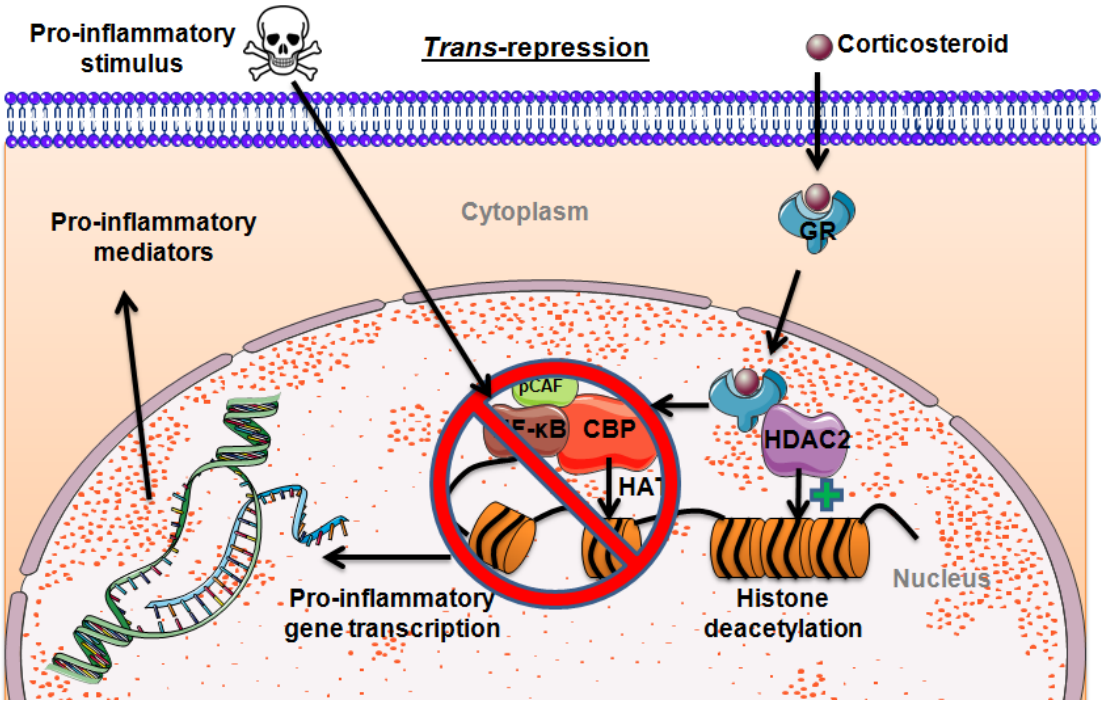


Figure 3: Transrepression of activated inflammatory genes. Inflammatory stimuli such as IL-1β and TNF-α stimulate an inflammatory cascade, which activates NF-κB. NF-κB translocates to the nucleus and forms a pro-inflammatory complex with co-activators such as pCAF and CBP. These activators have intrinsic HAT activity and acetylate histone proteins, increasing inflammatory gene transcription. Corticosteroids interfere with this process by binding to GR and translocating to the nucleus. In the nucleus they bind to co-activators, inhibiting HAT activity and also directly recruit HDAC-2. HDAC-2 deactylates histone proteins, suppressing gene transcription. NF-κB= nuclearfactor-kappaB; pCAF= p300 and p300-CBP associated factor; CBP= cyclic AMP response element binding (CREB) binding protein; HAT= histone acetyl transferase; HDAC= Histone deacetylase. Diagram redrawn adapted from Barnes, 2006.

6.1.4 Inhaled corticosteroid insensitivity

Resistance or insensitivity to corticosteroids exists in about 5% of the asthmatic population. Full corticosteroid resistance is rare and most patients lie on a spectrum of responsiveness (Szefler *et al*, 2002). Insensitivity to inhaled corticosteroids often results in higher doses being used which carry an increased risk of side effects (Schäcke *et al*, 2002). In patients completely unresponsive to inhaled corticosteroids, short bursts of oral corticosteroids are used to control symptoms (Chan *et al*, 1998). These are associated with extensive side effects and are therefore not desirable for long term control. Patients that are unresponsive to corticosteroid treatment are

more likely to have poorly controlled asthma and present considerable management and economic problems.

Patients with corticosteroid insensitive asthma display increases in mast cells, macrophages and lymphocytes. Increased levels of ECP, IL-8 and MPO are also observed and correspond with increases in eosinophils and neutrophils (Jatakanon *et al*, 1999). There is considerable heterogeneity in the presence of these 2 cells between individuals, suggesting several sub-groups of patients may exist (Wenzel *et al*, 1999). Whether these differences are due to variability in individuals' corticosteroid treatment is currently unknown. This is possible though as neutrophils are known to be less corticosteroid responsive then eosinophils (Strickland *et al*, 2001). The presence of neutrophils in corticosteroid insensitive asthmatics has led to the suggestion that the activation of the innate immune system is critical in its development. In particular, activation of interferon and TLR4 pathways during viral and LPS/bacterial induced asthma exacerbations have been implicated (Yang *et al,* 2009). Corticosteroid-insensitive asthmatics demonstrate inflammatory patterns characteristic of LPS activation, coinciding with high levels of LPS in lavage fluid (Goleva *et al*, 2008). Additionally, LPS induces neutrophilic inflammation which is unresponsive to inhaled corticosteroid treatment in healthy volunteers and asthmatics (Trapp *et al*, 1998; Michel *et al*, 2000). However, despite these observations the full relationship between LPS-induced asthma exacerbations and corticosteroid resistance remains to be fully characterised.

6.1.5 Mechanisms corticosteroid insensitivity

Several mechanisms have been implicated in corticosteroid insensitive asthma. These include decreased GR expression, decreased affinity of ligands for GR, decreased ability of GR to bind DNA, increased expression of pro-inflammatory transcription factors and decreased expression and activity of co-repressor proteins. Cellular mechanisms including Th17 lymphocytes and neutrophils have also been

proposed. The potential mechanisms are shown in Figure 4. The importance of these mechanisms probably varies between patients. It is know that the presence of corticosteroid insensitivity in asthma is not associated with any abnormality of cortisol levels or Addison's disease.

6.1.5.1 GR BINDING

A reduction in GR binding affinity to corticosteroids has been found in corticosteroid insensitive asthmatics. In most patients this defect was found to be reversible *in vitro* without the presence of IL-2 or IL-4 suggesting that the change is not intrinsic (Sher *et al*, 1994). One potential mechanism for this is increased expression of GR-β which can decrease the affinity of ligands for GR-α by the formation of hetrodimer complexes (Oakley *et al*, 1999). The combination of IL-2 and IL-4 or IL-13 alone increases the expression of GR-β but with no change in GR-α levels (Leung and Bloom, 2003). Increased expression of GR-β has been shown in macrophages of corticosteroid insensitive patients (Goleva *et al*, 2006). The high expression of GR-β in neutrophils, prevalent in corticosteroid insensitive asthma may also explain their insensitivity to steroids (Strickland *et al*, 2001). However, increased GR-β levels have not been found in all patients so may represent just one of many pathways that lead to corticosteroid insensitivity (Gagliardo *et al*, 2000; Butler *et al*, 2012). Other factors such as p38 MAPK and nitric oxide can also decrease GR activity, although the mechanism of this remains uncertain (Irusen *et al*, 2002; Galigniana *et al*, 1999).

6.1.5.2 GR NUCLEAR TRANSLOCATION AND GRE BINDING

Other groups of corticosteroid insensitive patients have shown decreased nuclear translocation of GR in human monocytes (Matthews *et al*, 2002). As with GR ligand affinity, p38 MAPK may also mediate this process (Irusen *et al*, 2002). The JAK-STAT pathway may also play a role in decreased nuclear translocation. Treatment of murine cells with IL-2 induced corticosteroid insensitivity which was reversed by a JAK3 inhibitor. The JAK3 inhibitor prevented GR and STAT5 binding, preventing nuclear translocation of GR (Goleva *et al*, 2002). However, Matthews *et al*, (2002)

demonstrated that in a separate group of patients nuclear translocation was normal but the ability of steroids to *trans*-activate certain genes was decreased.

6.1.5.2 COFACTOR ASSOCIATION

Corticosteroid insensitivity may also be due to an inability to repress pro-inflammatory signalling. Both AP-1 and NF-κB are redox sensitive proteins which demonstrate increased activity with increasing oxidative burden, as seen in asthma exacerbations or severe chronic inflammation (Rahman & MacNee, 1998). Increased levels of AP-1 are observed in corticosteroid insensitive asthma and concordantly a reduction in the ability of steroids to repress its pro-inflammatory activity (Adcock *et al*, 1995). A decrease in the ability to recruit co-repressor proteins such as HDAC may also be important in corticosteroid insensitivity. Many patients demonstrate no decrease in nuclear translocation or corticosteroid side effects (mediated by *trans*-activation) but decreased HDAC-2 function (Ito *et al*, 2006; Hew *et al*, 2006). The restoration of HDAC-2 activity by the drug theophylline restores corticosteroid sensitivity in alveolar macrophages *ex vivo* and *in vivo* (Ito *et al*, 2002; Ford *et al*, 2010). Decreased HDAC-2 activity may be direct or indirect.

Indirect routes include the PI3K/Akt pathway which is increased during oxidative stress. The PI3K-δ subtype is particularly important in the development of corticosteroid-insensitivity as mice without this protein are resistant to cigarette smoke-induced corticosteroid insensitivity (Marwick *et al*, 2009). Inhibition of this pathway by the PI3K inhibitor LY-294002 attenuates allergic inflammation, AHR and reverses corticosteroid resistance in mice (Barthel & Klotz, 2005). More selective inhibitors of PI3K-δ such as Nortriptyline have similar biological actions but lower incidence of toxic side-effects (Mercado *et al*, 2011). Directly, reactive oxygen species such as hydrogen peroxide and SIN-1 (a peroxynitrate generator) are able to reduce HDAC-2 activity and increased tyrosine nitiration of HDAC-2 (Ito *et al*, 2004). However, a recent study has called into question the importance of this mechanism in a majority of patients (Butler *et al*, 2012).

6.1.5.4 CELLULAR MECHANISMS

Neutrophils are implicated in the development of corticosteroid insensitive asthma. Their survival is increased in severe asthma and apoptosis inhibited by corticosteroid treatment (Uddin *et al*, 2009; Cox, 1995). This is associated with high constitutive levels of GR-β in circulating neutrophils and decreased expression of GR-α upon entering the airway (Strickland *et al*, 2001; Plumb *et al*, 2012). This particular modification may make airway neutrophils especially insensitive to corticosteroid treatment. Continued exposure to neutrophil promoting factors may facilitate neutrophil tissue damage and further oxidative stress induced modifications to corticosteroid responsiveness (Leung & Bloom, 2003).

Th17 cells and their cytokines have also been implicated in corticosteroid insensitive asthma. IL-17, a hallmark cytokine of Th17 responses is increased with neutrophilia in asthma (Bullens *et al*, 2006). Th17 cells are insensitive to corticosteroid both *in vitro* and *in vivo.* Additionally, Th17 cell transfer decreased responsiveness to corticosteroids in animals previously sensitive to their effects (Mckinley *et al*, 2008). Whether IL-17 represents a distinct pathway of corticosteroid insensitivity or can occur alongside the other potential mechanisms is unknown.

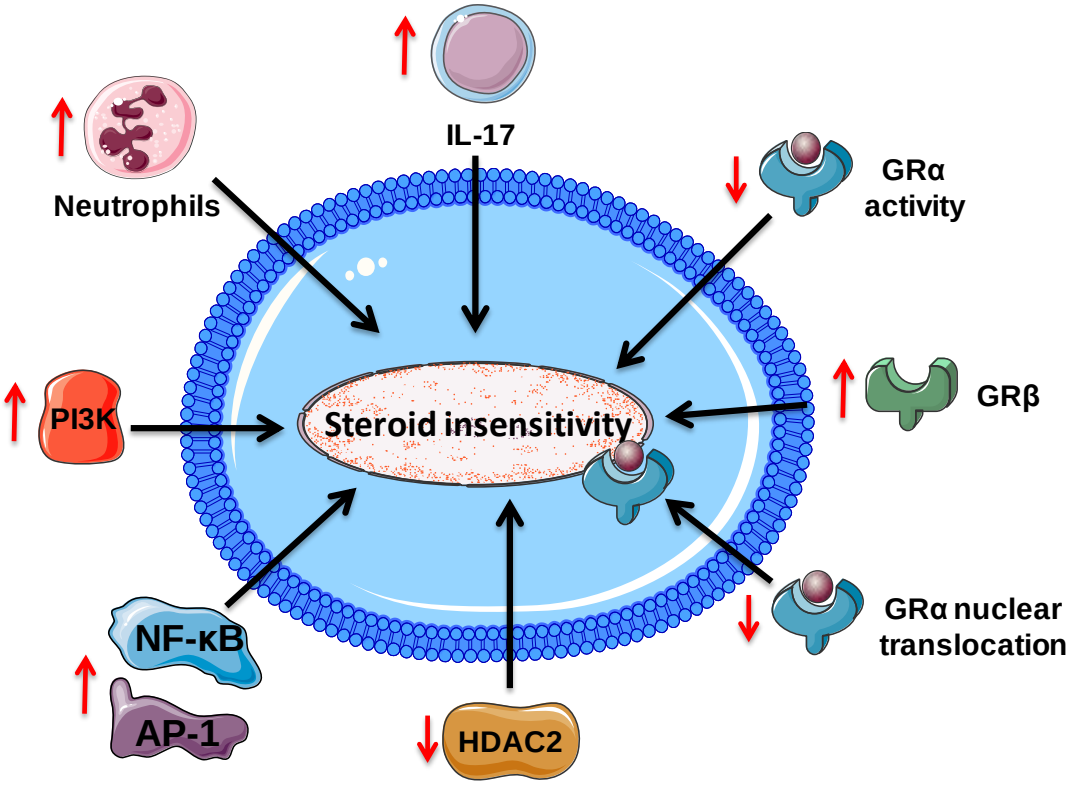


Figure 4: Diagram of the potential mechanisms of corticosteroid insensitivity in asthma.

6.1.5 Effect of inhaled corticosteroids in animal models

Inhaled corticosteroids such as fluticasone propionate (FP) have a range of effects on animal models of asthma. Evans *et al*, (2012) demonstrated that inhaled FP abolishes the late asthmatic response but has no effect on the early asthmatic response in guinea-pigs. Coinciding with this was a suppression of AHR and a significant reduction in eosinophilia and lymphocytes. These effects in guinea-pigs have also been confirmed by other groups (Lawrence *et al*, 1998).

Few groups have examined the effect of inhaled corticosteroids on asthma exacerbations. Animal models demonstrating partial insensitivity to inhaled corticosteroids have been developed. Ito *et al*, (2008) demonstrated that low level Ova challenge for 4 weeks followed by a large exacerbating dose can evoke partially inhaled corticosteroid insensitive inflammation and fully insensitive AHR in mice. This was associated with a decrease In HDAC-2 activity. Singam *et al*, (2006)

demonstrated similar results in mice using respiratory syncytial virus infection superimposed on allergen challenge. Inhaled FP did not reduce AHR, lymphocytes and neutrophils but did reduce eosinophils. Essilfie *et al,* (2012) demonstrated that *H influenza* before allergen sensitisation in mice increases neutrophilia and Th17 lymphocyte responses but decreased AHR, eosinophilia, total cell counts and Th2 cytokines. Treatment with a dose of intranasal dexamethasone, effective on Ova evoked responses alone does not reduce the *H.influenza* induced changes. Presently, no one has examined the effect of inhaled steroids in a model demonstrating exacerbation of functional and allergic responses to LPS.

6.2 HYPOTHESIS

'LPS exposure will decrease the inhaled corticosteroid sensitivity of functional and inflammatory responses in an acute ovalbumin model of asthma'

6.3 AIMS AND OBJECTIVES

The aim of this chapter was to determine the sensitivity to the inhaled corticosteroid, fluticasone propionate, of functional and inflammatory responses in ovalbumin, LPS and ovalbumin and LPS models. The initial aim was to establish an inhaled fluticasone propionate dose which significantly attenuates the late asthmatic response, AHR and cellular inflammation in an ovalbumin model. The effective dose was then used to establish the corticosteroid sensitivity of these responses in LPS alone and ovalbumin and LPS combination models. The effect of inhaled fluticasone propionate on lavage fluid protein and lung cytokines was also established. Whether the timing of LPS exposure alters the corticosteroid sensitivity of responses was also assessed by comparing 2 ovalbumin and LPS models used in chapter 5.

6.4 METHODS

Methods describing the measurement of lung function, AHR, airway oedema, airway inflammation and cytokine levels can be found in more detail in chapter 2.

6.4.1 OVALBUMIN PROTOCOLS

6.4.1.1 SENSITISATION

Guinea-pigs (200-300g, Dunkin-Hartley, male) were sensitised by a bilateral intra-peritoneal injection of a solution containing 150µg ovalbumin (Ova) and 100mg aluminium hydroxide ($Al(OH)_3$) in normal saline on day 1, 4 and 7.

6.4.1.2 ACUTE OVALBUMIN MODEL

Guinea-pigs were exposed to inhaled Ova (0.03%) for 1 hour on day 21 in a Perspex container as described in section 6.4.1.2. The lung function response to Ova was assessed over 12 hours. The bronchoconstrictor response to 0.3mM histamine was assessed on day 15 and 22 as described in section 6.4.5. FP (0.05, 0.1, 0.5 and 1mg/ml per day split into twice daily doses) was administered by inhalation in a Perspex chamber for 15 minutes, twice a day from day 16-21. On day 21 FP was administered 30 minutes pre- and 6 hours post-Ova challenge. A diagram of this protocol is shown in Figure 5.

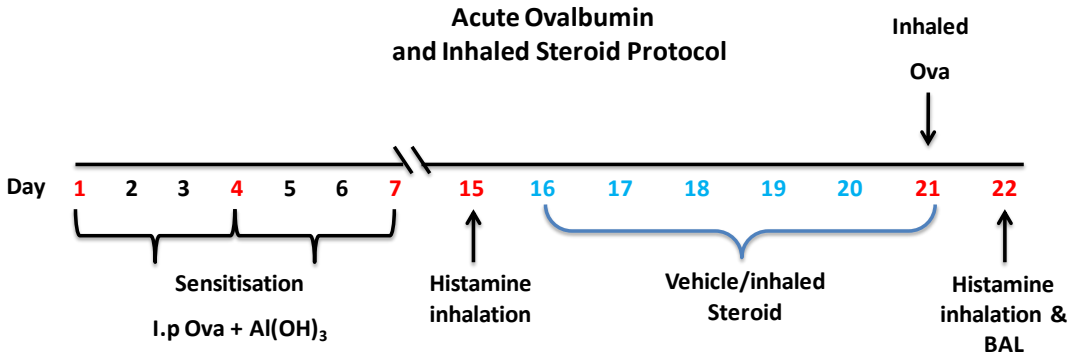


Figure 5: The acute ovalbumin (Ova) protocol. Guinea-pigs were challenged with inhaled Ova (0.03% on day 21). Fluticasone propionate (FP) was dosed twice daily for 6 days

6.4.1.3 LPS Pre-Acute Ovalbumin Exposure Protocol

Sensitised guinea-pigs were exposed to inhaled LPS (30µg/ml) 24 hours before Ova challenge for 1 hour in a Perspex box. On day 21 guinea-pigs were exposed to Ova (0.03%) for one hour. The bronchoconstrictor response to histamine was assessed on day 15 and day 22. FP (0.5mg/ml per day, split into twice daily doses) was administered by inhalation in a Perspex chamber for 15 minutes, from day 16-21. On day 20 and 21 FP was administered 30 minutes pre- and 6 hours post-Ova or LPS challenge. A diagram of this protocol is shown in Figure 6.

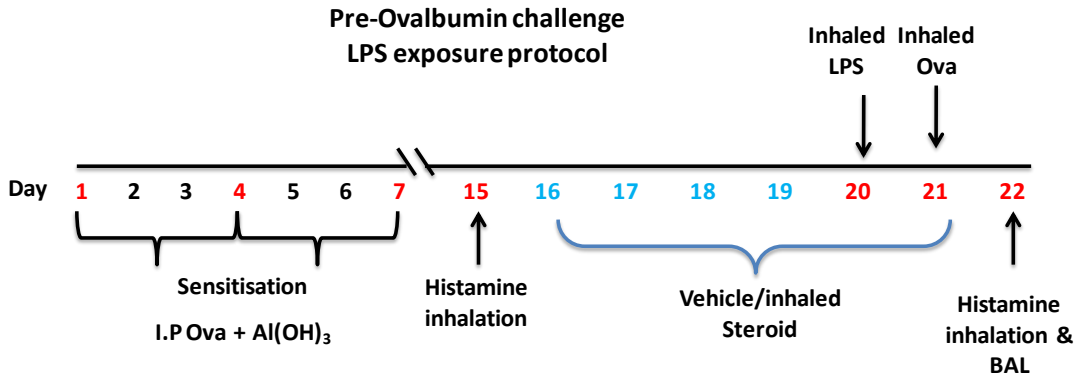


Figure 6: Diagram of the pre-ovalbumin (Ova) challenge LPS exposure protocol. Guinea-pigs were challenged with LPS (30µg/ml) 24 hours pre-Ova exposure. Fluticasone propionate (FP) was dosed twice daily for 6 days

6.4.1.4 Acute Ova and LPS co-exposure model

Sensitised guinea-pigs were exposed to inhaled LPS (30µg/ml) 48 hours before Ova challenge in an exposure chamber and both LPS (30µg/ml) and Ova (0.03%) co-administered on day 21. The bronchoconstrictor response to histamine was assessed on day 15 and day 22. FP (0.5 or 1mg/ml per day, split into twice daily doses) was administered by inhalation in a Perspex chamber for 15 minutes, from day 16-21. On day 19 and 21 FP was administered 30 minutes pre- and 6 hours post-Ova or LPS challenge. A diagram of this protocol is shown in shown in Figure 7.

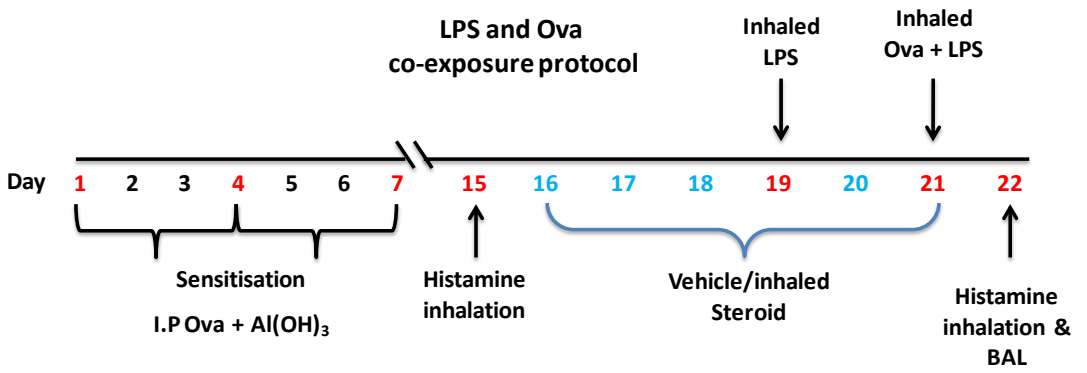


Figure 7: Diagram of the LPS and Ova co-exposure protocol. Guinea-pigs were exposed to LPS (30µg/ml) on day 19 and both Ova and LPS co-administered on day 21. Fluticasone propionate (FP) was dosed twice daily for 6 days

6.4.2 LPS EXPOSURE PROTOCOL

Non-sensitised guinea-pigs were exposed to LPS (30µg/ml) on day 5 and 7 of the protocol. The bronchoconstrictor response to histamine was assessed on day 1 and day 8. FP (0.5 or 1mg/ml per day, split into twice daily doses) was administered by inhalation in a Perspex chamber for 15 minutes, from day 2-7. On day 5 and 7 FP was administered 30 minutes pre- and 6 hours post-LPS challenge. A diagram of this protocol is shown in Figure 8

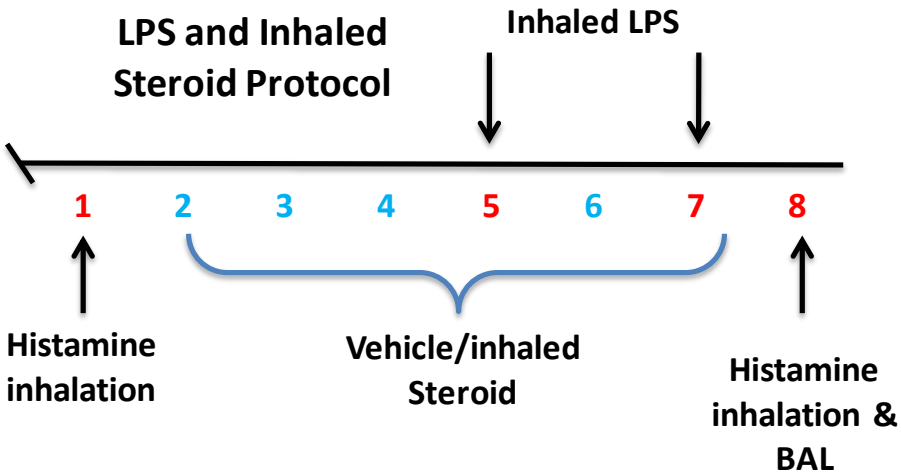


Figure 8: Diagram of the LPS alone exposure protocol. Guinea-pigs were exposed to 30µg/ml of LPS on day 5 and 7. Fluticasone propionate (FP) was dosed twice daily for 6 days.

6.4.3 Drug administration

FP was dissolved in ethanol (30%), DMSO (30%) and saline (40%) and nebulised for 15 minutes into a Perspex exposure chamber using a DeVilbiss nebuliser.

6.4.4 Measurement of lung function

Lung function was measured by whole body plethysmography following final Ova, LPS or Ova /LPS exposure in all protocols. Lung function was recorded hourly for 12 hours and every 15 minutes during the first hour of measurements. A final measurement was also taken 24 hours post-final challenge. All values were expressed as a percentage of the baseline reading, taken before the final challenge. A negative percentage change in baseline value represents a bronchoconstriction. In Ova protocols, early (0-6 hours) and late (6-12 hours) asthmatic responses were expressed as the peak bronchoconstriction during that period (displayed as a histogram next to a time course plot) and the area under the curve. The duration of the early phase was expressed as the time taken to recover to 50% of peak early phase bronchoconstriction values. In the LPS only protocol, lung function was measured for 12 hours after the second LPS exposure. The peak bronchoconstriction during this period was expressed on a histogram next to the time course plot.

6.4.5 Airways Hyperresponsiveness assessment.

AHR was determined using whole body plethysmography. This was performed by measuring the bronchoconstrictor response to histamine before inhaled corticosteroid treatment and 24 hours post-final LPS, Ova, LPS or Ova challenge. Histamine was delivered as described in chapter 2. Lung function was measured just before histamine inhalation and at 0, 5 and 10 minutes post-histamine exposure. The peak bronchoconstriction during this period was expressed on a histogram.

6.4.6 Assessment of airways inflammation

Following final histamine exposure guinea-pigs were sacrificed by an intra-peritoneal overdose of sodium pentobarbitone, the lungs excised and lavaged. Total and differential cell counts were then performed as described in chapter 2.

6.4.7 Quantification of cytokine levels using enzyme-linked immunosorbent assay (ELISA)

Levels of IL-8, IL-17 and IL-13 were measured in diluted homogenised lung samples using ELISA supplied by R&D systems. Cytokine levels were adjusted for total lung protein and expressed as weight per mg of lung as described in section 2.2.3.2.

6.4.8 Assessment of airway oedema

Protein content in lavage fluid was determined as a measure of airway oedema by BCA protein assay as per the manufacturer's instructions (Pierce protein biology).

6.5 Results

6.5.1 Sensitivity of ovalbumin responses to inhaled Fluticasone propionate

Guinea-pigs treated with 0.05mg/ml FP (-68.9±3.3%), 0.1mg/ml FP (-71.7±2.9%) and vehicle (-69.4±7.5%) demonstrated an immediate bronchoconstriction. FP had no effect on the time course of the EAR. A further bronchoconstriction was observed in all groups between 6-12 hours including 0.05mg/ml FP at 8 hours (-17.2±5.7%), 0.01mg/ml FP (-15.2±4.6%) and vehicle at 7 hours (-19.7±5.7%) (Figure 9, time course. No significant change in peak EAR, LAR or 24 values were observed with 0.05mg/ml and 0.1mg/ml FP treatment (Figure 9, histogram).

Guinea-pigs treated with 0.5mg/ml FP (-59.0±4.6%), 1mg/ml FP (-67.9±2.3%) and vehicle (-69.4±7.5%) demonstrated an immediate bronchoconstriction. Treatment with FP did not significantly change the EAR time course. A further bronchoconstriction was observed in vehicle treated guinea-pigs between 6-12 hours, at 7 hours the sGaw value was -19.7±5.7%. Guinea-pigs treated with 0.5mg/ml or 1mg/ml FP demonstrated significantly smaller bronchoconstrictions than vehicle between 7 and 10 hours; at 7 hours sGaw values were 2.8±3.2% and -1.3±2.8% respectively (Figure 10, time course). Peak EAR responses were not significantly changed by FP treatment. Peak LAR were significantly attenuated with 0.5mg/ml and 0.1mg/ml FP (-10.2±1.9% and -8.6±2.4%) compared with vehicle (-26.8±3.8%). No significant difference in sGaw values was observed 24 hours post-Ova challenge (Figure 10, histogram)

No significant difference in the total and EAR AUC were observed with FP treatment, although a non-significant trend for decreased EAR was observed with the 0.5 and 1mg/ml FP. LAR area under the curve decreased in a dose-dependent manner with

FP. This reached significance in guinea-pigs treated with 0.5mg/ml FP (16.8±6.2%.hr compared to 72.3±10.5%.hr). Guinea-pigs treated with 1mg/ml FP demonstrated a trend for decreased LAR area but this did not reach significance (Figure 11).

Vehicle treated guinea-pigs demonstrated a significant increase in the bronchoconstrictor response to histamine post-Ova compared to pre-Ova challenge (-25.7±5.6% compared to 3.4±3.3%). At 5 minutes post-histamine challenge, this bronchoconstriction was no longer significant compared to pre-challenge sGaw values (-6.8±5.0% compared to -2.3±1.9%). Guinea-pigs treated with 0.05mg/ml and 0.1mg/ml FP demonstrated significant increases in the bronchoconstrictor responses to histamine post-Ova challenge (-21.4±7.5% at 5 minutes compared to pre-challenge, 5.3±1.5%,; -14.8±3.8% at 0 minutes compared to pre-challenge -4.8±1.7%, respectively). Both these bronchoconstrictions returned to pre-challenge levels after 10 minutes. Guinea-pigs treated with 0.5 or 1mg/ml FP demonstrated no bronchonstrictor response to histamine post-Ova compared to pre-challenge values. Figure 12F demonstrates the peak bronchoconstrictor response to histamine both pre- and post-Ova challenge. Vehicle, 0.05mg/ml FP and 0.1mg/ml FP treated animals demonstrated a significant increase in bronchonstrictor response to histamine post- compared to pre-Ova challenge (-21.4±6.3% compared to -1.7±3.8%; -18.9±4.1% compared to -6.7±1.8%; -28.5±8.1% compared to -4.7±2.8% respectively) (Figure 12).

The total protein content in lavage fluid, of guinea-pigs challenged with Ova and treated with 0.05, 0.1, 0.5 or 1mg/ml FP or vehicle demonstrated a dose-dependent decrease with FP treatment but did significantly attenuate this response even at the highest FP dose, 1mg/ml (Figure 13).

0.05mg/ml FP treatment did not significantly reduce any cell type measured. Both 0.5mg/ml and 1mg/ml FP significantly decreased total cell numbers, compared to vehicle (5.2±0.4x10^6/ml, 4.5±0.4x10^6/ml compared to 9.0±0.45x10^6/ml respectively). This decrease was further characterised as both a significant decrease in eosinophils (2.0±0.23x10^6/ml, 1.8±0.17x10^6/ml compared to 3.6±0.235x10^6/ml respectively) and macrophages (2.74±0.28x10^6/ml, 2.1±0.19x10^6/ml compared to 4.38±0.24x10^6/ml respectively). Macrophage numbers were also reduced in 0.1mg/ml FP treated group (3.07±0.34x10^6/ml). Lymphocytes demonstrated a significant dose-dependent decrease with increasing FP dose, for 0.5 and 1mg/ml FP it was 0.07±0.02x10^6/ml and 0.08±0.01x10^6/ml respectively, compared to vehicle (0.23±0.01x10^6/ml). Neutrophil numbers were unchanged from vehicle level in FP treatment groups (Figure 14).

IL-13 levels, increased in Ova challenged and vehicle treated guinea-pigs (13.9±1.4ng/mg) compared to naïve (8.8±0.9ng/mg), decreased non-significantly with increasing FP dose. IL-17 levels were also elevated in vehicle groups (306.1±17.9pg/mg compared to 123.6±6.5) were not significantly reduced by FP in any group, although 0.5 and 1mg/ml FP did tend to produce lower IL-17 levels. IL-8 was not detectable in samples and is not shown (Figure 15).

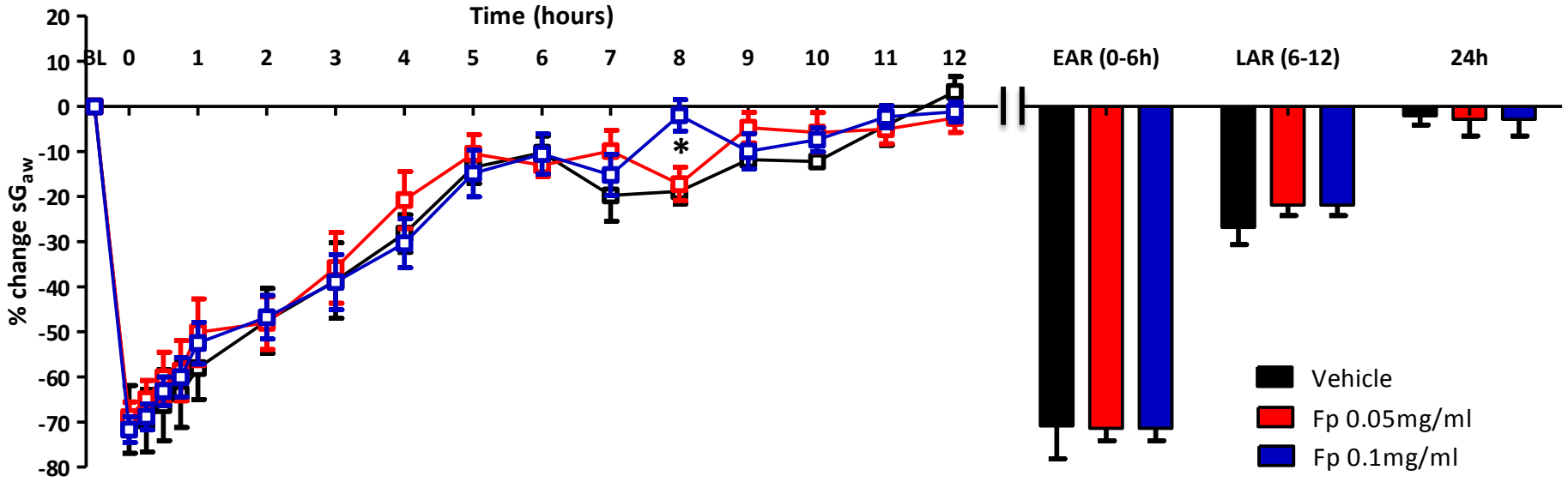


Figure 9: The mean time-course values of sGaw in ovalbumin (Ova) sensitised and challenged guinea-pigs treated with either inhaled fluticasone propionate (FP, 0.05mg/ml or 0.1mg/ml), split into twice daily doses. The histogram represents the maximum bronchoconstriction values recorded during the early asthmatic response (EAR) (0-6 hours), late asthmatic response (LAR) (6-12 hours) and 24 hours readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline prior to Ova challenge. A negative value represents a bronchoconstriction. N=5 (Ova + vehicle), N=10 (Ova + 0.05mg/ml FP), N=9 (Ova + 0.1mg/ml FP). *Significantly different from vehicle treatment $p<0.05$, performed with one-way analysis of variance followed by Dunnet's post-test.

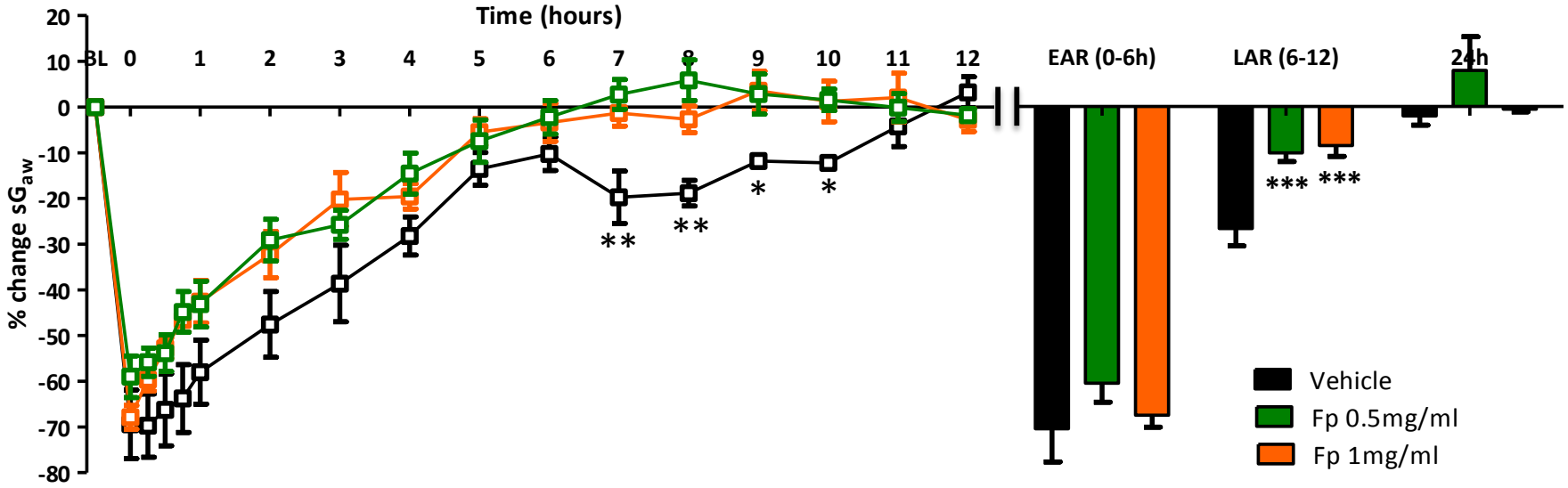


Figure 10: The mean time-course sGaw values in ovalbumin (Ova) sensitised and challenged guinea-pigs treated with either inhaled fluticasone propionate (FP, 0.5mg/ml or 1mg/ml), split into twice daily doses. The histogram represents the maximum bronchoconstriction values recorded during the early asthmatic response (EAR) (0-6 hours), late asthmatic response (LAR) (6-12 hours) and 24 hours readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline prior to Ova challenge. A negative value represents a bronchoconstriction. N=5 (Ova + vehicle), N=9 (Ova + 0.5mg/ml FP), N=6 (Ova + 1mg/ml FP). *Significantly different from vehicle treatment $p<0.05$, ** $p<0.01$; *** $p<0.001$; performed with one-way analysis of variance followed by Dunnet's post-test.

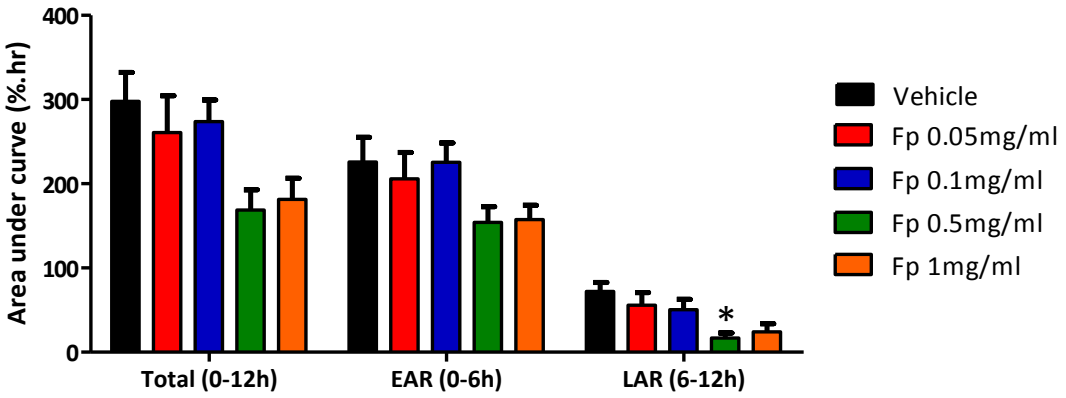


Figure 11 Area under the curve analysis of sGaw values over 12 hours following ovalbumin (Ova) challenge in guinea-pigs treated with either inhaled vehicle or fluticasone propionate (FP; 0.05mg/ml, 0.1mg/ml, 0.5mg/ml or 1mg/ml) split into twice daily doses. For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-12 hours, early asthmatic response (EAR) includes values from 0-6 hours and late asthmatic response (LAR) includes values from 6-12 hours. Area under the curve is expressed as %.hour. N=5 (vehicle), N=10 (0.05mg/ml FP), N=9 (0.1mg/ml FP), N=9 (0.5mg/ml FP), N=6 (1mg/ml FP); *significantly different from vehicle treatment $p<0.05$; performed with one-way analysis of variance followed by Dunnet's post-test.

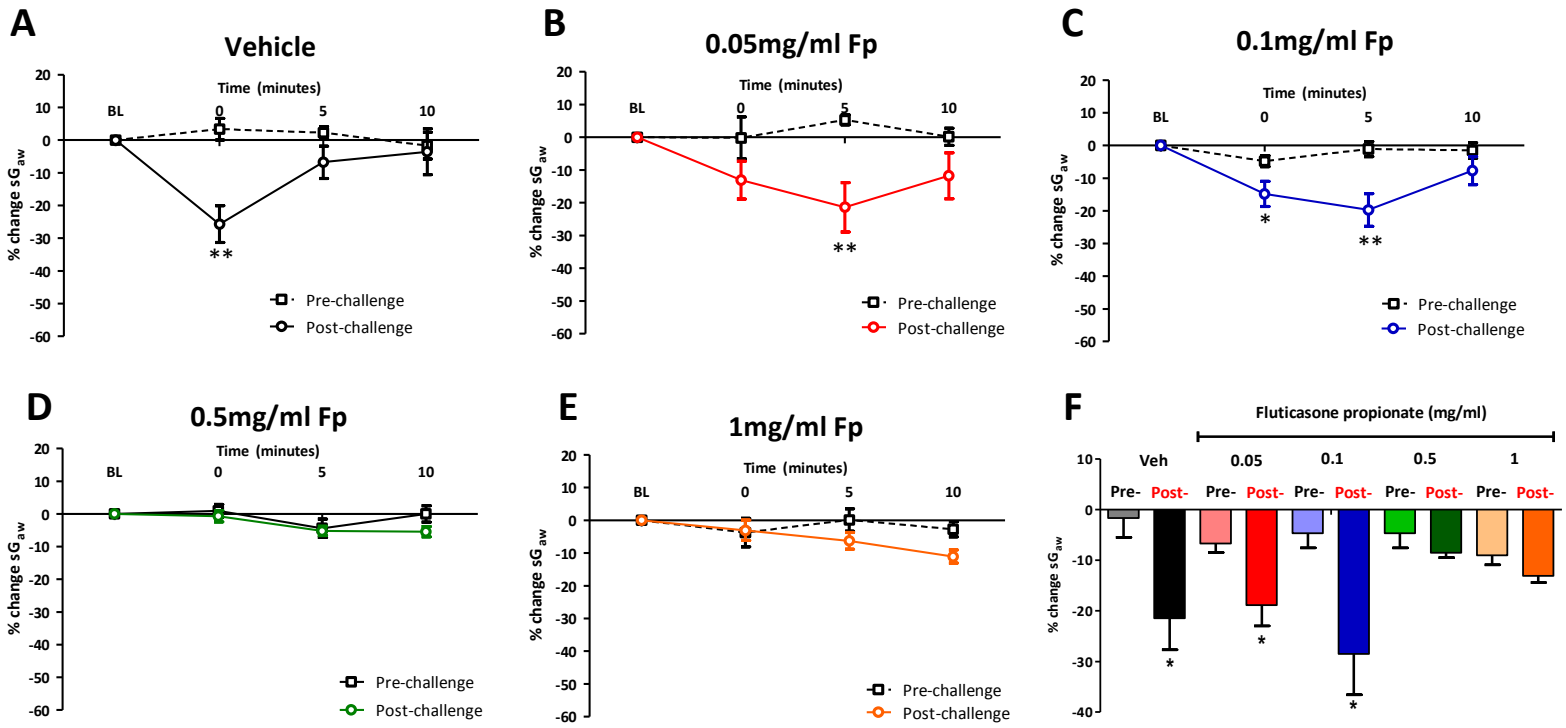


Figure 12: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles and 0.5LPM flow per chamber over 2 minutes, 1 minute drying period,) in ovalbumin (Ova) sensitised and challenged guinea-pigs treated with either inhaled **A)** vehicle or fluticasone propionate (FP) (**B)** 0.05mg/ml, **C)** 0.1mg/ml, **D)** 0.5mg/ml or **E)** 1mg/ml split into twice daily doses. **F)** Represents the peak bronchoconstriction to histamine pre- and post-Ova challenge. Values were recorded 7 days pre- and 24 hours post-Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=5 (vehicle), N=10 (0.05mg/ml FP), N=9 (0.1mg/ml FP), N=9 (0.5mg/ml FP), N=6 (1mg/ml FP). *Significantly different from time paired pre-Ova challenge values $p<0.05$, ** $p<0.01$; performed with a two tailed T-test.

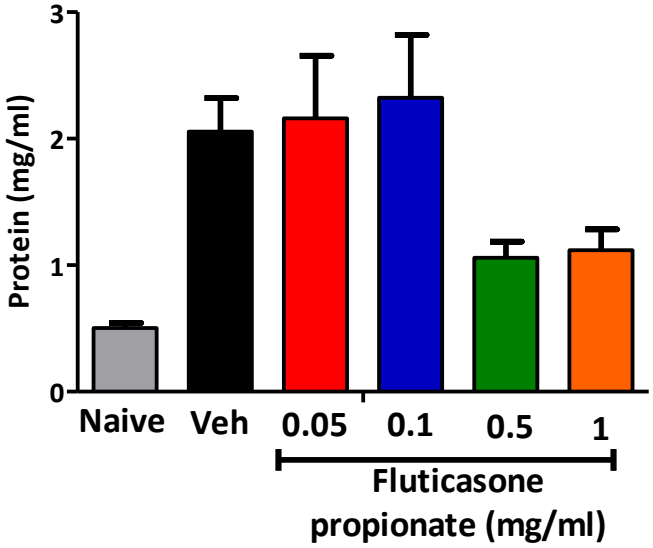


Figure 13: Total lavage fluid protein in ovalbumin (Ova) sensitised and challenged guinea-pigs treated with either inhaled vehicle or fluticasone propionate (FP) (0.05mg/ml, 0.1mg/ml, 0.5mg/ml or 1mg/ml) split into twice daily doses. N=5 (naïve and vehicle), N=10 (0.05mg/ml FP), N=9 (0.1mg/ml FP), N=9 (0.5mg/ml FP), N=6 (1mg/ml FP); performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

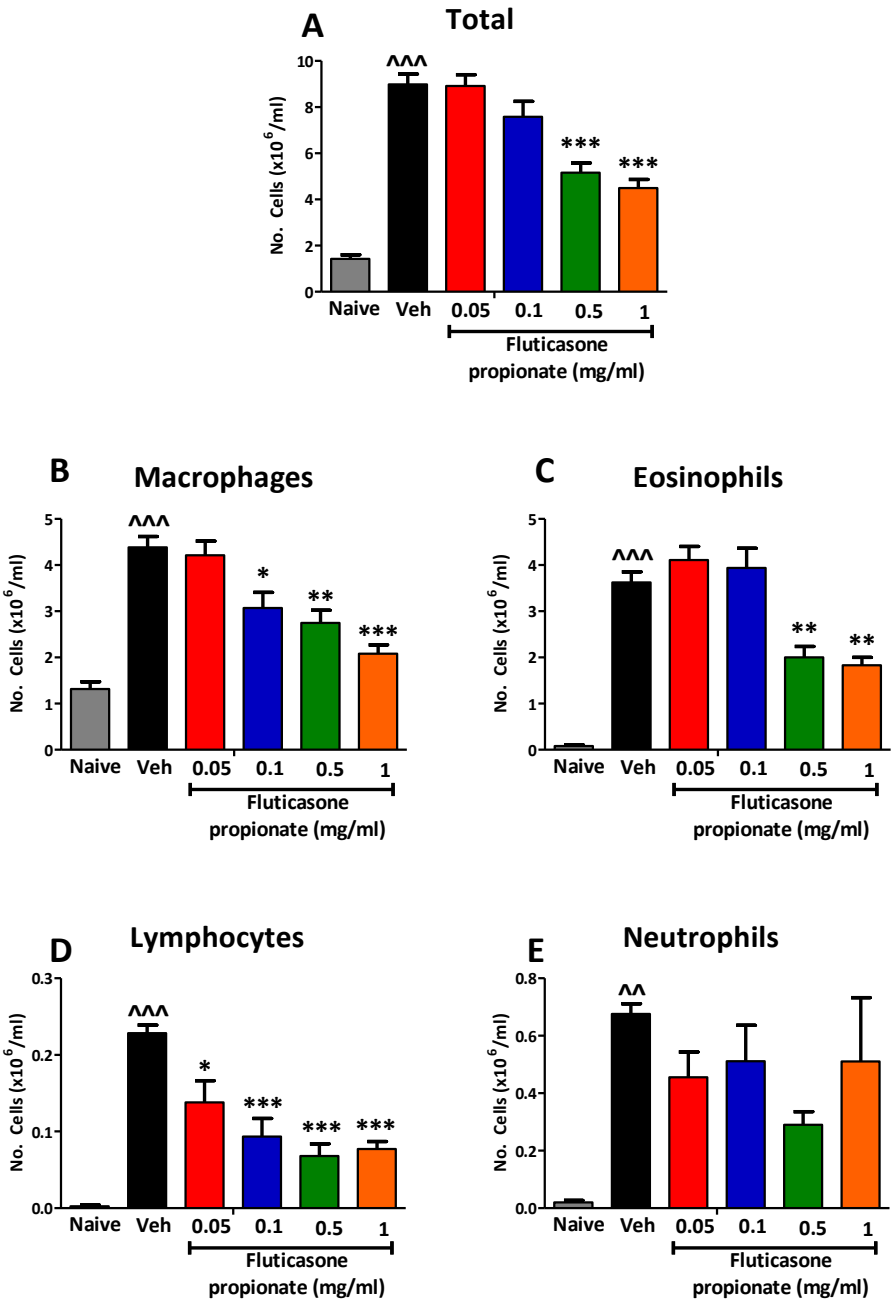


Figure 14 The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in bronchoalveolar fluid in ovalbumin (Ova) sensitised and challenged guinea-pigs treated with vehicle or inhaled vehicle fluticasone propionate (FP) (0.05mg/ml, 0.1mg/ml, 0.5mg/ml or 1mg/ml) split into twice daily doses. ^^ significantly different from naïve, $p<0.01$; ^^^ $p<0.001$; *significantly different from vehicle treatment $p<0.05$, ** $p<0.01$; *** $p<0.001$; N=5 (vehicle), N=10 (0.05mg/ml FP), N=9 (0.1mg/ml FP), N=9 (0.5mg/ml FP), N=6 (1mg/ml FP); performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

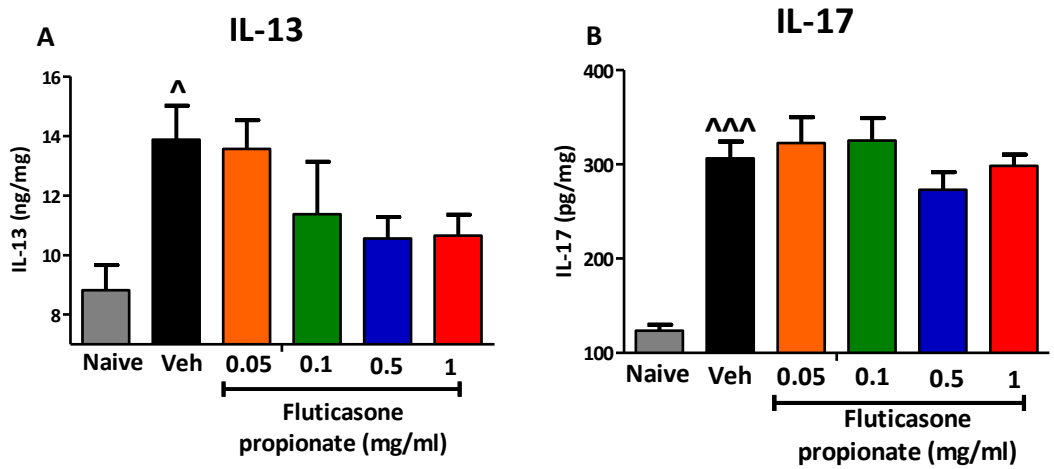


Figure 15: The concentration of A) IL-13 B) IL-17 in the lungs of ovalbumin (Ova) sensitised and challenged guinea-pigs treated with either inhaled vehicle fluticasone propionate (FP) (0.05mg/ml, 0.1mg/ml, 0.5mg/ml or 1mg/ml) split into twice daily doses. Results are expressed as mean±SEM; N=5 (vehicle), N=10 (0.05mg/ml FP), N=9 (0.1mg/ml FP), N=9 (0.5mg/ml FP), N=6 (1mg/ml FP); ^ significantly different from naïve, $p<0.05$; ^^^ $p<0.001$ performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

6.5.2 Sensitivity to inhaled Fluticasone propionate of Acute ovalbumin responses with LPS exposure 24 hours before

Both vehicle and FP treated guinea-pigs demonstrated early phase responses peaking within the first hour (-35.2±8.0% and -29.8±4.5%, respectively, 30 minutes post-Ova). This bronchoconstriction began to return to baseline within the first 6 hours and returned to baseline sGaw values by 12 hours. No significant difference in bronchoconstriction to Ova was observed at any time point in FP or vehicle treated guinea pigs. Figure 16 also represents the peak EAR, LAR and 24 sGaw values (Figure 16, time course). No significant difference in peak early and late phase responses (-22.0±3.9%, FP compared -20.3±2.8%, vehicle) was observed with FP treatment (Figure 16, histogram). No significant difference in the total, EAR and LAR area under the curve with FP treatment were observed (Figure 17).

Vehicle treated guinea-pigs demonstrated a significant increase in the bronchoconstrictor response to histamine 0 minutes post-Ova challenge compared to the pre-challenge sG_{aw} values (-19.3±7.5% and -0.04±0.9%). This bronchoconstriction returned to baseline by 10 minutes post-histamine challenge. In FP treated guinea-pigs, a significant bronchoconstriction to histamine was seen at 0 minutes (-16.9±5.0% compared to -1.3±1.3%), 5 minutes (-19.0±4.1% compared to -1.1±2.6%) and 10 minutes post-histamine challenge (-20.8±5.9% compared to -2.9±1.4%) (Figure 18). Figure 18 also presents the peak bronchoconstriction to histamine exposure pre- and post- Ova challenge. Guinea-pigs treated with vehicle demonstrated a significant increase in the peak bronchoconstrictor response to histamine post-Ova challenge (-20.7±6.8%) compared to pre-Ova challenge (4.2±2.1%). Guinea pigs treated with FP also demonstrated a significant increase in the peak bronchonstrictor response to histamine post-Ova challenge (-27.9±5.1% compared to -5.2±1.3%). No significant difference between peak post-Ova bronchoconstrictions were found.

Total cell numbers, increased in Ova challenged and vehicle treated animals ($14.1\pm0.8x10^6$/ml compared to naïve, $1.5\pm0.05x10^6$/ml were unchanged by FP treatment ($15.5\pm2.7x10^6$/ml). No change in eosinophils, macrophages and neutrophils cell population numbers were observed with FP treatment (Figure 19).

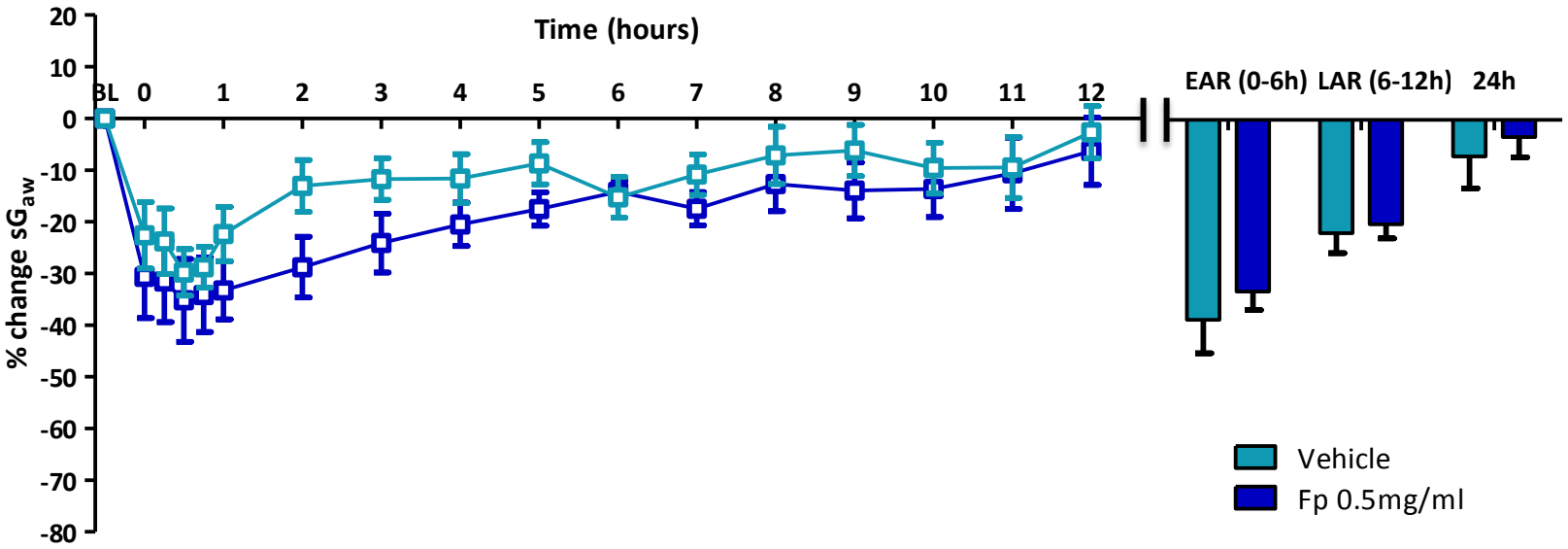


Figure 16: The mean time-course values of sGaw in ovalbumin (Ova) sensitised and challenged guinea pigs exposed to 30µg/ml LPS for 1 hour, 24 hours pre-Ova challenge and treated with vehicle and fluticasone propionate (FP) (0.5mg/ml, split into 2 twice daily 15 minute inhalations). The histogram represents the maximum bronchoconstriction values recorded during the early asthmatic response (EAR) (0-6 hours), late asthmatic response (LAR) (6-12 hours) and 24 hours readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline prior to Ova challenge. A negative value represents a bronchoconstriction. A negative value represents a bronchoconstriction. N=6 (vehicle), N=9 (FP); performed with two tailed T-test.

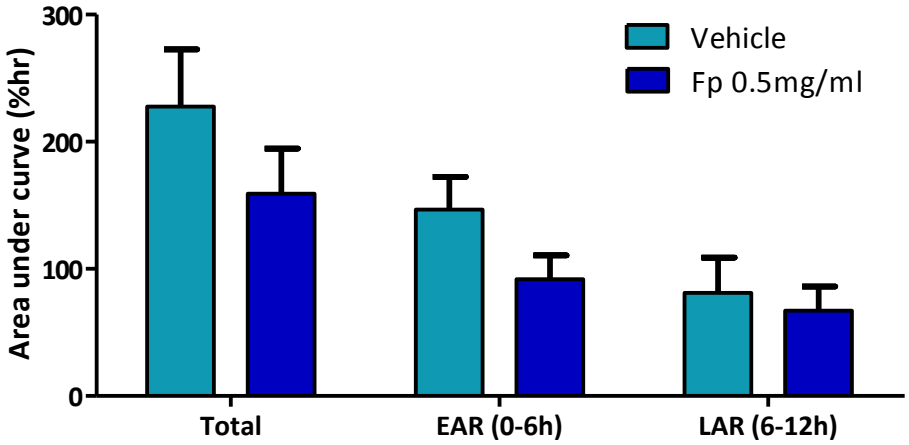


Figure 17: Area under the curve analysis of sGaw values over 12 hours following ovalbumin (Ova) challenge in guinea-pigs exposed to 30µg/ml LPS for 1 hour, 24 hours pre-Ova challenge and treated with vehicle or fluticasone propionate (FP) (0.5mg/ml, split into 2 twice daily 15 minute inhalations). For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-12 hours, early asthmatic response (EAR) includes values from 0-6 hours and late asthmatic response (LAR) includes values from 6-12 hours. Area under the curve is expressed as %.hour. N=6 (vehicle), N=9 (FP); performed with two tailed T-test.

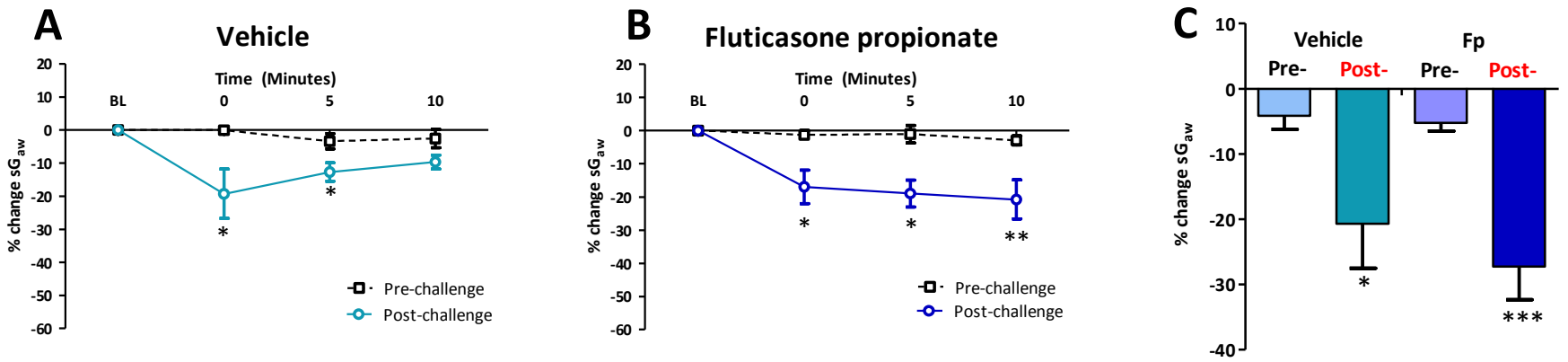


Figure 18: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles and 0.5LPM flow per chamber over 2 minutes, 1 minute drying period) in guinea-pigs exposed to 30µg/ml LPS for 1 hour, 24 hours pre-Ova challenge and treated with A) vehicle or B) fluticasone propionate (FP) (0.5mg/ml, split into 2 twice daily 15 minute inhalations). D) represents the peak sGaw values pre- and post-Ova challenge. Values were recorded 7 days pre- and 24 hours post-Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=6 (vehicle), N=9 (FP). *Significantly different from vehicle treatment $p<0.05$, ** $p<0.01$; *** $p<0.001$; performed with two tailed T-test.

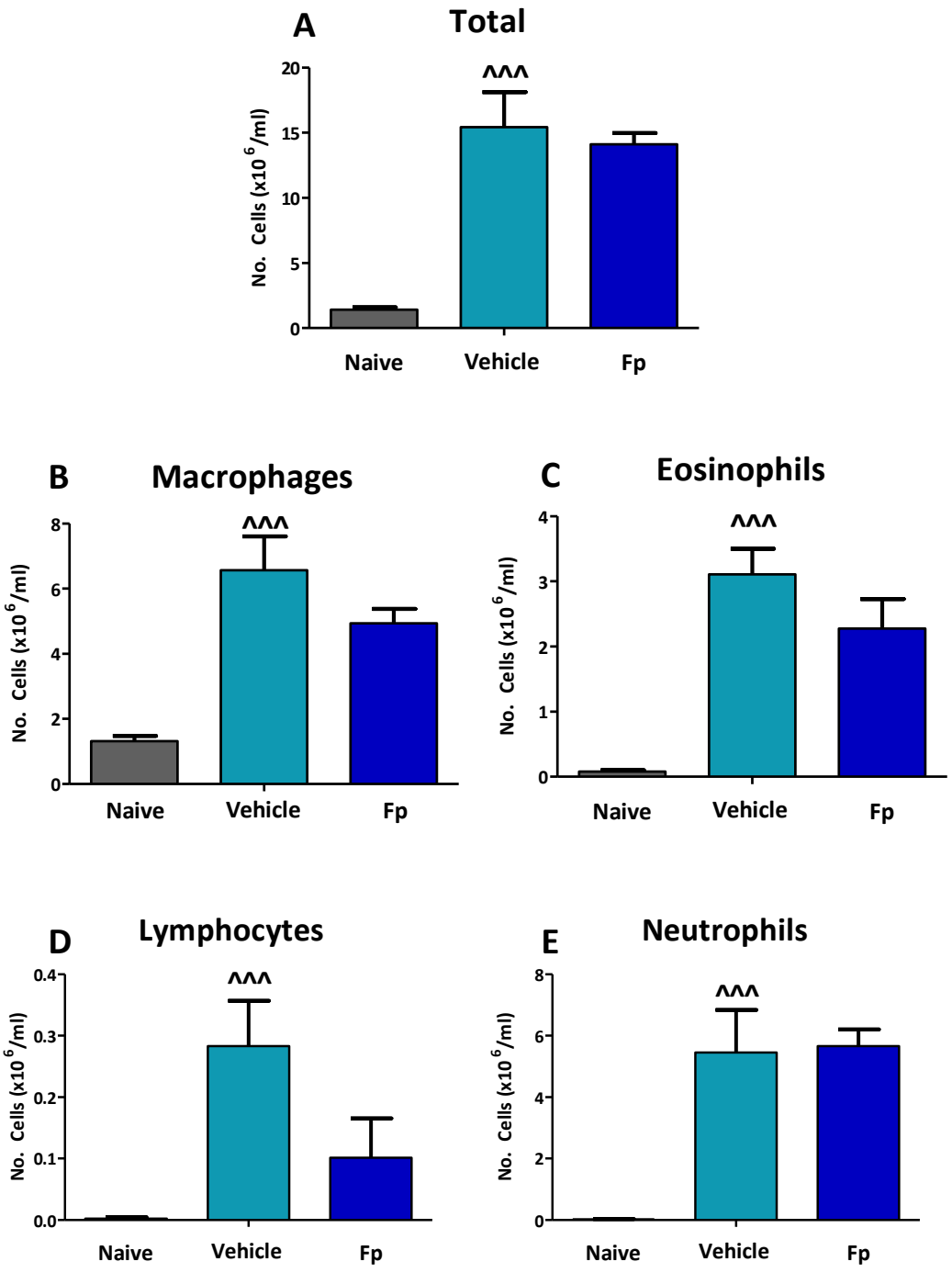


Figure 19: The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in bronchoalveolar fluid in guinea-pigs exposed to 30µg/ml LPS for 1 hour, 24 hours pre-Ova challenge and treated with vehicle or fluticasone propionate (FP) (0.5mg/ml, split into 2 twice daily 15 minute inhalations). N=6 (naïve and vehicle), N=9 (FP); ^significantly different from naive $p<0.05$, ^^^ $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

5.5.3 Sensitivity of ovalbumin and LPS co-administration protocol responses to inhaled Fluticasone propionate

Both vehicle and FP treated groups demonstrated EAR peaking in the first hour and returning to baseline values between 6-8 hours. No significant differences were observed between these groups. Vehicle and 0.5mg/ml FP treated guinea-pigs demonstrated a second delayed response peaking at 10 hours (-17.6±2.4% and -18.9±5.1%, respectively). This response was reduced but not significantly attenuated in 1mg/ml FP treated guinea-pigs at the same time point (-2.6±5.4%) (Figure 20, time course). No difference in the peak EAR was observed between all treatment groups. Peak LAR demonstrated a trend for a decrease with 0.5 and 1mg/ml FP treatment but did not reach significance. (-28.0±6.0% and -15.3±3.7% compared to vehicle, -34.6±6.8% respectively) No significant bronchoconstriction at the 24 hour reading was observed (Figure 20, histogram). No significant difference in total, EAR and LAR area under the curve were observed between all treatment groups (Figure 21). 0.5 or 1mg/ml FP not did not significantly reduce the time taken for the early phase to return to 50% of peak bronchoconstriction (3.7±0.4h, 4.0±0.4h compared to vehicle, 4.2±0.7h respectively) (Figure 22).

Vehicle treated guinea-pigs produced an immediate non-significant increase in the bronchoconstrictor response to histamine, post-Ova challenge. The bronchoconstriction continued to increase at 5 (-28.1±8.2% compared to pre-Ova: -1.8±3.3%) and 10 minutes (-34.7±8.5% compared to pre-Ova: -0.1±2.1%). 0.5 and 1mg/ml FP treatment did not significantly alter this response with significant constrictions still present at 10 minutes (-34.2±8.3% compared to pre-Ova: -0.1±2.0%; -31.7±11.8% compared to pre-Ova -4.5±1.9%, respectively) (Figure 23) Figure 23D also presents the peak bronchoconstriction to histamine pre- and post-Ova challenge. Guinea-pigs treated with vehicle, 0.5mg/ml FP and 1mg/ml FP demonstrated a significant increase in the peak bronchoconstriction to histamine

following Ova challenge (-33.3±4.5% compared to -9.4±7.1%; -40.2±9.0% compared to 6.1±1.6%; -41.2±10.6% compared to -9.9±2.0% respectively).

Lavage protein levels, elevated in vehicle treated guinea-pigs (3.05±0.1mg/ml, compared to naïve, 0.5±0.05mg/ml) were significantly reduced by 0.5 and 1mg/ml FP (1.4±0.1mg/ml and 1.8±0.4mg/ml respectively) compared to vehicle (Figure 24).

Total cells, increased in vehicle treated guinea pigs ($21.6\pm1.3x10^6$/ml, compared to naïve $1.4\pm0.2x10^6$/ml) were not significantly reduced by 0.5 or 1mg/ml FP ($21.6\pm1.3x10^6$/ml, and $18.0\pm2.6x10^6$/ml respectively). No specific cell population was reduced by FP treatment (Figure 25).

IL-8 and IL-17 levels were significantly increased in Ova challenged and vehicle treated guinea-pigs compared to naïve animals (16.5±1.6ng/mg compared to undetectable; 183.4±36.4ng/mg compared to 61.2±3.2ng/mg, respectively). IL-8 and IL-17 were not reduced by FP treatment. IL-13 was significantly decreased by both 0.5mg/ml FP and 1mg/ml (6.1±0.6ng/mg, 6.4±0.3ng/mg compared to vehicle, 8.6±0.6ng/mg) (Figure 26).

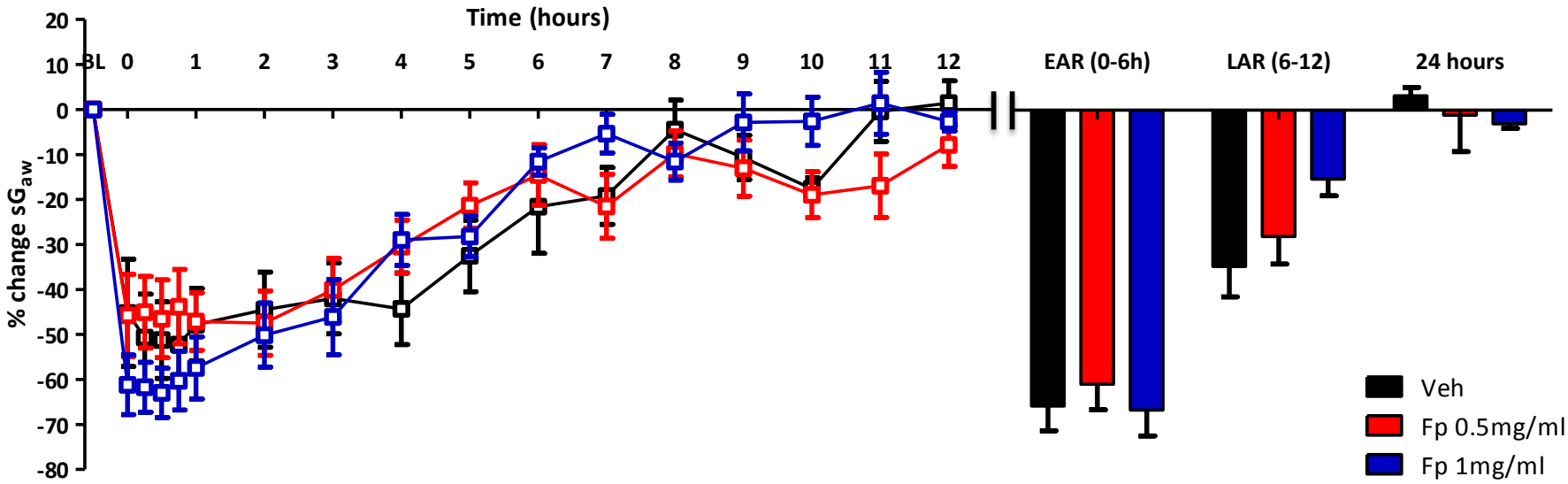


Figure 20: The mean time-course values of sGaw in ovalbumin (Ova) challenged guinea-pigs exposed to 30µg/ml LPS for 1 hour, 48 hours before Ova challenge and co-administered with Ova and treated with fluticasone propionate (FP) (0.05mg/ml or 1mg/ml, split into 2 twice daily 15 inhalations,) or vehicle. The histogram represents the maximum bronchoconstriction values recorded during the early asthmatic response (EAR) (0-6 hours), late asthmatic response (LAR) (6-12 hours) and 24 hours readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline prior to Ova challenge. A negative value represents a bronchoconstriction. N=7 (vehicle), N=7 (0.5mg/ml FP), N=5 (1mg/ml FP; performed with one-way analysis of variance followed by Dunnet's post-test.

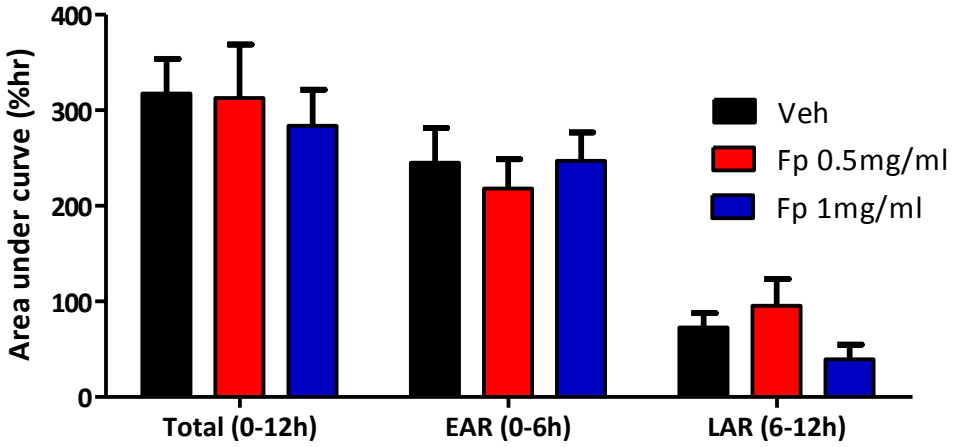


Figure 21: Area under the curve analysis of sGaw values over 12 hours in ovalbumin (Ova) challenged guinea-pigs exposed to 30µg/ml LPS for 1 hour, 48 hours before Ova challenge and co-administered with Ova and treated with fluticasone propionate (FP) (0.05mg/ml or 1mg/ml, split into 2 twice daily 15 inhalations) or vehicle. For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-24 hours, early asthmatic response (EAR) includes values from 0-6 hours and late asthmatic response (LAR) includes values from 6-12 hours. Area under the curve is expressed as %.hour. N=7 (vehicle), N=7 (0.5mg/ml FP), N=5 (1mg/ml FP); performed with one-way analysis of variance followed by Dunnet's post-test.

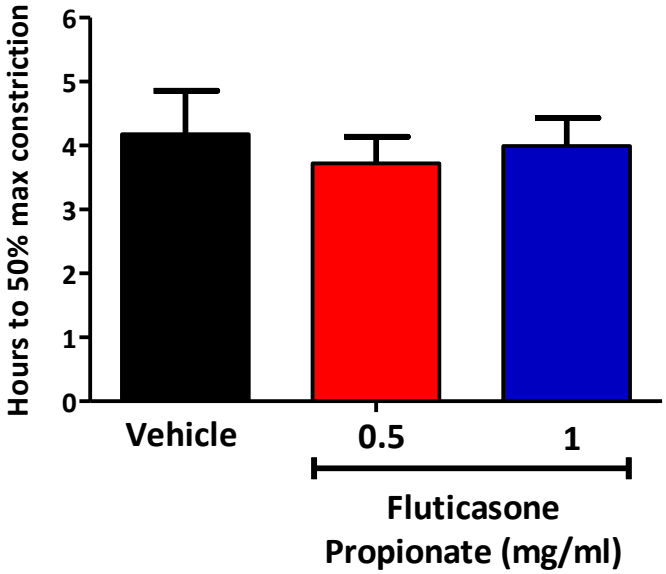


Figure 22: Analysis of the time taken for early asthmatic responses (EAR) to recover to 50% of peak bronchoconstriction values in ovalbumin (Ova) challenged guinea-pigs exposed to 30µg/ml LPS for 1 hour, 48 hours before Ova challenge and co-administered with Ova and treated with fluticasone propionate (FP) (0.05mg/ml or 1mg/ml, split into 2 twice daily 15 inhalations) or vehicle. N=7 (vehicle), N=7 (0.5mg/ml FP), N=5 (1mg/ml FP); performed with one-way analysis of variance followed by Dunnet's post-test.

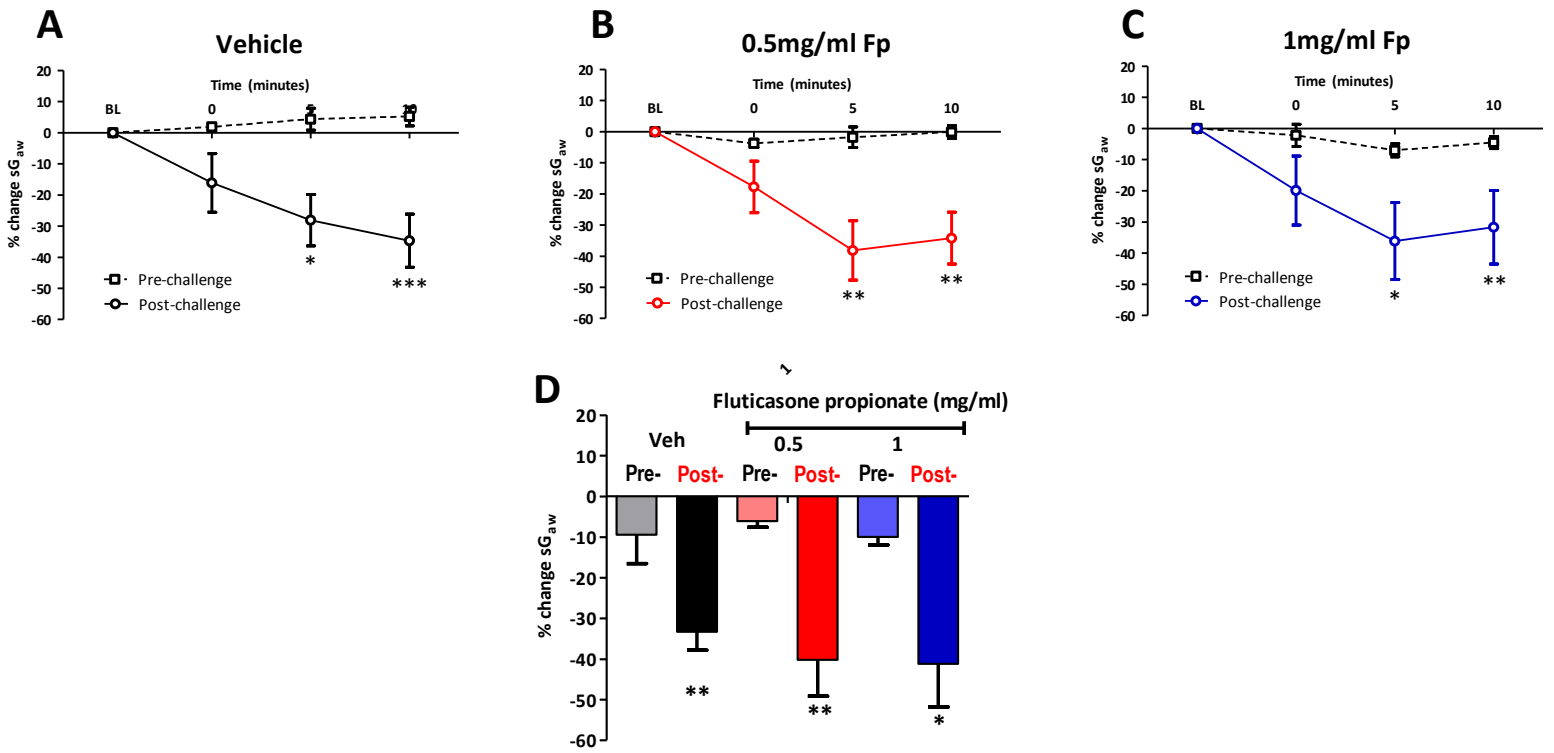


Figure 23: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles and 0.5LPM flow per chamber over 2 minutes, 1 minute drying period) in ovalbumin (Ova) challenged guinea-pigs exposed to 30µg/ml LPS for 1 hour, 48 hours before Ova challenge and co-administered with Ova and treated with A) vehicle or fluticasone propionate (FP) (B) 0.05mg/ml or C) 1mg/ml, split into 2 twice daily 15 inhalations). D) represents the peak sGaw values pre- and post-Ova challenge. Values were recorded 7 days pre- and 24 hours post-Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=7 (vehicle), N=7 (0.5mg/ml FP), N=5 (1mg/ml FP). *Significantly different from time paired pre-Ova challenge values $p<0.05$, ** $p<0.01$; *** $p<0.001$; performed with a two tailed T-test.

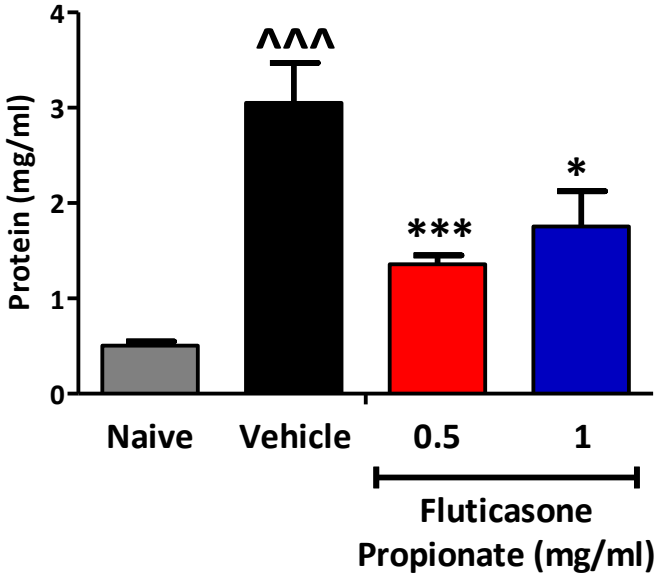


Figure 24: Total lavage fluid protein in ovalbumin (Ova) challenged guinea-pigs exposed to 30µg/ml LPS for 1 hour, 48 hours before Ova challenge and co-administered with Ova and treated with fluticasone propionate (FP) (0.05mg/ml or 1mg/ml, split into 2 twice daily 15 inhalations) or vehicle. Protein content was determined by BCA protein assay. N=6 (naïve), N=7 (vehicle), N=7 (0.5mg/ml FP), N=5 (1mg/ml FP). ^^^significantly different from naive $p<0.001$;*significantly different from vehicle treatment $p<0.05$, ** $p<0.01$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

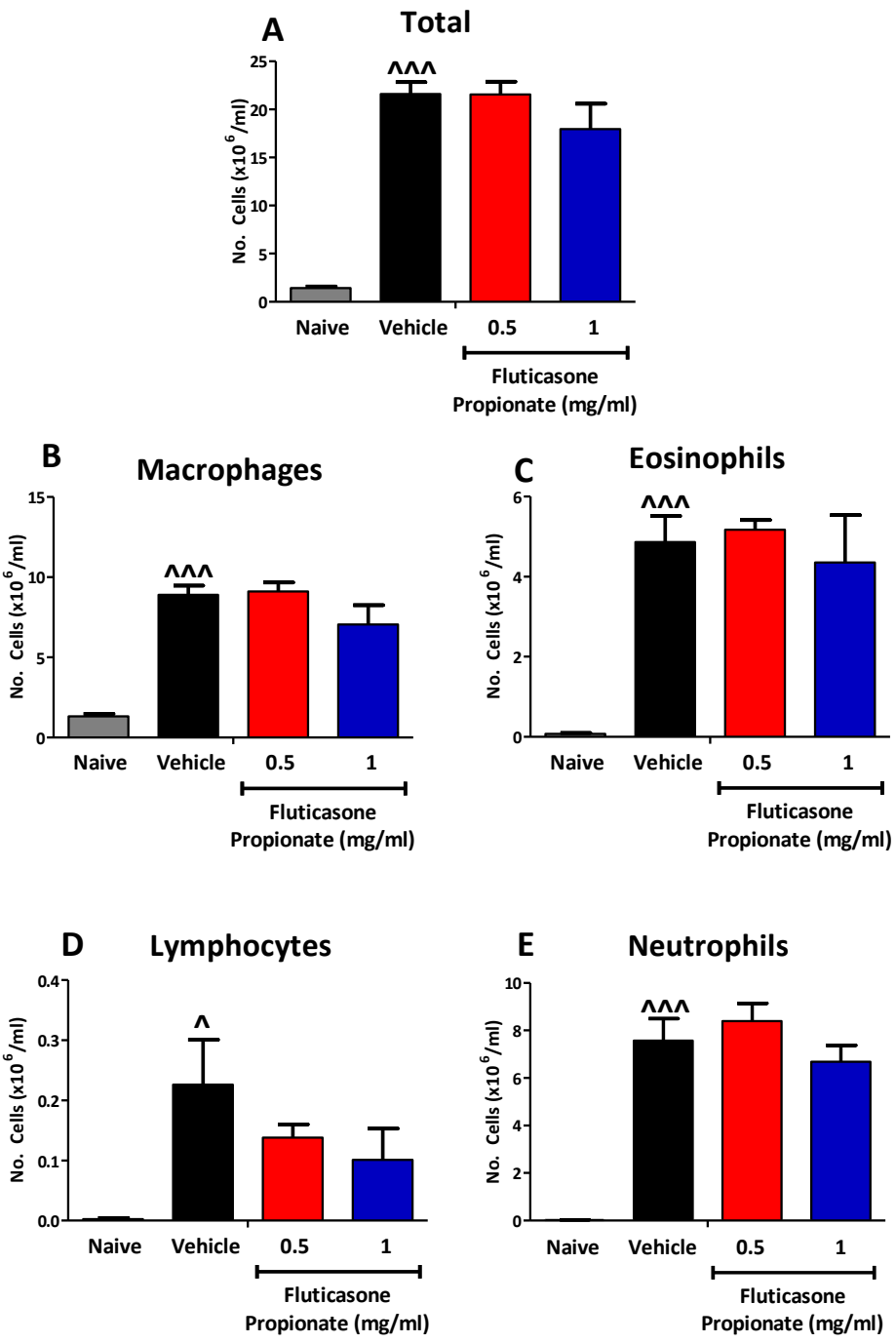


Figure 25: The total cell (A), macrophages (B), eosinophils (C), lymphocytes (D) and neutrophils (E) counts in bronchoalveolar fluid in ovalbumin (Ova) challenged guinea-pigs exposed to 30µg/ml LPS for 1 hour, 48 hours before Ova challenge and co-administered with Ova and treated with fluticasone propionate (FP) (0.05mg/ml or 1mg/ml, split into 2 twice daily 15 inhalations) or vehicle. N=6(naïve), N=7 (vehicle), N=7 (0.5mg/ml FP), N=5 (1mg/ml FP). ^significantly different from naive $p<0.05$, ^^^ $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

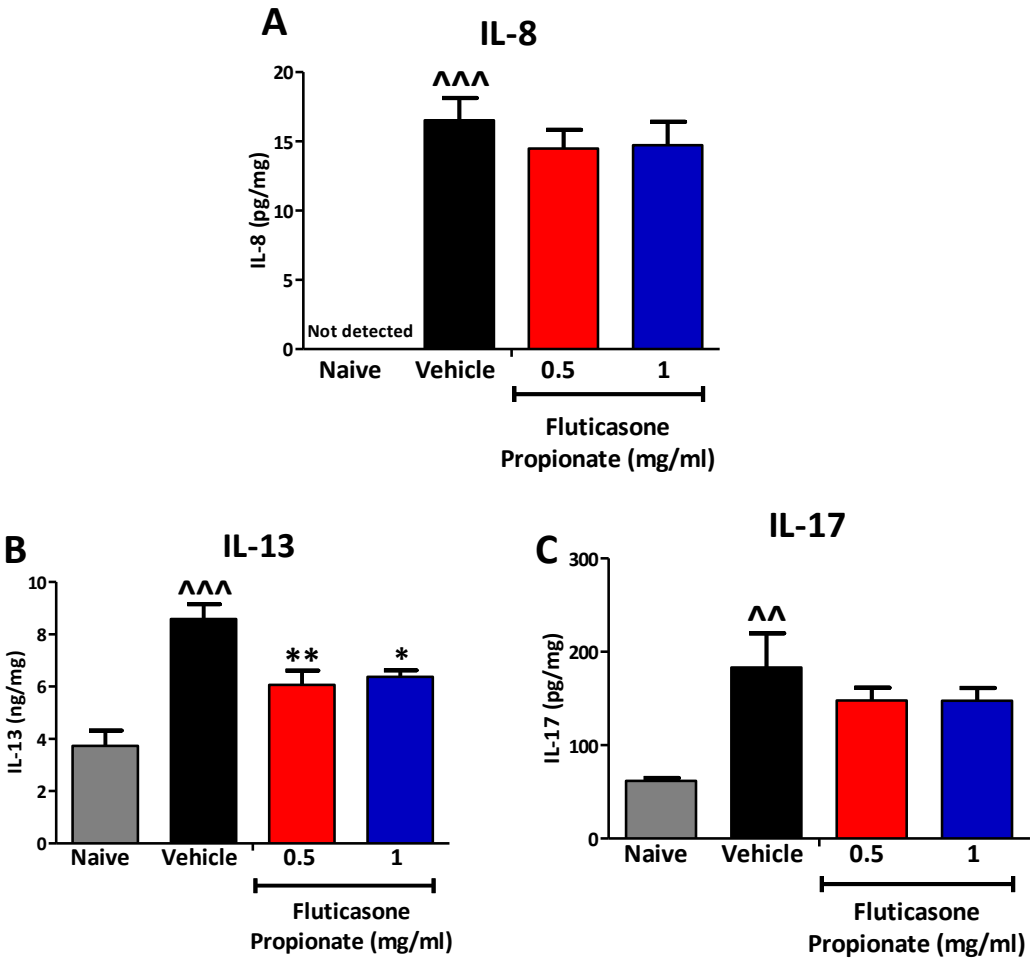


Figure 26: The concentration of A) IL-8 B) IL-13 C) IL-17 in the lungs of ovalbumin (Ova) sensitised guinea-pigs exposed to 30µg/ml LPS for 1 hour, 48 hours before Ova challenge and co-administered with Ova and treated with fluticasone propionate (FP) (0.05mg/ml or 1mg/ml, split into 2 twice daily 15 inhalations or vehicle. N=6 (naïve), N=7 (vehicle), N=7 (0.5mg/ml FP), N=5 (1mg/ml FP). ^^significantly different from naive $p<0.01$, ^^^ $p<0.001$; *significantly different from vehicle treatment $p<0.05$, ** $p<0.01$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

6.5.4 Sensitivity of LPS responses to inhaled Fluticasone propionate

Both vehicle and FP treated groups showed a progressive increase in bronchoconstriction during the 1st hour post-LPS exposure, reaching its greatest extent at 3 hours (vehicle: -22.9±5.4%; 0.5mg/ml FP: -24.3±6.1%; 1mg/ml -19.4±2.0%). The bronchoconstriction then dissipated and returned to near baseline sGaw levels by 5 hours (Figure 27, time course). Vehicle treated guinea pigs demonstrated a peak bronchoconstriction of -32.9±2.9% which was not significantly changed by FP treatment, despite a trend (0.5mg/ml: -28.9±4.4%; 1mg/ml -24.6±3.8%). A small bronchoconstriction was observed at 24 hours in vehicle treated guinea-pigs and unaltered by FP treatment (Figure 27, histogram). FP treatment did not significantly change the area under the curve between 0-12 hours (Figure 28)

In vehicle treated guinea-pigs a non-significant increase in the response to histamine was observed post-LPS challenge throughout the 10 minutes post-histamine challenge, reaching significance at 10 minutes (-16.5±1.9%) compared to pre-challenge (-5.6±1.9 %). Guinea-pigs treated with 0.5mg/ml and 1mg/ml FP demonstrated non-significant increase in the bronchoconstrictor response to histamine following LPS exposure. In guinea-pigs treated with 1mg/ml FP this was significant at 5 minutes post-histamine (-16.2±3.4%) compared to pre-LPS (-1.6±5.3%) (Figure 29). Figure 29D shows peak bronchoconstrictor responses pre- and post-LPS exposure. A significant increase in the peak bronchoconstrictor response to histamine was seen in vehicle treated guinea-pigs (-22.0±5.2% post-LPS, compared to pre-LPS -7.0±1.8%). Guinea-pigs treated with FP demonstrated a non-significant trend for an increase in the bronchoconstrictor response to histamine post-LPS.

LPS increased protein levels in lavage fluid (1.9±0.3mg/ml, compared to naïve 0.5±0.05mg/ml) which were not significantly reduced by FP treatment (0.5mg/ml: 2.2±0.5mg/ml and 1mg/ml: 1.6±0.3mg/ml) (Figure 30).

Total cell numbers, increased in vehicle treated groups ($22.5\pm2.3x10^6$/ml compared to naïve, $1.4\pm0.2x10^6$/ml) were not significantly reduced by treatment with 0.5 or 1mg/ml FP ($20.9\pm2.8x10^6$ and $20.5\pm3.7x10^6$/ml, respectively). Macrophages, eosinophils, lymphocytes and neutrophils were all significantly increased by LPS exposure but not significantly reduced by either dose of FP (Figure 31).

IL-8 levels, increased in guinea-pigs challenged with Ova and treated with vehicle were not significantly reduced by either does of FP used (0.5mg/ml FP: 21.9±1.4pg/ml: 1mg/mg FP: 29.5±6.4pg/mg and vehicle: 18.6±1.3pg/mg) (Figure 32).

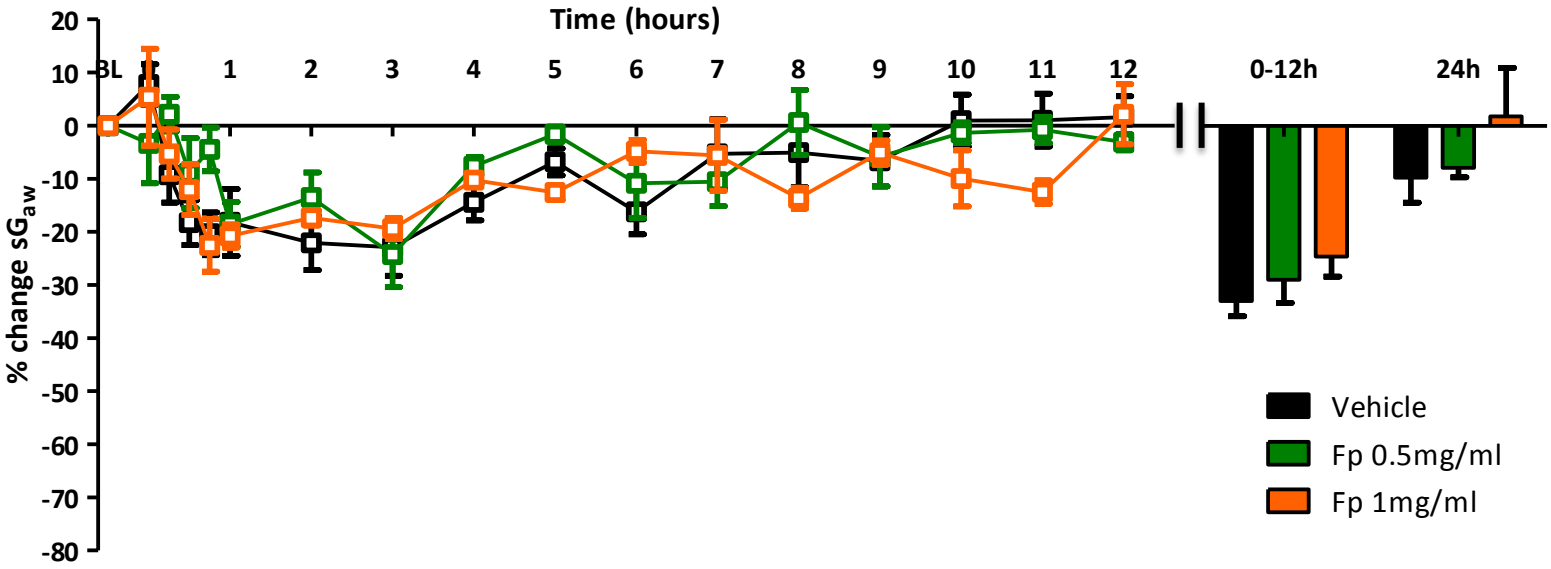


Figure 27: The mean time-course values of sGaw in guinea-pigs exposed to 30µg/ml LPS twice and treated with fluticasone propionate (FP, 0.5 or 1mg/ml) or vehicle. The histogram represents the maximum bronchoconstriction values recorded during the 0-12 hours and 24 hours readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline prior LPS challenge. A negative value represents a bronchoconstriction. Results shown are after the second LPS exposure. N=6 (veh and 0.5mg/ml FP), N=4 1mg/ml FP; performed with one-way analysis of variance followed by Dunnet's post-test.

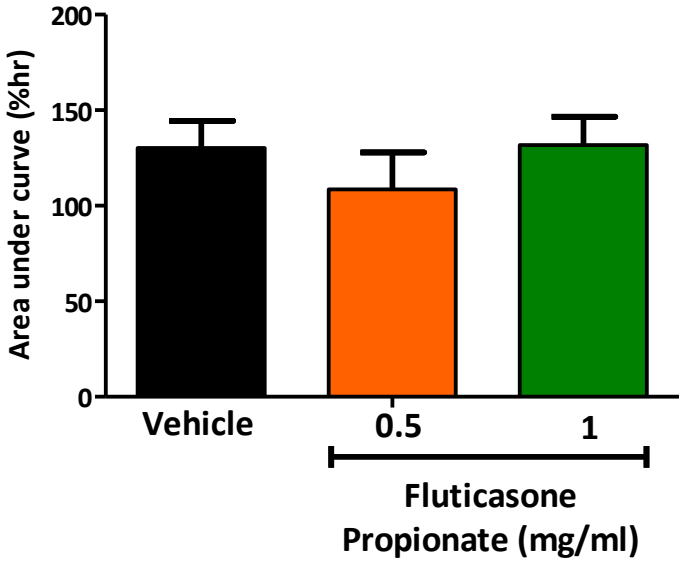


Figure 28: Total area under the curve analysis of sGaw values in guinea-pigs exposed to 30µg/ml LPS twice and treated with fluticasone propionate (FP, 0.5 or 1mg/ml) or vehicle. For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-12 hours. Area under the curve is expressed as %.hour. N=6 (veh and 0.5mg/ml FP), N=4 1mg/ml FP; performed with one-way analysis of variance followed by Dunnet's post-test.

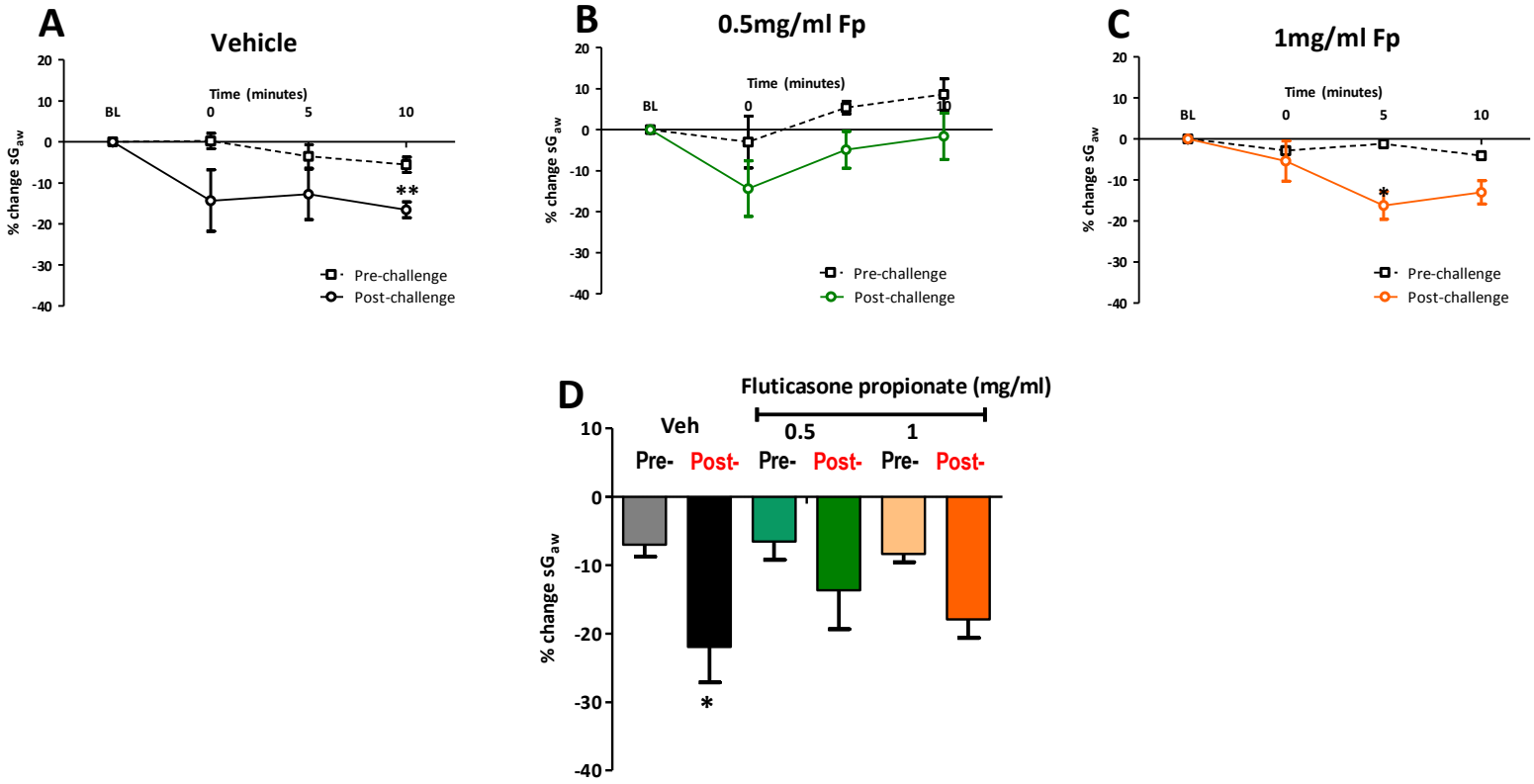


Figure 29: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles and 0.5LPM flow per chamber over 2 minutes, 1 minute drying period) in guinea-pigs exposed to 30µg/ml LPS twice and treated with A) vehicle or fluticasone propionate (FP, B) 0.5 or C) 1mg/ml). D) represents the peak sGaw values pre- and post-Ova challenge. Values were recorded 7 days pre- and 24 hours post-2nd LPS challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=6 (veh and 0.5mg/ml FP), N=4 1mg/ml FP *Significantly different from time paired pre-LPS challenge values $p<0.05$, ** $p<0.01$; performed with a two tailed T-test.

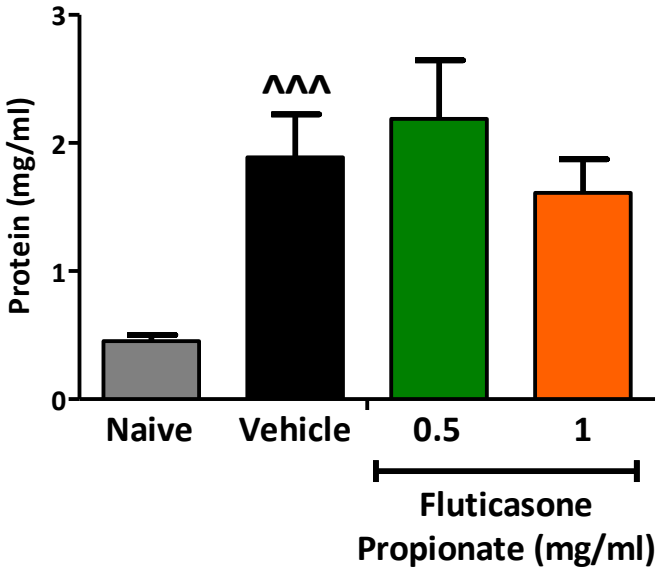


Figure 30: Total lavage fluid protein in guinea-pigs exposed to 30µg/ml LPS twice and treated with fluticasone propionate (FP, 0.5 or 1mg/ml) or vehicle. Protein content was determined by BCA protein assay. N=6 (Naïve, veh and 0.5mg/ml FP), N=4 1mg/ml FP; ^^^significantly different from naive $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

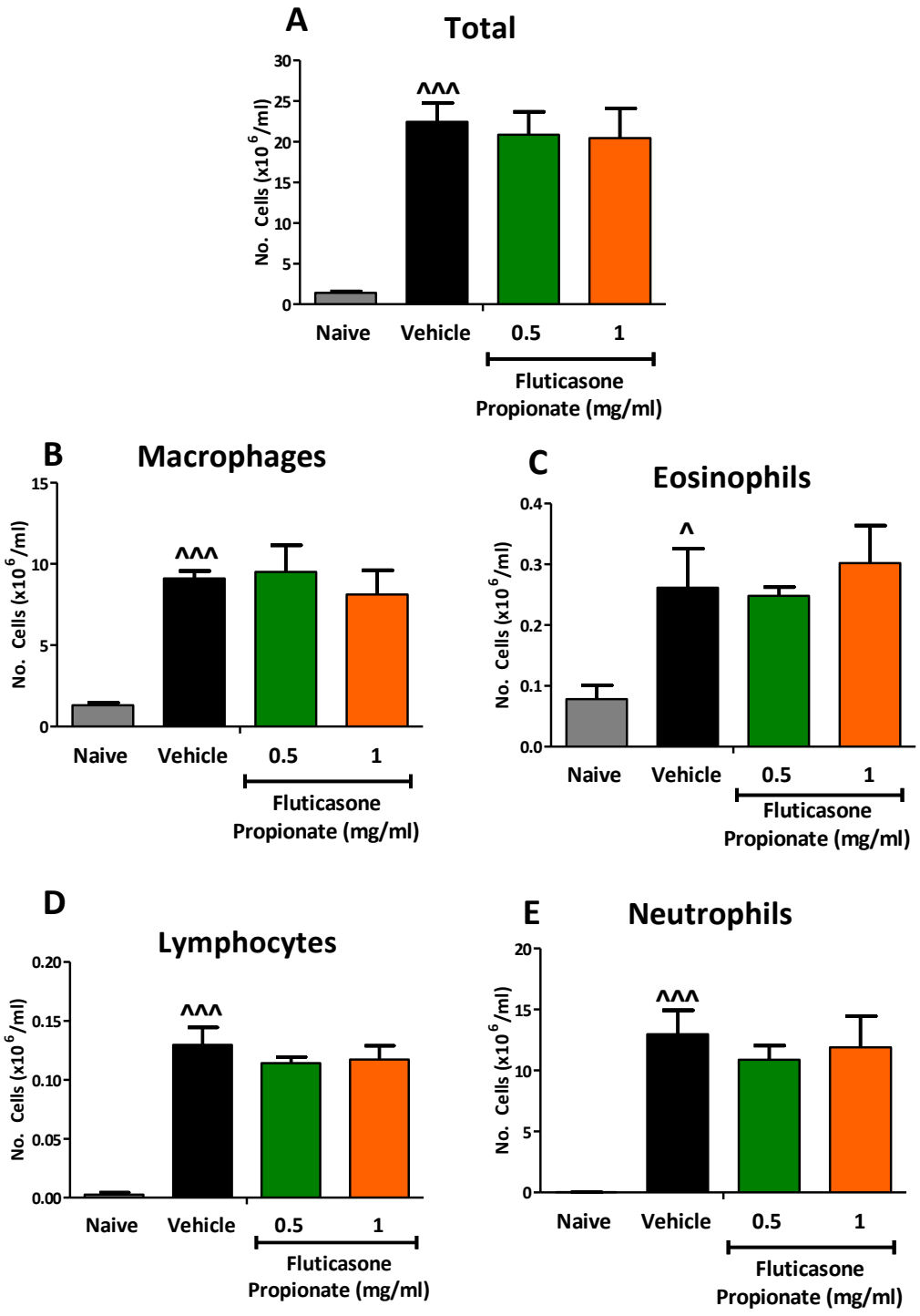


Figure 31: The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in bronchoalveolar fluid in guinea-pigs exposed to 30µg/ml LPS twice and treated with fluticasone propionate (FP, 0.5 or 1mg/ml) or vehicle; N=6 (naïve, veh and 0.5mg/ml FP), N=4 1mg/ml FP ^significantly different from naive $p<0.05$; ^^^ $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

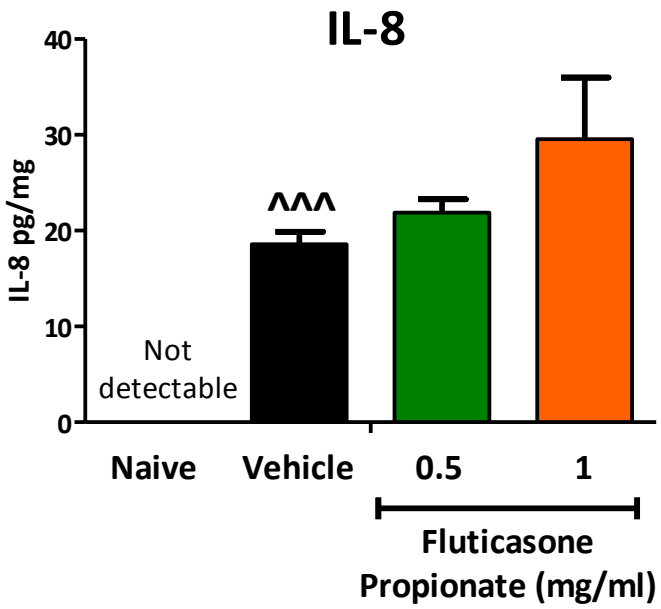


Figure 32: The concentration of IL-8 in the lungs of guinea-pigs exposed to 30µg/ml LPS twice for 1 hour and treated with vehicle or fluticasone propionate (FP, 0.5 or 1mg/ml). Results are expressed as mean±SEM; N=6 (Naïve, veh and 0.5mg/ml FP), N=4 1mg/ml FP ^^^significantly different from naive $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

6.6 DISCUSSION

Inhaled corticosteroids have wide ranging effects on both inflammatory and functional attributes of asthma. Initial work in this chapter established a dose of fluticasone propionate (FP) that significantly attenuated the LAR, AHR and cellular influx by dose-response curve. This dose was then used on 2 ovalbumin and LPS models developed in chapter 5. A higher dose of FP was also tried on the Ova and LPS combination model to assess the extent of inhaled corticosteroid insensitivity. Finally, these 2 doses of FP were used to assess the corticosteroid sensitivity of bronchoconstrictor and inflammatory responses in a double LPS exposure model.

6.6.1 THE EFFECT OF INHALED FLUTICASONE PROPIONATE ON EARLY AND LATE ASTHMATIC RESPONSES

In the current study it was shown that FP does not significantly affect the EAR in guinea-pigs challenged with Ova alone. The EAR in both humans and animal models has been shown to be insensitive to corticosteroids (Booij-Noord *et al.*, 1971; Evans *et al*, 2012). This would be expected as the EAR is mainly mediated by mast cell degranulation and corticosteroids are not known to have any effect on this process. The prolonged EAR induced by LPS co-administered with ovalbumin was also found to be FP insensitive. This would suggest that the mechanisms that mediate the increased duration of the EAR are also insensitive to inhaled corticosteroid. Thus enhanced activation of mast cells or neutrophils by LPS may be the cause (Masuda *et al*, 2002; Reino *et al*, 2012). Neutrophils are known to be relatively corticosteroid insensitive, especially in the lung and have been implicated in the EAR (Plumb *et al*, 2012). However, their levels do not increase until 3 hours post-allergen challenge, so neutrophils are not likely to be involved in the induction of the EAR but may be responsible for its elongation of it (Danahay *et al*, 1999). In the current study, LPS alone also induced a bronchoconstriction which developed slowly over 3 hours and was insensitive to inhaled FP. Similarly, the attenuated but prolonged EAR in guinea-pigs exposed to LPS 24 hours before Ova challenge was also unresponsive to inhaled

FP. These both coincided with an increase in lavage fluid neutrophils 24 hours later, further suggesting the role of the neutrophil in elongated EARs.

The LAR was attenuated with 0.5mg/ml inhaled FP in ovalbumin challenged guinea-pigs. The LAR was not suppressed by the 5-fold lower dose of FP (0.1mg/ml), nor was there increased suppression at the 2-fold higher dose of 1mg/ml. This indicates that the LAR has a fairly steep dose-response relationship with inhaled FP. This is similar in humans where higher doses of inhaled corticosteroid do not provide increased clinical benefit above lower effective doses (Adam *et al*, 2001). The attenuation of the LAR by Inhaled FP would be expected as corticosteroids are able to suppress this response in both humans and animal models (Palmqvist *et al*, 2005; Evans *et al*, 2012). The LAR has been linked to a range of inflammatory cells and mediators including lymphocytes, eosinophils and IL-5, all of which are suppressed by the wide ranging anti-inflammatory activity of corticosteroids (Belvisi, 2004).

Contrasting with effects of inhaled FP on Ova alone, in guinea-pigs exposed to either of the Ova and LPS protocols, the LAR was not significantly decreased by 0.5mg/ml FP. This indicates that LPS exposure has decreased the sensitivity of the Ova induced LAR to inhaled FP. It also indicates that the development of corticosteroid insensitivity is not dependent on the presence of a full EAR. Guinea-pigs exposed to LPS 24 hours before Ova challenge, displayed an attenuated EAR, merging into a LAR which was not reduced by FP treatment. To examine the extent of inhaled FP insensitivity of the LAR in the Ova and LPS co-administration model, a higher dose of 1mg/ml FP, effective on the LAR of Ova challenged guinea-pigs, was used. This dose of FP was not able to significantly attenuate the LAR but did demonstrate a trend for decreased sGaw values. This would suggest that the insensitivity to inhaled FP is due to a rightward shift in the dose response curve. Higher doses of FP were not tried due to issues with FP solubility.

6.6.2 The effect of inhaled fluticasone propionate on Airways hyperresponsiveness

Inhaled FP (0.5 and 1mg/ml) significantly attenuated the development of AHR following Ova challenge. Contrastingly, the 2 lower doses of FP used did not suppress the development of AHR. This was an expected effect as inhaled corticosteroids are well known clinically to suppress the development of AHR (Palmqvist *et al*, 2005). In contrast, inhaled FP demonstrated only modest suppressive effects on AHR to LPS alone. Even at 1mg/ml FP AHR was not completely abolished. This may indicate that LPS-induced AHR is inherently insensitive to inhaled FP. Although the precise mechanisms of AHR are still unknown, a general link to inflammation has been made and more specifically eosinophils. Eosinophil granule release can cause epithelial damage, increasing the exposure of underlying nerves to bronchoconstrictor stimuli (Gleich *et al*, 1993; Laitinen *et al*, 1987). Regardless of the specific inflammatory mediators of AHR, corticosteroids broad anti-inflammatory action is likely to be responsible for the suppression of AHR.

In contrast with the effectiveness of inhaled FP on Ova induced AHR, in guinea-pigs also exposed to LPS, 0.5mg/ml did not attenuate the development of AHR. In the case of guinea-pigs exposed to LPS 24 hours before Ova challenge, 0.5mg/ml FP actually potentiated the length of the bronchoconstrictor response to histamine compared to vehicle. Additionally, a higher dose of FP (1mg/ml) was also unable to suppress the development of AHR in Ova/LPS co-administration groups. The failure to suppress AHR would further suggest the development of insensitivity to this inhaled corticosteroid in the combined Ova and LPS model. It would also support the hypothesis that AHR in the model is mediated by inflammation. Differences in the inflammatory responses may account for the differing responsiveness of AHR to inhaled FP between Ova and Ova/LPS models. In particular, neutrophils, which are prominent in Ova and LPS models, have been implicated in the development of AHR (Essilfie *et al,* 2012; Ito *et al*, 2008). Additionally, neutrophils demonstrate increased survival in the presence of corticosteroids (Cox, 1995). This could explain why

inhaled FP potentiated AHR in guinea-pigs exposed to LPS 24 hours before Ova challenge. This could increase exposure to neutrophil granule products, increasing airway damage. However, a potentiation of AHR was not seen in LPS challenged guinea-pigs, also displaying increased neutrophilia indicating that this process is more complex and requires the presence of other factors induced by Ova. This is supported by Yang *et al*, (2009) who found that the development of corticosteroid-resistant AHR is dependent on exposure to both Ova and LPS. It was also found that macrophage but not neutrophil depletion was able to prevent the development of AHR. This may indicate that macrophages are an important cell type in the development of corticosteroid resistant AHR. However, this study produced these effects on a Th1 inflammatory background, contrasting with the Th2 inflammatory background present in the current study. This may mean that different molecular pathways are involved in AHR, making it difficult to make comparisons. Overall, the lack of insensitivity of all inflammatory cells measured does not allow any conclusions as to which particular inflammatory cell is mediating these effects.

6.6.3 The effect of inhaled fluticasone propionate on airway inflammation and cytokines

Inhaled FP reduced the total cells, macrophages and lymphocytes in lavage fluid in a dose-dependent manner in Ova challenged guinea-pigs. 0.5 and 1mg/ml FP reduced eosinophils significantly but 0.05 and 0.1mg/ml FP had no effect on numbers. This indicates a steep dose response effect of inhaled FP on eosinophilia. IL-13 a typical Th2 cytokine implicated in the development of AHR was also reduced. The effectiveness of inhaled FP in reducing Ova induced inflammation would be expected as corticosteroids are known to be potent suppressants of allergic inflammation (Belvisi, 2004).

LPS responses have been shown to be less sensitive to single and multiple doses of inhaled corticosteroids (Trapp *et al*, 1998; Michel *et al*, 2000). These results were confirmed in the present study. Neither dose of FP was able to significantly reduce

LPS-induced increases in total cells, macrophages and neutrophils. IL-8, an important chemoattractant for neutrophils was also not reduced by inhaled FP treatment, indicating that this cytokine may be partly responsible for the continued presence of neutrophils even in the presence of FP. Whether the combination of Ova and LPS would result in inflammation that was inhaled corticosteroid insensitive was previously unknown. The present study showed that guinea-pigs exposed to LPS and Ova demonstrate no decrease in any cell type with 0.5mg/ml FP treatment, regardless of the timing of LPS exposure. Increasing the inhaled FP dose to 1mg/ml did not significantly decrease any cell type in the Ova and LPS co-administration model. However, a non-significant trend for a decrease was seen, further suggesting a rightward shift in corticosteroid dose-response effects.

The mechanism behind insensitivity to inhaled FP in this study remains to be investigated. However, several hypotheses exist. Neutrophils are known to be less sensitive to corticosteroid treatment then other cell types, so inhaled FP not reducing neutrophils would be expected (Uddin *et al*, 2009). The observation that eosinophils, macrophages and lymphocytes are all not significantly decreased is less expected as these cells are typically corticosteroid sensitive. Essilfie *et al,* (2012) also demonstrated that *H. Influenza* can cause inhaled corticosteroid insensitivity in eosinophils and lymphocytes. This contrasts with the findings of Ito *et al*, (2008) who demonstrated that eosinophils and lymphocytes are both significantly decreased with inhaled FP, while neutrophils and AHR are unaffected in repeat, low level allergen challenge model. This suggests that more widespread corticosteroid insensitivity is evoked by the TLR4 pathway which is activated by LPS.

Corticosteroid insensitivity in eosinophils, macrophages and lymphocytes in Ova and LPS challenged guinea-pigs may be as a result of the upregulation of GR-β. Increases in GR-β in macrophages, eosinophils and lymphocytes have been found in other allergic diseases (Hamilos *et al*, 2001). GR-β does not bind corticosteroids but can interfere with GR-α binding at DNA sites, as well as forming transcriptionally less active heterodimers with GR-α (Oakley *et al*, 1999). Thus, an increase in GR-β would

make cells less sensitive to corticosteroids. Alternatively, mechanisms such as a decrease in HDAC-2 as a result of increased AP-1 and NF-κB signalling, activation of the PI3K/Akt pathway and decreased HDAC-2 activity could all be involved. Pro-inflammatory signalling and the oxidative burden of the lungs increase during an asthma exacerbation which activates these pathways and decreases corticosteroid activity (Ito *et al*, 2004).

Interestingly, IL-13 levels were decreased despite there not being a significant decrease in any of the inflammatory cells measured. This may indicate that other cell types e.g. epithelial cells, not measured are producing IL-13 and are being suppressed by corticosteroids (Temann *et al*, 2007). Alternatively, a decrease in Th2 lymphocytes which secrete IL-13 may be masked by the corticosteroid insensitivity of other lymphocyte subpopulations. In the present study although no significant decrease in lymphocytes was seen, there is a trend for decreasing numbers with increasing FP dose. Analysis of the sensitivity of different lymphocyte populations may find that Th2 cells are still corticosteroids sensitive, as is typically reported (Yang *et al*, 2009).

IL-17 levels in the lung were unaffected by inhaled FP treatment in Ova challenged guinea-pigs. Similarly, FP did not significantly decrease IL-17 levels in LPS and Ova challenged guinea-pigs. This is in accordance with studies that have shown IL-17 responses to be corticosteroid insensitive (Mckinley *et al*, 2008). This has led to the suggestion that IL-17 and Th17 cells, a major source of IL-17 may have a role in corticosteroid insensitivity. As IL-17 levels did not increase with the addition of LPS to Ova (data shown in chapter 5) it seems unlikely that IL-17 is mediating corticosteroid-insensitivity in this model. However, other cytokines such as IL-17F and IL-22 have been implicated in Th17 responses. These cytokines along with Th17 cell numbers are unknown in the present study meaning its not possible to fully rule out a role of IL-17 in the inhaled FP insensitivity observed.

6.6.4 The effect of inhaled fluticasone propionate on airway oedema

Total protein levels in lavage fluid, an indicator of airway oedema decreased with FP treatment (although not significantly) in Ova challenged guinea-pigs. They were also decreased in Ova and LPS challenged guinea-pigs indicating that although this model demonstrates corticosteroid insensitivity, not all corticosteroid effects are reduced. This also indicates that results seen in this model are not due to impaired delivery of inhaled FP. This reduction in total lavage fluid protein levels may be due to the vasoconstrictive effects of corticosteroids (Mendes *et al*, 2003). This is thought to be by decreased uptake of noradrenaline at sympathetic synapses, potentiating noradrenaline's action at α1 adrenoreceptors (Wanner *et al*, 2004). Contrastingly, guinea-pigs exposed to LPS demonstrated no reduction in total lavage fluid protein levels with inhaled FP. A lack of corticosteroid effect on plasma extravasation has been demonstrated before with systemic corticosteroids (O'Leary *et al*, 1996). The reason behind a lack of corticosteroid effect on LPS induced increases in lavage fluid protein levels is unknown.

6.6.5 General conclusions

This study has demonstrated that the LAR, AHR, airway inflammation and airway oedema induced by Ova alone is sensitive to inhaled FP. Moreover, it has been shown that exposure to LPS and Ova together decreases the response of the LAR, AHR and airway inflammation to inhaled FP. The timing of LPS exposure (24 hours pre- vs 48 hours pre-Ova and co-administered with Ova) does not affect the development of inhaled corticosteroid insensitivity. LPS alone was shown to produce a bronchoconstriction, inflammation and airway oedema which is also insensitive to inhaled FP. Whether a systemically administered corticosteroid will have the same effect is unknown and will be investigated in the following chapter.

CHAPTER 7

The Effect Of Systemic Dexamethasone On Functional And Inflammatory Responses To Ovalbumin And LPS

Chapter 7

7.1 INTRODUCTION

7.1.1 SYSTEMIC CORTICOSTEROIDS

Systemic corticosteroids are used as last resort therapy in patients who do not achieve symptomatic control with high dose inhaled corticosteroids, long acting β_2-adrenoreceptor agonists or alternative therapies. Systemic corticosteroids such as prednisone and dexamethasone are given orally while hydrocortisone is generally given by intravenous injection. Side effects with systemic corticosteroids are extensive and include osteoporosis, cataracts, various cardiovascular and gastrointestinal disturbances and growth retardation in children (Schäcke *et al*, 2002). For this reason, systemic corticosteroids are only used in short bursts (1-2 weeks). Oral corticosteroids are indicated in the treatment of asthma exacerbations whereas intravenous therapy is used in acute asthma, with patients displaying <30% of predicted lung function and not responsive to a β_2-adrenoreceptor agonist (Chan *et al*, 1998). Their biological action and mechanisms are similar to inhaled corticosteroids. A description of these can be found in chapter 6. Systemic corticosteroids do have the added effect of suppressing bone marrow haematopoiesis (Mao *et al*, 2004). This decreases the maturation of inflammatory progenitor cells, especially eosinophils. Corticosteroids do this by suppressing CCL11 (eotaxin-1) and IL-5 induced differentiation of CD34+ cells into eosinophils (Ben *et al*, 2008). Inhaled corticosteroids can also suppress bone marrow inflammatory cell maturation but probably only with sustained treatment and higher doses (Wood *et al*, 1999; Shen *et al*, 2002).

7.1.2 SYSTEMIC CORTICOSTEROID RESISTANCE

A description of proposed mechanisms of corticosteroid insensitivity can be found in chapter 6. Clinically, a patient is defined as fully corticosteroid resistant if they demonstrate a failure to increase FEV_1 more than >15% after 14 days of prednisone treatment, despite demonstrating the same increase with a β_2 agonist. Although insensitivity to corticosteroids is reported to exist in about 5% of the asthmatic

population, full resistance to systemic corticosteroids is rare (Szefler *et al*, 2002). The development of full corticosteroid resistance is associated with longer disease duration, increased airway inflammation and more frequent exacerbations (Bumbacea *et al*, 2004). This suggests that repeated asthma exacerbations on a chronic inflammatory background can progressively increase corticosteroid insensitivity in an individual. Asthma exacerbations, although not particularly sensitive to inhaled corticosteroids seem to retain some sensitivity to systemic corticosteroids. Matsuse *et al*, 2012 reported that patients with virally induced asthma exacerbations demonstrate reduced time to clear symptoms with oral corticosteroid treatment. However, this study did not use any measures to assess improvement so it is not possible to know what aspects of the asthma exacerbation responded to corticosteroid therapy. A recent study by Hodgson *et al*, (2012) suggests that inhaled corticosteroid-insensitive patients with eosinophilic or mixed granulocytic airway inflammation retain sensitivity to systemic corticosteroid treatment. Oral prednisone treatment increased lung function and decreased eosinophil numbers. LPS induced inflammatory responses also seem to retain some sensitivity to systemic corticosteroid, despite not displaying any towards inhaled corticosteroids (Trapp *et al*, 1998).

7.1.3 The effect of systemic corticosteroids in animal models

Systemic corticosteroids such as dexamethasone have been shown to attenuate the LAR, AHR and inflammation in a guinea-pig Ova model (Toward & Broadley, 2004). Asthma exacerbation models have differing sensitivity to systemic corticosteroids depending on the nature of the exacerbation. Latent adenovirus infection in combination with Ova sensitisation and challenge results in systemic corticosteroid resistant eosinophils and sensitive lymphocyte subpopulations (Yamada *et al*, 2000). Contrastingly, combined Ova and LPS induced inflammation is sensitive and AHR insensitive to treatment with systemic dexamethasone in mice (Komlosi *et al,* 2006). LPS responses without the presence of Ova seem to be sensitive to systemic dexamethasone. Single or multiple LPS exposures cause systemic corticosteroid

sensitive airway inflammation and goblet cell hyperplasia, but insensitive lung function (O'leary *et al* 1996; Toward & Broadley 2002). The effect of systemic corticosteroid on early and late asthmatic responses has not been investigated in a model of asthma exacerbation. Moreover, a comparison between the sensitivity of asthma exacerbation models to inhaled and systemic corticosteroid has not been made.

7.2 HYPOTHESIS

'LPS exposure will decrease the systemic corticosteroid sensitivity of functional and inflammatory responses in an acute ovalbumin model of asthma'

7.3 AIMS AND OBJECTIVES

The aim of this chapter will be to determine the systemic corticosteroid sensitivity of functional and inflammatory responses to ovalbumin, LPS and combined ovalbumin and LPS models. Initially a dose of systemic dexamethasone which reduces the LAR, AHR and airways inflammation after Ova challenge was established. The sensitivity of LPS and ovalbumin and LPS responses to this dose of systemic dexamethasone was also tested. The effect of systemic dexamethasone on cytokines and lavage fluid protein was also established.

7.4 METHODS

Methods describing the measurement of lung function, AHR, airway oedema, airway inflammation, cytokine levels and can be found in more detail in chapter 2.

7.4.1 OVALBUMIN PROTOCOLS

7.4.1.1 SENSITISATION

Guinea-pigs (200-300g, Dunkin-Hartley, male) were sensitised by a bilateral intra-peritoneal injection of a solution containing 150µg Ovalbumin (Ova) and 100mg aluminium hydroxide ($Al(OH)_3$) in normal saline on day 1, 4 and 7.

7.4.1.2 ACUTE OVALBUMIN MODEL

Sensitised guinea-pigs were exposed to inhaled Ova (0.03%) for 1 hour on day 21 in a Perspex container as described in section 7.4.1.2. The lung function response to Ova was assessed over 12 hours. The bronchoconstrictor response to 0.3mM histamine was assessed on day 15 and 22 as described in section 7.4.5. Dexamethasone (5, 10 or 20mg/kg) was given by intraperitoneal injections, daily from day 16-21. On day 21, dexamethasone was administered 30 minutes pre-Ova challenge. A diagram of this protocol is shown Figure 1.

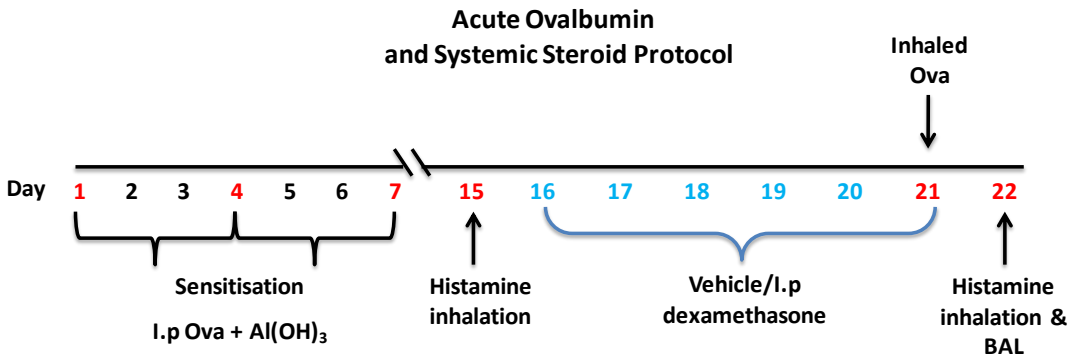


Figure 1: The acute Ovalbumin (Ova) protocol. Guinea-pigs were challenged with inhaled Ova (0.03% on day 21). Dexamethasone was administered once a day by intraperitoneal injection.

7.4.1.3 Acute Ova and LPS co-exposure model

Sensitised guinea-pigs were exposed to inhaled LPS (30µg/ml) 48 hours before Ova challenge in an exposure chamber and both LPS (30µg/ml) and Ova (0.03%) co-administered on day 21. The bronchoconstrictor response to histamine was assessed on day 15 and day 22. Dexamethasone (5, 10 or 20mg/kg) was given by intraperitoneal injections, daily from day 16-21. On day 21, dexamethasone was administered 30 minutes pre-Ova challenge. A diagram of this protocol is shown in shown in Figure 2.

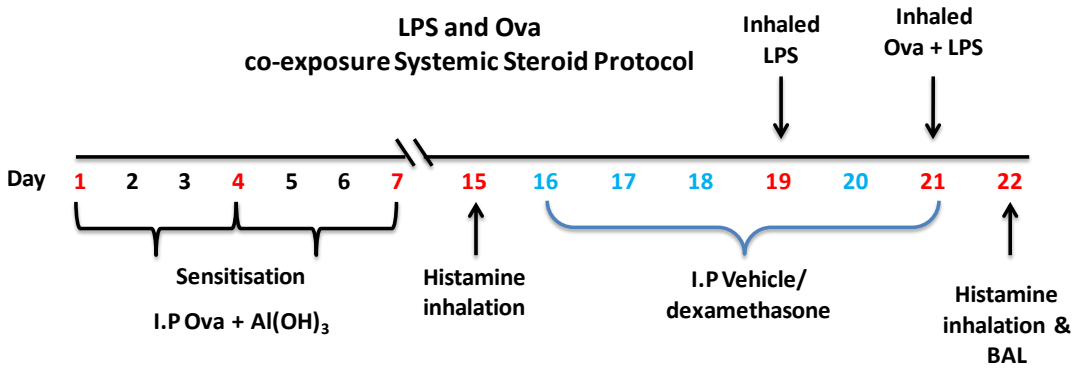


Figure 2: Diagram of the LPS and Ova co-exposure protocol. Guinea-pigs were exposed to LPS (30µg/ml) on day 19 and both Ova and LPS co-administered on day 21. Dexamethasone was administered once a day by intraperitoneal injection.

7.4.2 LPS exposure protocol

Non-sensitised guinea-pigs were exposed to LPS (30µg/ml) on day 5 and 7 of the protocol. The bronchoconstrictor response to histamine was assessed on day 1 and day 8. Dexamethasone (5, 10 or 20mg/kg) was given by intraperitoneal injections, daily from day 16-21. On day 5 and 7, dexamethasone was administered 30 minutes pre-LPS challenge. A diagram of this protocol is shown in Figure 3.

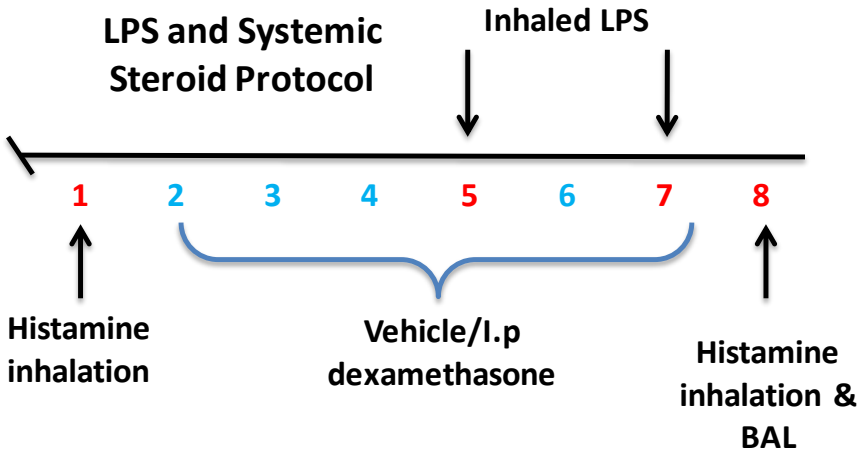


Figure 3: Diagram of the LPS alone exposure protocol. Guinea-pigs were exposed to 30µg/ml of LPS on day 5 and 7. Dexamethasone was administered once a day by intra peritoneal injection.

7.4.3 Drug administration

Dexamethasone was dissolved in 25% DMSO and 75% saline and administered by a bilateral intraperitoneal injection.

7.4.4 Measurement of lung function

Lung function was measured by whole body plethysmography following final Ova, LPS or combined Ova and LPS exposure in all protocols. Lung function was recorded hourly for 12 hours and every 15 minutes during the first hour of measurements. A final measurement was also taken 24 hours post-final challenge. All values were expressed as a percentage of the baseline reading, taken before the final challenge. A negative percentage change in baseline value represents a bronchoconstriction. In Ova protocols, early (0-6 hours) and late (6-12 hours) asthmatic responses were expressed as the peak bronchoconstriction during that period (displayed as a histogram next to a time course plot) and area under the curve. The duration of the early phase was expressed as the time taken to recover to 50% of peak early phase bronchoconstriction values. In the LPS only protocol lung function was measured for 12 hours after the second LPS exposure. The peak bronchoconstriction during this period was expressed on a histogram next to the time course plot.

7.4.5 Airways Hyper-responsiveness assessment.

AHR was determined using whole body plethysmography. This was performed by measuring the bronchoconstrictor response to histamine before inhaled corticosteroid treatment and 24 hours post-final LPS, Ova, LPS or Ova challenge. Histamine was delivered as described in chapter 2. Lung function was measured just before histamine inhalation and at 0, 5 and 10 minutes post-histamine exposure. The peak bronchoconstriction during this period was expressed on a histogram.

7.4.6 Assessment of airways inflammation

Following final histamine exposure guinea-pigs were sacrificed by an intra-peritoneal overdose of sodium pentobarbitone, the lungs excised and lavaged. Total and differential cell counts were then performed as described in chapter 2.

7.4.7 Quantification of cytokine levels using enzyme-linked immunosorbent assay (ELISA)

Levels of IL-8, IL-17 and IL-13 were measured in diluted homogenised lung samples using ELISA supplied by R&D systems. Cytokine levels were adjusted for total lung protein and expressed as weight per mg of lung as described in section 2.2.3.2.

7.4.8 Assessment of airway oedema

Protein content in lavage fluid was determined as a measure of airway oedema by BCA protein assay as per the manufacturer's instructions (Pierce protein biology).

7.5 Results

7.5.1 The effect of systemic dexamethasone on acute Ovalbumin responses

Vehicle treated guinea-pigs displayed an immediate bronchoconstriction to Ova at 0 minutes (-74.4±1.5%) which returned to baseline sGaw values by 6 hours. Guinea-pigs treated with 5mg/kg dexamethasone also displayed an immediate bronchoconstriction to Ova (-66.0±5.5%) and significantly less of a bronchoconstriction then vehicle at 45 minutes (-32.8±3.2% and -58.7±2.7% respectively). 10mg/kg dexamethasone treated guinea-pigs consistently demonstrated less of a bronchoconstriction to Ova during the first 4 hours, at 1 hour it was (-33.6±3.3%) compared to vehicle (-56.7±4.3%) and at 2 hours it was (-20.8±4.3%) compared to vehicle (-51.9±3.8%). 20mg/kg dexamethasone did not significantly alter the early phase response to Ova. Vehicle treated guinea-pigs demonstrated a second, delayed response at 7 hours (-21.4±2.2%), which was unchanged by 5mg/kg dexamethasone but significantly attenuated by both 10 and 20mg/kg dexamethasone (-3.1±3.5% and 2.0±4.0% respectively). sGaw values returned to baseline levels in all groups by 12 hours (Figure 4, time course).

Dexamethasone treatment did not significantly alter the peak EAR compared to vehicle. 20 but not 5 and 10mg/kg dexamethasone significantly reduced the peak LAR (-10.2±1.7%, -21.4±7.4% and -21.3±2.0% respectively) compared to vehicle (-22.6±1.9%). No significant differences between the 24 hour sGaw values were found (Figure 4, histogram). The total area under the curve (AUC) was significantly reduced by 5, 10 and 20mg/kg dexamethasone (187.6±22.9%.hr, 177.4±17.8%.hr and 194.1±28.4%.hr respectively) compared to vehicle (316.6±28.3%.hr). Early asthmatic response AUC was significantly decreased by 10mg/kg dexamethasone (139.1±12.4%.hr) compared to vehicle (248.6±19.7%.hr). 5 and 20mg/kg dexamethasone did not significantly reduce the early asthmatic response AUC. Late asthmatic response AUC was significantly attenuated by 5, 10 and 20mg/kg

dexamethasone (20.7±6.5%.hr, 39.5±9.6%.hr and 11.8±3.5%.hr respectively) compared to vehicle (68.1±12.9%.hr) (Figure 5).

In vehicle treated guinea-pigs histamine produced a significant increase in bronchoconstrictor response to histamine post-Ova challenge (-20.3±3.7%) compared to pre-Ova (-0.02±5.2%). At 5 and 10 minutes, the bronchoconstriction was still significantly increased over pre-Ova values (-23.7±3.4% compared to pre-Ova, -2.8±5.9%; -23.3±2.9% compared to pre-Ova, -0.2±1.9%, respectively). Treatment with 5, 10 and 20mg/kg dexamethasone reduced the bronchoconstrictor response to histamine, with no significant difference between pre and post-Ova responses (Figure 6). The peak bronchoconstrictor response to histamine was significantly increased in guinea-pigs treated with vehicle post-Ova treatment (-21.0±4.8%) compared to pre-Ova (-4.9±3.3%). All doses of dexamethasone significantly decreased this response (Figure 6).

Guinea-pigs challenged with Ova and treated with vehicle demonstrated a significant increase in protein (1.74±0.1mg/ml) compared to naïve levels (0.4±0.01mg/ml). 10 and 20 but not 5mg/kg dexamethasone significantly reduced protein levels (1.2±0.2mg/ml, 1.1±0.2mg/ml and 1.7±0.2mg/ml respectively) (Figure 7)

Total cell numbers were increased in Ova challenged and vehicle treated guinea-pigs ($8.6\pm0.5x10^6$/ml) compared to naïve $1.4\pm0.2x10^6$/ml). Dexamethasone reduced total cell numbers in a dose-dependent manner; 20mg/kg dexamethasone significantly decreased total cell numbers ($5.6\pm0.5x10^6$/ml). Macrophages were also significantly decreased by 20mg/kg dexamethasone ($2.9\pm0.3x10^6$/ml compared to vehicle, $4.0\pm0.3x10^6$/ml). Eosinophils were significantly reduced by both 10 and 20mg/kg dexamethasone ($2.2\pm0.5x10^6$/ml and $2.3\pm0.3x10^6$/ml compared to vehicle, $4.3\pm0.5x10^6$/ml). Additionally 10mg/kg dexamethasone significantly decreased

lymphocyte numbers ($0.03\pm0.01x10^6$ compared to vehicle $0.8\pm0.02x10^6$). A non significant trend for decreased neutrophils with increasing dexamethasone dose was also observed (Figure 8).

IL-13 levels were significantly increased in Ova challenged and vehicle treated guinea-pigs (13.9±1.14ng/ml) compared to naïve (9.8±0.6ng/mg). IL-13 was significantly decreased by treatment with 5, 10 and 20mg/kg dexamethasone compared to vehicle (10.2±0.42ng/mg, 10.1±0.8ng/ml and 10.0±0.6ng/ml respectively). IL-17 levels were also increased in Ova challenged and vehicle treated guinea-pigs (143.5±6.5pg/mg) compared to naïve (112±5.0pg/mg). IL-17 levels were decreased by dexamethasone treatment in a dose-dependent manner. With 5, 10 and 20mg/kg dexamethasone IL-17 levels were 107.6±5.7pg/mg, 103.0±4.0pg/mg and 98.2±6.1pg/mg respectively. IL-8 was not detectable in samples and is not shown (Figure 9).

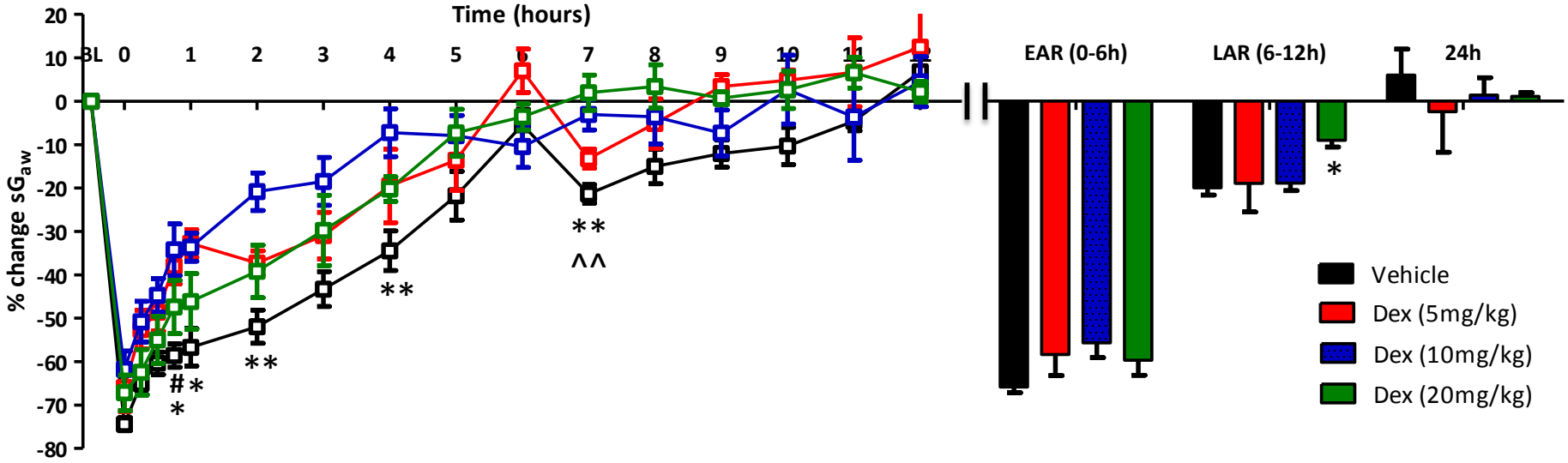


Figure 4: The mean time-course values of sGaw in ovalbumin (Ova) sensitised and challenged guinea-pigs treated with either intra-peritoneal vehicle or dexamethasone (Dex, 5mg/kg, 10mg/kg and 20mg/kg) dosed daily for 6 days. The histogram represents the maximum bronchoconstriction values recorded during the early asthmatic response (EAR) (0-6 hours), late asthmatic response (LAR) (6-12 hours) and 24 hours readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline prior to Ova challenge. A negative value represents a bronchoconstriction. N=6 (vehicle), N=4 (Dex 5mg/kg), N=6 (Dex 10mg/kg), N=7 (Dex 20mg/kg). *Dex 10mg/kg significantly different from vehicle treatment $p<0.05$, ** $p<0.01$; ^^ Dex 20mg/kg significantly from vehicle treatment $p<0.01$; # Dex 5mg/kg significantly different from vehicle treatment $p<0.05$ performed with one-way analysis of variance followed by Bonferroni post-test.

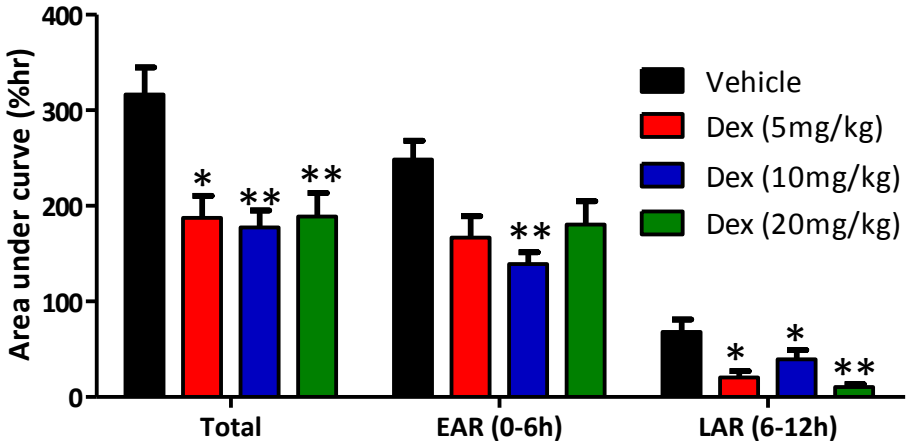


Figure 5: Area under the curve analysis of sGaw values over 12 hours following ovalbumin (Ova) challenge in sensitised guinea-pigs treated with either intra-peritoneal vehicle or dexamethasone (Dex, 5mg/kg, 10mg/kg and 20mg/kg) dosed daily for 6 days. For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-12 hours, early asthmatic response (EAR) includes values from 0-6 hours and late asthmatic response (LAR) includes values from 6-12 hours. Area under the curve is expressed as %.hour N=6 (vehicle), N=4 (Dex 5mg/kg), N=6 (Dex 10mg/kg), N=7 (Dex 20mg/kg).*significantly different from vehicle treatment $p<0.05$, ** $p<0.01$; performed with one-way analysis of variance followed by Dunnet's post-test.

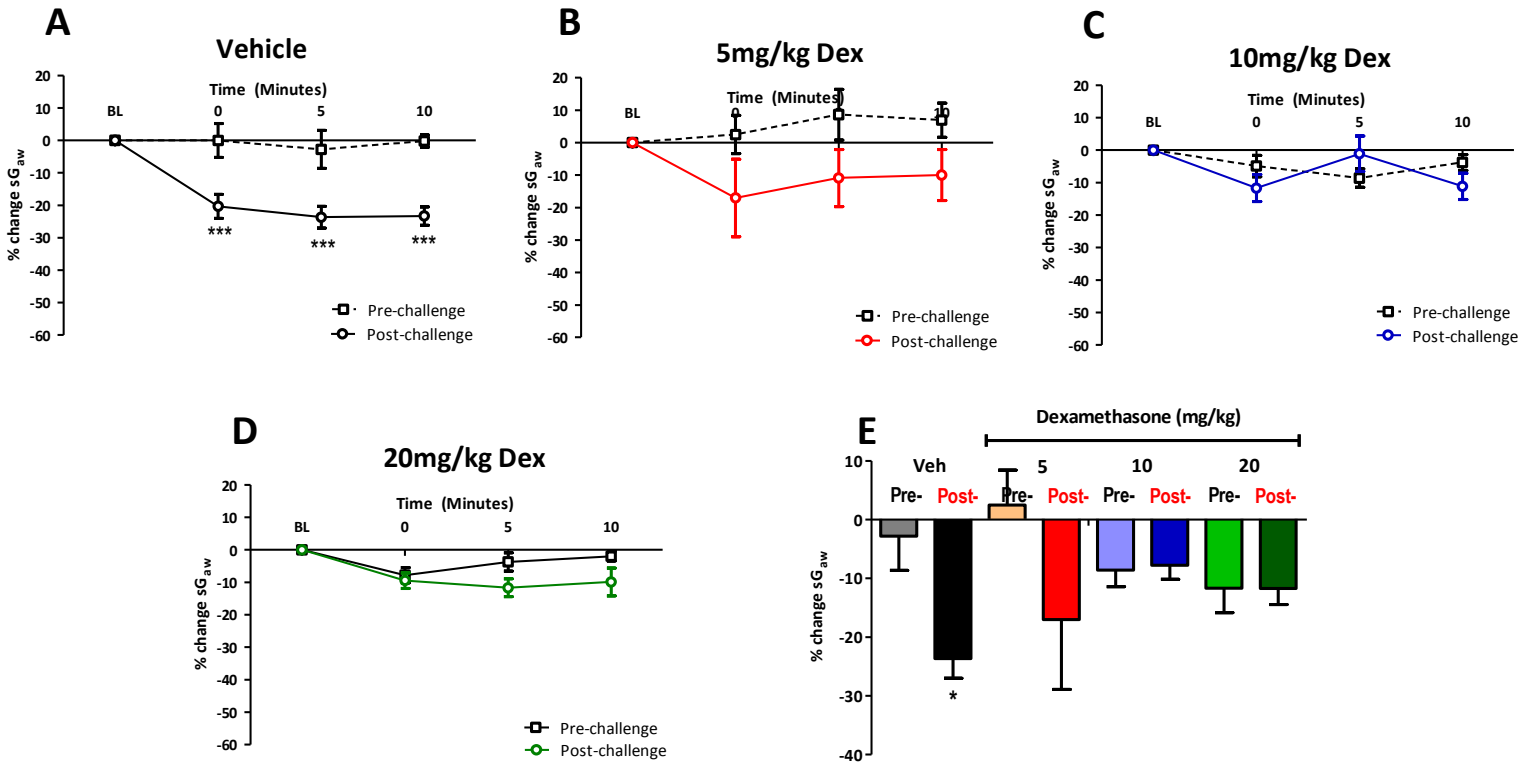


Figure 6: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles and 0.5LPM flow per chamber over 2minutes, 1 minute drying period) in ovalbumin (Ova) sensitised and challenged guinea-pigs treated with either **A)** intra-peritoneal vehicle or dexamethasone (**B)** 5mg/kg **C)** 10mg/kg **D)** 20mg/kg**)** dosed daily for 6 days. **E)** Represents the peak the bronchoconstriction pre- and post-Ova following histamine challenge. Values were recorded 7 days pre- and 24 hours post-Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=6 (vehicle), N=4 (Dex 5mg/kg), N=6 (Dex 10mg/kg), N=7 (Dex 20mg/kg). ***significantly different from vehicle treatment $p<0.001$; performed with performed with a two tailed T-test.

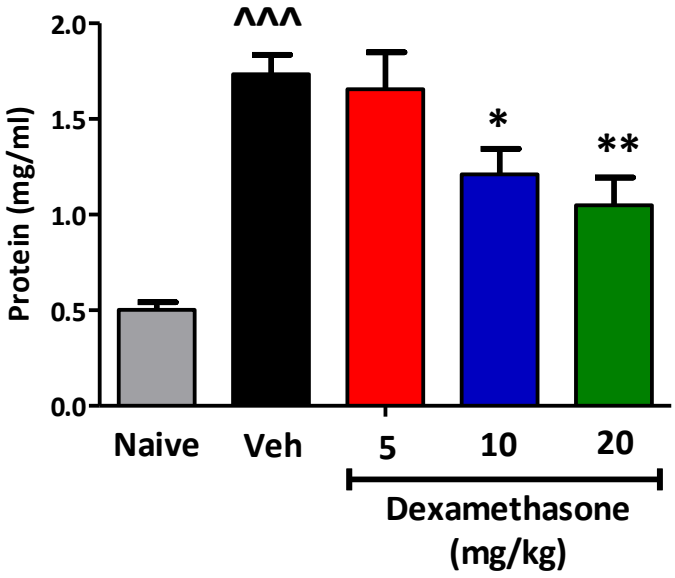


Figure 7: Total lavage fluid protein in ovalbumin (Ova) sensitised and challenged guinea-pigs treated with either intra-peritoneal vehicle or dexamethasone (Dex, 5mg/kg, 10mg/kg and 20mg/kg) dosed daily for 6 days. Protein content was determined by BCA protein assay. N=6 (vehicle), N=4 (Dex 5mg/kg), N=6 (Dex 10mg/kg), N=7 (Dex 20mg/kg).*significantly different from vehicle treatment $p<0.05$, ** $p<0.01$; ^^^significantly different from naïve, $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

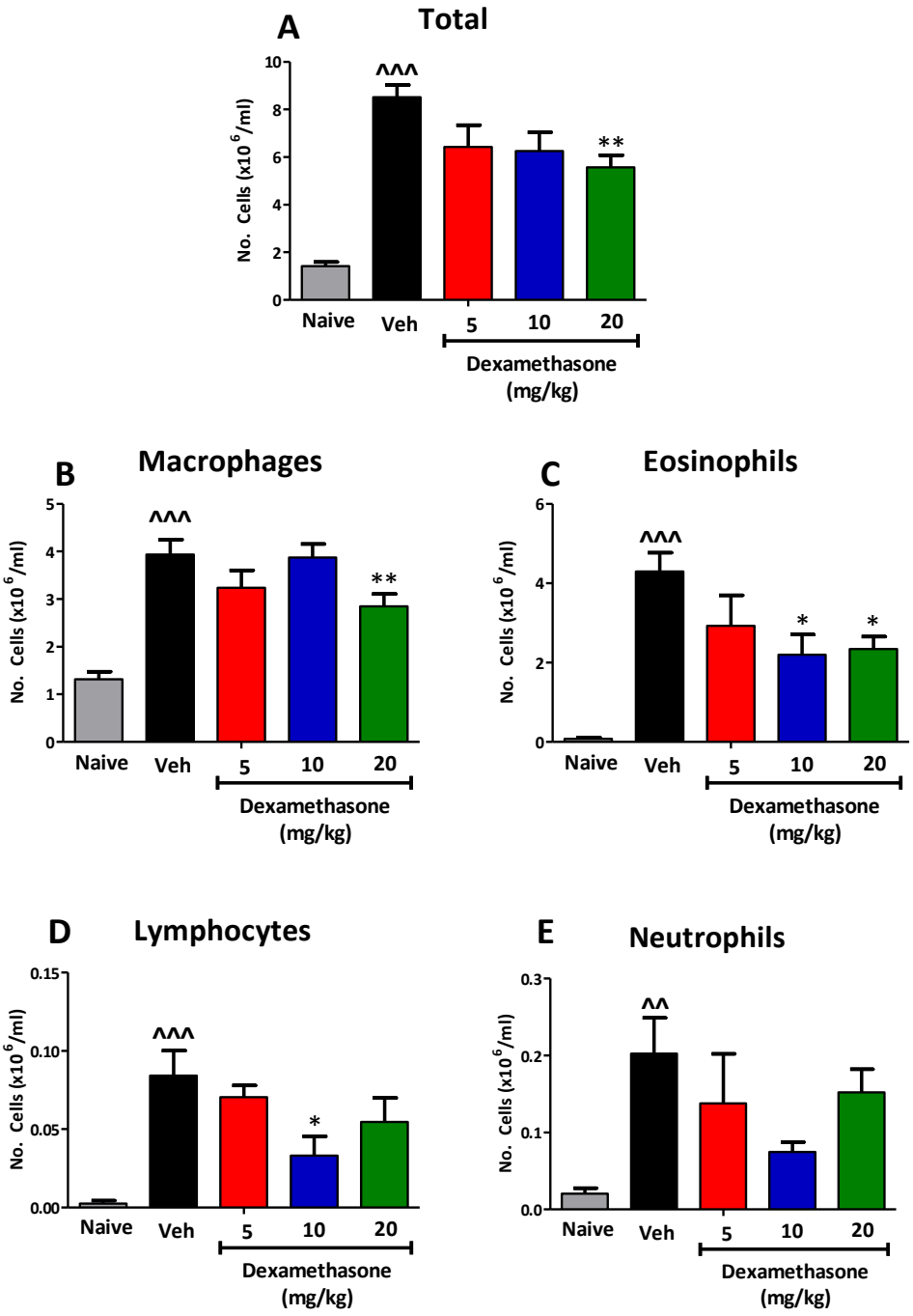


Figure 8: The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in bronchoalveolar fluid in ovalbumin (Ova) sensitised and challenged guinea-pigs treated with intra-peritoneal vehicle or dexamethasone (Dex, 5mg/kg, 10mg/kg and 20mg/kg) dosed daily for 6 days. N=6 (vehicle), N=4 (Dex 5mg/kg), N=6 (Dex 10mg/kg), N=7 (Dex 20mg/kg).*significantly different from vehicle treatment $p<0.05$, ** $p<0.01$; ^^^ significantly different from naive $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

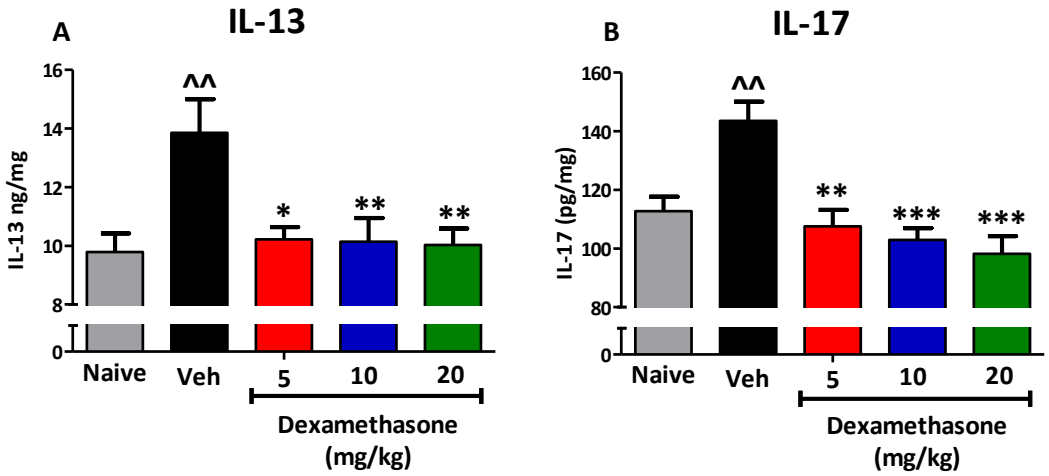


Figure 9: The concentration of A) IL-13 B) IL-17 in the lungs of ovalbumin (Ova) sensitised and challenged guinea-pigs treated with intra-peritoneal vehicle or dexamethasone (Dex, 5mg/kg, 10mg/kg and 20mg/kg) dosed daily for 6 days. N=6 (vehicle), N=4 (Dex 5mg/kg), N=6 (Dex 10mg/kg), N=7 (Dex 20mg/kg).*significantly different from vehicle treatment $p<0.05$, ** $p<0.01$; *** $p<0.001$; ^^ significantly different from naive $p<0.01$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

7.5.2 The effect of systemic dexamethasone on acute Ovalbumin and LPS co-exposure responses

Ova challenged and vehicle treated guinea-pigs demonstrated an immediate bronchoconstriction to Ova at 0 minutes (-54.3±6.6%) which did not return to baseline sGaw values until 7 hours. Dexamethasone (20mg/kg) treated animals also demonstrated an immediate bronchoconstriction (-46.1±11.2%). During the first 6 hours following Ova exposure, dexamethasone treated animals demonstrated significantly less of a bronchoconstriction than vehicle, including at 3 (-15.4±10.0% compared to -55.6±4.6%) 4 (-7.9±8.8% compared to -41.9±5.2%) and 5 hours (-11.5±8.2% compared to -35.1±3.4%). Vehicle treated guinea-pigs displayed a late asthmatic response at 9 hours (-21.9±9.9%) which was not significantly reduced by dexamethasone treatment (-9.0±9.7%) (Figure 10, time course). No difference in the peak early or late phase responses was observed between vehicle and dexamethasone treatment groups (Figure 10, histogram). The area under the curve for early phase responses was significantly reduced by dexamethasone treatment (208.0±59.3%.hr compared to vehicle, 357.6±44.0%.hr). No significant difference in late phase area under the curve was observed (Figure 11). Intra-peritoneal dexamethasone (20mg/kg) significantly increased the recovery from early asthmatic response bronchoconstriction (2.1±0.5h compared to 4.8±0.5h respectively) (Figure 12).

Vehicle treated guinea-pigs demonstrated an immediate increase in the bronchoconstrictor response to histamine, post-Ova challenge (-34.8±5.8% compared to pre-Ova, -2.0±1.3%). This bronchoconstriction continued to increase over 10 minutes. At 10 minutes it was -43.2±5.1% compared to pre-challenge, -6.9±3.1%. Treatment with 20mg/kg dexamethasone did not significantly attenuate the development of airways-hyperresponsiveness. At 5 and 10 minutes the bronchoconstriction to histamine post-Ova challenge was -36.0±12.3% compared to

pre-Ova, -3.9±4.1%; -35.3±9.9% compared to pre-Ova, -1.1±2.6% respectively) although the immediate response to histamine was attenuated (Figure 13). The peak bronchoconstriction to histamine increased post-Ova/LPS challenge in both vehicle (-43.2±5.1% compared to pre-Ova, -7.0±5.1%) and dexamethasone (-36.0±12.3% compared to pre-Ova, -6.3±1.8%).

Lavage fluid protein levels, increased in vehicle treated guinea-pigs (2.4±0.4mg/ml) compared to naïve (0.4±0.1). Lavage fluid protein levels were reduced in dexamethasone (20mg/kg) treated guinea pigs, nearly back to naïve levels (0.7±0.2mf/ml) (Figure 14).

Total cell numbers, increased In Ova/LPS challenged guinea-pigs (25.8±1.6x10^6/ml) compared to naïve (1.4±0.2x10^6/ml), were significantly reduced by dexamethasone (20mg/kg) treatment (11.2±1.2x10^6/ml). All cell types measured were reduced by dexamethasone treatment including macrophages (5.5±0.5x10^6/ml compared to vehicle 10.5±0.8x10^6/ml), eosinophils (2.4±0.7x10^6/ml compared to vehicle 8.2±1.2x10^6/ml), lymphocytes (0.1±0.04x10^6/ml compared to vehicle 0.6±0.2x10^6/ml) and neutrophils (3.2±0.8x10^6/ml compared to vehicle 6.8±0.8x10^6/ml) (Figure 15).

IL-8, IL-13 and IL-17 levels were significantly elevated in Ova/LPS challenged and vehicle treated guinea-pigs (45±7.3pg/mg, 16.9±1.0ng/mg and 105.6±19.8pg/mg, respectively) compared to naïve (not detectable, 8.7±2.9ng/mg and not detectable respectively). None of these cytokines were significantly reduced by dexamethasone treatment (Figure 16).

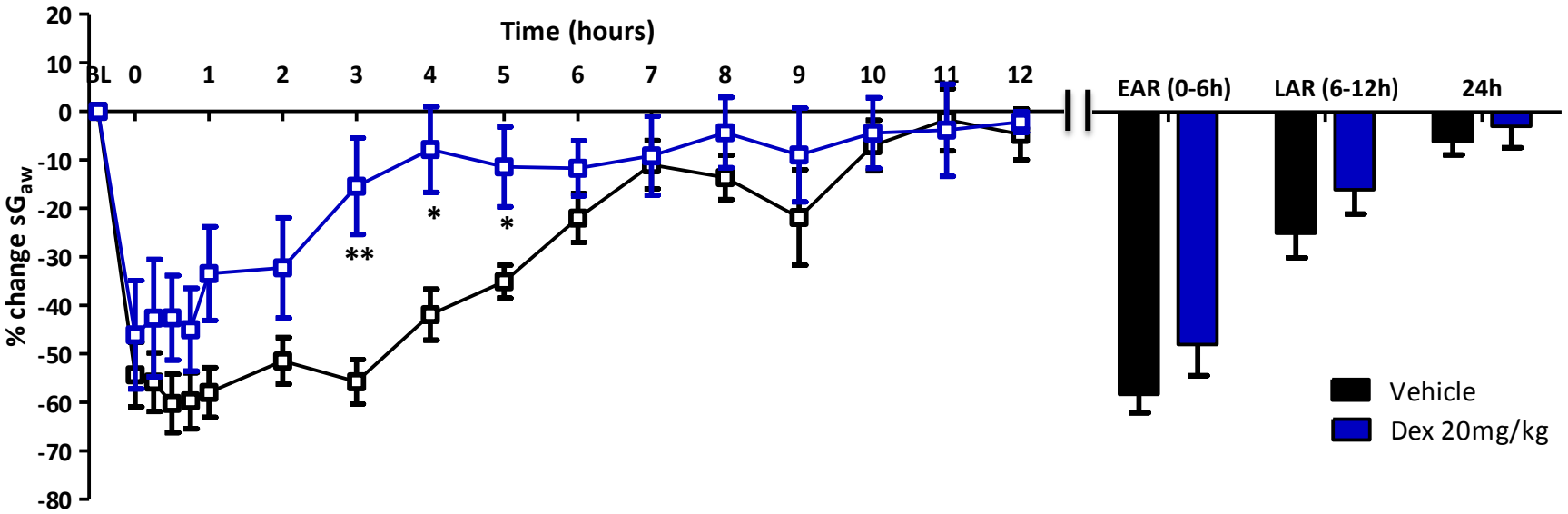


Figure 10: The mean time-course values of sGaw in ovalbumin (Ova) and LPS challenged guinea-pigs treated with intra-peritoneal vehicle or dexamethasone (Dex, 20mg/kg) dosed daily for 6 days. The histogram represents the maximum bronchoconstriction values recorded during the early asthmatic response (EAR) (0-6 hours), late asthmatic response (LAR) (6-12 hours) and 24 hours readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline prior to Ova challenge. A negative value represents a bronchoconstriction. N=5 (vehicle), N=6 (Dex) *significantly different from vehicle treatment $p<0.05$, ** $p<0.01$; performed with a two tailed T-test.

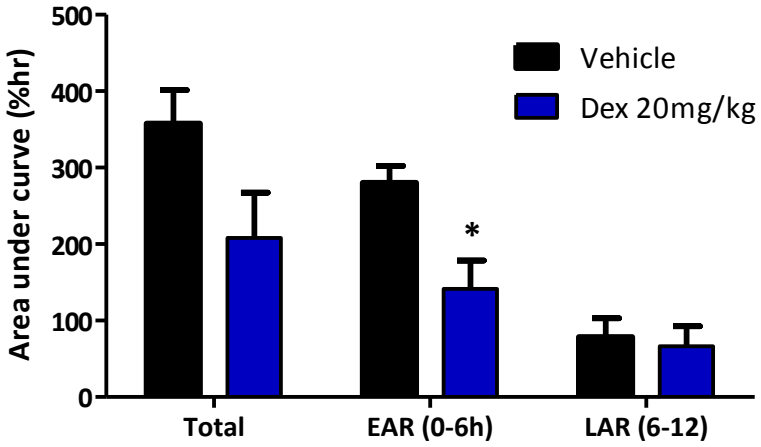


Figure 11: Area under the curve analysis of sGaw values over 12 in ovalbumin (Ova) and LPS challenged guinea-pigs treated with intra-peritoneal vehicle or dexamethasone (Dex, 20mg/kg) dosed daily for 6 days. For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-24 hours, early asthmatic response (EAR) includes values from 0-6 hours and late asthmatic response (LAR) includes values from 6-12 hours. Area under the curve is expressed as %.hour. N=5 (vehicle), N=6 (Dex) *significantly different from vehicle treatment $p<0.05$; performed with a two tailed T-test.

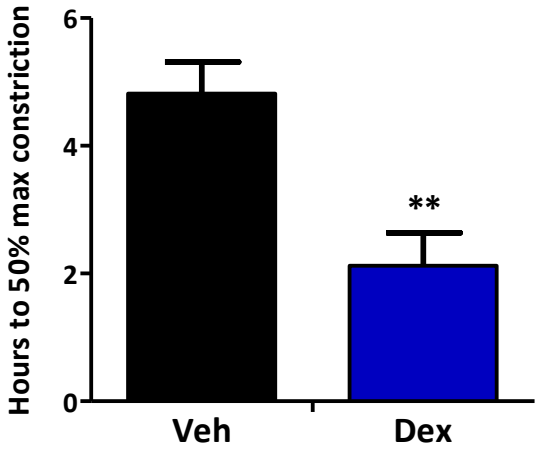


Figure 12: Analysis of the time taken for early asthmatic responses (EAR) to recover to 50% of peak bronchoconstriction values in ovalbumin (Ova) and LPS challenged guinea-pigs treated with intra-peritoneal vehicle or dexamethasone (Dex, 20mg/kg) dosed daily for 6 days. N=5 (vehicle), N=6 (Dex) **significantly different from vehicle treatment $p<0.01$; performed with a two tailed T-test.

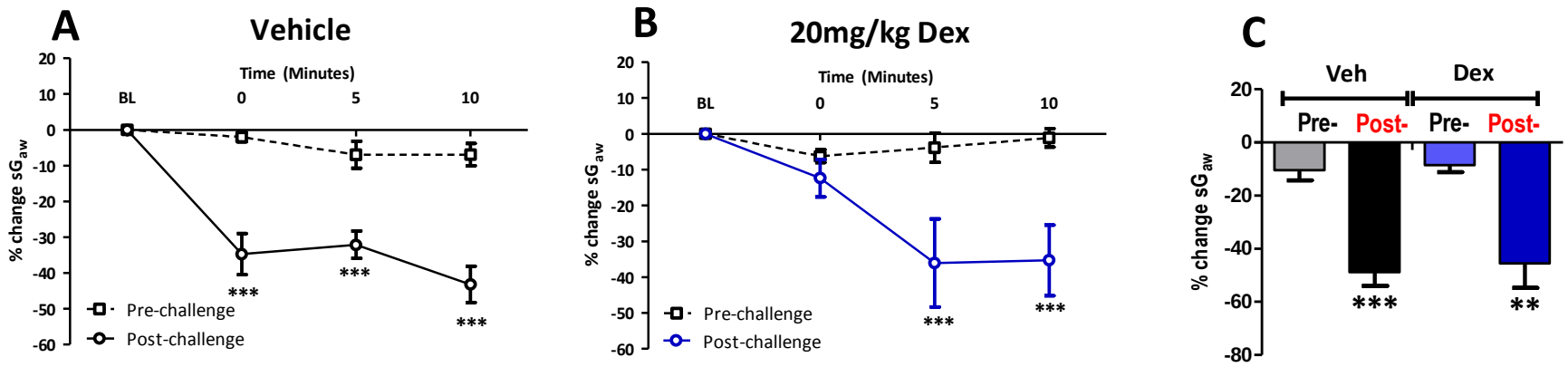


Figure 13: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles 0.5LPM flow per chamber over 2 minutes, 1 minute drying period) in ovalbumin (Ova) and LPS challenged guinea-pigs treated with intra-peritoneal A) vehicle or B) dexamethasone (Dex, 20mg/kg) dosed daily for 6 days. **C)** Represents the peak the bronchoconstriction pre- and post-Ova following histamine challenge. Values were recorded 7 days pre- and 24 hours post-Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=5 (vehicle), N=6 (Dex) **significantly different from time paired pre-Ova challenge values $p<0.01$; *** $p<0.001$; performed with a two tailed T-test.

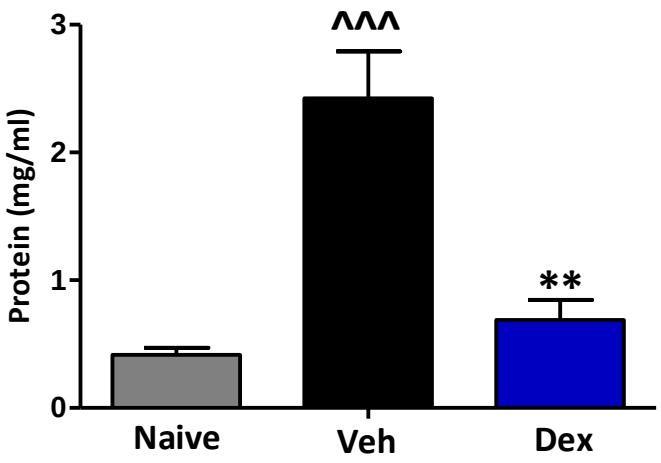


Figure 14: Total lavage fluid protein in ovalbumin (Ova) and LPS challenged guinea-pigs treated with intra-peritoneal vehicle or dexamethasone (Dex, 20mg/kg) dosed daily for 6 days. Protein content was determined by BCA protein assay. N=5 (vehicle), N=6 (Dex) **significantly different from vehicle treatment $p<0.01$; ^^^significantly different from naïve, $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

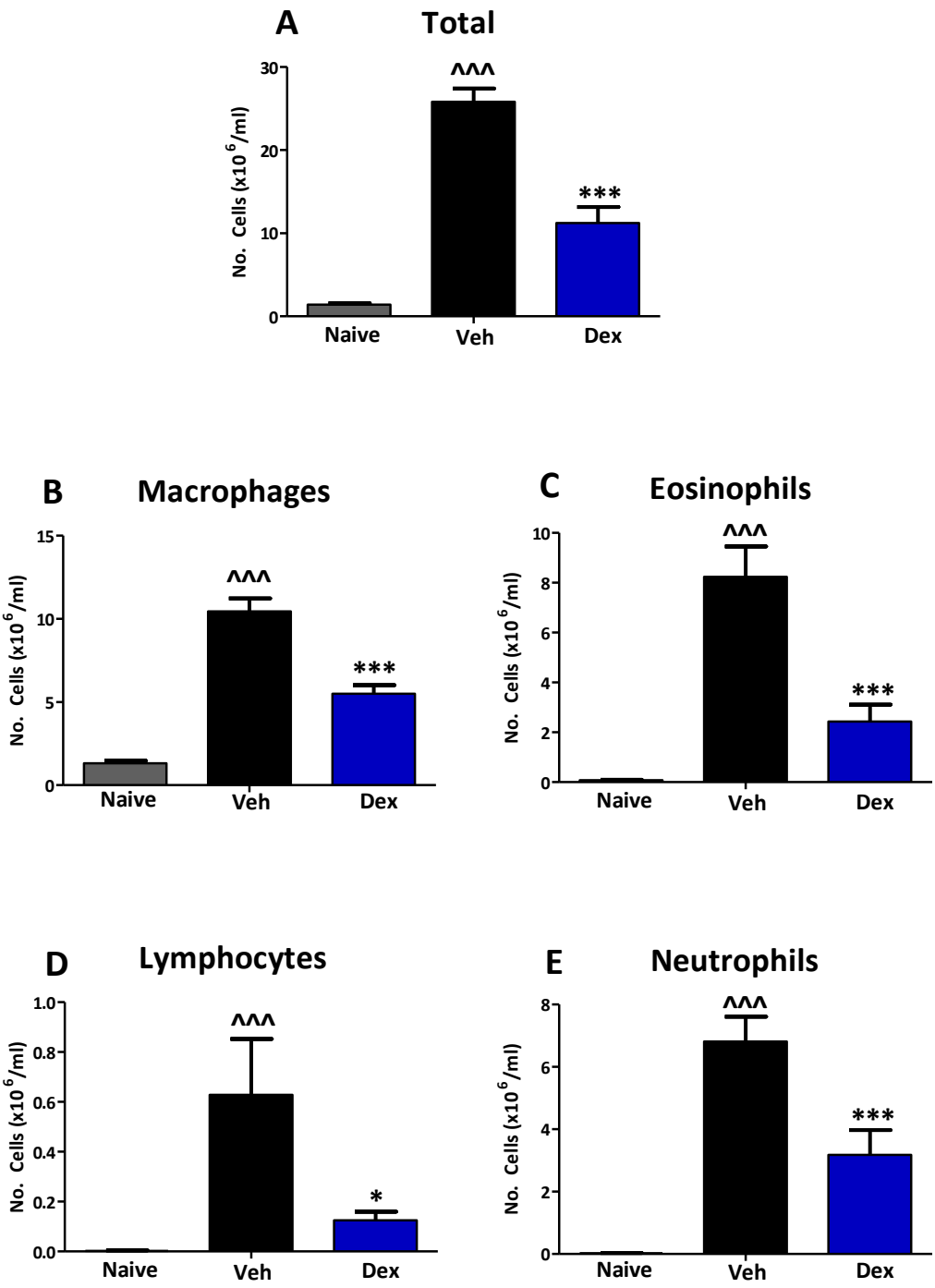


Figure 15: The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in bronchoalveolar fluid of ovalbumin (Ova) and LPS challenged guinea-pigs treated with intra-peritoneal vehicle or dexamethasone (Dex, 20mg/kg) dosed daily for 6 days. N=5 (vehicle), N=6 (Dex) *significantly different from vehicle treatment $p<0.05$,; *** $p<0.001$; ^^^significantly different from naïve, $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

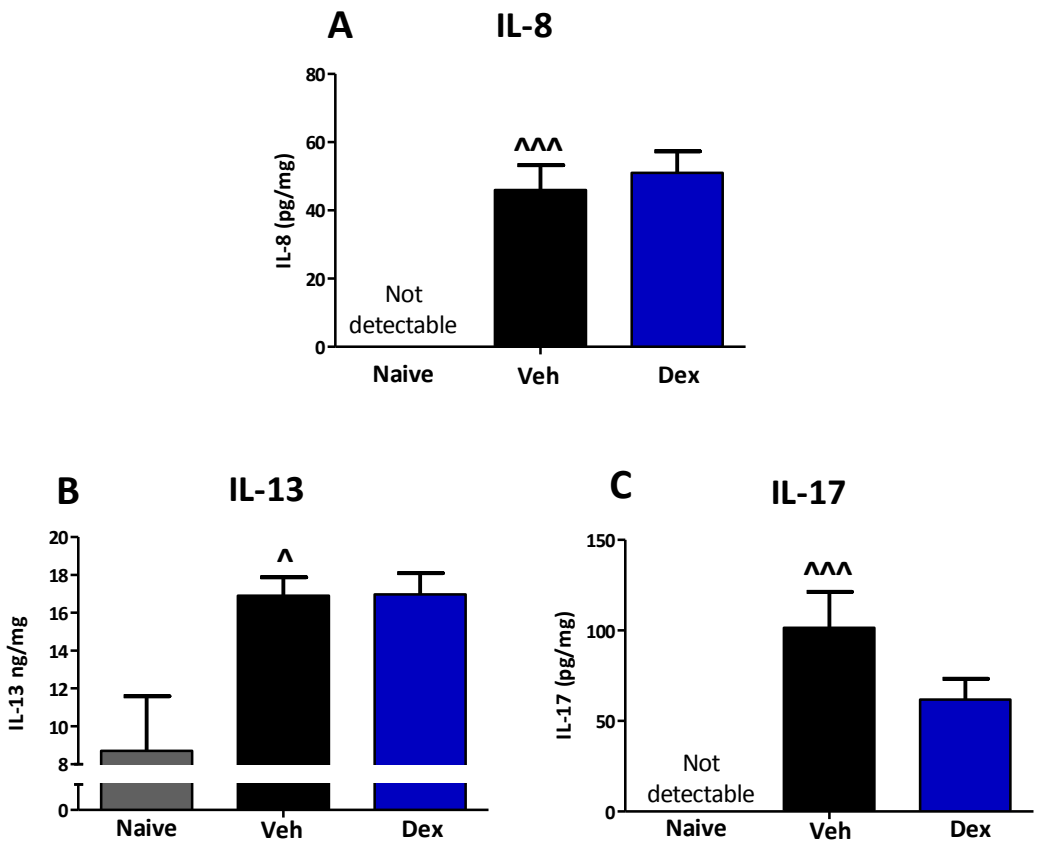


Figure 16: The concentration of A) IL-8 B) IL-13 C) IL-17 in the lungs of ovalbumin (Ova) sensitised guinea-pigs treated with intra-peritoneal vehicle or dexamethasone (Dex, 20mg/kg) dosed daily for 6 days. N=5 (vehicle), N=6 (Dex) ^significantly different from naive $p<0.05$, ^^^ $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

8.5.3 THE EFFECT OF SYSTEMIC DEXAMETHASONE ON LPS EXPOSURE RESPONSES

Vehicle treated animals demonstrated a progressive increase in bronchoconstriction during the 1st hour post-LPS exposure, reaching its peak at 3 hours (-36.0±3.5%). Dexamethasone (20mg/kg) significantly attenuated the LPS induced bronchoconstriction during this time period. At 1 hour the bronchoconstriction was -9.5±7.1% compared to vehicle -31.8±2.8%, 2 hours -7.9±5.1% compared to vehicle, -29.1±3.4% and 3 hours -7.8±5.9% compared to -36.0±3.5%. In vehicle treated animals the bronchoconstriction returned to baseline sGaw levels at 5 hours. No further significant difference between vehicle and dexamethasone treated groups was seen (Figure 17, time course). Vehicle treated guinea pigs demonstrated a peak constriction between 0-12 hours of -38.5±3.2% which was significantly reduced by dexamethasone treatment (-17.6±5.6%) (Figure 17, histogram). Dexamethasone treatment significantly reduced the area under the curve between 0-12 hours compared to vehicle (212.6±34.1%.hr compared to 93.0±30.4%.hr) (Figure 18).

LPS increased protein levels in lavage fluid (1.5±0.1mg/ml) compared with naïve (0.5±0.05mg/ml) was significantly reduced by dexamethasone (20mg/kg) treatment to near naïve levels (0.6±0.1mg/ml and 0.5±0.1mg/ml respectively) (Figure 19).

Vehicle treated guinea-pigs demonstrated a significant increase in the bronchoconstrictor response at 0 minutes to histamine post-LPS exposure (-15.8±3.1%) compared to pre-LPS (-2.1±3.5%). At 5 minutes post-histamine challenge a significant bronchoconstriction was still present (-12.3±2.3%) compared to pre-LPS (5.6±2.4%). The bronchoconstriction was no longer significant at 10 minutes. Dexamethasone treated guinea-pigs also displayed no increase in bronchoconstrictor response to histamine (Figure 20). LPS challenged and vehicle treated guinea pigs also demonstrated an increase in the peak bronchoconstriction to histamine (-

16.5±2.7%) compared to pre-LPS (-7.4±1.7%). Dexamethasone treated guinea-pigs demonstrated no change in peak bronchoconstriction.

LPS increased total cell numbers (19.4±0.8x10^6/ml) compared to naïve (1.1±00.2x10^6/ml) and were significantly reduced by treatment with dexamethasone (13.5±0.9x10^6/ml). This decrease corresponded with a decrease in the number of eosinophils and neutrophils (0.1±0.02x10^6/ml compared with vehicle, 0.6±0.2x10^6/ml; 5.5±0.7x10^6/ml compared with vehicle, 10.5±0.4x10^6/ml). All other cell types remained unchanged by dexamethasone treatment (Figure 21).

IL-8 levels increased in vehicle treated guinea–pigs (190.8±34.4pg/mg) compared to naïve (not detectable) and was significantly reduced by treatment with dexamethasone (91.4±15.8pg/mg) (Figure 22).

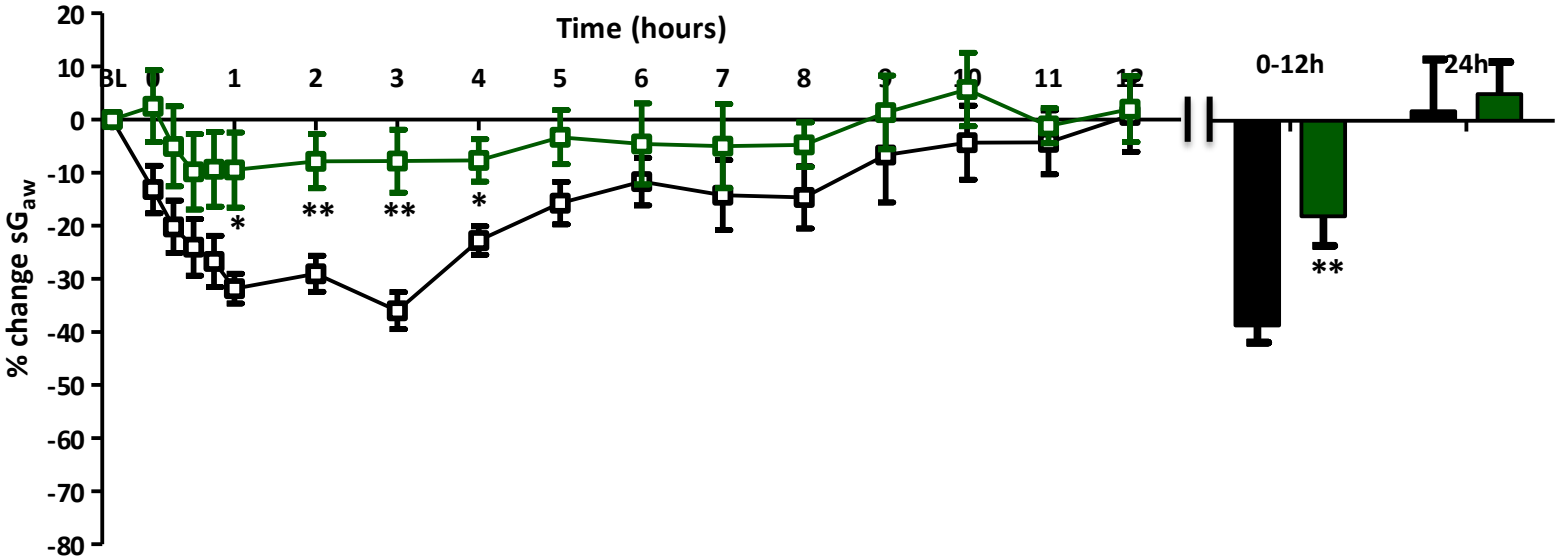


Figure 17: The mean time-course values of sGaw in guinea-pigs exposed to LPS twice and treated with an intra-peritoneal injection of vehicle or 20mg/kg dexamethasone, dosed daily for 6 days. The histogram represents the maximum bronchoconstriction values recorded during 0-12 hours and 24 hours readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline prior LPS challenge. A negative value represents a bronchoconstriction. Results shown are after the second LPS exposure. N=6 *significantly different from vehicle treatment $p<0.05$, ** $p<0.01$; performed with a two tailed T-test.

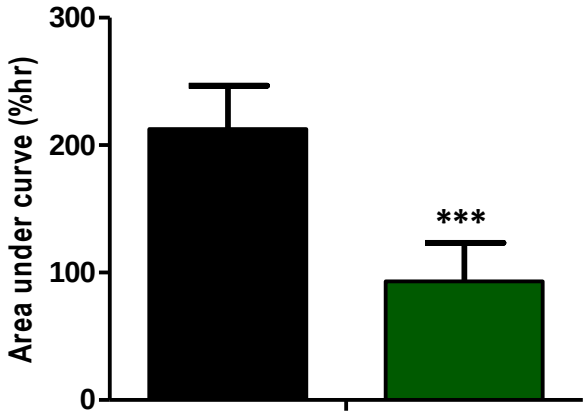


Figure 18: Total area under the curve analysis of sGaw values in guinea-pigs exposed to LPS twice and treated with an intra-peritoneal injection of vehicle or 20mg/kg dexamethasone, dosed daily for 6 days. For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-12 hours. Area under the curve is expressed as %.hour. N=6 ***significantly different from vehicle treatment p<0.001; performed with a two tailed T-test.

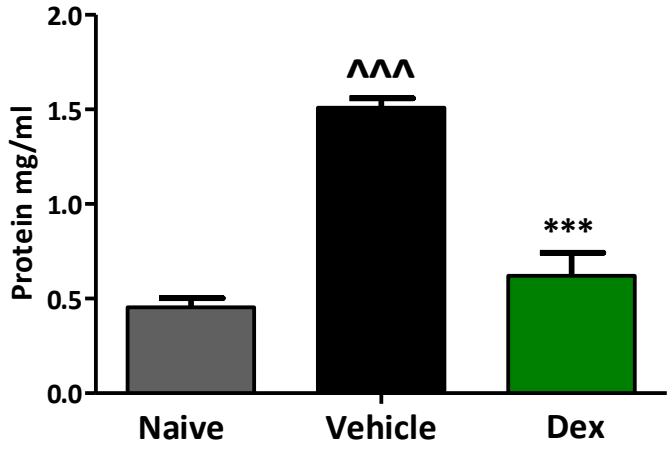


Figure 19: Total lavage fluid in guinea-pigs exposed to LPS twice and treated with an intra-peritoneal injection of vehicle or 20mg/kg dexamethasone, dosed daily for 6 days. Protein content was determined by BCA protein assay. N=6 ***significantly different from vehicle treatment p<0.001; ^^^ significantly different from naive p<0.001; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

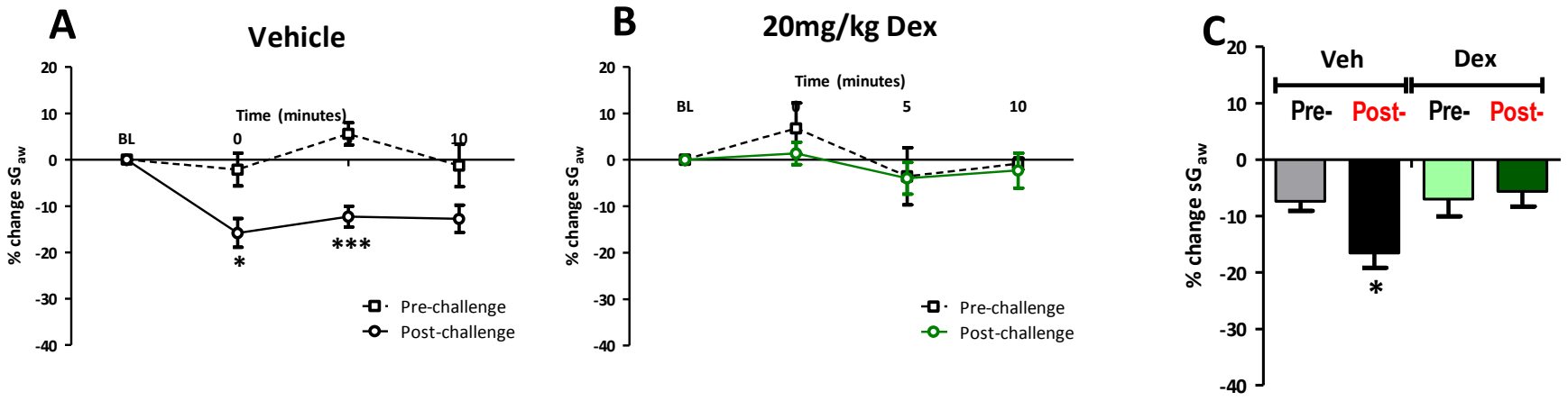


Figure 20: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles 0.5LPM flow per chamber over 2 minutes, 1 minute drying period,) in guinea-pigs exposed to LPS twice and treated with an intra-peritoneal injection of A) vehicle or B) 20mg/kg dexamethasone, dosed daily for 6 days. **C)** Represents the peak the bronchoconstriction pre- and post-Ova following histamine challenge. Values were recorded 7 days pre- and 24 hours post-Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=6 *significantly different from time paired pre-LPS challenge values $p<0.05$; *** $p<0.001$; performed with a two tailed T-test.

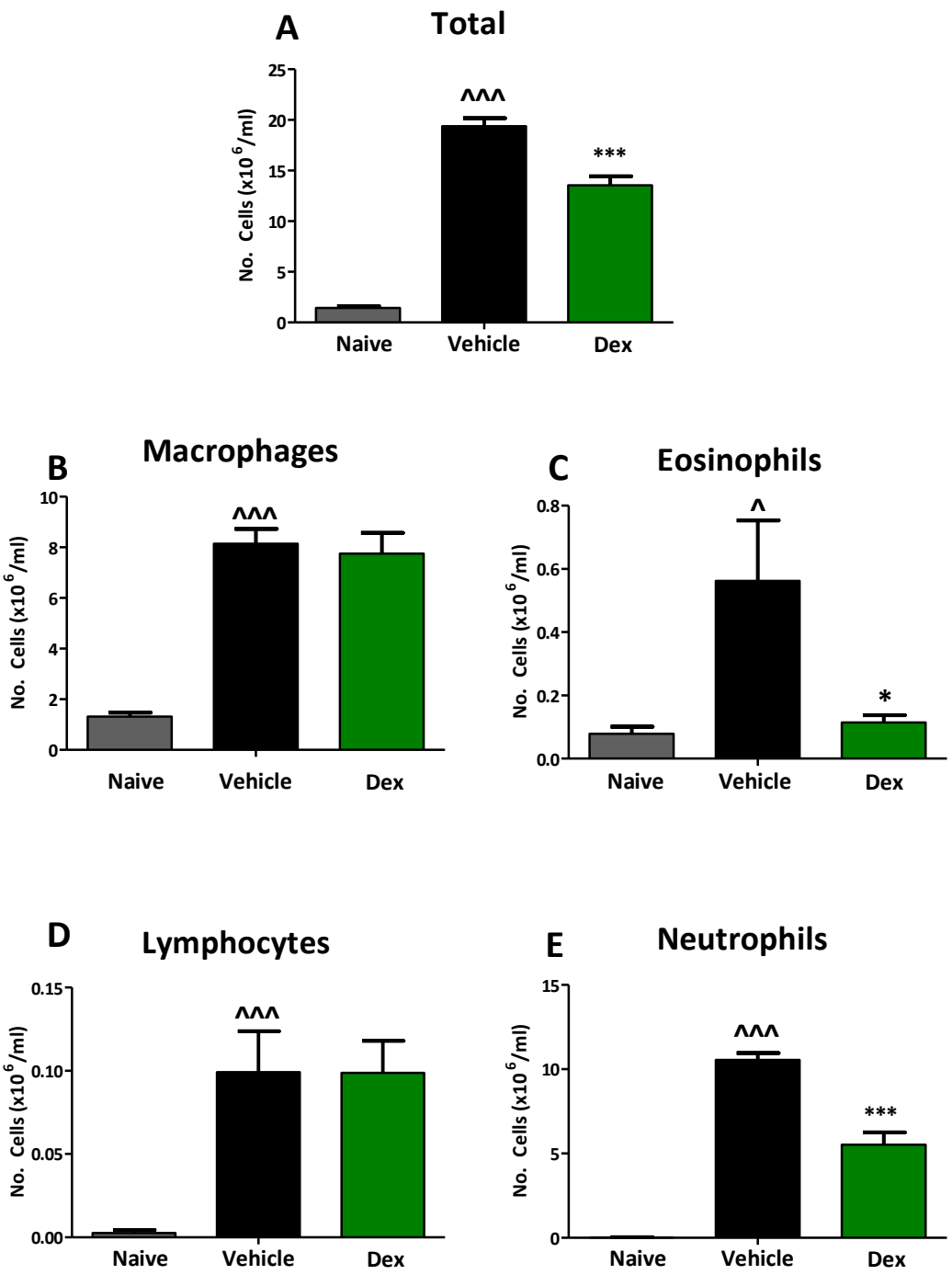


Figure 21: The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in bronchoalveolar in guinea-pigs exposed to LPS twice and treated with an intra-peritoneal injection of vehicle or 20mg/kg dexamethasone, dosed daily for 6 days. N=6 ***significantly different from vehicle treatment $p<0.001$; ^significantly different from naïve, $p<0.05$; ^^^ $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

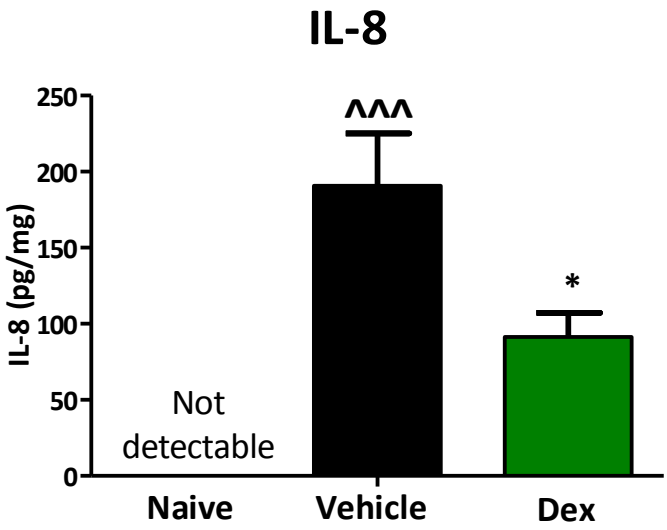


Figure 22: The concentration of IL-8 in the lungs of guinea-pigs exposed to LPS twice and treated with an intra-peritoneal injection of vehicle or 20mg/kg dexamethasone, dosed daily for 6 days. N=6 *significantly different from vehicle treatment $p<0.05$, ^^^ significantly different from naive $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

7.6 DISCUSSION

Initially, a dose of systemic dexamethasone which reduced the LAR, AHR and airway inflammation induced by Ova alone was sought. This dose was then used to test the systemic corticosteroid sensitivity of the Ova and LPS exacerbation model developed in chapter 5. The sensitivity of LPS induced responses to this same dose was also investigated.

7.6.1 THE EFFECT OF SYSTEMIC DEXAMETHASONE ON EARLY AND LATE ASTHMATIC RESPONSES

Systemic dexamethasone demonstrated some effect on the EAR. The peak of the EAR was not affected by any dose of systemic dexamethasone but the area under the curve during this time period was reduced. In the case of 10mg/kg dexamethasone this was a statistically significant reduction. This indicates that systemic dexamethasone can reduce the duration of the EAR. This effect of dexamethasone is supported by a study by Toward & Broadley, 2004, who also demonstrated that systemically administered dexamethasone reduces the length of the EAR. This response coincided with a reduction in lavage fluid histamine levels and increased nitric oxide 1 hour after allergen challenge. This indicates that a reduction in histamine release from mast cells and increased nitric oxide production may mediate this effect in the present study. This is further supported by the observation that L-NAME, a nitric oxide synthase inhibitor increases the duration of the EAR (Schuiling *et al*, 1998).

Systemic dexamethasone also reduced the prolonged EAR in Ova and LPS challenged guinea-pigs. This may suggest that the EAR in Ova/LPS groups consists of separate systemic corticosteroid sensitive and insensitive components. This could be mediated by the selective decrease in mast cells and histamine release. James *et al*, (2012) recently demonstrated that inhaled corticosteroids can selectively decrease epithelial and smooth muscle mast cells but not sub mucosal mast cells. A similar

selective decrease in the mast cells with a systemic corticosteroid may account for a decrease in the prolonged EAR in Ova/LPS groups. The reduction could also be due to a decrease in neutrophils in lavage fluid. Systemic dexamethasone treatment in LPS exposed guinea-pigs also decreased LPS-induced bronchoconstrictions and neutrophil numbers. Neutrophils increase during the EAR and can release bronchoconstrictive substances (Danahay *et al*, 1995; Reino *et al*, 2012). However, sensitivity to systemic corticosteroid of LPS-induced bronchoconstrictions and neutrophils has not been shown in all studies. Lefort *et al*, (2001) demonstrated that LPS-induced bronchoconstrictions were systemic corticosteroid insensitive while lavage neutrophils were not. However, no decrease in neutrophil numbers was found in the airway structure with histological assessment, not making it possible to rule out the role of neutrophils. Instead this study suggested that macrophages may be the mediators of immediate bronchoconstrictions to LPS. However, in the present study macrophage numbers were not reduced with systemic dexamethasone treatment not supporting this conclusion. The reasons for this disparity are unclear, but differences in the LPS exposure protocol and the use of C57BL/6 mice may account for this.

The LAR was reduced in a dose-dependent manner; being significantly attenuated by the highest dose of dexamethasone (20mg/kg) used in Ova challenged guinea-pigs. The ability of systemic dexamethasone to reduce the LAR is well established (Toward & Broadley, 2004). In contrast, systemic dexamethasone did not significantly reduce the LAR in Ova and LPS challenged guinea-pigs. This is slightly surprising as the LAR is linked to the presence of airway inflammation but in the current study inflammation is reduced by dexamethasone treatment (Yoshida *et al*, 2005; Gauvreau *et al*, 2000). However, the specific component of the inflammatory response that promotes the LAR is unknown and consequently dissociation has been observed in several animal models (Smith & Broadley, 2007). Additionally, despite the reduction in airway inflammations by systemic dexamethasone, the absolute number of cells found in lavage fluid is still high (10 million total). Thus the reduction

in airway inflammation may not be sufficient to suppress the LAR. The observation that IL-13 levels, typically corticosteroid sensitive are not reduced by systemic dexamethasone would suggest these remaining cells are still actively secreting mediators.

7.6.2 The effect of systemic dexamethasone on Airways hyperresponsiveness

All doses of systemic dexamethasone reduced the development of AHR following Ova challenge. This confirms the results of previous studies from this laboratory in guinea-pigs (Toward & Broadley, 2004; Smith & Broadley, 2010). Systemic dexamethasone (20mg/kg) also reduced LPS-induced AHR at 24 hours. This confirms that the AHR suppressive effects of systemic dexamethasone at 1 hour extend to the 24 hour time point (Toward & Broadley, 2001).

In contrast, even the highest dose (20mg/kg) of systemic dexamethasone did not significantly reduce the development of AHR in guinea-pigs exposed to Ova and LPS. This result is supported by a study by Komlosi *et al,* (2006) who also found that Ova and LPS induced systemic corticosteroid insensitive AHR. Although AHR is generally associated with inflammation, in the present study total and differential cell counts are all reduced by systemic dexamethasone in Ova/LPS animals. This suggests that the reduction in inflammation is not sufficient to completely abolish the development of AHR in these animals. In support of this is the observation that although inflammation is reduced by systemic dexamethasone treatment, cell numbers are still significantly higher than naïve levels. Thus neutrophils and macrophages may still be active and releasing mediators which cause epithelial damage. The damage may increase the access and prevent the breakdown of bronchoconstrictive substances such as exogenous histamine. Some mediators such as IFN-γ and IL-13 are also directly implicated in AHR (Cockcroft & Davis, 2006; Yang *et al*, 2009). In the current study, IL-13 levels are not significantly reduced by systemic dexamethasone in Ova/LPS challenged guinea-pigs. However, IL-13 is

unlikely to be the main mediator of the prolonged bronchoconstrictor response in this model since levels do not increase over those in Ova challenged guinea-pigs

7.6.3 The effect of systemic dexamethasone on airway inflammation and cytokines

Systemic dexamethasone reduced the total number of lavage cells in a dose-dependent manner in Ova challenged guinea-pigs. Eosinophils, macrophages and lymphocytes were all reduced by the highest dose (20mg/kg) of systemic dexamethasone. This confirms the findings of Toward & Broadley, (2004) and Smith & Broadley, (2010) who also used systemic dexamethasone in a guinea-pig allergen challenge model. Total and differential cells were also reduced by systemic dexamethasone in Ova and LPS challenged guinea-pigs, indicating that the inflammation in this model is sensitive to systemic corticosteroids. This confirms the findings of Komlosi *et al,* (2006) who also demonstrated significant decreases in these cell populations with systemic dexamethasone in mice. IL-13 was significantly reduced by systemic dexamethasone in both Ova and Ova and LPS groups. Contrastingly, in the present study IL-13 levels were only decreased in Ova but not Ova/LPS challenged groups. The reason for this difference is unknown but species differences, protocols and the medium used to assess IL-13 (lavage fluid vs lung) may account for the difference.

The reason inflammation is sensitive to systemic corticosteroid in the Ova and LPS model is likely to be due to the compartmentalisation of corticosteroid insensitivity in the lung. Also, systemic corticosteroids have additional biological effects over inhaled corticosteroids. Systemic corticosteroids work not only by reducing inflammation in the lungs but also by suppressing bone marrow derived haemopoietic cells (Mao *et al*, 2004; Ben *et al*, 2008). Since LPS is administered by inhalation to the lungs the main corticosteroid insensitivity inducing effects are likely localised to this region of the body. Although systemic exposure to LPS from inhalation is also likely, the levels of exposure will be lower than that of the lung. At

higher doses or increased numbers of LPS exposures, corticosteroid insensitivity may become more generalised in the body. Goleva *et al*, (2008) showed that *in vitro* prolonged exposure to LPS reduces the effect of dexamethasone on human monocytes. *In vivo* a high dose of intranasal LPS in mice can cause systemic corticosteroids insensitivity (Lefort *et al*, 2001). This contrasts with the current study where fewer and lower doses of LPS induced inflammation that was sensitive to systemic dexamethasone.

Neutrophils have different sensitivity to corticosteroids in the systemic circulation and the lungs. Although neutrophils in the lung are relatively corticosteroid insensitive due to decreased expression of GR-α, in the blood they demonstrate increased expression of GR-α, rendering them sensitive to suppression by corticosteroids (Plumb *et al*, 2012). This may account for the decrease in neutrophils with systemic dexamethasone in Ova/LPS and LPS challenged guinea-pigs. Systemic administration of corticosteroids suppresses neutrophil recruitment to the lung because of high circulating levels of the drug (Ben *et al*, 2008). However, blood neutrophils are still relatively insensitive to corticosteroids, probably explaining why systemic dexamethasone does not completely eliminate them. Lung neutrophils in conditions of high oxidative stress may become even more corticosteroid insensitive by decreasing the activity of HDAC-2 and increasing GR-β (Hamilos *et al*, 2001; Ito *et al*, 2004). Thus the elevated neutrophil numbers still present in Ova and LPS challenged guinea-pigs may represent a distinct corticosteroid insensitive lung neutrophil population. This population may be supported by the increased levels of IL-8 in the lungs, which are not suppressed by systemic dexamethasone. IL-17 levels in lung were unaffected by systemic dexamethasone treatment in Ova and LPS but decreased in Ova challenged groups. This indicates that IL-17-based responses have become less responsive to systemic corticosteroid treatment. This may be as a result of IL-17 responses being localised to the lung, where cells secreting IL-17 are less corticosteroid sensitive with Ova and LPS exposure.

7.6.4 The effect of systemic dexamethasone on airway oedema

Systemic dexamethasone significantly decreased total lavage fluid protein levels in guinea-pigs challenged with Ova, LPS/Ova and LPS. The decrease in total protein levels correlated with decreases in inflammation which may indicate that these effects are due to decreased levels of pro-inflammatory mediators. However, corticosteroids are known to have extra-genomic effects such as vasoconstriction so these effects are likely to also be important (Mendes *et al*, 2003).

7.6.5 General conclusions

This study has demonstrated that Ova induced LAR, AHR, airway inflammation, lung cytokines and lavage fluid protein levels are sensitive to systemic dexamethasone. Exposure to LPS reduces the effect of systemic dexamethasone on the LAR, AHR and cytokines. However, the elongated EAR, inflammatory cell influx and lavage protein levels are still sensitive to systemic dexamethasone. These results demonstrate that the Ova and LPS model may be clinically relevant. Patients who are insensitive to inhaled corticosteroids frequently demonstrate some sensitivity to systemic corticosteroids (Hodgson *et al*, 2012; Massuse *et al*, 2012). To fully confirm inhaled corticosteroid insensitivity in this model, the next chapter will use dexamethasone as an inhaled preparation.

CHAPTER 8

The Effect Of Inhaled Dexamethasone On Functional And Inflammatory Responses To Ovalbumin And LPS

Chapter 8

8.1 INTRODUCTION

For a description of the use of inhaled corticosteroids, corticosteroid insensitivity and their mechanism please refer to Chapter 6.1.

8.1.1 CONFIRMATION OF INHALED CORTICOSTEROID INSENSITIVITY

Following on from the results of chapter 6 and 7, this chapter aimed to confirm that insensitivity to inhaled corticosteroids in the Ova and LPS combination model was not specific to just FP. Therefore dexamethasone, having been shown to be systemically active in reducing functional and allergic responses in the Ova and LPS combination model was given as an inhalation. Dexamethasone is normally administered systemically but a few studies have given it as an intranasal preparation and found that it reduces AHR, airway inflammation and Th2 cytokines (Essilfie *et al,* 2012). Only one previous study in animals has administered dexamethasone as an inhalation (Jungsuwadee *et al*, 2004). This study found that inhaled dexamethasone in mice was also effective at reducing Ova-induced AHR and airway inflammation. To find an effective dose at reducing these parameters and the EAR, LAR and oedema induced by Ova alone, several doses were initially investigated.

8.2 HYPOTHESIS

'LPS exposure will decrease the sensitivity of functional and inflammatory responses to inhaled dexamethasone in an ovalbumin model of asthma'

8.3 AIMS AND OBJECTIVES

The aim of this chapter was to establish whether LPS decreases the sensitivity of Ova-induced functional and inflammatory responses to inhaled dexamethasone. Initially a dose of inhaled dexamethasone effective at reducing the LAR, AHR and airway inflammation by Ova alone was established. This dose was then tested in the LPS and Ova/LPS models.

8.4 METHODS

Methods describing the measurement of lung function, AHR, airway oedema, airway inflammation and cytokine levels can be found in more detail in chapter 2.

8.4.1 OVALBUMIN PROTOCOLS

8.4.1.1 SENSITISATION

Guinea-pigs (200-300g, Dunkin-Hartley, male) were sensitised by a bilateral intra-peritoneal injection of a solution containing 150µg ovalbumin (Ova) and 100mg aluminium hydroxide ($Al(OH)_3$) in normal saline on day 1, 4 and 7.

8.4.1.23 ACUTE OVALBUMIN MODEL

Sensitised guinea-pigs were exposed to inhaled Ova (0.03%) for 1 hour on day 21 in a Perspex container as described in section 8.4.1.2. The lung function response to Ova was assessed over 12 hours. The bronchoconstrictor response to 0.3mM histamine was accessed on day 15 and 22 as described in section 8.4.5. Dexamethasone was administered by inhalation in a Perspex chamber for 15 minutes, once a day from day 16-21. On day 21, dexamethasone was administered 30 minutes pre-Ova challenge. A diagram of this protocol is shown in shown in Figure 1.

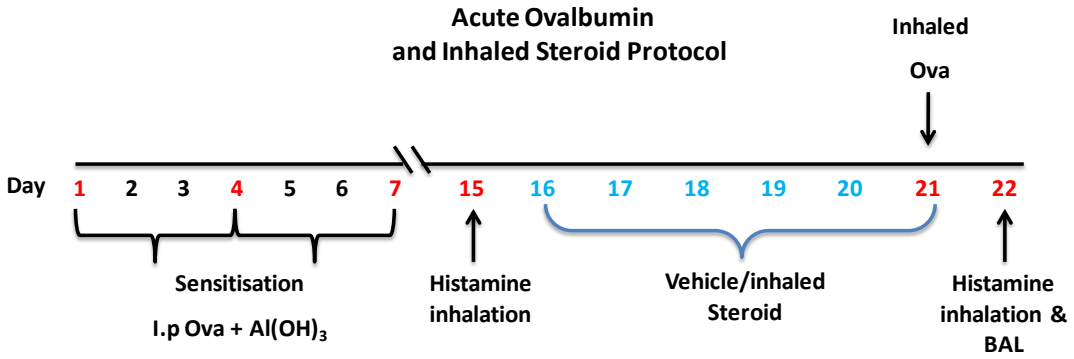


Figure 1: The acute ovalbumin (Ova) protocol. Guinea-pigs were challenged with inhaled Ova (0.03% on day 21). Dexamethasone (Dex) was dosed daily for 6 days.

8.4.1.3 Acute Ova and LPS co-exposure model

Sensitised guinea-pigs were exposed to inhaled LPS (30µg/ml) 48 hours before Ova challenge in an exposure chamber and both LPS (30µg/ml) and Ova (0.03%) co-administered on day 21. The bronchoconstrictor response to histamine was assessed on day 15 and day 22. Dexamethasone was administered by inhalation in a Perspex chamber for 15 minutes, once a day from day 16-21. On day 21, dexamethasone was administered 30 minutes pre-Ova challenge. A diagram of this protocol is shown in shown in Figure 2.

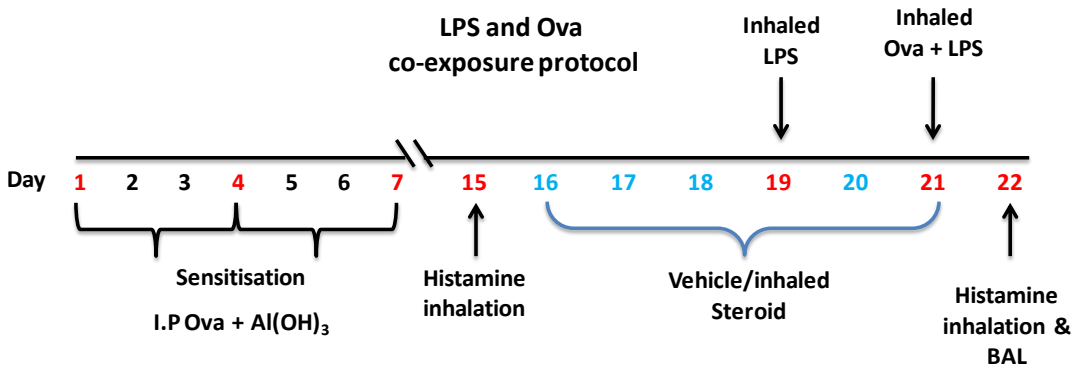


Figure 2 Diagram of the LPS and Ova co-exposure protocol. Guinea-pigs were exposed to LPS (30µg/ml) on day 19 and both Ova and LPS co-administered on day 21. Dexamethasone (Dex) was dosed daily for 6 days.

8.4.2 LPS exposure protocol

Non-sensitised guinea-pigs were exposed to LPS (30µg/ml) on days 5 and 7 of the protocol. The bronchoconstrictor response to histamine was assessed on day 1 and day 8. Figure 3 shows a diagram of this protocol. Dexamethasone was administered by inhalation in a Perspex chamber for 15 minutes, once a day from day 2-7. On day 7, dexamethasone was administered 30 minutes pre-LPS challenge. A diagram of this protocol is shown in Figure 2.

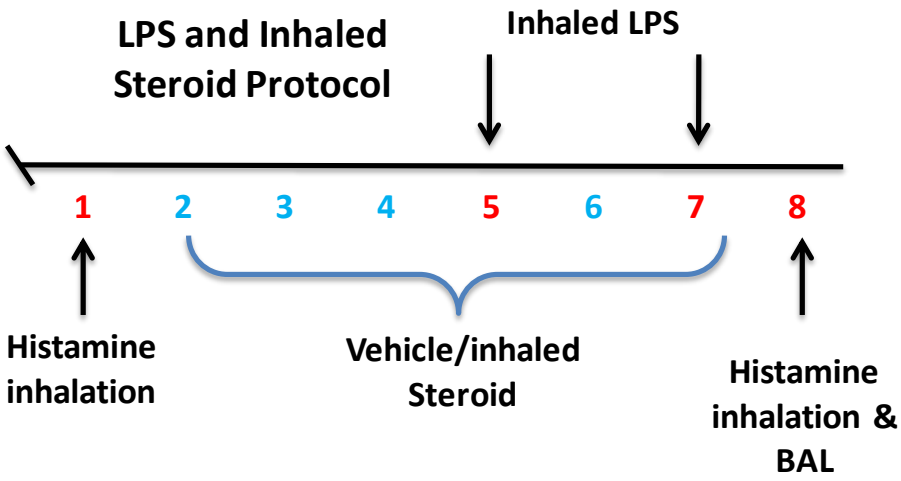


Figure 3: Diagram of the LPS alone exposure protocol. Guinea-pigs were exposed to 30µg/ml of LPS on day 5 and 7. Dexamethasone (Dex) was dosed daily for 6 days.

8.4.3 Drug administration

Dexamethasone was dissolved in 25% DMSO and 75% saline and administered as an inhalation for 15 minutes in a Perspex exposure chamber using a DeVilbiss nebuliser.

8.4.4 Measurement of lung function

Lung function was measured by whole body plethysmography following final Ova, LPS or Ova/LPS exposure in all protocols. Lung function was recorded hourly for 12 hours and every 15 minutes during the first hour of measurements. A final measurement was also taken 24 hours post-final challenge. All values were expressed as a percentage of the baseline reading, taken before the final challenge. A negative percentage change in baseline value represents a bronchoconstriction. In Ova protocols, early (0-6 hours) and late (6-12 hours) asthmatic responses were expressed as the peak bronchoconstriction during that period (displayed as a histogram next to a time course plot) and area under the curve. The duration of the early phase was expressed as the time taken to recover to 50% of peak early phase bronchoconstriction values. In the LPS only protocol, lung function was measured for 12 hours after the second LPS exposure. The peak bronchoconstriction during this period was expressed on a histogram next to the time course plot.

8.4.5 Airways Hyperresponsiveness assessment.

AHR was determined using whole body plethysmography. This was performed by measuring the bronchoconstrictor response to histamine before inhaled corticosteroid treatment and 24 hours post-final LPS, Ova, LPS or Ova challenge. Histamine was delivered as described in chapter 2. Lung function was measured just before histamine inhalation and at 0, 5 and 10 minutes post-histamine exposure. The peak bronchoconstriction during this period was expressed on a histogram.

6.4.6 Assessment of airways inflammation

Following final histamine exposure guinea-pigs were sacrificed by an intra-peritoneal overdose of sodium pentobarbitone, the lungs excised and lavaged. Total and differential cell counts were then performed as described in chapter 2.

8.4.7 Quantification of cytokine levels using enzyme-linked immunosorbent assay (ELISA)

Levels of IL-8, IL-17 and IL-13 were measured in diluted homogenised lung samples using ELISA supplied by R&D systems. Cytokine levels were adjusted for total lung protein and expressed as weight per mg of lung as described in section 2.2.3.2.

8.4.8 Assessment of airway oedema

Protein content in lavage fluid was determined as a measure of airway oedema by BCA protein assay as per the manufacturer's instructions (Pierce protein biology).

8.5 Results

8.5.1 The effect of inhaled dexamethasone on acute ovalbumin

Inhaled vehicle (25% DMSO/75% saline) treated animals demonstrated an immediate bronchoconstriction to Ova at 0 minutes (-70.2±2.7%). This was attenuated in guinea-pigs treated with 20mg/ml but not 4mg/ml dexamethasone (-57.7±3.6% and -72.8±2.7%, respectively). Animals treated with 20mg/ml dexamethasone demonstrated significantly less of a bronchoconstriction than vehicle during the first hour of readings, at 1 hour the sGaw value was -34.0±5.1% compared to vehicle, -56.2±5.4%. This was also the case at 3 hours (-26.1±5.8%) compared to vehicle (-46.5±5.5%). Both vehicle and dexamethasone treated group's early phase bronchoconstrictions returned to baseline sGaw values by 6 hours. Both vehicle and 4mg/ml dexamethasone treated animals demonstrated late asthmatic responses between 7-10 hours (-18.3±3.7% and -18.7±2.6% respectively). No clear LAR was seen in 20mg/ml treated animals during this period (-6.2±3.5%) (Figure 4, time course).

Vehicle treated guinea-pigs demonstrated a peak EAR of -70.8±2.6%, unchanged by 4mg/ml dexamethasone (-73.2±2.7%) but significantly attenuated by 20mg/ml dexamethasone (-57.9±3.6%). Late asthmatic response in animals treated with 4mg/ml dexamethasone were unchanged from vehicle (-21.3±2.6 compared to -23.3±2.6%) but were significantly reduced by 20mg/ml dexamethasone (-11.1±2.0%). No significant differences between sGaw values at 24 hours were observed (Figure 4, histogram). 4mg/ml dexamethasone did not significantly reduce the total or early asthmatic response area under the curve compared to vehicle. 20mg/ml dexamethasone significantly reduced both total (169.2±31.9%.hr compared to vehicle, 302.1±32.1%.hr) and EAR area (145.5±25.9%.hr compared to vehicle, 235.9±24.4%.hr). LAR area was reduced by 20mg/ml dexamethasone but not significantly (Figure 5).

Vehicle treated guinea-pigs demonstrated a significant increase in the bronchoconstrictor response to histamine post-Ova compared to pre-Ova challenge (-17.1±6.2% compared to 0.3±2.9%). At 5 minutes post-histamine challenge, sGaw values began to return to pre-challenge values (-12.3±4.5% compared to 8.2±2.4%). At 10 minutes the bronchoconstriction was still significant (-11.4±4.2% compared to pre-challenge, 6.8±4.0%). Guinea pigs treated with both 4 and 20 mg/ml dexamethasone demonstrated no significant change in histamine bronchoconstrictor response post-Ova challenge (Figure 6).

Lavage fluid protein levels increased following Ova challenge and vehicle treatment (2.6±0.5mg/ml) and was reduced non-significantly by both 4 and 20mg/ml dexamethasone treatment (1.5±0.3mg/ml and 1.7±0.2mg/ml respectively) (Figure 7).

Ova challenge with vehicle treatment increased total (9.1±1.1x10^6/ml) compared to naïve (1.4±0.2x10^6/ml) and differential cell counts. Guinea-pigs treated with dexamethasone or vehicle. Both 4 and 20mg/ml dexamethasone treatment significantly reduced total cell numbers compared to vehicle (6.1±0.7x10^6/ml and 5.3±0.3x10^6/ml). Both 4 and 20mg/ml dexamethasone also significantly reduced eosinophil numbers (2.1±0.3x10^6/ml, 1.8±0.2x10^6/ml compared to vehicle, 4.1±0.8x10^6/ml respectively). Additionally, 20mg/ml dexamethasone significantly reduced macrophage cell numbers compared to vehicle levels (3.1±0.3x10^6/ml compared to 4.8±0.4x10^6/ml). 4 but not 20mg/ml dexamethasone significantly reduced neutrophil numbers compared to vehicle (0.2±0.1x10^6/ml compared with 0.5±0.1x10^6/ml respectively) (Figure 7).

IL-13 levels, increased in Ova challenged and vehicle treated guinea-pigs (32.0±5.0%) compared to naïve (2.8±0.8ng/mg), demonstrated a non-significant trend for a decrease with increasing dose of dexamethasone. IL-17 levels were also increased in vehicle treated animals (106.6±17.7pg/mg) compared to naïve (41.9±15.4pg/mg)

and decreased non-significantly by dexamethasone, IL-8 was not detectable and is not shown (Figure 9).

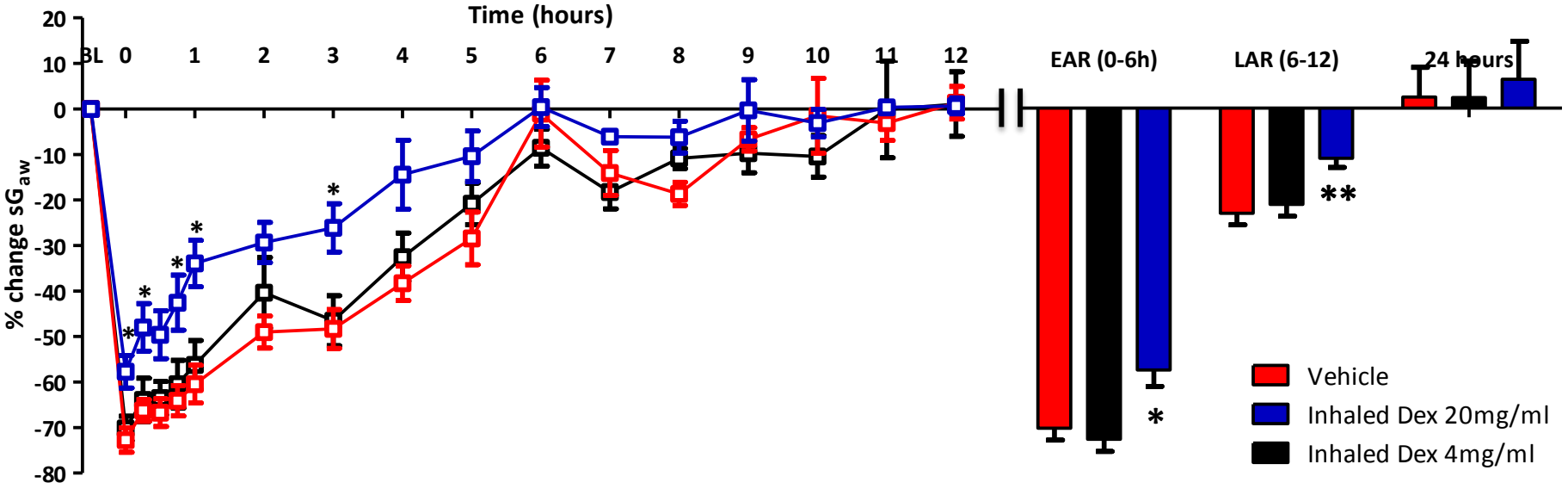


Figure 4: The mean time-course values of sGaw in ovalbumin (Ova) sensitised and challenged guinea-pigs treated with either inhaled vehicle or dexamethasone (Dex, 4mg/ml or 20mg/ml), dosed daily for 6 days. The histogram represents the maximum bronchoconstriction values recorded during the early asthmatic response (EAR) (0-6 hours), late asthmatic response (LAR) (6-12 hours) and 24 hours readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline prior to Ova challenge. A negative value represents a bronchoconstriction. N=6 *significantly different from vehicle treatment $p<0.05$, ** $p<0.01$; performed with one-way analysis of variance followed by Dunnet's post-test.

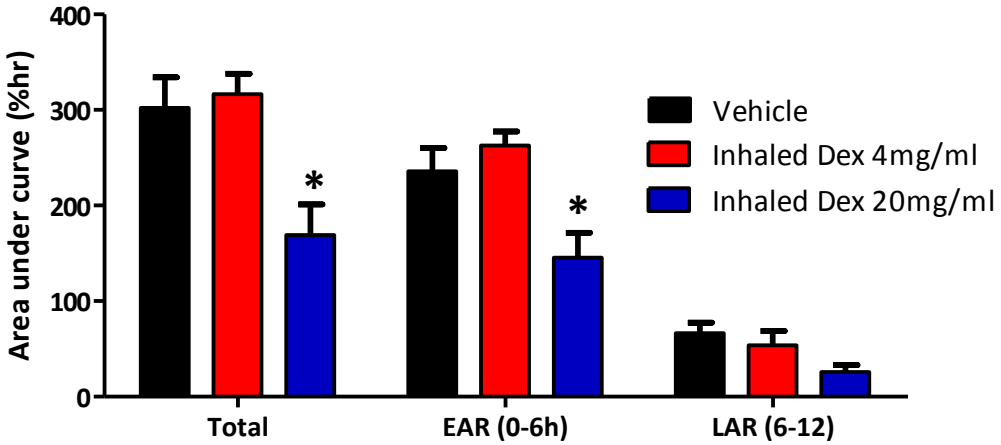


Figure 5: Area under the curve analysis of sGaw values over 12 hours following ovalbumin (Ova) challenge in guinea-pigs treated with either inhaled vehicle or dexamethasone (Dex, 4mg/ml or 20mg/ml), dosed daily for 6 days. For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-12 hours, early asthmatic response (EAR) includes values from 0-6 hours and late asthmatic response (LAR) includes values from 6-12 hours. Area under the curve is expressed as %.hour. N=6 *significantly different from vehicle treatment $p<0.05$; performed with one-way analysis of variance followed by Dunnet's post-test.

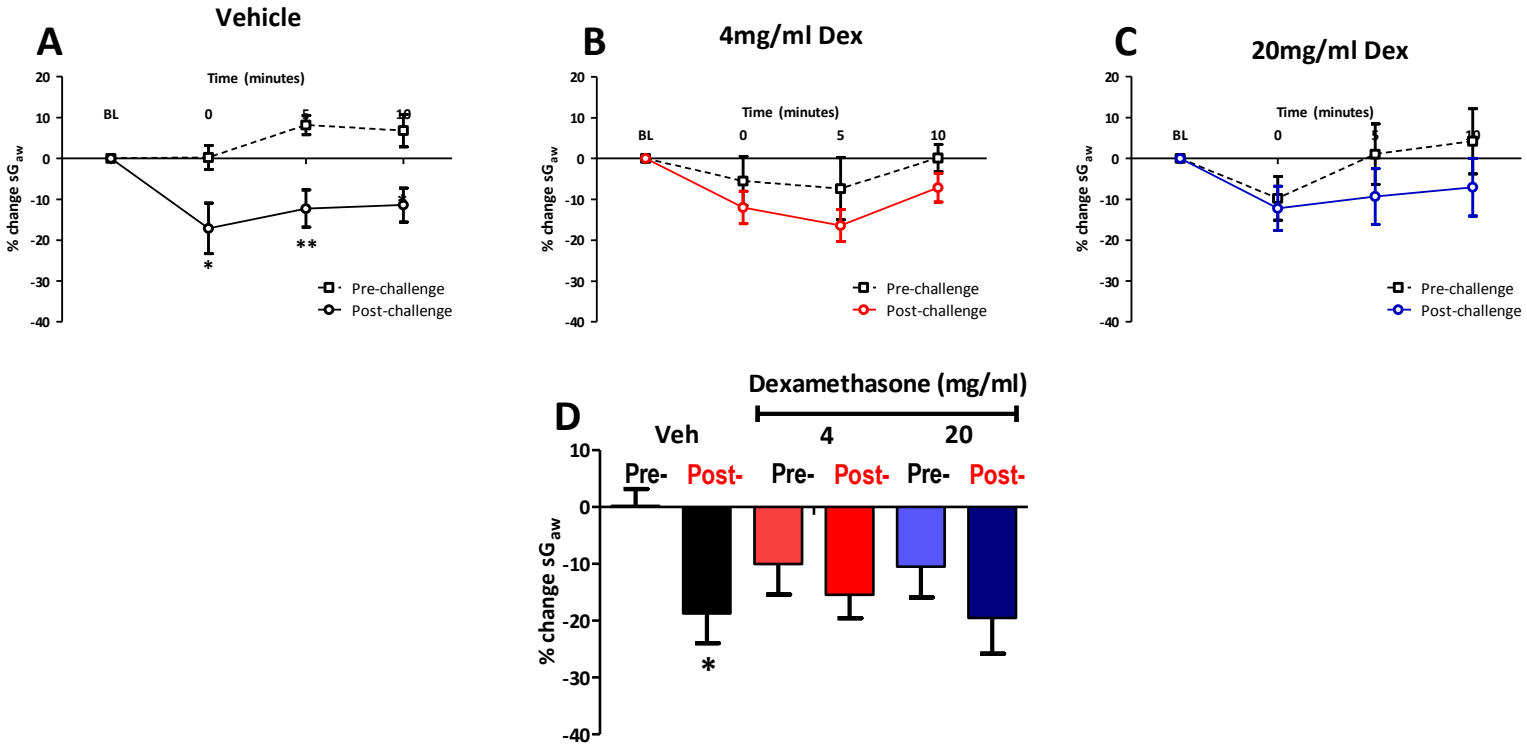


Figure 6: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles and 0.5LPM flow per chamber over 2 minutes, 1 minute drying period) in ovalbumin (Ova) sensitised and challenged guinea-pigs treated with either inhaled **A)** vehicle (**B)** 4mg/ml dexamethasone (Dex) **C)** 20mg/ml dexamethasone dosed daily for 6 days. **D)** Represents the peak the bronchoconstriction pre- and post-Ova following histamine challenge. Values were recorded 7 days pre- and 24 hours post-Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=6, *Significantly different from time paired pre-Ova challenge values $p<0.05$, ** $p<0.01$; performed with a two tailed T-test.

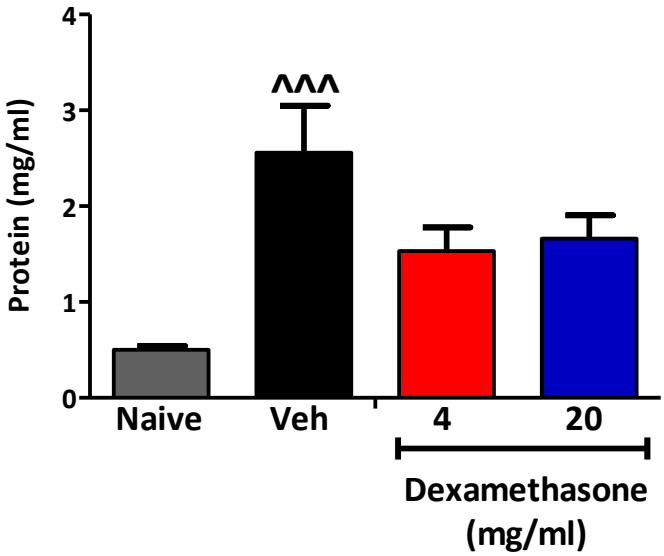


Figure 7: Total lavage fluid protein in ovalbumin (Ova) sensitised and challenged guinea-pigs treated with either inhaled vehicle or dexamethasone (Dex, 4mg/ml or 20mg/ml), dosed daily for 6 days. ^^^significantly different from naïve $p<0.001$; N=6, performed with one-way analysis of variance followed by selected groups Bonferroni post-test

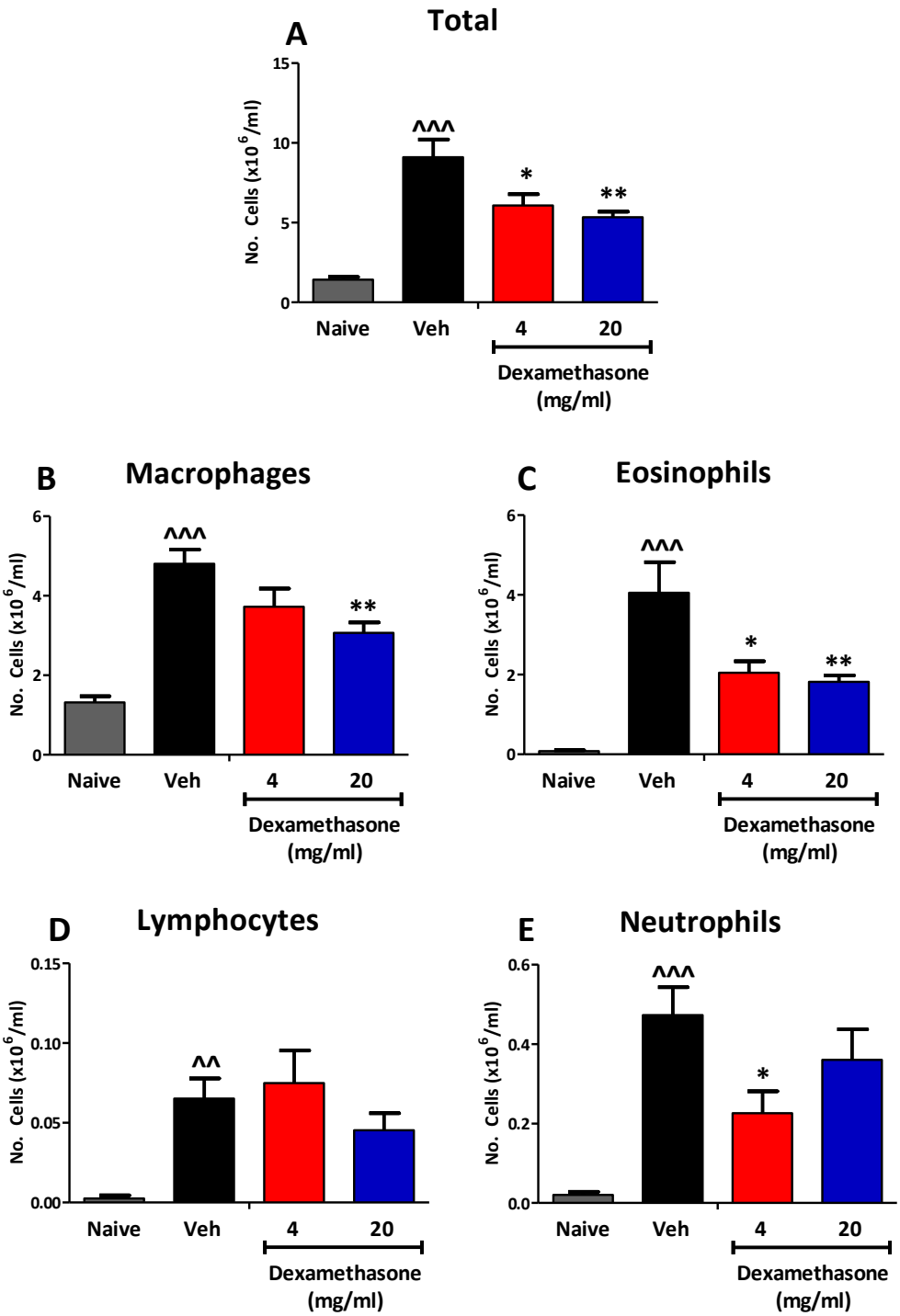


Figure 8: The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in bronchoalveolar fluid in ovalbumin (Ova) sensitised and challenged guinea-pigs treated with either inhaled vehicle or dexamethasone (Dex, 4mg/ml or 20mg/ml), dosed daily for 6 days. N=6 *significantly different from vehicle treatment $p<0.05$, ** $p<0.01$; ^^significantly different from naïve $p<0.01$; ^^^ $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test

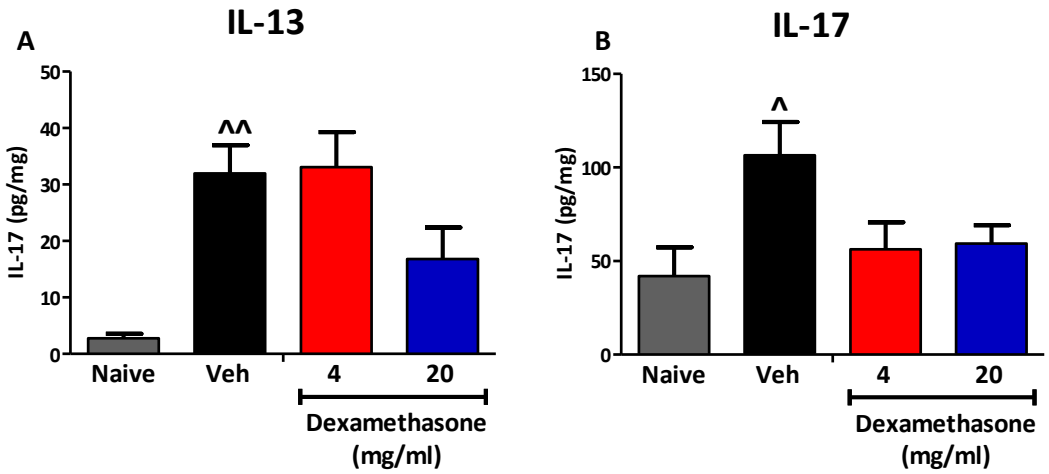


Figure 9: The concentration of A) IL-13 B) IL-17 in the lungs of ovalbumin (Ova) sensitised and challenged guinea-pigs treated with either inhaled vehicle or dexamethasone (Dex, 4mg/ml or 20mg/ml), dosed daily for 6 days, N=6 ^^significantly different from naive $p<0.01$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

7.5.6 The effect of inhaled dexamethasone on acute ovalbumin and LPS co-exposure.

Vehicle treated guinea-pigs demonstrated an immediate bronchoconstriction to Ova/LPS at 0 minutes (-50.6±4.9%) which returned to baseline sGaw values at 6 hours. Dexamethasone (20mg/ml) treated animals also demonstrated an immediate bronchoconstriction (-55.5±2.2%). During the first hour following Ova exposure, dexamethasone treated animals demonstrated significantly more of a bronchoconstriction then vehicle, including at 15 (-61.8±3.1% compared to -48.3±3.1%), 30 (-61.1±2.8% compared to -46.9±4.2%) and 45 minutes (-57.2±3.1% compared to -47.2±2.8%). Vehicle treated guinea-pigs displayed a late asthmatic response from 7 hours (-18.2±5.9%) which was not significantly reduced by dexamethasone treatment (-15.0±2.6%). No significant difference in LAR responses was seen from 8-12 hours (Figure 10, time course). No difference in the peak early or late phase responses was observed between vehicle and dexamethasone treatment groups (Figure 10, histogram). No significant difference in total, early phase, late phase area under the curve was observed (Figure 11). Inhaled dexamethasone treatment did not significantly change the length of the early phase bronchoconstriction (2.9±02h) compared to vehicle (3.8±0.9h) (Figure 12).

Vehicle treated guinea-pigs demonstrated an immediate increase in the bronchoconstrictor response to histamine, post-Ova/LPS challenge but this was not significant. This bronchoconstriction continued to increase and at 5 minutes was significant compared to pre-Ova challenge sGaw values (-17.7±6.0%) compared to pre-challenge (-3.1±1.2%). By 10 minutes post-histamine challenge the bronchoconstriction was -30.2±10.2% compared to pre-Ova, -0.2±2.1%. Treatment with dexamethasone (20mg/ml) did not significantly attenuate the development of airway hyperresponsiveness. At 0 minutes the bronchoconstriction to histamine was significantly increased (-13.0±4.6%) compared to pre-Ova (-2.0±1.3%). This bronchoconstriction continued to increase and at 5 and 10 minutes the

bronchoconstriction to histamine post-Ova challenge was -20.5±4.3% compared to pre-Ova, -4.1±2.8% and -23.3±5.0% compared to pre-Ova, -4.0±2.4% respectively (Figure 13).

Protein levels were increased in vehicle treated guinea-pigs (1.6±0.3mg/ml) compared to naïve (0.4±0.06mg/ml). Protein levels were significantly reduced in dexamethasone treated guinea pigs, (0.8±0.1mg/ml) (Figure 14).

Total cell numbers were significantly increased in Ova/LPS challenged and vehicle treated guinea-pigs ($18.2\pm1.0x10^6$/ml) compared to naïve ($1.4\pm0.2x10^6$/ml). Dexamethasone treatment significantly increased total cell numbers ($21.3\pm0.8x10^6$/ml). Correspondingly, macrophage numbers were also increased by dexamethasone treatment ($10.5\pm0.4x10^6$/ml compared to vehicle $8.6\pm0.5x10^6$/ml). Dexamethasone treatment did not significantly decrease eosinophils, lymphocytes and neutrophils (Figure 15).

IL-8, IL-13 and IL-17 levels increased in Ova/LPS challenged and vehicle treated guinea-pigs. IL-8, IL-13 and IL-17 levels were not significantly reduced by dexamethasone treatment (Figure 16).

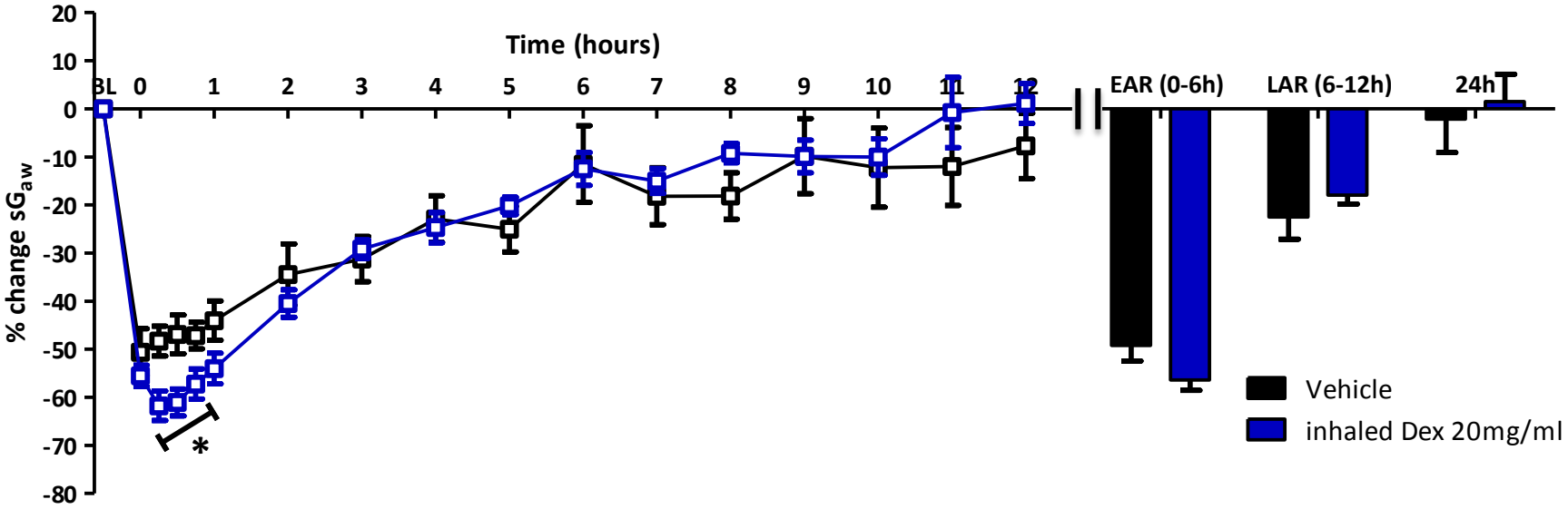


Figure 10: The mean time-course values of sGaw in ovalbumin (Ova) and LPS challenged guinea-pigs treated with inhaled vehicle or dexamethasone (Dex, 20mg/ml) dosed daily for 6 days. The histogram represents the maximum bronchoconstriction values recorded during the early asthmatic response (EAR) (0-6 hours), late asthmatic response (LAR) (6-12 hours) and 24 hours readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline prior to Ova challenge. A negative value represents a bronchoconstriction. N=6 *significantly different from vehicle treatment $p<0.05$; performed with a two tailed T-test.

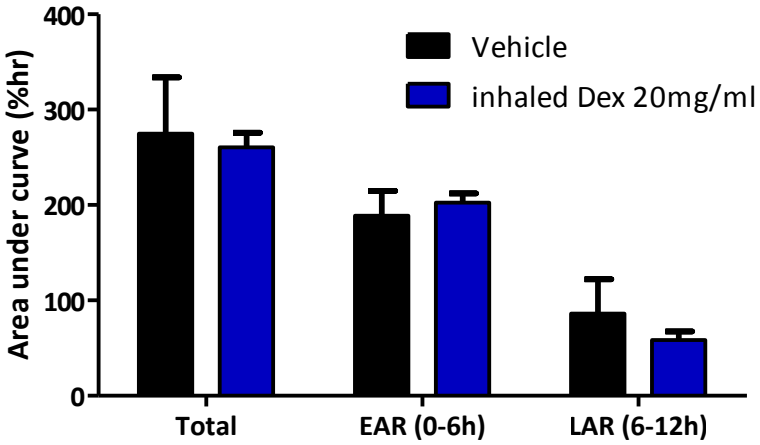


Figure 11: Area under the curve analysis of sGaw values over 12 in ovalbumin (Ova) and LPS challenged guinea-pigs treated with inhaled vehicle or dexamethasone (Dex, 20mg/ml) dosed daily for 6 days. For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-24 hours, early asthmatic response (EAR) includes values from 0-6 hours and late asthmatic response (LAR) includes values from 6-12 hours. Area under the curve is expressed as %.hour. N=6 performed with a two tailed T-test.

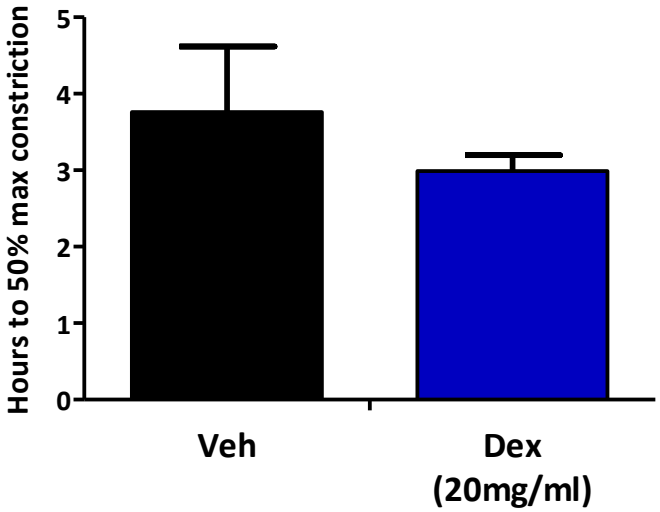


Figure 12: Analysis of the time taken for early asthmatic responses (EAR) to recover to 50% of peak bronchoconstriction values in ovalbumin (Ova) and LPS challenged guinea-pigs treated with inhaled vehicle or dexamethasone (Dex, 20mg/ml) dosed daily for 6 days. N=6; performed with a two tailed T-test.

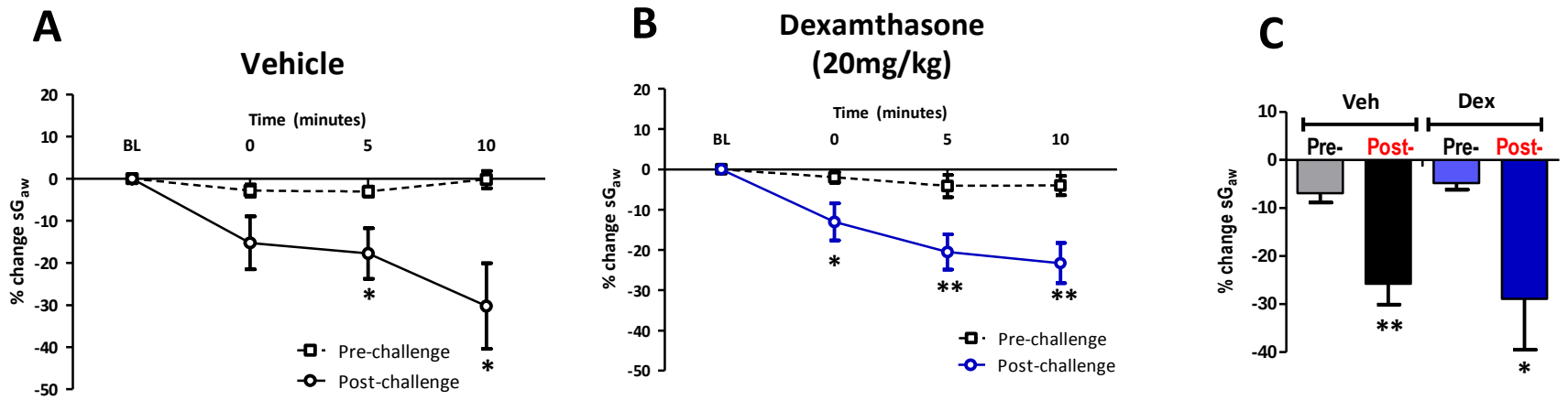


Figure 13: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles and 0.5LPM flow per chamber over 2 minutes, 1 minute drying period) in ovalbumin (Ova) and LPS challenged guinea-pigs treated with inhaled vehicle or dexamethasone (Dex, 20mg/ml) dosed daily for 6 days. Values were recorded 7 days pre- and 24 hours post-Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=6 *significantly different from time paired pre-Ova challenge values $p<0.05$, ** $p<0.01$; performed with a two tailed T-test.

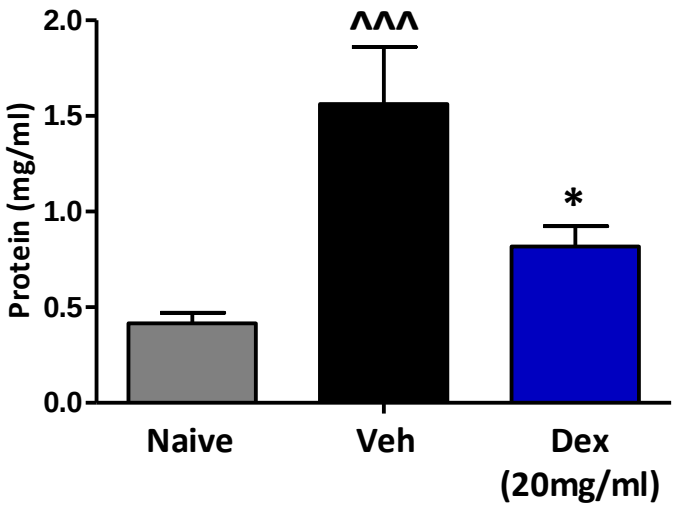


Figure 14: Total lavage fluid protein in ovalbumin (Ova) and LPS challenged guinea-pigs treated with inhaled vehicle or dexamethasone (Dex, 20mg/ml) dosed daily for 6 days. Protein content was determined by BCA protein assay. N=6 *significantly different from vehicle treatment $p<0.05$, ^^^significantly different from naïve, $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

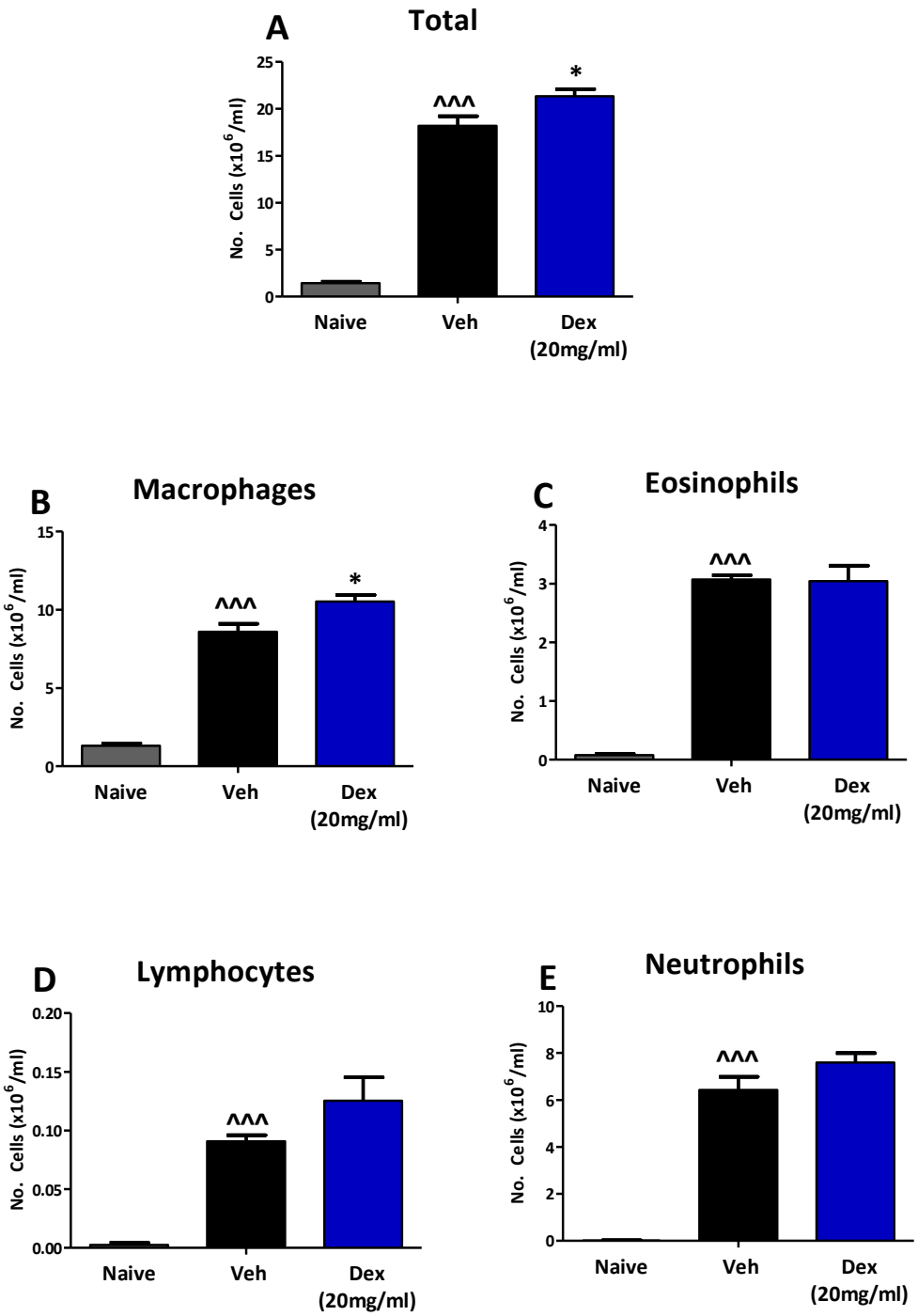


Figure 15: The total cell (A), macrophage (B), eosinophil (C), lymphocyte (D) and neutrophil (E) counts in bronchoalveolar fluid of ovalbumin (Ova) and LPS challenged guinea-pigs treated with inhaled vehicle or dexamethasone (Dex, 20mg/ml) dosed daily for 6 days. N=6 *significantly different from vehicle treatment $p<0.05$; ^^^significantly different from naive $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

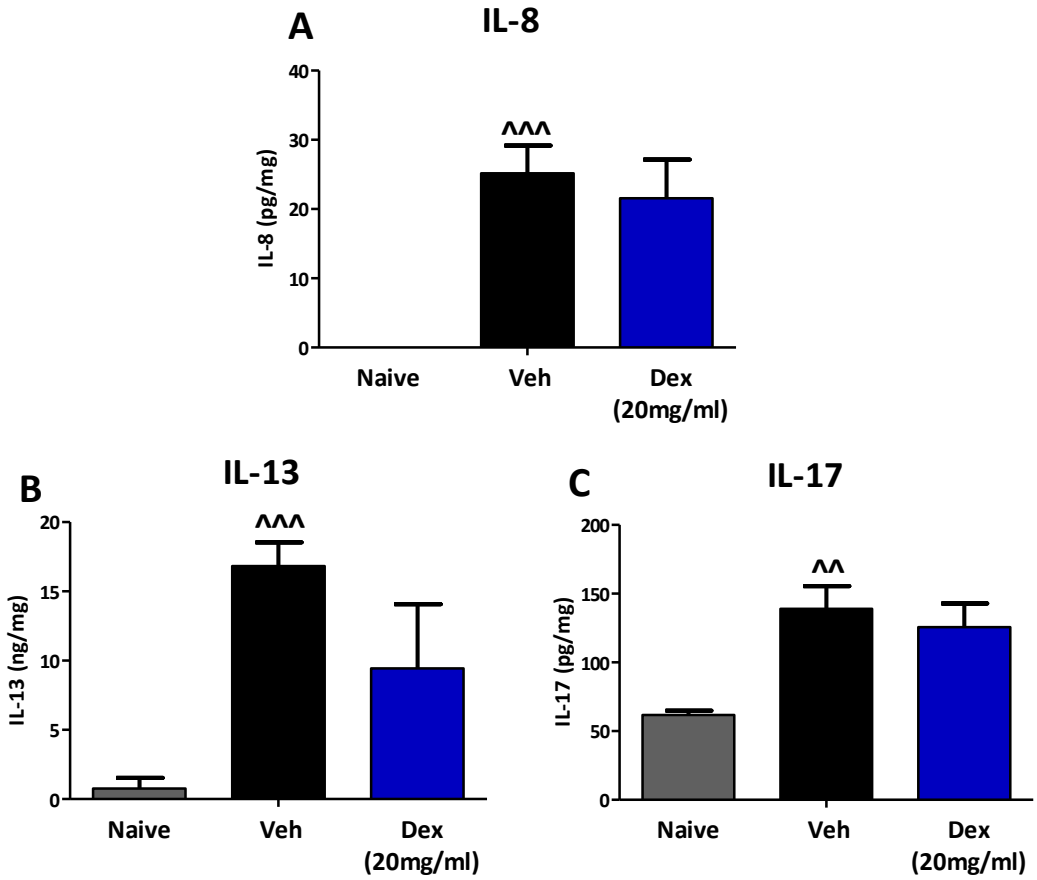


Figure 16: The concentration of A) IL-8 B) IL-13 C) IL-17 in the lungs of ovalbumin (Ova) sensitised guinea-pigs treated with inhaled vehicle or dexamethasone (Dex, 20mg/ml) dosed daily for 6 days. N=6 ^^^significantly different from naive $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

7.5.7 THE EFFECT OF INHALED DEXAMETHASONE ON LPS EXPOSURE.

Both vehicle and dexamethasone (20mg/ml) treated animals demonstrated a progressively increasing bronchoconstriction during the 1st hour post-LPS exposure, reaching its peaking at 3 hours (vehicle: -20.1±6.0%; dexamethasone: -19.8±5.5%). The bronchoconstrictions then dissipated and returned to near baseline levels by 7 hours. Figure 17 also presents the mean peak bronchoconstrictions between 0-12 hours and at 24 hours. Vehicle treated guinea pigs demonstrated a peak bronchoconstriction of -30.8±4.0% which was not significantly changed by dexamethasone treatment (-26.0±5.5%) (Figure 17, time course). Dexamethasone treatment did not significantly change the area under the curve between 0-12 hours (Figure 18).

In Ova challenged and vehicle treated guinea-pigs a significant increase in the response to histamine was observed post-LPS challenge (-21.9±5.4% compared to pre-challenge: -1.6±1.8%). A significant increase in histamine response was also present at 10 minutes (-18.5±5.2% compared to pre-challenge: -4.6±2.3%). Guinea-pigs treated with dexamethasone demonstrated a bronchoconstriction to histamine at 0 minutes following LPS exposure (-5.7±1.6%) compared to pre-LPS (2.7±3.0%). At 10 minutes a significant bronchoconstriction was still present (-9.4±2.8%) compared to pre-LPS (3.9±2.8%). Dexamethasone treatment significantly reduced the peak bronchoconstriction to histamine post-LPS challenge (-11.1±2.8%) compared to vehicle (-26.3±3.8%) (Figure 19).

LPS increased protein levels in lavage fluid (1.7±0.1mg/ml) compared to naïve (0.5±0.05mg/ml), which were not significantly reduced by dexamethasone treatment (1.3±0.2mg/ml) (Figure 20).

LPS increased total cell numbers ($18.9\pm1.0x10^6$/ml) compared to naïve ($1.4\pm0.2x10^6$/ml). These were not significantly reduced by treatment with dexamethasone (20mg/ml) ($20.5\pm1.6x10^6$/ml). Macrophages, eosinophils, lymphocytes and neutrophils were all increased by LPS exposure but not significantly reduced by dexamethasone (Figure 21).

IL-8 levels were increased in Ova challenged and vehicle treated guinea-pigs (85.0±14.7pg/mg) compared to naïve (undetectable) and were not significantly reduced by dexamethasone (20mg/ml) treatment (Figure 22).

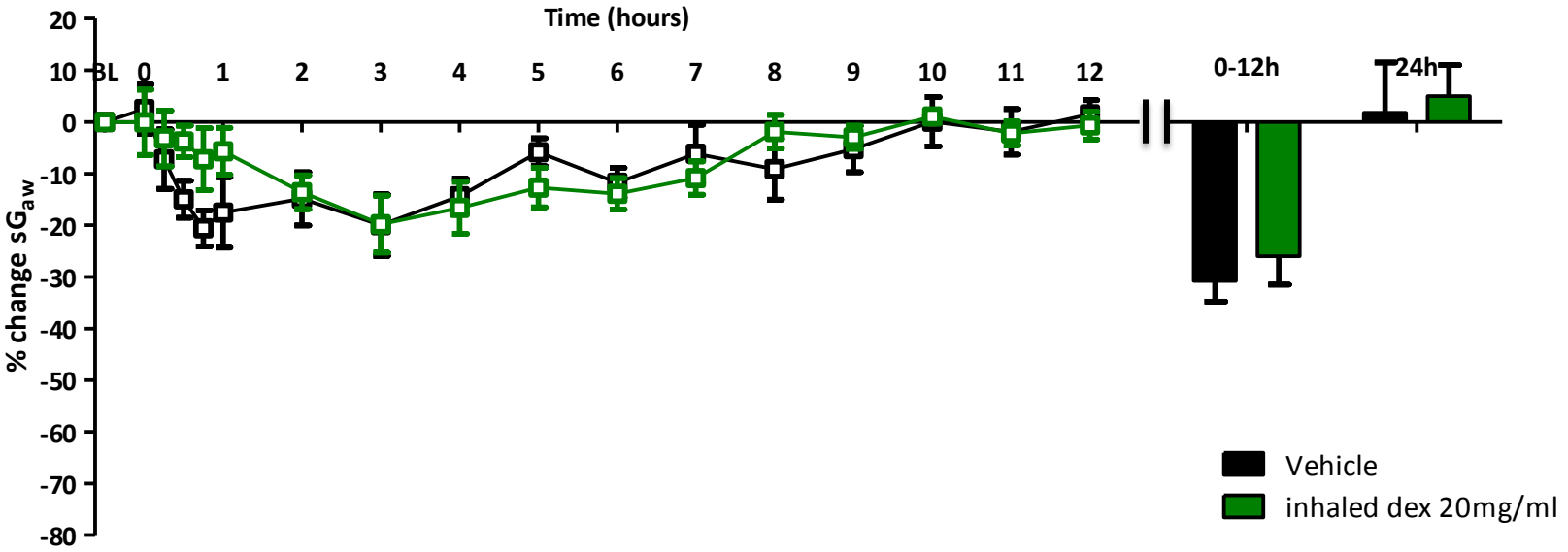


Figure 17: The mean time-course values of sGaw in guinea-pigs exposed to LPS twice and treated with inhaled vehicle or 20mg/ml dexamethasone, dosed daily for 6 days. The histogram represents the maximum bronchoconstriction values recorded during 0-12 hours and 24 hours readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline prior LPS challenge. A negative value represents a bronchoconstriction. Results shown are after the second LPS exposure. N=6; performed with a two tailed T-test.

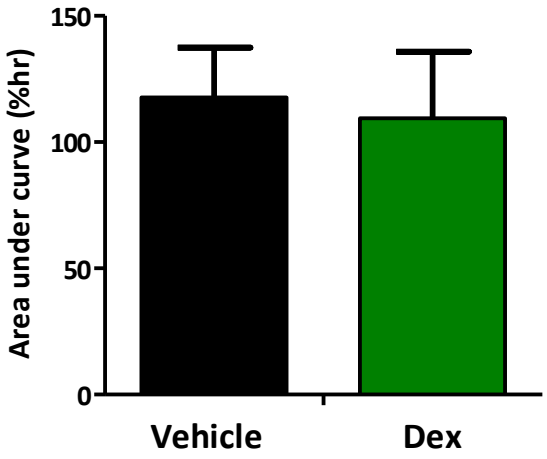


Figure 18: Total area under the curve analysis of sGaw values in guinea-pigs exposed to LPS twice and treated with inhaled vehicle or 20mg/ml dexamethasone, dosed daily for 6 days. For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-12 hours. Area under the curve is expressed as %.hour. N=6; performed with a two tailed T-test.

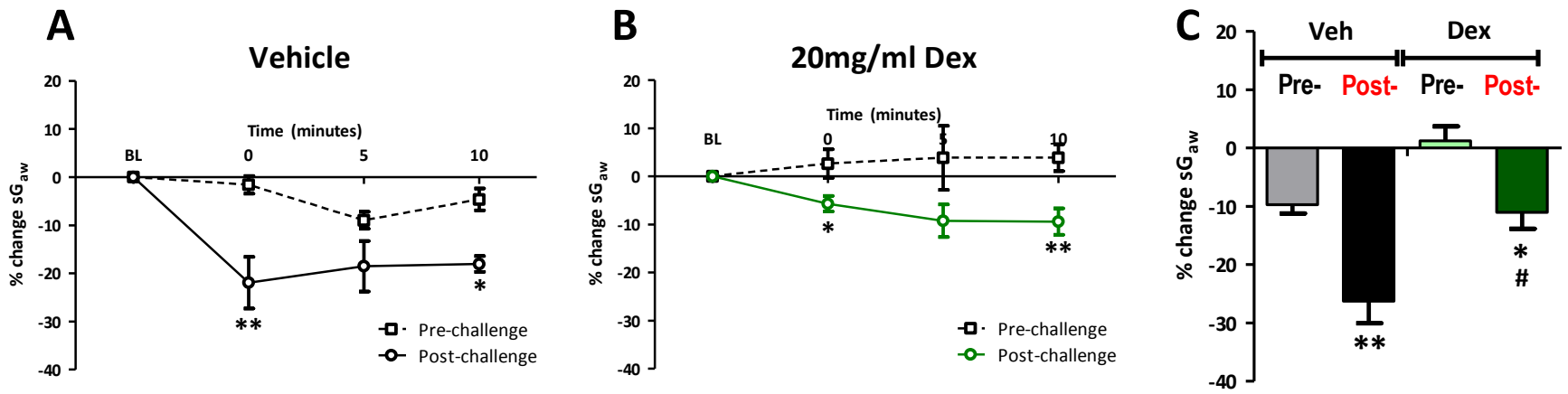


Figure 19: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles and 0.5LPM flow per chamber over 2 minutes, 1 minute drying period) in guinea-pigs exposed to LPS twice and treated with inhaled vehicle or 20mg/ml dexamethasone, dosed daily for 6 days. C) Represents the peak the bronchoconstriction pre- and post-Ova following histamine challenge. Values were recorded 7 days pre- and 24 hours post-Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=6 *Significantly different from time paired pre-LPS challenge values $p<0.05$, ** $p<0.01$; # significantly different from post-LPS + veh; A & B) performed with a two tailed T-test.; C) performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

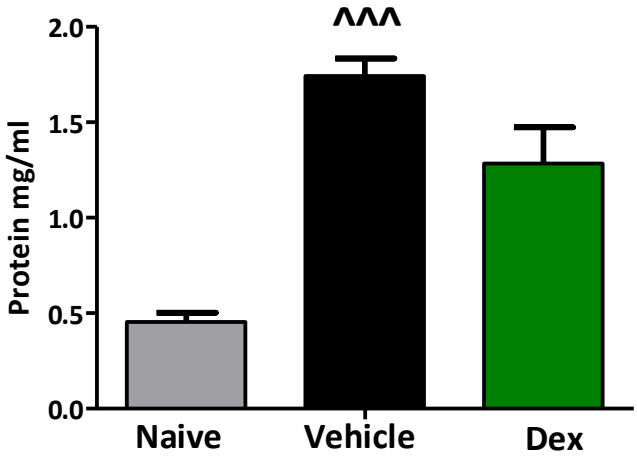


Figure 20: Total lavage fluid protein in guinea-pigs exposed to LPS twice and treated with inhaled vehicle or 20mg/ml dexamethasone, dosed daily for 6 days. Protein content was determined by BCA protein assay. N=6 ^^^significantly different from naïve, $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

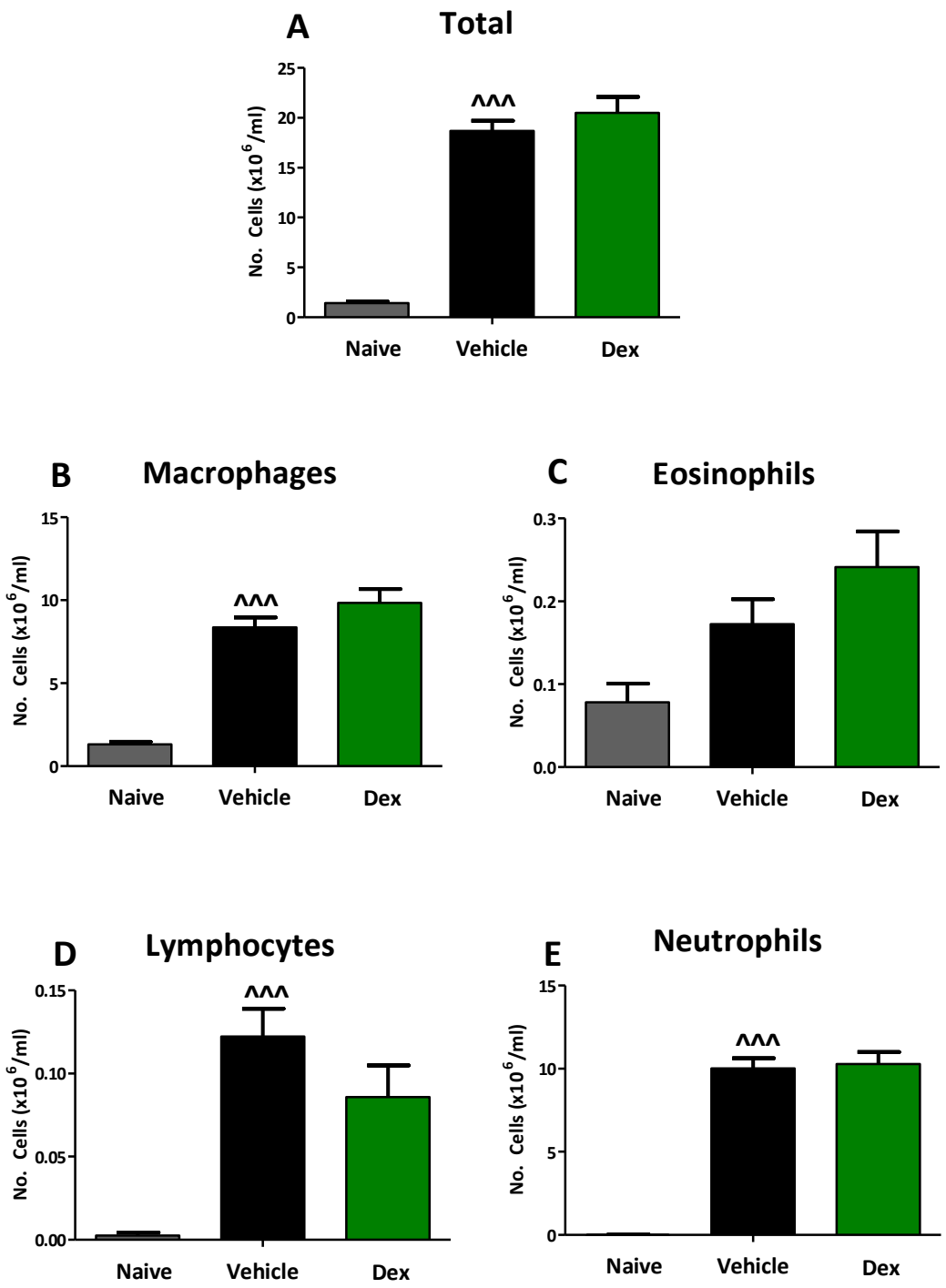


Figure 21: The total cell (A), macrophages (B), eosinophils (C), lymphocytes (D) and neutrophils (E) counts in bronchoalveolar fluid in guinea-pigs exposed to LPS twice and treated with inhaled vehicle or 20mg/ml dexamethasone, dosed daily for 6 days. N=6 ^^^significantly different from naïve, $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

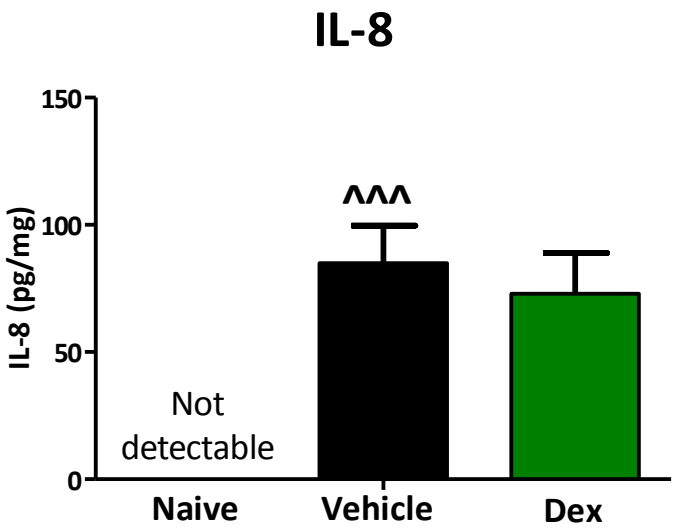


Figure 22: The concentration of IL-8 in the lungs of guinea-pigs exposed to LPS twice and treated with inhaled vehicle or 20mg/ml dexamethasone, dosed daily for 6 days. N=6 ^^^significantly different from naïve $p<0.001$; performed with one-way analysis of variance followed by selected groups Bonferroni post-test.

8.5 DISCUSSION

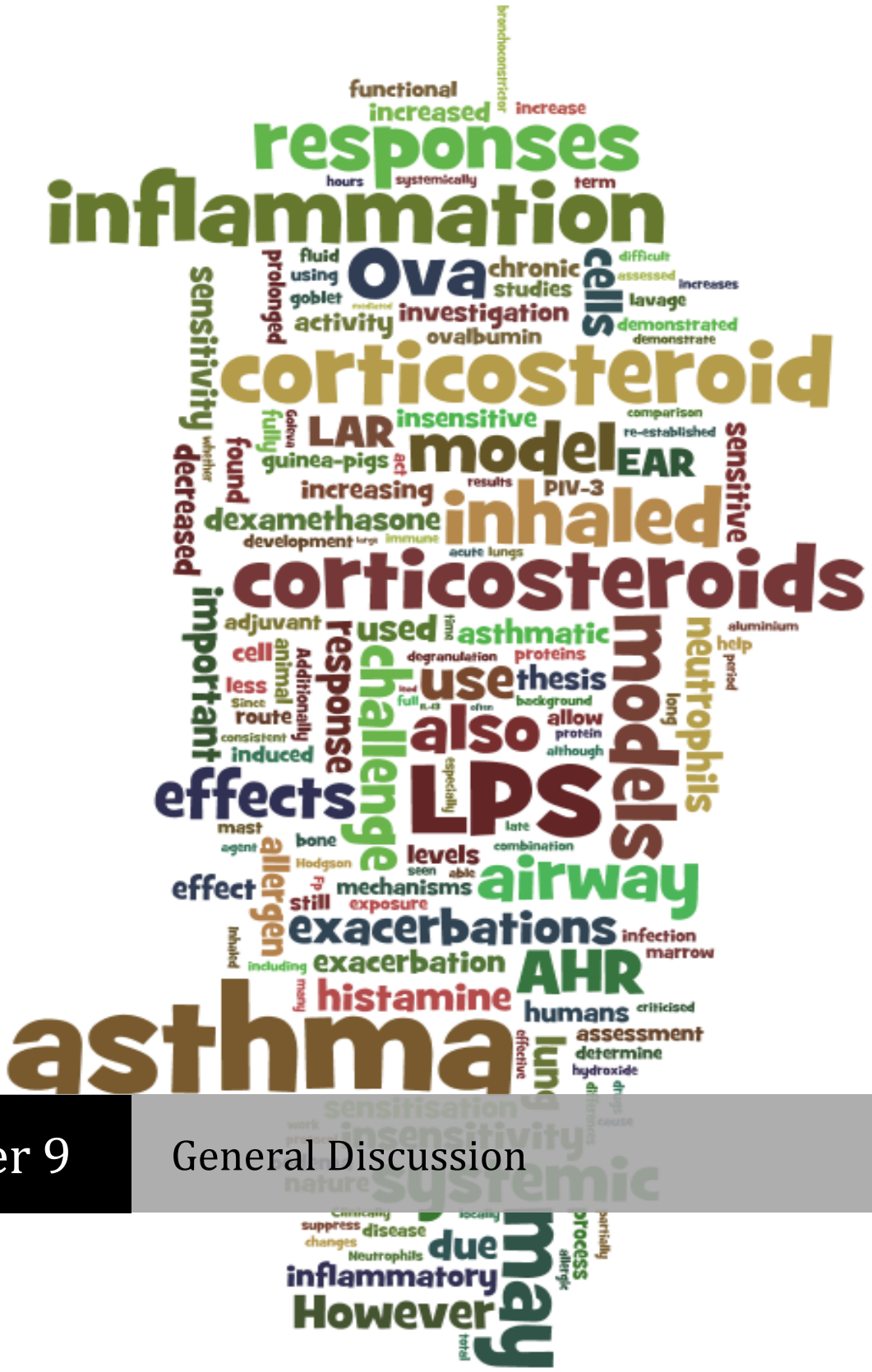
8.5.1 THE EFFECT OF INHALED DEXAMETHASONE ON OVALBUMIN, LPS AND OVALBUMIN AND LPS INDUCED RESPONSES.

Dose-response assessment of inhaled dexamethasone's effect on Ova responses revealed that 20mg/ml was an effective dose at decreasing the LAR, AHR, total cells, macrophages, eosinophils and lavage fluid protein levels, as inhaled FP (0.5 and 1mg/ml) did in chapter 6. Also like inhaled FP, inhaled dexamethasone demonstrated a trend for a reduction in lymphocytes and IL-13 levels. Unlike inhaled FP, IL-17 levels were also decreased, although non-significantly. These results confirm the sensitivity of Ova induced responses to inhaled corticosteroid and the report in the literature by Jungsuwadee *et al*, (2004). The effects of inhaled dexamethasone on LPS alone were also similar to inhaled FP. Inhaled dexamethasone did not reduce the immediate bronchoconstriction to LPS, total and differential cells and lavage fluid protein. Similar to inhaled FP, inhaled dexamethasone also reduced but did not abolish AHR 24 hours after the second LPS exposure. This indicates that inhaled LPS responses in the airways are insensitive to inhaled corticosteroids.

In the combined Ova /LPS challenged groups, inhaled dexamethasone did not reduce the prolonged EAR, LAR and AHR, confirming the results from chapter 6. However lavage fluid protein levels were also decreased indicating that this was not due to drug delivery issues. Unlike inhaled FP which did not change total and differential cell counts, dexamethasone increased total cells and macrophages. Additionally, although IL-13 levels demonstrated a trend for a reduction with inhaled dexamethasone, they were not significantly reduced as in inhaled FP groups. This may be due to the issues of using the lung to assess cytokine changes. Levels may be higher or lower in different regions of the lung. Although the part of the lung that was homogenised was standardised, discrepancies are possible. These results confirm that 2 inhaled LPS exposures decrease the inhaled corticosteroid sensitivity of Ova induced functional and allergic responses. In the LPS alone challenged groups,

Inhaled dexamethasone did not reduce the bronchoconstriction, inflammation or lavage fluid protein levels. Thus these results confirm the lack of sensitivity of LPS responses to inhaled corticosteroids.

These results suggest that the induction of inhaled corticosteroid insensitivity in Ova and LPS challenged guinea-pigs may be due to the inherent inhaled corticosteroid insensitivity of LPS responses. However, the insensitivity of responses such as the LAR and eosinophilia suggests that on an allergic background LPS renders allergic responses also insensitive, suggesting a synergistic effect. This study revealed some slight differences in the effect of inhaled FP and dexamethasone on Ova and LPS responses. The most interesting finding was that total cells and more specifically macrophages increased with inhaled dexamethasone treatment. This increase in macrophages could represent an as yet unidentified pro-survival effect of corticosteroids on macrophages in conditions of increased oxidative and nitric stress. Neutrophils are known to demonstrate this effect in the presence of corticosteroid. Strickland *et al*, (2001) observed decreased neutrophil apoptosis due to upregulation of GR-β in the presence of dexamethasone *in vitro*. Why this effect was not seen with inhaled FP is unknown but it may represent a dose related effect. The dose of FP used in this thesis was close to the threshold for suppression of the LAR, AHR and airway inflammation whereas the dose of inhaled dexamethasone was fairly high. Alternatively, this may be due to differences in the pharmadynamic and pharmacokinetic properties of inhaled corticosteroids. Differences in the GR receptor binding affinity, particle size, bioavailability, pulmonary residence size, systemic availability and excretion could account for the differences in the effects between inhaled FP and inhaled dexamethasone (Derendorf *et al*, 2006; Schaaf *et al*, 2005). Further studies are required to explore the mechanisms behind this effect in macrophages.



Chapter 9 General Discussion

Chapter 9

9.1 Main aims

Exacerbations of asthma are a serious economic and social burden, accounting for the majority of asthma hospitalisations and deaths. Currently they are poorly treated due to their decreased responsiveness to frontline inhaled corticosteroid therapy. In the long term consistent asthma exacerbations can lead to a more severe form of disease, refractory to even oral corticosteroid treatment. There is currently a need for animal models to identify targets and develop novel therapeutics. Most models of asthma exacerbation only examine inflammatory components of asthma and ignore the functional changes in the lungs. This is important since inflammatory and functional parameters in these models can be dissociated (Smith & Broadley, 2007). Moreover, differences in the sensitivity to inhaled and systemic corticosteroids in these models have not been assessed. Clinically this is important as patients who are insensitive to inhaled corticosteroids demonstrate some responsiveness to systemic corticosteroids (Hodgson *et al*, 2012). The main aim of this thesis was to create a model of asthma displaying an exacerbation of functional and allergic responses. The second aim was to establish the corticosteroid sensitivity of this model in comparison to a standard model of asthma. The parameters that were assessed were early and late asthmatic responses (EAR & LAR, respectively), airway hyperresponsiveness (AHR), cellular inflammation, lung and lavage fluid cytokines, lavage fluid protein and goblet cell hyperplasia.

9.2 Outcomes

9.2.1 Model of asthma

At the start of the program of work the established Ova sensitisation and challenge model was found to no longer induce a LAR or AHR (Evans *et al*, 2012). By cumulative modifications to the sensitisation and challenge protocols, these responses together with the EAR and cellular influx were established. AHR to histamine was re-established by increasing the Ova challenge dose 3-fold. The LAR was re-established by increasing

the amount of Ova and number of sensitisation injections from 2 to 3 and either increasing aluminium hydroxide sensitisation dose or increasing the length of time between Ova sensitisation and challenge. The latter protocol was better tolerated and was used in all subsequent chapters. Thus, following modification this Ova model demonstrated an EAR, LAR, AHR and eosinophilic airway inflammation. These studies demonstrated that full functional and allergic responses can be restored by increasing the severity of both sensitisation and challenge procedures. The reasons behind the decreased response to the ovalbumin protocol are unknown but not believed to be related to seasonal effects, infection or changes in environment or transportation (Wiley & Evans, 2009).

9.2.2 Asthma Exacerbation Models

Viral infection is an important cause of asthma exacerbations in humans (Johnston *et al*, 1995). The effects of parainfluenza-3 (PIV-3) alone and in combination with ovalbumin challenge were established. Initially, PIV-3 induced AHR to histamine, airway inflammation and oedema. However, this response was not consistent with repeat experiments, coinciding with decreased viral titres in lavage fluid. PIV-3 inoculation in Ova sensitised and challenged guinea-pigs showed no change in early or late asthmatic responses or total inflammation, with small increases in eosinophilia and AHR to histamine. This result was not consistent with Broadley *et al*, (2008), who demonstrated that PIV-3 caused merging of the EAR and LAR and increased total inflammation (especially neutrophils). The unreliability of PIV-3 effects in guinea-pigs led to the investigation of the TLR-3 agonist, poly I:C as a potential alternative exacerbating agent. TLR-3 activates similar pathways to viral infection. However, poly I:C did not induce AHR to histamine, airway inflammation or oedema so was not further investigated.

LPS is also important in asthma exacerbations and the development of corticosteroid insensitivity (Goleva *et al*, 2008). Two LPS exposures induced a bronchoconstriction, immediately following the 2nd exposure, increased airway inflammation and oedema,

goblet cell hyperplasia and a small increase in AHR. In combination with Ova it increased the duration of the EAR, prolonged the bronchoconstrictor response to histamine and increased total cells, neutrophils, macrophages, goblet cells and IL-8 levels. This effect was found to be dependent on the timing of LPS exposure. The aforementioned effects were only found when the second LPS exposure was co-administered with Ova challenge. LPS exposure 24 hours before Ova challenge decreased the EAR and AHR, while increasing airway inflammation and goblet cell numbers. The temporal differences in the effect of LPS may be due to its ability to activate mast cells, causing degranulation (Masuda *et al*, 2002). Since the production of new granules may take over 24 hours, mast cells challenged with allergen within this period may not be able to fully respond to allergen challenge (Dvorak, 2005).

9.2.3 Corticosteroid Sensitivity

The dose-response effects of corticosteroids on Ova induced responses were determined to establish effective doses at reducing the LAR, AHR, airway inflammation and oedema. Inhaled fluticasone propionate (Fp), inhaled dexamethasone and systemically administered dexamethasone all attenuated the development of the LAR, AHR, airway inflammation, lung IL-13 levels and oedema induced by ovalbumin. In contrast, responses to combined Ova and LPS challenge were found to be insensitive to inhaled Fp. No decrease in the prolonged EAR, LAR, prolonged bronchoconstrictor response to histamine and cellular inflammation was seen. Inhaled Fp was still effective at decreasing lavage fluid protein levels and IL-13. These results were confirmed by the use of inhaled dexamethasone, indicating that LPS reduces the sensitivity of Ova responses to inhaled corticosteroids. However, the combined Ova and LPS-induced responses were found to be partially sensitive to systemically administered dexamethasone. Systemic dexamethasone reduced airway inflammation (including neutrophils), the prolonged EAR and lavage fluid protein but not AHR, the LAR and

cytokine levels. Responses to LPS alone were also found to be insensitive to inhaled corticosteroids but fully sensitive to systemic dexamethasone.

These results indicate that corticosteroid insensitivity in asthma may be a localised phenomenon and that the route of corticosteroid administration can alter the sensitivity of asthma exacerbation responses. This is important as many studies have assumed that corticosteroids always have the same effect regardless of the route of administration. A good example of this is in the discussion of Trapp *et al*, (1998), where the researchers comment on their results being contrary to O'leary *et al*, (1996), despite the former using the inhaled route and the latter systemic.

Inhaled corticosteroids mainly act by suppressing asthmatic responses directly in the lung due to their poor systemic bioavailability (Wood *et al*, 1999; Shen *et al*, 2002). Systemic corticosteroids also work locally in the lungs but due to their systemic distribution can also suppress bone marrow derived hemopoietic cells, which are recruited to the lung in asthma (Mao *et al*, 2004; Ben *et al*, 2008). During asthma exacerbations, asthmatic responses are less sensitive to corticosteroids due to a range of modifications to proteins responsible for mediating the effects of these drugs. The corticosteroid insensitivity inducing effects of asthma exacerbations are likely to be local to the site of the exacerbation i.e. the lungs. Therefore, inhaled corticosteroids which act mainly in the lungs are unable to reduce asthmatic responses during an exacerbation, whereas systemic corticosteroids which can act via the lungs, blood and bone marrow are still partially effective.

A particular problem in asthma exacerbations is the large increase in neutrophils commonly observed. Neutrophils by their very nature are less corticosteroid sensitive than other inflammatory cell types (Strickland *et al*, 2001). However, their response to corticosteroid seems to differ depending on their location within the body. Neutrophils in the blood are more sensitive than in the lung due to differences in glucocorticoid receptors (Plumb *et al*, 2012). Therefore, the large increase in lung neutrophils during

asthma exacerbations may represent an inherently inhaled corticosteroid insensitive population. On the other hand, systemic corticosteroids can suppress neutrophil recruitment before sequestering to the lungs. However, since not all neutrophils are prevented from lung recruitment, those that make it into the airways may undergo extensive neutrophil degranulation. This may increase the oxidative/nitric stress of the lungs, in turn modifying the expression of proteins important in corticosteroid effects in a range of cells (Goleva *et al*, 2006; Ito *et al*, 2004; Hamilos *et al*, 2001). This may also account for the persistence of functional effects such as AHR despite reductions in inflammation seen with systemic dexamethasone.

The development of steroid insensitivity may represent an evolutionary mechanism to locally suppress the effects of endogenous corticosteroids during periods of short term immune activation i.e. during a chronic bacterial infection. However, during chronic bombardment by antigens, especially in the modern world this process may in the long term be counter-productive. Unchecked inflammation can cause excessive damage to the afflicted site, leading to processes such as airway remodelling, which cause permanent airflow obstruction.

9.3 Experimental limitations

The use of animal models in the research of what is almost an exclusively human disease has been criticised (Holmes *et al*, 2011). However, the use of animals in asthma research is not a problem as long as it is remembered that they cannot replicate the full human disease, which is the extensive product of interacting genetic and environmental factors. Instead animal models offer the opportunity to model and examine certain aspects of asthma such as the late asthmatic responses and inflammation. They are complementary to studies examining the underlying causes of asthma in humans. However, the use of animals is not without drawbacks. The guinea-pig has several disadvantages including IgG mediated type 1 hypersensitivity reactions and axonal

reflexes not present in humans. However, their benefits outweigh their disadvantages, especially in comparison to the more commonly used mouse.

The systemic route of allergen sensitisation in ovalbumin models is often criticised. Humans are probably sensitised to allergen via the respiratory mucosa which in theory could lead to differences in immune response. Since the development of asthma is complex and not understood it is very difficult to fully replicate this process. Additionally, no study to compare the difference between models using the systemic and inhaled route has been conducted. The use of adjuvants such as aluminium hydroxide has also been criticised as they may alter the mechanism of allergen sensitisation. Ova protocols which do not use a separate adjuvant do exist but generally take longer to achieve sensitisation or are not able to fully reproduce functional responses e.g. the LAR (Hessel *et al*, 1997; Dale *et al*, 2012). Additionally, these protocols contain quantities of LPS, which acts as an adjuvant and has been shown to be critical for Ova sensitisation (Eisenbarth *et al*, 2002). Other allergens now used in models such as *Aspergillus fumigatus* and house dust mite also contain substances such as LPS and proteases which act as adjutants (Canning & Chou, 2008; Cates *et al*, 2007). Instead the nature of the adjuvant may be a more important factor (Matzinger, 1994). The use of aluminium hydroxide with Ova alters the nature of allergen responses (Nakae *et al*, 2007). This is important to bear in mind when making comparisons between animal models and humans.

One other drawback of the models used throughout this thesis is their acute nature. Acute models tend to only use a single allergen challenge, with no pre-existing background of inflammation. Since asthma exacerbations likely occur on a background of chronic inflammation, the model employed in this thesis probably does not fully reflect the situation in humans. However, the model does still demonstrate many features of asthma such as an EAR, LAR, AHR and airway inflammation. Thus the acute model used still represents a good starting point for the investigation of asthma exacerbations and for early trials of novel drug therapy.

9.4 FURTHER WORK

There are many opportunities for further work from this thesis. Some of these suggestions were originally planned to be included in this thesis but due to time and financial constraints they were unable to be completed. Some studies are very difficult to perform in guinea-pigs due to a scarcity of molecular tools. Ideally a full assessment of all the models used in this thesis would be made using PCR/gene array technology which would allow for an unbiased assessment of molecular changes.

Further investigation of the temporal effects of LPS on allergen-induced responses is warranted. It would be interesting to investigate whether the decrease in the EAR seen with LPS delivered 24 hours before allergen exposure is due to partial mast cell degranulation. Microscopic investigation of mast cells after LPS exposure and measurement of histamine following allergen challenge may be able to help determine this (Vannier *et al*, 1991). Additionally, although airway inflammation still increases in these guinea-pigs the nature of the inflammatory response may be different. The use of FACS to identify lymphocyte subpopulations and immunohistochemistry could help achieve this, although such techniques are difficult with guinea-pigs due to a lack of available reagents. Further characterisation of the exacerbating effects of LPS on allergic inflammation would also be of interest; specifically the mechanisms behind the prolonged bronchoconstrictor response to histamine. This process may be mediated by decreased levels of diamine oxidase, which breaks down histamine or by increased nitric oxide (Stroheimer *et al*, 2001). Whether this effect is non-specific to the spasmogen would also be of interest. The use of alternative bronchoconstrictive agents such as methacholine could help investigate this. The use of an iNOS inhibitors could help determine this. A determination of neutrophil and eosinophil activity by elastase and EPO may also determine whether changes in the activity of these cells are involved.

The mechanisms that mediate inhaled corticosteroid insensitivity in the Ova and LPS combination model require further investigation. Many mechanisms have been

proposed to mediate this process including decreased GR-α, increased GR-β, decreased activity of HDAC-2 and increased PI3K activity (Leung & Bloom, 2003). The levels/activity of these proteins could be measured using ELISA assays and western blotting. These effects may be localised to specific cell populations, making it necessary to use immunohistochemistry and cell isolation techniques. Gross assessment of lung levels may give a false picture as to the importance of any mechanism. Additionally, a comparison of the activity of these proteins in airway cells with those in the circulation may explain the differing sensitivity of the Ova and LPS models sensitivity to inhaled and systemic corticosteroids. The use of drugs such as curcumin (HDAC-2 activator) and nortriptyline (PI3K inhibitor) could also help determine the mechanisms of corticosteroid insensitivity.

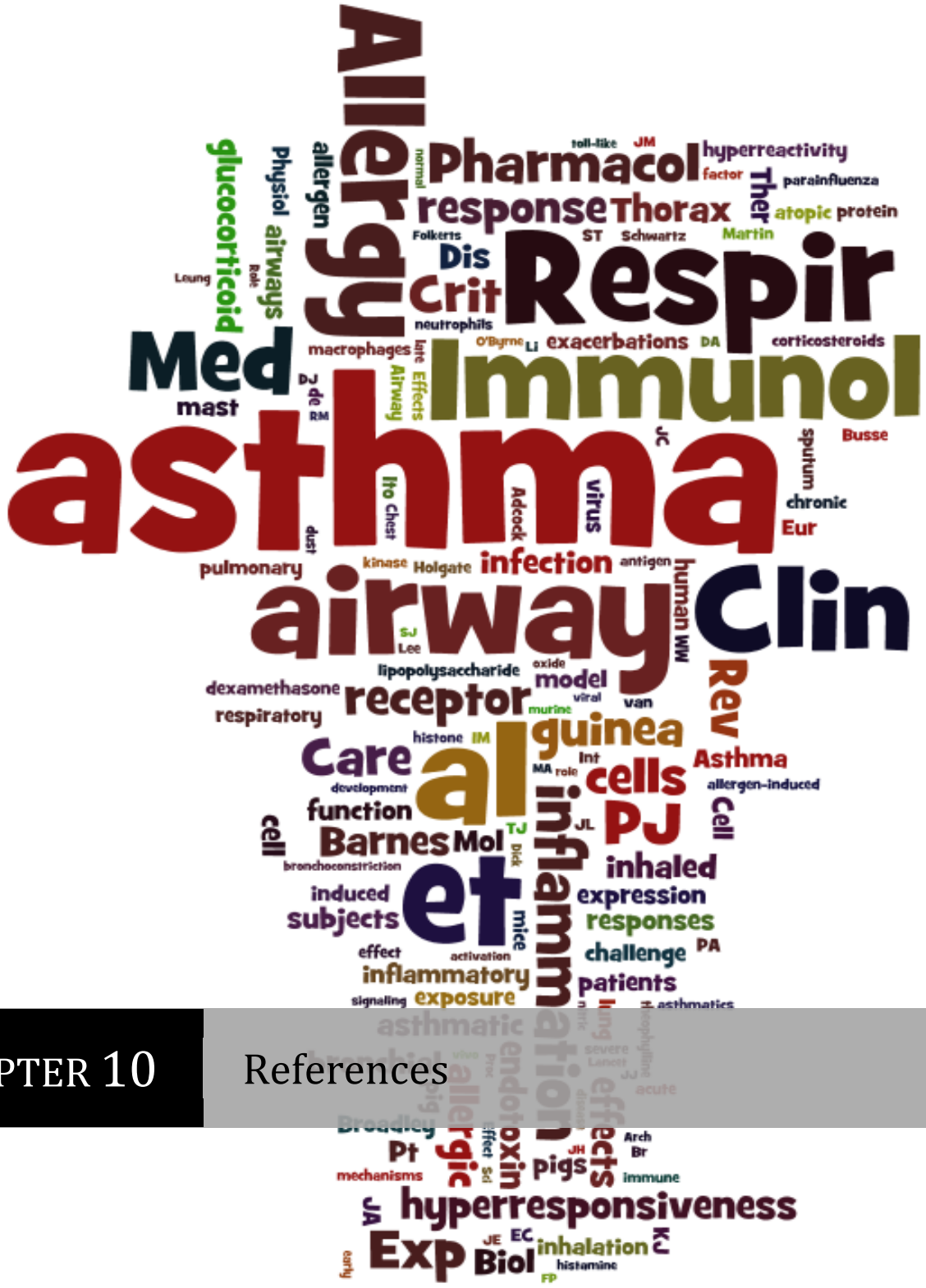
The importance of neutrophils in corticosteroid insensitivity also requires further investigation. Neutrophils are known to be less corticosteroid sensitive than other inflammatory cells and are also present in other corticosteroids insensitive lung diseases such as COPD (Strickland *et al*, 2001). The use of a pharmacological agent such as vinblastine, which depletes neutrophils in bone marrow would be useful for this. Finally a proper determination of the effect of inhaled and systemic corticosteroids on bone marrow in the models used in this thesis is warranted. Measurement of the plasma concentrations of circulating corticosteroid, as well as activity on bone marrow haematopoiesis would allow this.

Further studies could also assess the effect of LPS on airway responses in a chronic model of asthma. These models reflect the chronic nature of inflammation in humans and would allow for the assessment of the effect of LPS on a pre-existing inflammatory background. Chronic models also demonstrate airway remodelling which would allow for the long term assessment of the effect of repeat exacerbations on this process (Evans *et al*, 2012). Differences in corticosteroid insensitivity between acute and chronic models could also be assessed. Additionally, whether corticosteroid insensitivity resolves after a period of time or is permanent is unknown. Allowing a period of

resolution of Ova and LPS challenge before starting corticosteroid treatment would allow this.

9.5 Conclusions and clinical relevance

LPS inhalation exacerbates Ova-induced functional and inflammatory responses, decreasing their sensitivity to inhaled and partially to systemic corticosteroids. Clinically, LPS is a relevant agent for asthma exacerbations and corticosteroid insensitivity as it's found ubiquitously in the environment (Hunt *et al*, 1994; Goleva *et al*, 2008). The observation that responses in an asthma exacerbation model are nearly fully insensitive to inhaled corticosteroid is important. Clinically, asthmatic patients are less sensitive to inhaled corticosteroids during an asthma exacerbation and in more severe forms of the disease (in't Veen *et al*, 1999; Hodgson *et al*, 2012). However, they often show some clinical benefit with oral corticosteroids, which act systemically (Matsuse *et al*, 2012; Hodgson *et al*, 2012). Reflecting this, the asthma exacerbation model demonstrated some sensitivity to a systemic corticosteroid. Further work is required to better characterise this animal model and understand its applicability to asthma. None the less, this model may be useful as a preclinical model for investigating mechanisms and developing novel drugs for asthma exacerbations and corticosteroid-resistant asthma.



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A1.1 EQUIPMENT

Cannula - Bio-Med healthcare, India

Capillary tube - Roche, Switzerland

Cell culture flasks - Fisher Scientific UK, Loughborough, UK.

Centrifuge (Mistral 3000) - Mistral, UK.

Cold Plate - Fisher Scientific UK, Loughborough, UK.

Coverslip - Fisher Scientific UK, Loughborough, UK.

Digital Camera (Leica DC500) - Leica Microsystems, Germany

ELISA Plates - Sigma, Poole, UK.

Exposure Chamber - Buxco Research Systems, Winchester, UK.

Glass Slides - Surgipath Europe Ltd, Peterborough, UK.

Histology Cassettes - Surgipath Europe Ltd, Peterborough, UK.

Microscope (Olympia BH-2) - Olympus, London, UK.

Microscope (Leica DMRAZ) - Leica Microsystems, Germany

Microtome - Mistral, UK.

Neubauer Haemocytometer - Supe-rior, Marienfeld, Germany.

Nuclease free tubes - Invitrogen, USA

Polysine slides - Thermo Scientific, UK

Cyclegene thermo cycler - Technene, Staffordshire, UK

Thermo light cycler - Roche, Switzerland

Tissue homogeniser - Precellys, UK

Tissue homogeniser tubes - Precellys, UK

Wax Dispenser - Fisher Scientific UK, Loughborough, UK.

Whole Body Plethysmograph (complete set up) - Buxco Research Systems, Winchester, UK.

Wright Nebuliser (Pulmostar) - Sunrise Medical Ltd., Wollaston, UK.

A1.2 MATERIALS

Acetic acid - Sigma, Poole, UK.

Alcian blue powder - Sigma, Poole, UK.

Aluminium ammonium sulphate - Fisher Scientific UK, Loughborough, UK.

Aluminium hydroxide - Sigma, Poole, UK.

Aprotinin - Sigma, Poole, UK.

Aqueous acetic acid - Sigma, Poole, UK.

BCA protein assay Kit - Pierce protein biology, Thermo Scientific, UK

BCA protein assay chemical A

BCA protein assay chemical B

BCA protein assay standard

BSC-1 cells - European collection animal cell culture (ECACC)

BSC-1 cell culture reagents

EMEM - Invitrogen, USA

Glutamine - Sigma, Poole, UK.

Non-essential amino acids - Sigma, Poole, UK.

Foetal bovine serum - Sigma, Poole, UK.

cDNA primers - Primer design Ltd

Chloroform - Sigma, Poole, UK.

Chloral hydrate - Fisher Scientific UK, Loughborough, UK.

Citric acid - Sigma, Poole, UK.

Cytokine assay reagents (Capture and detection Ab, protein standard (Duoset) - R&D systems, UK

Cytokine assay diluent - R&D systems, UK

Cytokine assay TMB substrate solutions A & - R&D systems, UK

Cytokine assay Stepavidin-HRP - R&D systems, UK

Dexamethasone 21-phosphate salt - Sigma, Poole, UK.

Dimethyl sulfoxide - Sigma, Poole, UK.

EGTA disodium salt - Sigma, Poole, UK.

Eosin - Surgipath Europe Ltd, Peterborough, UK.

Ethanol - Sigma, Poole, UK.

Fluticasone propionate - Sigma, Poole, UK.

Formaldehyde - Fisher Scientific UK, Loughborough, UK.

Guinea Pigs - Harlan, UK and Charles River, Germany

Haematoxylin - Fisher scientific, UK

Histamine - Sigma, Poole, UK.

Histoclear - Fisher scientific, UK

Histomount - Fisher scientific, UK

Hydrochloric acid - Sigma, Poole, UK.

Leishman's Powder - Sigma, Poole, UK.

Leupeptin - Sigma, Poole, UK.

Lipopolysaccharide from *E. coli* - Sigma, Poole, UK.

Magnesium sulphate - Fisher Scientific UK, Loughborough, UK.

Mayer's haematoxylin - Surgipath Europe Ltd, Peterborough, UK.

Mepyramine - Sigma, Poole, UK.

Methanol - Fisher Scientific UK, Loughborough, UK.

Ovalbumin - VWR International Ltd., Leicestershire, UK.

Paraffin wax - Surgipath Europe Ltd, Peterborough, UK.

Periodic acid - Surgipath Europe Ltd, Peterborough, UK.

PMSF - Sigma, Poole, UK.

Phenylarsine oxide - Sigma, Poole, UK.

Picric acid - Sigma, Poole, UK.

Potassium alum - Fisher Scientific UK, Loughborough, UK.

Saline tablets - Fisher Scientific UK, Loughborough, UK.

Schiff's reagent - Surgipath Europe Ltd, Peterborough, UK.

Sirius red - Sigma, Poole, UK.

Sodium bicarbonate - Fisher Scientific UK, Loughborough, UK.

Sodium chloride - Sigma, Poole, UK.

Sodium Fluoride - Sigma, Poole, UK.

Sodium iodate - Fisher Scientific UK, Loughborough, UK.

Sodium molybdate - Sigma, Poole, UK.

Sodium pentobarbitone (Euthatal) - Cardiff University, UK

$NaVO_4$ - Sigma, Poole, UK.

Sulphuric acid - Sigma, Poole, UK.

Superscript first-strand synthesis system kit - Invitrogen, USA

Triton x - Sigma, Poole, UK.

Trizma base - Sigma, Poole, UK.

Tween 20 - Sigma, Poole, UK.

Viral ribonucleic acid isolation kit - Roche, Switzerland

Contains: **Proteinase K -** (reconstituted in 5ml Elution buffer)

Poly A - (reconstituted in 500µl Elution buffer)

Binding buffer - premade

Inhibitor removal buffer - (reconstituted in 20ml ethanol)

Wash buffer - (reconstituted in 40ml ethanol)

Elution buffer - (Nuclease-free, sterile, double distilled water).

All made up as per the manufactures instructions

Light cycler TaqMan master kit (contains master mix) - Roche, Switzerland

A1.3 SOLUTIONS MADE

Alcian blue-

1g Alcian blue powder

100ml 3% acetic acid

BCA protein assay working reagent -Pierce protein biology, Thermo scientific, UK

50 parts BCA protein assay chemical A

1 part BCA protein assay chemical B

Leishman's solution - 0.5% w/v Leishman's powder in 100% methanol

Lipopolysaccharide - Made up in normal saline

Lysis buffer -

Component A - 50mM Trizma base, 5mM EGTA disodium salt, 150mM NaCl, 1% Triton and 100ml $_{D}H_2O$.

Component B - 0.4mM $NaVO_{4,}$ 50mM NaF, 1mM PMSF, 20µM Phenylarsine oxide, 10mM Sodium molybdate, 10µg/ml Leupeptin, 10µg/ml Aprotinin in lysis buffer component A.

Mayer's haematoxylin -

1g haematoxylin, 50g Aluminium ammonium sulphate and 0.2g sodium iodate dissolved in 1L distilled water with warming. 1g citric acid and 50g chloral hydrate added, the solution boiled for 5 minutes, cooled then filtered.

Normal saline - 1 saline tablet per 100ml of dH_2O

Ovalbumin - made up in normal saline

Phosphate buffered saline - 1 PBS tablet to 100µl $_{d}H20$

Phosphate buffered saline-tween 20 - 25µl tween 20 in 500ml of PBS.

Scotts tap water - 20g sodium bicarbonate, 3.5g magnesium sulphate, 1 distilled water.

Sirius red stain - (pH 8-9)- 2g Direct Red 80, 180ml Distilled water, 200ml 100% ethanol.

A1.4 COMPUTER PROGRAMS

Data acquisition - Fine point software, Buxco

GraphPad Instat 3 - GraphPad software, Inc., La Jolla, CA, USA.

GraphPad Prism 5 - GraphPad software, Inc., La Jolla, CA, USA.

ImageJ - National Institute of Health - http://rsbweb.nih.gov/ij/index.html

Microsoft Excel - Microsoft corporation

Leica QWin software - Leica Microsystems, Germany

A2.1 THE MEASUREMENT OF SGAW

Specific airway conductance (sGaw) is derived from the changes in volume between animal body and plethysmograph chamber that occur as a result of ventilation. Flow based whole body plethysmograph chambers such as the one used in the present thesis are divided into nasal and thoracic components. The plethysmograph chamber measures the difference between the rate of change in nasal flow and thoracic displacement. The volume and rate of these flows is affected by both conditioning and resistance.

To minimise the effect of conditioning, a reading is taken at the end of inspiration, where conditioning (which is equal to flow) is zero as there is no flow at this point.

At this point box pressure is proportional to

Resistance (R) x thoracic gas volume (TGV) x Difference in flow (dF) x difference in time (dt)

The negative pressure in lungs during inspiration pulls air into the lungs against airway resistance so

Negative pressure of the lungs (P_{alv}) = R x F

The rate of pressure change (dPb/dt) may also be expressed as

TGV x dP_{alv}/dt at the flow zero crossings.

Additionally, when there is zero flow dF/dt is generally large as there is a delay in transition between inspiratory to expiratory thoracic flow.

Thus, when there is zero flow the resistance component of the plethysmograph signal dominates, despite conditioning being a large component for the rest of the waveform.

During a bronchoconstriction the nasal flow lags behind the thoracic flow (Figure 1). When conditioning is low as when there is zero flow the time delay between the 2 flows is related to specific airway resistance (sRaw) and sGaw. The reciprocal of Raw, airway conductance

(Gaw), is considered a better measurement of airway function as it accounts for change in lung tissue tension and transpulmonary pressure.

Gaw = 1/ Raw

sGaw = Gaw/TGV

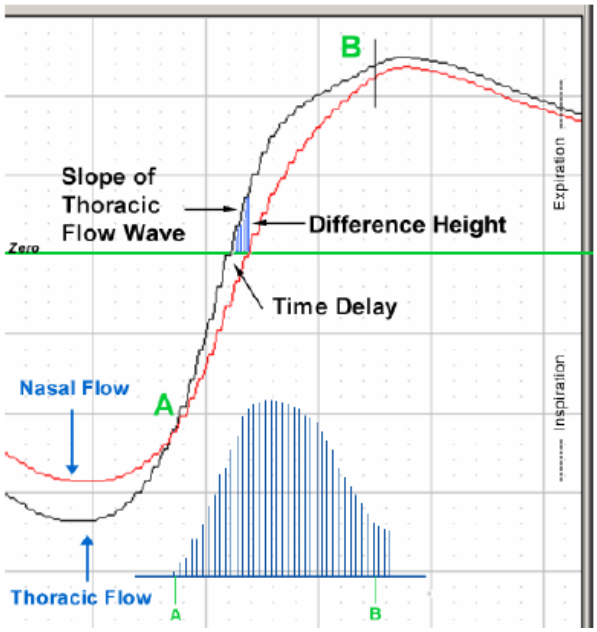

Figure 1- Waveform trace showing nasal and thoracic flows during a bronchoconstrictions

Adapted from Lamask, (2005).

A3.1 IL-4

GUINEA PIG SEQUENCE

1 MGLVPQLTVI LFCLLACASA VVRGCNHHTL QEIIQHLNTL SREKSPCAEL LVTDVFADPQ
61 GPASGDLCTA ATVLHHTAYL RGPQSCPNRE GDPLYPSVLR QVFRNLRSMA QSNCPVSELR
121 QTTLKDFLEN LKRIMQKRYS KCRR

HUMAN SEQUENCE

GENE ID: 3565 IL4 | interleukin 4 [Homo sapiens]

Score = 88.6 bits (218), Expect = 2e-20, Method: Compositional matrix adjust.
Identities = 68/153 (44%), Positives = 89/153 (58%), Gaps = 13/153 (8%)

```
Query  1    MGLVPQLTVILFCLLACASAVVRG--CNHHTLQEIIQHLNTLSREKSPCAELLVTDVFAD  58
            MGL  QL   LF LLACA   V G  C+  TLQEII+ LN+L+ +K+ C EL VTD+FA
Sbjct  1    MGLTSQLLPPLFFLLACAGNFVHGHKCDI-TLQEIIKTLNSLTEQKTLCTELTVTDIFAA  59

Query  59   PQGPASGD-LCTAATVL-----HHTAYLR--GPQSCPNREGDPLYPSVLRQVFRNLRSMA  110
            +    + C AATVL     HH    R  G +       L   +L+++ RNL  +A
Sbjct  60   SKNTTEKETFCRAATVLRQFYSHHEKDTRCLGATAQQFHRHKQLIR-LLKRLDRNLWGLA  118

Query  111  QSN-CPVSELRQTTLKDFLENLKRIMQKRYSKC  142
             N CPV E  Q+TL++FLE LK IM+++YSKC
Sbjct  119  GLNSCPVKEANQSTLENFLERLKTIMREKYSKC  151
```

MOUSE SEQUENCE

GENE ID: 16189 Il4 | interleukin 4 [Mus musculus]

Score = 85.9 bits (211), Expect = 1e-19, Method: Compositional matrix adjust.
Identities = 53/142 (37%), Positives = 77/142 (54%), Gaps = 4/142 (3%)

```
Query  1    MGLVPQLTVILFCLLACASAVVRGCNHHTLQEIIQHLNTLSREKSPCAELLVTDVFADPQ  60
            MGL PQL VIL  L C  + + GC+ + L+EII  LN ++ E +PC E+ V +V    +
Sbjct  1    MGLNPQLVVILLFFLECTRSHIHGCDKNHLREIIGILNEVTGEGTPCXEMDVPNVLTATK  60

Query  61   GPASGDL-CTAATVLHHTAYLRGPQSCPNREGDPLYPSVLRQVFRNLRSMAQS-NCPVSE  118
               +L C A+ VL      G   C  +   L    L+++FR  R +  S +C ++E
Sbjct  61   NTTESELVCRASKVLRIFYLKHGKTPCLKKNSSVLME--LQRLFRAFRCLDSSISCTMNE  118

Query  119  LRQTTLKDFLENLKRIMQKRYS  140
            +  +LKDFLE+LK IMQ  YS
Sbjct  119  SKSXSLKDFLESLKSIMQMDYS  140
```

A3.2 IL-5

GUINEA PIG SEQUENCE

1 MRVLLQLGLL ALGAVCVCAI PKQSATLRAL VRETLTLLST HRTLLKGNET LRISVPAHKN
61 HQLCIEEIFQ GIDTLKNQTT QGEALATLFQ NLSLIKKHID LQKQKCGEER RRVKQFLDYL
121 QEFLAVINTE WTIEG

HUMAN SEQUENCE

interleukin 5 (colony-stimulating factor, eosinophil) [Homo sapiens]

Score = 174 bits (440), Expect = 1e-54, Method: Compositional matrix adjust.
Identities = 87/119 (73%), Positives = 96/119 (81%), Gaps = 1/119 (1%)

Query 17 VCAIPKQSATLRALVRETLTLLSTHRTLLKGNETLRISVPAHKNHQLCIEEIFQGIDTLK 76
V AIP + T ALV+ETL LLSTHRTLL NETLRI VP HKNHQLC EEIFQGI TL+
Sbjct 17 VYAIPTEIPT-SALVKETLALLSTHRTLLIANETLRIPVPVHKNHQLCTEEIFQGIGTLE 75

Query 77 NQTTQGEALATLFQNLSLIKKHIDLQKQKCGEERRRVKQFLDYLQEFLAVINTEWTIEG 135
+QT QG + LF+NLSLIKK+ID QK+KCGEERRRV QFLDYLQEFL V+NTEW IE
Sbjct 76 SQTVQGGTVERLFKNLSLIKKYIDGQKKKCGEERRRVNQFLDYLQEFLGVMNTEWIIES 134

CAT SEQUENCE

interleukin 5 (colony-stimulating factor, eosinophil)
[Felis catus]

Score = 165 bits (418), Expect = 3e-51, Method: Compositional matrix adjust.
Identities = 84/119 (71%), Positives = 93/119 (78%), Gaps = 1/119 (1%)

Query 17 VCAIPKQSATLRALVRETLTLLSTHRTLLKGNETLRISVPAHKNHQLCIEEIFQGIDTLK 76
V AI QS R LV ETL LLSTHRTLL G+ L I P H NHQLCIEE+FQGIDTLK
Sbjct 17 VSAIAVQSPMNR-LVAETLALLSTHRTLLIGDGNLMIPTPEHNNHQLCIEEVFQGIDTLK 75

Query 77 NQTTQGEALATLFQNLSLIKKHIDLQKQKCGEERRRVKQFLDYLQEFLAVINTEWTIEG 135
N+T G+A+ LF+NLSLIK+HID QK+KCG ER RVK+FLDYLQ FL VINTEWTIEG
Sbjct 76 NRTVPGDAVEKLFRNLSLIKEHIDRQKKKCGGERWRVKKFLDYLQVFLGVINTEWTIEG 134

A3.3 INTERFERON GAMMA

GUINEA PIG SEQUENCE

```
1   MKYTSSILAL QFCIILSFSS YYCQSRFTNE IRILKNYFNA DNSDVGDNGT LFVGILKNCQ
61  EESERKIFQS QIVSFYFKLF EKHFTDNQTV QNSMNTIKEQ IITKFFKDNS SNKVQAFKNL
121 IQISVNDEHV QRQAIIELKK VIDDLSPNQR KRRRTQMLFQ SRRASK
```

HORSE SEQUENCE

Score = 191 bits (484), Expect = 3e-60, Method: Compositional matrix adjust.
Identities = 101/166 (61%), Positives = 125/166 (75%), Gaps = 4/166 (2%)

```
Query  1    MKYTSSILALQFCIILSFSSYYCQSRFTNEIRILKNYFNADNSDVGDNGTLFVGILKNCQ  60
            MKYTS ILA Q C IL  S+YYCQ+ F  EI  LK YFNA N DVGD G LF+ ILKN +
Sbjct  1    MKYTSFILAFQLCAILGSSTYYCQAAFFKEIENLKEYFNASNPDVGDGGPLFLDILKNWK  60

Query  61   EESERKIFQSQIVSFYFKLFEKHFTDNQTVQNSMNTIKEQIITKFFKDNSSNKVQAFKNL  120
            E+S++KI QSQIVSFYFKLFE +  DNQ +Q SM+TIKE +  KFF ++S++K++ F+ L
Sbjct  61   EDSDKKIIQSQIVSFYFKLFE-NLKDNQVIQKSMDTIKEDLFVKFF-NSSTSKLEDFQKL  118

Query  121  IQISVNDEHVQRQAIIELKKVIDDLSP--NQRKRRRTQMLFQSRRA  164
            IQI VND  VQR+AI EL KV++DLSP  N RKR+R+Q  F+ RRA
Sbjct  119  IQIPVNDLKVQRKAISELIKVMNDLSPKANLRKRKRSQNPFRGRRA  164
```

HUMAN SEQUENCE

GENE ID: 3458 IFNG | interferon, gamma [Homo sapiens] (Over 100 PubMed links)

Score = 175 bits (443), Expect = 5e-54, Method: Compositional matrix adjust.
Identities = 96/168 (57%), Positives = 119/168 (71%), Gaps = 4/168 (2%)

```
Query  1    MKYTSSILALQFCIILSFSSYYCQSRFTNEIRILKNYFNADNSDVGDNGTLFVGILKNCQ  60
            MKYTS ILA Q CI+L     YCQ  +  E   LK YFNA +SDV DNGTLF+GILKN +
Sbjct  1    MKYTSYILAFQXCIVLGSLGCYCQDPYVKEAENLKKYFNAGHSDVADNGTLFLGILKNWK  60

Query  61   EESERKIFQSQIVSFYFKLFEKHFTDNQTVQNSMNTIKEQIITKFFKDNSSNKVQAFKNL  120
            EES+RKI QSQIVSFYFKLF K+F D+Q++Q S+ TIKE +  KFF  N   K   F+ L
Sbjct  61   EESDRKIMQSQIVSFYFKLF-KNFKDDQSIQKSVETIKEDMNVKFFNSNKK-KRDDFEKL  118

Query  121  IQISVNDEHVQRQAIIELKKVIDDLSPNQR--KRRRTQMLFQSRRASK  166
              SV D +VQR+AI EL +V+ +LSP  +  KR+R+QMLF+ RRAS+
Sbjct  119  TNYSVTDLNVQRKAIHELIQVMAELSPAAKTGKRKRSQMLFRGRRASQ  166
```

A3.4 IL-10

Guinea pig sequence

```
1   MPSSALLCCL VLLAGVKASQ GTNTQSEDSC AHFPAGLPHM LRELRAAFGR VKTFFQTQDQ
61  LDNVLLNKSL LEDFKGYLGC QALSEMIQFY LVEVMPKAEN HDPDIKEHVS SLGEKLKTLR
121 LRLRRCHRFL PCENKSKAVE QVKNTFNKLQ EKGVYKAMSE FDIFINYIEA YMTRKLTN
```

Mouse sequence

GENE ID: 16153 Il10 | interleukin 10 [Mus musculus] (Over 100 PubMed links)

Score = 303 bits (777), Expect = 1e-104, Method: Compositional matrix adjust.
Identities = 150/178 (84%), Positives = 163/178 (92%), Gaps = 0/178 (0%)

```
Query  1    MPGSALLCCLALLAGVKASQGTNTQSEDSCAHFPAGLPHMLRELRAAFGRVKTFFQTQDQ  60
            MPGSALLCCL LL G++ S+G  ++ +++C HFP G  HML ELR AF +VKTFFQT+DQ
Sbjct  1    MPGSALLCCLLLLTGMRISRGQYSREDNNCTHFPVGQSHMLLELRTAFSQVKTFFQTKDQ  60

Query  61   LDNVLLNKSLLEDFKGYLGCQALSEMIQFYLVEVMPQAEKHGPEIKEHLNSLGEKLKTLR  120
            LDN+LL  SL++DFKGYLGCQALSEMIQFYLVEVMPQAEKHGPEIKEHLNSLGEKLKTLR
Sbjct  61   LDNILLTDSLMQDFKGYLGCQALSEMIQFYLVEVMPQAEKHGPEIKEHLNSLGEKLKTLR  120

Query  121  MRLRRCHRFLPCENKSKAVEQVKSDFNKLQDQGVYKAMNEFDIFINCIEAYMMIKMKS  178
            MRLRRCHRFLPCENKSKAVEQVKSDFNKLQDQGVYKAMNEFDIFINCIEAYMMIKMKS
Sbjct  121  MRLRRCHRFLPCENKSKAVEQVKSDFNKLQDQGVYKAMNEFDIFINCIEAYMMIKMKS  178
```

A3.5 IL-12 p70 (p40 & p35)

Guinea pig p40 subunit sequence

```
1    MCHRQLISSW LSLVLLASPL LAMWELKKDV YVVELDWHTD APGETVVLTC NTAEEDGITW
61   TSDRKSDILG SGKTLTIQVK EFEDAGGYTC HKGGEVLSRS QLLLHKKEDE IWSTDILKEQ
121  KGSNGKTFLK CEARSYSGRF TCWWLTAFGT DVKFSVKGSR GSSDPSGVTC GEAERVSGDN
181  QEYKYSVECQ EDSACPTAEE SLPIEVVVDA IHKFKYENYT SSFYIRDIIK PDPPKNLQLK
241  PSVNSQQVEV SWEYPDTWST PHSYFSLTFL VQTHGKNKNR REKKYELFTD KTSATVSCHK
301  ISKVEVRARD RYYSSSWSEW ASVSCSEVSV SR
```

Human p40 subunit sequence

gb|AAA59938.1| natural killer cell stimulatory factor [Homo sapiens]
Length=328

GENE ID: 3593 IL12B | interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40) [Homo sapiens]
(Over 100 PubMed links)

Score = 512 bits (1319), Expect = 0.0, Method: Compositional matrix adjust.
Identities = 258/330 (78%), Positives = 283/330 (86%), Gaps = 6/330 (2%)

```
Query  1    MCHRQLISSWLSLVLLASPLLAMWELKKDVYVVELDWHTDAPGETVVLTCNTAEEDGITW  60
            MCH+QL+ SW SLV LASPL+A+WELKKDVYVVELDW+ DAPGE VVLTC+T EEDGITW
Sbjct  1    MCHQQLVISWFSLVFLASPLVAIWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITW  60

Query  61   TSDRKSDILGSGKTLTIQVKEFEDAGGYTCHKGGEVLSRSQLLLHKKEDEIWSTDILKEQ  120
            T D+ S++LGSGKTLTIQVKEF DAG YTCHKGGEVLS S LLLHKKED IWSTDILK+Q
Sbjct  61   TLDQSSEVLGSGKTLTIQVKEFGDAGQYTCHKGGEVLSHSLLLLHKKEDGIWSTDILKDQ  120

Query  121  KGSNGKTFLKCEARSYSGRFTCWWLTAFGTDVKFSVKGSRGSSDPSGVTCG----EAERV  176
            K   KTFL+CEA++YSGRFTCWWLT  TD+ FSVK SRGSSDP GVTCG    AERV
Sbjct  121  KEPKNKTFLRCEAKNYSGRFTCWWLTTISTDLTFSVKSSRGSSDPQGVTCGAATLSAERV  180

Query  177  SGDNQEYKYSVECQEDSACPTAEESLPIEVVVDAIHKFKYENYTSSFYIRDIIKPDPPKN  236
            GDN+EY+YSVECQEDSACP AEESLPIEV+VDA+HK KYENYTSSF+IRDIIKPDPP N
Sbjct  181  RGDNKEYEYSVECQEDSACPAAEESLPIEVMVDAVHKLKYENYTSSFFIRDIIKPDPPNN  240

Query  237  LQLKPSVNSQQVEVSWEYPDTWSTPHSYFSLTFLVQTHGKNKNRREKKYELFTDKTSATV  296
            LQLKP  NS+QVEVSWEYPDTWSTPHSYFSLTF VQ  GK+K  REKK  +FTDKTSATV
Sbjct  241  LQLKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSK--REKKDRVFTDKTSATV  298

Query  297  SCHKISKVEVRARDRYYSSSWSEWASVSCS  326
             C K + + VRA+DRYYSSSWSEWASV CS
Sbjct  299  ICRKNASISVRAQDRYYSSSWSEWASVPCS  328
```

MOUSE SEQUENCE

gb|AAF22556.1|AF128215_1 interleukin 12b p40 subunit [Mus musculus]
Length=335

GENE ID: 16160 Il12b | interleukin 12b [Mus musculus] (Over 100 PubMed links)

Score = 396 bits (1017), Expect = 4e-136, Method: Compositional matrix adjust.
Identities = 208/336 (62%), Positives = 249/336 (74%), Gaps = 16/336 (5%)

```
Query  1    MCHRQLISSWLSLVLLASPLLAMWELKKDVYVVELDWHTDAPGETVVLTCNTAEEDGITW  60
            MC ++L  SW ++VLL SPL+AMWEL+KDVYVVE+DW  DAPGETV LTC+T EED ITW
Sbjct  1    MCPQKLTISWFAIVLLVSPLMAMWELEKDVYVVEVDWTPDAPGETVNLTCDTPEEDDITW  60

Query  61   TSDRKSDILGSGKTLTIQVKEFEDAGGYTCHKGGEVLSRSQLLLHKKEDEIWSTDILKEQ  120
            TSD++  ++GSGKTLTI VKEF DAG YTCHKGGE LS S LLLHKKE+ IWST+ILK
Sbjct  61   TSDQRHGVIGSGKTLTITVKEFLDAGQYTCHKGGETLSHSHLLLHKKENGIWSTEILKNF  120

Query  121  KGSNGKTFLKCEARSYSGRFTCWWLTAFGTDVKFSVKGSRGSSDPSGVTCG----EAERV  176
            K   KTFLKCEA +YSGRFTC WL    D+KF++K S  S D   VTCG    AE+V
Sbjct  121  K---NKTFLKCEAPNYSGRFTCSWLVQRNMDLKFNIKSSSSSPDSRAVTCGTASLSAEKV  177
```

```
Query  177  SGDNQEY-KYSVECQEDSACPTAEESLPIEVVVDAIHKFKYENYTSSFYIRDIIKPDPPK  235
            + D ++Y KYSV CQED  CPTAEE+LPIE+ ++A  + KYENY++SF+IRDIIKPDPPK
Sbjct  178  TLDQRDYEKYSVSCQEDVTCPTAEETLPIELALEARQQNKYENYSTSFFIRDIIKPDPPK  237

Query  236  NLQLKPSVNSQQVEVSWEYPDTWSTPHSYFSLTFLVQTHGKNKNRR------EKKYELFT  289
            NLQ+KP  NS QVEVSWEYPD+WSTPHSYFSL F V+   K +  +      +K  L
Sbjct  238  NLQMKPLKNS-QVEVSWEYPDSWSTPHSYFSLKFFVRIQRKKEKMKETEEGCNQKGALLV  296

Query  290  DKTSATVSCHKISKVEVRARDRYYSSSWSEWASVSC  325
            +KTS  V C K   V V+A+DRYY+SS S+WA V C
Sbjct  297  EKTSTEVQC-KGGNVCVQAQDRYYNSSCSKWACVPC  331
```

GUINEA PIG IL-12 P35 SUBUNIT

```
1   MSPLRKCLLL TSLVLLVSCS LARNLPRASP APVTEPVQCF NHSQTLLRAV NSELHKAIQM
61  LAVYSCTPEE IDHEDITKDK TSTVKACVPL ELVKNESCLA SGHISFTTNG SCLASGKTSF
121 MMALCLNSIY EDLKLYQLEF KNMNAQLLMD PQRQIFLDQN MLSAIDELIQ ALNGSDVTVP
181 QKLSLEEPDF YKIKMKLCIL LHAFRIRAVT IDRVMSYLTS S
```

HUMAN SEQUENCE

gb|AAA35694.1| cytotoxic lymphocyte maturation factor 35 kDa subunit [Homo sapiens]
Length=219

GENE ID: 3592 IL12A | interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35) [Homo sapiens]
(Over 100 PubMed links)

Score = 305 bits (780), Expect = 1e-108, Method: Compositional matrix adjust.
Identities = 153/202 (76%), Positives = 169/202 (84%), Gaps = 2/202 (1%)

```
Query  20   SLARNLPRASPAPVTEPVQCFNHSQTLLRAVNSELHKAIQMLAVYSCTPEEIDHEDITKD  79
            SLARNLP A+P P   P  C +HSQ LLRAV++ L KA Q L  Y CT EEIDHEDITKD
Sbjct  20   SLARNLPVATPDPGMFP--CLHHSQNLLRAVSNMLQKARQTLEFYPCTSEEIDHEDITKD  77

Query  80   KTSTVKACVPLELVKNESCLASGHISFTTNGSCLASGKTSFMMALCLNSIYEDLKLYQLE  139
            KTSTV+AC+PLEL KNESCL S   SF TNGSCLAS KTSFMMALCL+SIYEDLK+YQ+E
Sbjct  78   KTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIYEDLKMYQVE  137

Query  140  FKNMNAQLLMDPQRQIFLDQNMLSAIDELIQALNGSDVTVPQKLSLEEPDFYKIKMKLCI  199
            FK MNA+LLMDP+RQIFLDQNML+ IDEL+QALN +  TVPQK SLEEPDFYK K+KLCI
Sbjct  138  FKTMNAKLLMDPKRQIFLDQNMLAVIDELMQALNFNSETVPQKSSLEEPDFYKTKIKLCI  197

Query  200  LLHAFRIRAVTIDRVMSYLTSS  221
            LLHAFRIRAVTIDRV SYL +S
Sbjct  198  LLHAFRIRAVTIDRVTSYLNAS  219
```

MOUSE SEQUENCE

gb|AAF22552.1|AF128211_1 interleukin 12a [Mus musculus]
Length=116

GENE ID: 16159 Il12a | interleukin 12a [Mus musculus] (Over 100 PubMed links)

Score = 87.4 bits (215), Expect = 1e-24, Method: Compositional matrix adjust.
Identities = 41/77 (53%), Positives = 55/77 (71%), Gaps = 0/77 (0%)

```
Query  32   PVTEPVQCFNHSQTLLRAVNSELHKAIQMLAVYSCTPEEIDHEDITKDKTSTVKACVPLE  91
            PV+ P +C + S+ LL+  +  +  A + L  YSCT E+IDHEDIT+D+TST+K C+PLE
Sbjct  26   PVSGPARCLSQSRNLLKTTDDMVKTAREKLKHYSCTAEDIDHEDITRDQTSTLKTCLPLE  85

Query  92   LVKNESCLASGHISFTT  108
            L KNESCLA+  S TT
Sbjct  86   LHKNESCLATRETSSTT  102
```

A3.6 IL-17 (IL-17A)

GUINEA PIG SEQUENCE: PREDICTED

```
  1 MVIKVDQEKS KSIVGVRAEE KIIGSLQSLQ RAPSWKGTRS PYAFPPTART FRSLLLLSLM
 61 ATVKAGIPIP RNPGCPTATE GKNFLQNVKL NLSIFNPLTQ NVNSRRSSDY YKRSTSPWTL
121 HRNENPNRYP PVIWEAECRY SGCVNAAGKE DHHVSSVPIQ QEILVLQREP QNCPLSFRLE
181 KMKVTVGCTC VTPIVRHVG
```

HUMAN SEQUENCE

ref|XP_003474811.1| PREDICTED: interleukin-17A-like [Cavia porcellus]
Length=199

GENE ID: 100735572 LOC100735572 | interleukin-17A-like [Cavia porcellus]

Score = 205 bits (521), Expect = 1e-65, Method: Compositional matrix adjust.
Identities = 102/146 (70%), Positives = 118/146 (81%), Gaps = 2/146 (1%)

```
Query  12   LLLLSLEAIVKAGITIPRNPGCPNS-EDKNFPRTVMVNLNIHNRNT-NTNPKRSSDYYNR  69
            LLLLSL A VKAGI IPRNPGCP + E KNF + V +NL+I N  T N N +RSSDYY R
Sbjct  54   LLLLSLMATVKAGIPIPRNPGCPTATEGKNFLQNVKLNLSIFNPLTQNVNSRRSSDYYKR  113

Query  70   STSPWNLHRNEDPERYPSVIWEAKCRHLGCINADGNVDYHMNSVPIQQEILVLRREPPHC  129
            STSPW LHRNE+P RYP VIWEA+CR+ GC+NA G  D+H++SVPIQQEILVL+REP +C
Sbjct  114  STSPWTLHRNENPNRYPPVIWEAECRYSGCVNAAGKEDHHVSSVPIQQEILVLQREPQNC  173

Query  130  PNSFRLEKILVSVGCTCVTPIVHHVA  155
            P SFRLEK+ V+VGCTCVTPIV HV
Sbjct  174  PLSFRLEKMKVTVGCTCVTPIVRHVG  199
```

A3.7 IL-13

GUINEA PIG SEQUENCE- PREDICTED

1 MAFWVTVVLA LACLGGLTAP GPVPVSSTVA VSIKELLGEL VNITQDQKTP LCNSSMVWSV
61 NLTAGLWYCA ARESLINVSN CSALQRTQKI LSGLCQHKAS AGVSSLRSPD TKIEVAEFVK
121 KLRIHVQCLY RHGKFH

HUMAN SEQUENCE

ref|XP_003464584.1| PREDICTED: interleukin-13-like [Cavia porcellus]
Length=136
GENE ID: 100720118 LOC100720118 | interleukin-13-like [Cavia porcellus]

Score = 171 bits (433), Expect = 3e-53, Method: Compositional matrix adjust.
Identities = 85/137 (62%), Positives = 108/137 (79%), Gaps = 6/137 (4%)

Query 1 MALLLTTVIALTCLGGFASPGPVPPST----ALRELIEELVNITQNQKAPLCNGSMVWSI 56
MA +T V+AL CLGG +PGPVP S+ +++EL+ ELVNITQ+QK PLCN SMVWS+
Sbjct 1 MAFWVTVVLALACLGGLTAPGPVPVSSTVAVSIKELLGELVNITQDQKTPLCNSSMVWSV 60

Query 57 NLTAGM-YCAALESLINVSGCSAIEKTQRMLSGFCPHKVSAGQFSSLHVRDTKIEVAQFV 115
NLTAG+ YCAA ESLINVS CSA+++TQ++LSG C HK SAG SSL DTKIEVA+FV
Sbjct 61 NLTAGLWYCAARESLINVSNCSALQRTQKILSGLCQHKASAG-VSSLRSPDTKIEVAEFV 119

Query 116 KDLLLHLKKLFREGQFN 132
K L +H++ L+R G+F+
Sbjct 120 KKLRIHVQCLYRHGKFH 136

MOUSE SEQUENCE

ref|XP_003464584.1| PREDICTED: interleukin-13-like [Cavia porcellus]
Length=136
GENE ID: 100720118 LOC100720118 | interleukin-13-like [Cavia porcellus]

Score = 167 bits (424), Expect = 4e-52, Method: Compositional matrix adjust.
Identities = 86/135 (64%), Positives = 103/135 (76%), Gaps = 4/135 (3%)

Query 1 MALWVTAVLALACLGGLAAPGPVPRSVSLPLTLKELIEELSNITQDQ-TPLCNGSMVWSV 59
MA WVT VLALACLGGL APGPVP S ++ +++KEL+ EL NITQDQ TPLCN SMVWSV
Sbjct 1 MAFWVTVVLALACLGGLTAPGPVPVSSTVAVSIKELLGELVNITQDQKTPLCNSSMVWSV 60

Query 60 DLAAG-GFCVALDSLTNISNCNAIYRTQRILHGLCNRKAPTTVSSL--PDTKIEVAHFIT 116
+L AG +C A +SL N+SNC+A+ RTQ+IL GLC KA VSSL PDTKIEVA F+
Sbjct 61 NLTAGLWYCAARESLINVSNCSALQRTQKILSGLCQHKASAGVSSLRSPDTKIEVAEFVK 120

Query 117 KLLSYTKQLFRHGPF 131
KL + + L+RHG F
Sbjct 121 KLRIHVQCLYRHGKF 135

A4.1 PROTOCOL 5

Sensitisation: 3 intra-peritoneal injections of 150µg Ova and 100mg $Al(OH)_3$ in 1ml (protocol 4) or in 2ml (protocol 5) of saline

Ova challenge: 0.03% Ova

In both guinea-pig groups Ova challenge a significant bronchoconstriction was observed immediately (-60.7±4.1% and –61.0±5.5%, protocol 4 and 5 respectively). No significant difference was seen in the time course. No significant bronchoconstrictions were observed during the expected time of the LAR (6-12 hours) (Figure 1, time course). No significant difference in peak bronchoconstriction was seen during the EAR and LAR (Figure 1, histogram). No difference was observed between the two groups at total, EAR and LAR time points (Figure 2).

In guinea-pigs sensitised by protocol 5 there was a significant bronchoconstriction immediately following histamine challenge when compared to the sGaw values measured prior to Ova challenge (-34.2±18.4% compared to 6.1±7.3%). At 5 minutes post-histamine exposure sGaw values were not significantly different from pre-Ova challenge values. No significant difference in post-Ova response to histamine was found between the 2 groups (Figure 3).

No significant difference in total or differential cell counts was observed (Figure 4).

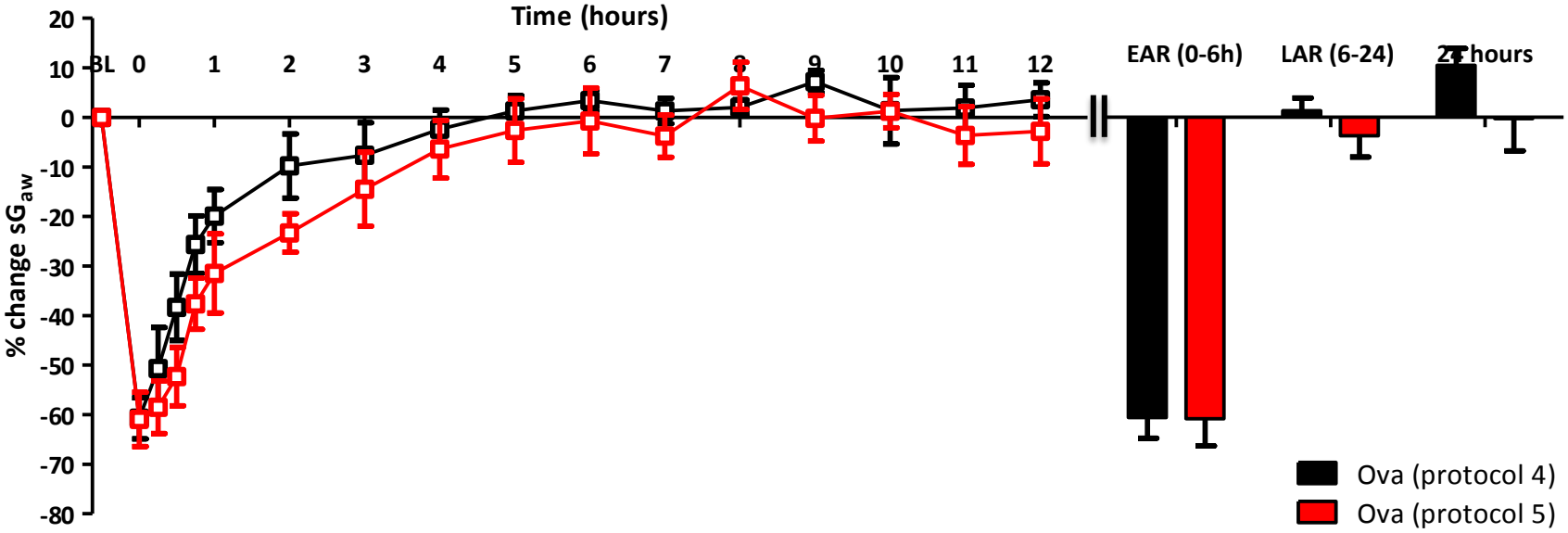

Figure 1: the mean time-course values of sGaw in guinea-pigs sensitised with 3 injections of either 1ml (protocol 4) or 2mls (protocol 5) of a suspension of 150ug ova and 100mg aluminium hydroxide and challenged with 0.03% Ova. The histogram represents the maximum bronchoconstriction values recorded during baseline, early asthmatic response (EAR) (0-6 hours), late asthmatic response (LAR) (6-12 hours) and 24 hours readings. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=5; performed with a two tailed t-test.

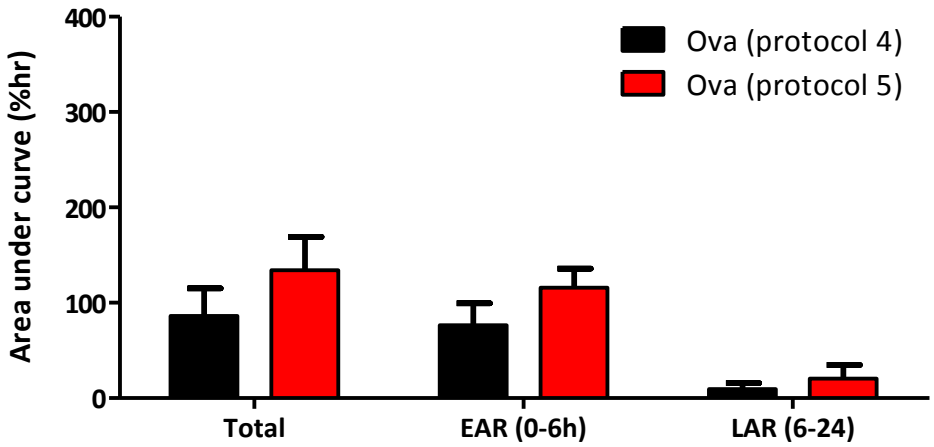

Figure 2: Area under the curve analysis of the bronchoconstrictor response of guinea-pigs sensitised with 3 injections of either 1ml (protocol 4) or 2mls (protocol 5) of a suspension of 150ug Ova and 100mg aluminium hydroxide and challenged with 0.03% Ova. For the purpose of analysis only negative peaks were considered and all positive sGaw values were excluded. Total includes all negative peaks from 0-24 hours, early asthmatic response (EAR) includes values from 0-6 hours and late asthmatic response (LAR) includes values from 6-12 hours. Area under the curve is measured in %.hour. N=5; performed with a two tailed T-test.

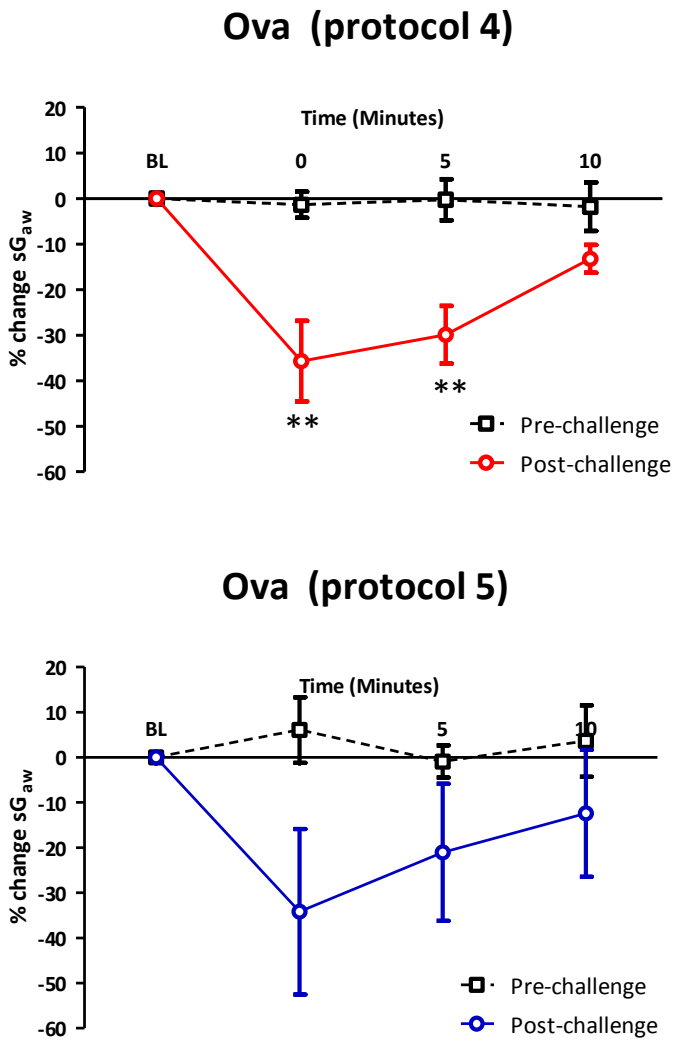

Figure 3: Response of the airways to nebulised histamine delivered in a plethysmograph (0.3mM, 10% duty cycles per camber over 2 minutes, 1 minute drying period, 0.5LPM flow per chamber) in guinea-pigs sensitised with 3 injections of either 1ml (protocol 4) or 2mls (protocol 5) of a suspension of 150ug Ova and 100mg aluminium hydroxide and challenged with 0.03% Ova. Values were recorded 24 hours before Ova challenge and at 24 hours post-Ova challenge. Mean changes in sGaw are expressed as mean±SEM percentage change from baseline. A negative value represents a bronchoconstriction. N=5; **significantly different from time paired pre-Ova challenge values $p<0.01$; performed with a two tailed T-test.

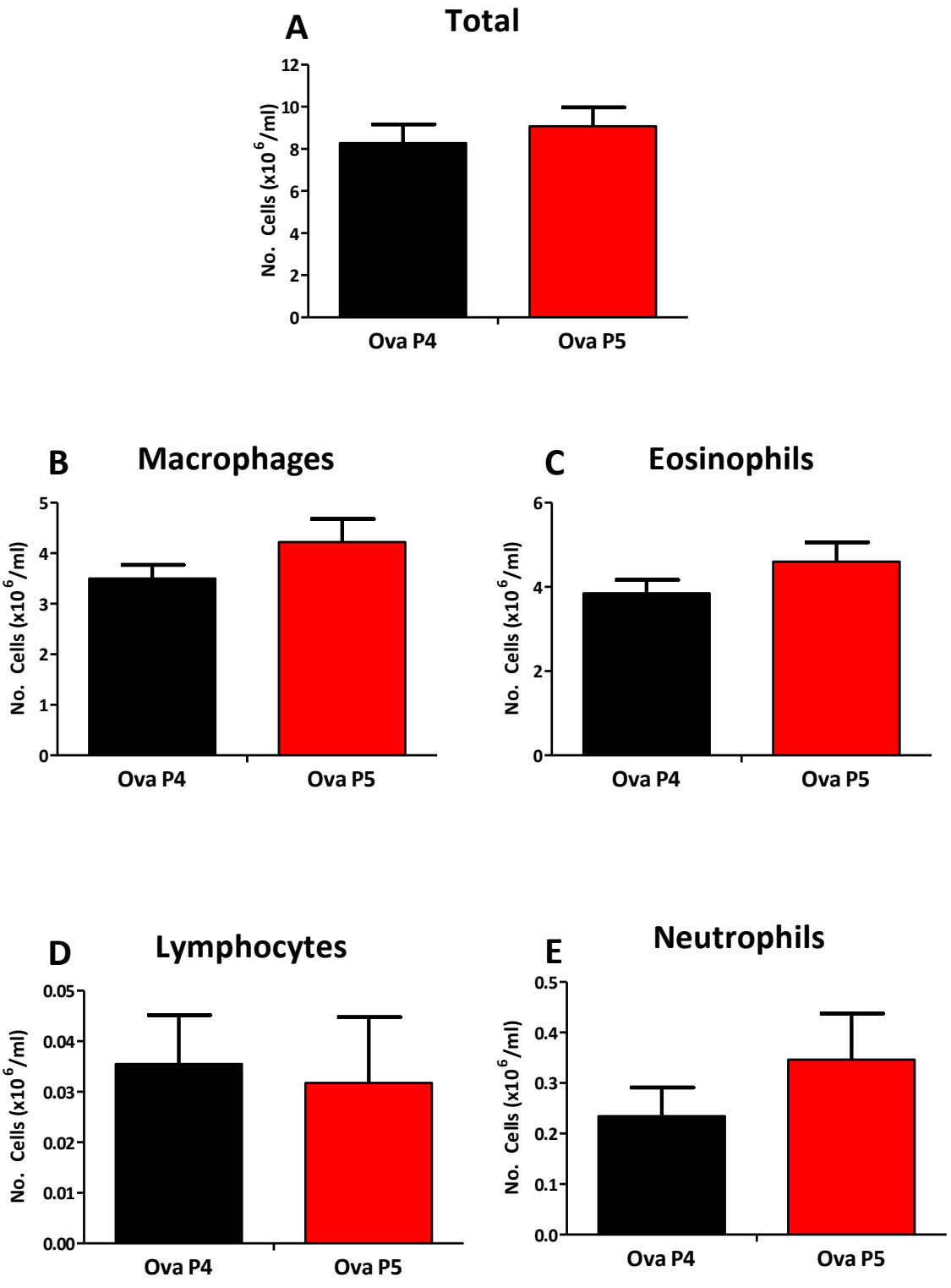

Figure 4: The total cell (A), macrophages (B), eosinophils (C), lymphocytes (D) and neutrophils (E) counts in bronchoalveolar fluid in guinea-pigs sensitised with 3 injections of either 1ml (protocol 4) or 2mls (protocol 5) of a suspension of 150ug Ova and 100mg aluminium hydroxide and challenged with 0.03% Ova. N=5; *; performed with a two tailed T-test.